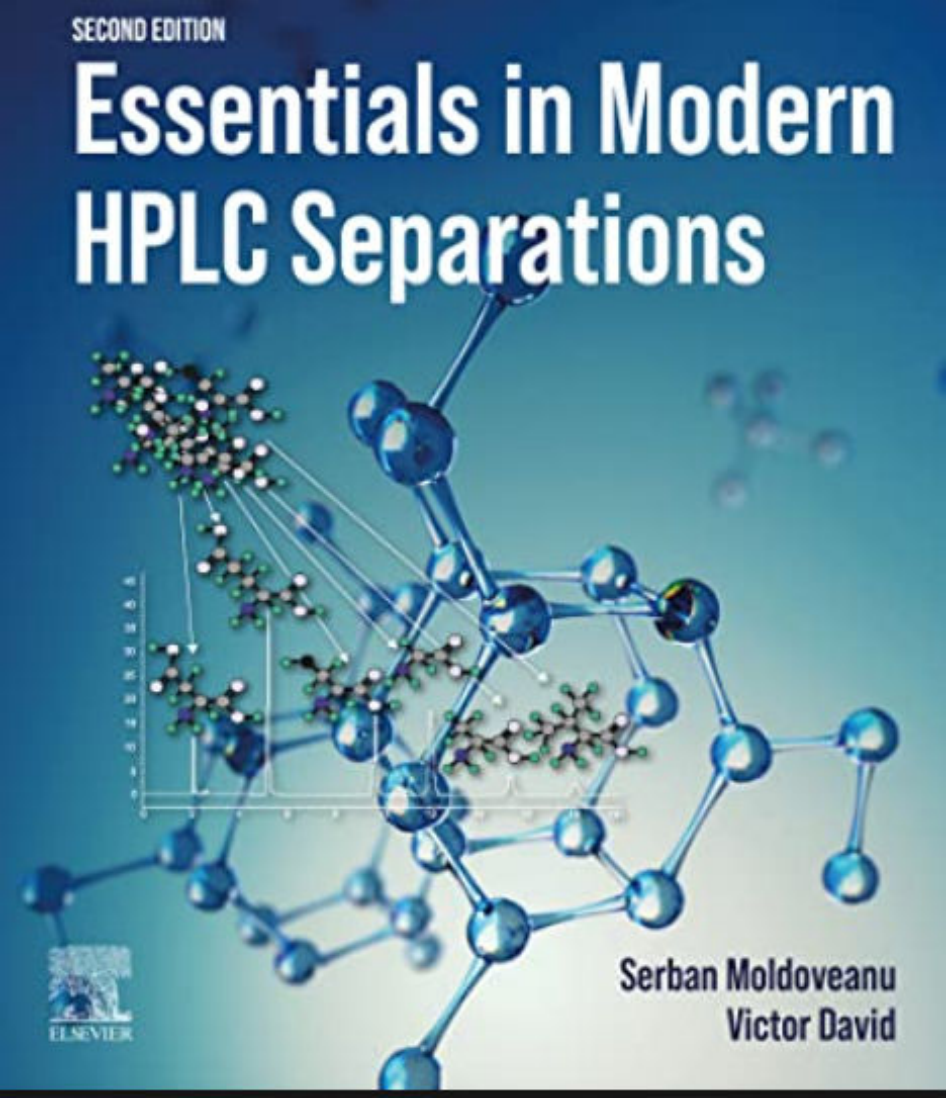
SECOND EDITION

Essentials in Modern HPLC Separations



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ELSEVIER

ESSENTIALS IN MODERN HPLC SEPARATIONS

SECOND EDITION

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Preface

High-performance liquid chromatography (HPLC) is the most utilized technique in chemical analysis of a variety of materials. As a result, a very large amount of information is available for HPLC in peer-reviewed journals, in books, in manufacturer catalogues, and on the internet. Besides the applications of HPLC for analytical purposes, there is also interest in using HPLC for the estimation of various physico-chemical characteristics of molecules and for the description of certain processes which can be studied with difficulty by other means. The goal of present book is to offer a coherent synthesis of the large amount of information about HPLC and to present the novel developments in the field. Separation is a key part of HPLC and the book is focused on the understanding of various aspects of it such as the distribution process between nonmiscible phases, the molecular aspects involved in the separation, the parameters that characterize the separation, the types and performances of materials used in the construction of chromatographic HPLC columns that provide the physical support for the separation, the role of mobile phase in the separations, etc. The material is presented having in mind the applicability of theory for the selection of the best HPLC analytical procedure, and besides the separation, other aspects of HPLC are also included such as instrumentation and utilization in practice of HPLC.

From the first edition, the book was subject to a significant number of changes. New literature sources were used, new references were added, and also the initial structure of the book was modified to make it more useful for practical applications. The book was organized in four parts, basic information, main types of HPLC separations, practice of HPLC analysis, and appendices. The large tables containing detailed data on the main commercial HPLC stationary phases and columns and on main properties of solvents used in the mobile phases for HPLC were placed as appendix to avoid breaking the text flow. This new edition of the book was written with the purpose to offer both a simple theoretical background about HPLC and guidance for the utilization of different types of HPLC in practice. No explicit methods for the analysis of specific analytes are described in the book (except for some examples), this field being much better covered in dedicated peer-reviewed literature.

The authors are indebted to the editorial team Mrs. Kathryn Eryilmaz, Ms. Emerald Li, and Mr. Rajan Vijay Bharath from Elsevier, for their assistance in publishing this book. Also, we are thankful to the referees of this new edition for their fair comments and useful recommendations.

Essentials in Modern HPLC Separations

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CHAPTER

1

Introductory information regarding HPLC

1.1 Preliminary discussion about HPLC

General comments

The steps in a chemical analysis involve both conceptual activities and also experimental ones and they can be schematically summarized as shown in Fig. 1.1.1 [1].

In the chain of activities indicated in Fig. 1.1.1, the most common core analysis, in current practice, is high-performance liquid chromatography (HPLC). The main advantages of HPLC as a method of analysis include the combination of a powerful separation step (that is presented in detail in this book) with a sensitive measurement capability, plus the possibility to be applied to a wide variety of molecules regardless of molecular weight of the analytes or of their volatility (required, for example, in gas chromatography (GC)). For these reasons, HPLC is an ideal analytical technique for many types of samples regardless how complex or simple they are.

What is chromatography and what is HPLC?

The term *chromatography* designates several similar techniques that allow the separation of different molecular species from a mixture. Applications of chromatography are numerous, and can be related to laboratory or industrial practices. Analytical chromatography uses a chromatographic separation of the compound from a *sample* and for the identification and/or measurement of these compounds uses a specific detection. The molecular species from the sample are indicated as *analytes* and *matrix*.

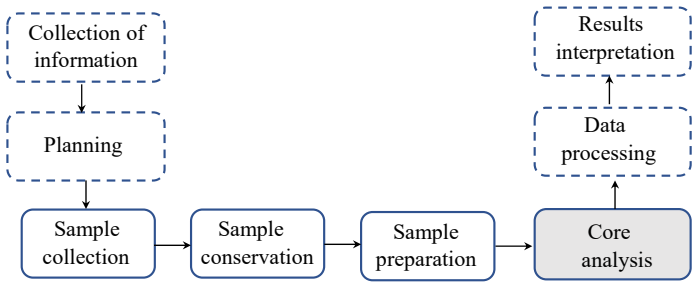

FIGURE 1.1.1 Steps in a chemical analysis (conceptual steps in dotted frame).

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The analytes are the molecular species of interest and the matrix forms the rest of components in the sample. For chromatographic separation, the sample components are introduced in a flowing *mobile phase* that passes a *stationary phase*. The stationary phase retains stronger or weaker different passing molecular species and releases them separately in time back into the mobile phase. When the mobile phase is a gas, the chromatography is indicated as *gas chromatography* (GC), and when it is a liquid, is indicated as *liquid chromatography* (LC). Other types of chromatography are known and they include supercritical fluid, countercurrent, electrochromatography, etc. When the sample is present as a solution its components are indicated as *solutes*. Sample dissolution and/or preliminary modifications are frequently necessary to have the analytes amenable for a chromatographic separation (e.g., Ref. [1]). In a common type of liquid chromatography, the stationary phase is in the form of a column packed with very small porous particles (1.7−10.0 μm in diameter), or a porous monolithic material, and the liquid mobile phase (or *eluent*) is moved through the column by a pump (at elevated pressure). This type of chromatography is indicated as high performance (or pressure) liquid chromatography (HPLC). In HPLC, the sample in liquid form (solution) is injected in the mobile phase as a small volume at the head of the *chromatographic column*. As the mobile phase flows, the sample constituents are separated and the eluted molecules that exit the column can be detected by various techniques. A schematic diagram of the separation process is shown in Fig. 1.1.2.

The eluted molecules from the chromatographic column differ from the mobile phase components by certain physico-chemical properties, which makes them detectable. Common properties used for detection in liquid chromatography are the density, the UV absorption, the fluorescence, the type of ions in a mass spectrometer, etc. The role of the detector in the chromatographic analysis is to produce an electrical signal which depends on the specific physicochemical property of the material eluting from the chromatographic column. The electrical signal caused by the detection of specific molecular species along a chromatographic separation (chromatographic run) is translated into a graphic output which is known as a *chromatogram*. The separated components of a mixture eluting at different times (known as retention times t_R) are displayed as *peaks* in the chromatogram. Due to diffusion (and other effects) in the chromatographic column, the peaks have ideally a Gaussian shape. Different peaks (or patterns) on the chromatogram belong to different components of the separated mixture. An example of an HPLC chromatogram obtained from a standard mixture of organic acids separated on a Synergi 4u Hydro-RP column with the retention times written above the peaks is shown in Fig. 1.1.3 [2].

As shown in Fig. 1.1.3, the separation of the peaks can be very good but also can be only partial. Some compounds may not be separated at all. Separated peaks may indicate individual compounds only when each peak corresponds

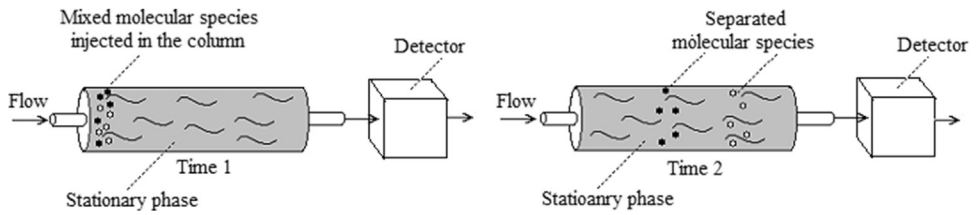

FIGURE 1.1.2 Schematic illustration of the separation process in chromatography showing the initial time (Time 1) and after the compounds started to be separated (Time 2) (the black and white stars indicate two different molecular species).

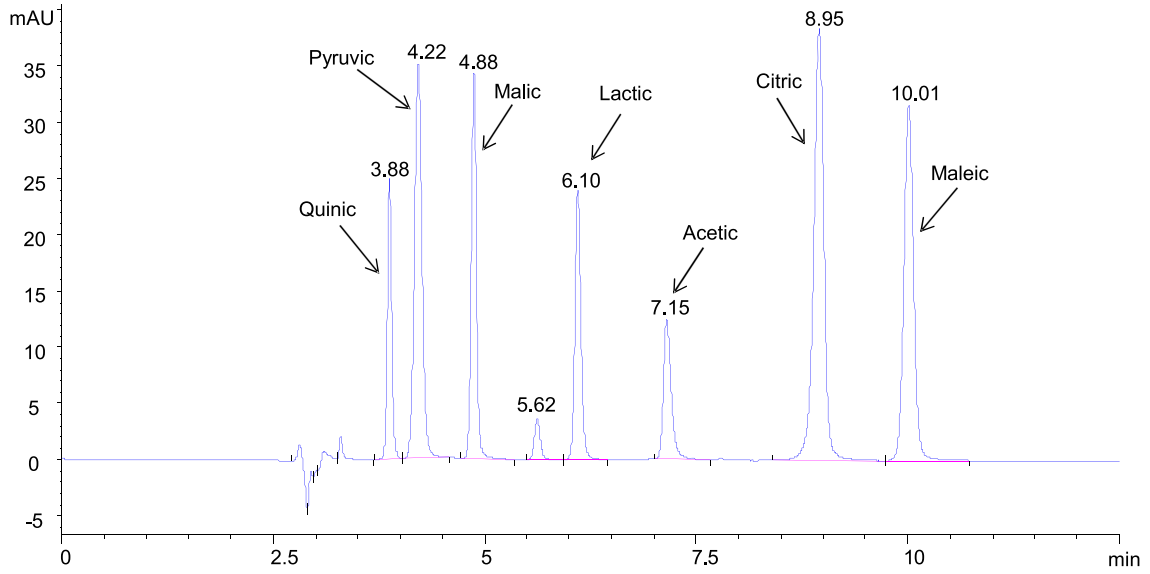

FIGURE 1.1.3 Picture of an HPLC chromatogram indicating the retention time for some of the peaks in a standard mixture of organic acids [2].

to a single molecular species. In HPLC, the analytes should be separated from each other and from the matrix, as well as possible. For example, in the chromatogram from Fig. 1.1.3, the first peaks belong to the separated matrix (their retention time is not indicated). The zones occupied by a specific analyte when it is eluted from the chromatographic column (peak width in a chromatogram) can be narrower or wider. The width of these zones affects the separation, and for two analytes with different retention times, the separation is better when the elution zones are narrower.

The peaks in the chromatogram may have different heights (and peak areas) depending on a number of factors such as the amount of compound in the mixture, amount of sample injected, and sensitivity of the detection procedure. Since peak areas are dependent on the amount of the compound injected into the column, HPLC can be used for quantitation after a proper calibration. In this way, HPLC became an excellent technique for separation and quantitation of compounds even in very complex mixtures and is currently the most widely used analytical technique ever practiced.

It can be seen from the previous short description of HPLC, that the technique has two distinct parts: (1) separation of the analytes and of the matrix, (2) detection and measurement of the analytes. The discussion about the separation is the main subject of this book. Based on the nature of the analytes, the separation process is achieved depending on the choice of a chromatographic column and a specific mobile phase. The detection step is achieved using one or more detectors, and the sensitivity, selectivity, and stability of these detectors are essential for the success of the HPLC analysis. Present book does not include a detailed discussion on detection and measurement of the analytes, and is focused mainly on their separation.

Separation by HPLC can also be used for semipreparative or preparative purposes, some with industrial applications. They differ to the analytical HPLC by the experimental design, mainly by using columns with larger dimensions

(length, diameter, and particle size that offer lower back pressures than analytical HPLC) and by the scale of samples, in order to isolate, collect, enrich, or purify the components of interest from higher amounts of samples. For example, the analytical columns packed with stationary phase particle sizes of 3–5 μm, while for preparative columns the particle size are larger than 10 μm. The separated compounds of interest are collected and possibly object of further purification [3]. However, the main focus of this book is analytical HPLC, and semipreparative and preparative HPLC are beyond the scope of the present material.

Types of equilibria in HPLC

The separation process in HPLC is based on an equilibrium established between the molecules present in the mobile phase and those retained in the stationary phase. The difference in the concentration of a molecular species in one phase and in another determines if the species is retained or eluted with the mobile phase. When the concentration of the solute (analyte) is higher in the mobile phase than in the stationary phase, the solute is eluted faster from the chromatographic column. The opposite happens when the concentration of the solute is higher in the stationary phase. In this case, the solute is more strongly retained and the elution takes place after a longer period of time.

Common types of equilibria for a molecular species between two phases include, for example, the distribution of a compound between two immiscible solvents (partition equilibrium). Another common type takes place during the retention of a compound from a fluid on an adsorbing material such as porous graphitic carbon. In a separation, it is possible that more than one type of equilibrium takes place although some equilibria can be characterized as mainly partition or mainly adsorption. Not all equilibria can be classified as partition or adsorption. Chemical equilibrium in a solution, e.g., between two ionic compounds, is also a known equilibrium type, although its classification refers more to the forces involved in the separation. A summary of main types of equilibria encountered in chromatography is given below:

(1) *Partition equilibrium.* This type of equilibrium takes place when the molecules of the solute are distributed between two liquid phases. In HPLC, one liquid phase is kept immobile on a solid material, and the other is mobile (the eluent). The immobilization of the liquid to become a stationary phase in partition chromatography is achieved, for example, when the liquid is highly polar and can establish hydrogen bonds with the solid support, which is also polar. One such example is water on a silica surface. In this case, the mobile phase should consist of a liquid less polar than water. However, the partition equilibrium can also be applied for a nonpolar stationary phase and a more polar mobile phase. The theory of separation in partition chromatography is based on liquid/liquid extraction (LLE) principles. The different molecular species, being in continuous equilibrium between the mobile and stationary phase, will be separated based on their tendency to exist in higher concentration in the mobile liquid or in the stationary liquid, in accordance with their affinity for these phases. A schematic description of the partition chromatography process is shown in Fig. 1.1.4. In partition chromatography, the concept of "immobile

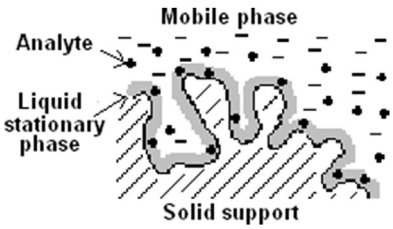

FIGURE 1.1.4 Schematic description of partition equilibrium.

liquid" is commonly approached in a "loose" manner. For example, a layer of adsorbed water on the surface of a silica solid support, or a layer of bonded organic moieties on a silica surface (such as in the common C18 chromatographic columns), or a layer of mechanically held polymer on an inert core are all considered liquid stationary phases for partition chromatography. The types of interactions in partition equilibria can be of different types including hydrophobic interactions, van der Waals interactions, ionic interactions, etc.

The possibility of performing chromatography using two liquid phases without having one liquid phase immobilized is exploited in countercurrent chromatography [4,5]. This subject is beyond the purpose of this book (for details, see e.g., Ref. [6]).

(2) *Adsorption equilibrium.* This type of equilibrium takes place when molecules are exchanged between a solid surface and a liquid mobile phase. For example, carbon black can adsorb nonpolar molecules on the solid stationary phase surface, while the more polar molecules are kept mainly in the mobile phase. Being in equilibrium between the solid and the liquid, the nonpolar molecules also elute from the chromatographic column, but later than the polar compounds. A schematic description of the adsorption chromatography process is shown in Fig. 1.1.5.

The partition and the adsorption are utilized basically as models for describing the type of equilibrium, but a difference between the two processes is not commonly apparent from thermodynamic point of view [7]. Also, in many instances, the separation can be viewed either as a partition or as an adsorption, the differentiation being made only with the purpose to estimate differently the separation parameters, while the classification has no effect on the real process that may take place with both types of equilibrium.

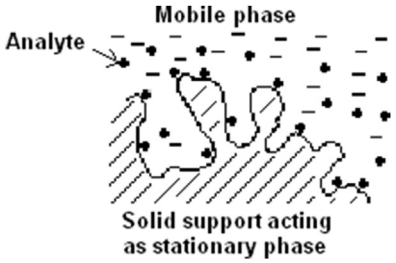

FIGURE 1.1.5 Schematic description of adsorption equilibrium.

(3) *Equilibria involving ions.* Equilibria between ions in solutions take place in numerous chemical reactions. For applications in HPLC, one ionic species must be immobilized, for example, by being connected through a covalent bond to a solid matrix. An example of this type of ion can be a sulfonic group connected to polystyrene. The ions from solution can be bound by ionic interactions to the immobilized counterion or may remain in solution. The equilibrium between solid phase and mobile phase, depending on the strength of the bond to the stationary phase, may provide a means for separation. This type of interaction is usually indicated as ion exchange. Although equilibria involving ions appear to be more referring to the type of interaction, the separation involving ion exchange is different from partition or adsorption. A schematic description of the interactions in ion exchange chromatography process is shown in Fig. 1.1.6.

(4) *Equilibria based on size exclusion.* Size exclusion uses a stationary phase that consists of a porous structure in which small molecules can penetrate and spend time passing through the long channels of the solid material, while large molecules cannot penetrate the pore system of the stationary phase and are not retained. Applied in HPLC, the large molecules elute earlier, while the small molecules are retained

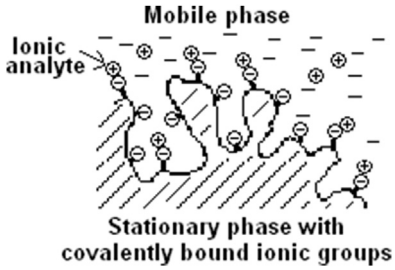

FIGURE 1.1.6 Schematic description of ion exchange process.

longer. An equilibrium can be envisioned between molecules in the mobile phase and those partly trapped in the solid matrix. A schematic description of size exclusion process is shown in Fig. 1.1.7.

As in the case of other types of separation, size exclusion process is frequently associated with other type of equilibrium than partition or adsorption.

(5) *Equilibria based on affinity interactions.* This type of interaction is also more referring to the interaction types and is typical for protein binding. This type of equilibria allows very specific separations. Examples of such interactions are protein−antibody, avidin−biotin, etc. Affinity chromatography is widely used at low pressure for protein purification, but is also applied in HPLC.

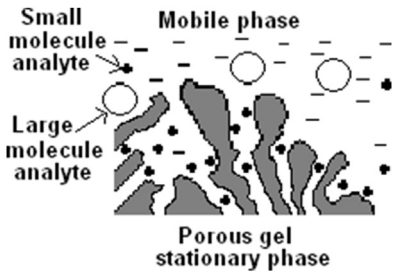

FIGURE 1.1.7 Schematic description of size exclusion process.

Role of polarity in HPLC

One common concept related to HPLC separations is that of "polarity." *Polarity* refers to an asymmetrical charge distribution in a molecule, which causes the molecule to act as an electric dipole. However, charge distribution is a very complex concept, and the calculation of the values for charge density, used to characterize charge distribution in a molecule, is usually a difficult task. Also, polarity can refer to the analytes, to the mobile phase or to the stationary phase. The compounds are typically indicated as polar when opposite partial charges are known to be present in the molecule, when specific physical properties such as water solubility or solubility on solvents miscible with water are known, or when specific functional groups known to be "polar" such as −COOH, or $-NH_2$ are present in the molecule. In addition to that, during molecular interactions, the charge distribution of the molecules suffers changes (expressed by *polarizability*). In any interaction, the polarizability affects the charge distribution of the molecules. For these reasons, comparing molecules or phases as "more polar" or "less polar" is not a quantitative assessment. The opposite to the polar character is the *hydrophobic character* (*or lipophilic character*; the two terms are not interchangeable, hydrophobicity being the physical property of a molecule to be repelled from water, while lipophilicity referring to the ability of a chemical compound to dissolve in fats, oils, lipids, and nonpolar solvents). Nonpolar compounds that do not have polar groups and are not water soluble, or materials on which surface the water does not adhere are commonly indicated as hydrophobic. Both the polar and the hydrophobic character of a compound are reasonably described by the partition constant (also indicated as partition coefficient) between octanol and water K_{ow} (or P_{ow}). The experimental values for K_{ow} are known for many compounds, and for their evaluation there are available several computer programs (e.g.,

MarvinSketch 5.4.0.1, ChemAxon Ltd., [8], EPI Suite [9]), as well as extensive tables [10,11]). Positive values for log K_{ow} indicate a hydrophobic character for the compound X, larger values indicating higher hydrophobicity. Molecules with low or negative values for K_{ow} are frequently indicated as polar, although there is not a direct relation between K_{ow} and the charge distribution in the molecule.

In the body of this book, the polarity will be further discussed and clarified. However, the concept of polar and nonpolar (hydrophobic) compounds or materials will be frequently used in the "imprecise" manner. For mobile phases, the extension of the concept of polarity is immediate, being based on the polarity of the molecules of the phase. For stationary phases, the polarity refers to the nature of the stationary phase surface or of its active part.

As a conclusion, HPLC techniques can be differentiated based on a number of criteria. Each selected criterion has advantages and disadvantages. For example, the type of interactions for a particular separation is not always well understood, and the "polar" or "nonpolar" nature of the interactions involved in the separation is sometimes difficult to quantify. Also, the physical criteria such as particle dimension in the chromatographic column or the scale of the HPLC equipment are available in a range and the limits used for the classification are subjective. For this reason, the HPLC classifications should be viewed mainly as an attempt to have models, and sometimes a particular HPLC type can be classified in more than one way.

Applications of HPLC in chemical analysis

The utility of HPLC is very diverse, including the utilization for analytical purpose, but also for preparative purposes. The subject of preparative HPLC is beyond the purpose of this book and it is reported in the literature in a variety of publications such as peer reviewed papers, books (see e.g., Refs. [12–14]), and information on the web. The main application of analytical HPLC is in quantitative analysis. However, qualitative analysis by HPLC is also common, although it has some limitations. Analytical HPLC is also used for other purposes than "analytical," such as the determination of certain physical parameters of compounds (e.g., octanol/water partition coefficient), and for other applications.

Qualitative analysis is based in HPLC on two different types of parameters, one type being related to the separation process and the other related to the detectors utilized. The main parameter from the separation process used in qualitative analysis is the retention time t_R which is specific for the analytes in a specific separation. The expected t_R for an analyte in a given separation can be determined using a standard, and the t_R can be used to assess the presence or absence of the analyte peak in the chromatogram of a sample. This procedure, although very useful in many applications, has several shortcomings, including the need to use standards in order to know at which t_R the analyte of interest is eluting and as a result the inability to identify an unknown compound, and the possibility that other compound may elute at the same t_R as the analyte which would lead to interferences and incorrect identifications. These make the use of t_R a relatively weak procedure for qualitative identification.

When the parameters related to the separation process are not sufficient (or fail) to provide qualitative identification of a compound, the information from the detection may provide the solution. A variety of detectors are used in HPLC, some being indicated as "universal" meaning they respond to all analytes but do not provide any qualitative information (such as refractive index detectors). Other detectors provide only partial information that is not in itself sufficient for positive identification of an analyte (e.g., UV adsorption). Detectors such as

mass spectrometers (MS) or MS/MS offer more detailed insight regarding qualitative peak identification. However, the lack of fragmentation of the analyte in LC/MS and the dependence on operational conditions of the mass spectra obtained using LC/MS/MS instrumentation make the process of compound identification still relatively difficult in HPLC. Progress in MS identification of unknown compounds has been done, for example, by using very high accuracy in mass measurement for the parent ion of the analyte and for its fragments (e.g., using Orbitrap or cyclotron technologies). Also, specific computer programs (Mass Frontier, SmileMS) provide help for the identification of unknown compounds. However, in many cases, the compound identification capability, even using MS or MS/MS detection, is not used for the discovery of the composition of an unknown compound, but for the positive identification of a known analyte, corroborated with the retention time of its standard, previously analyzed. The confirmation of the peak for a specific analyte in a chromatogram using MS detection (typically based on molecular ion and two or three confirmation ions) is an important and common procedure in HPLC practice. The use of standards with labeled isotopes for the analytes (e.g., deuterated analyte) spiked in the sample is also a common practice for peak identification in LC/MS and LC/MS/MS. Although the retention time of the isotope labeled standards may vary slightly from that of the analyte itself, peak identification is significantly facilitated using this technique.

Quantitative analysis is the main use of HPLC. Once separated, the concentration of the analytes in the sample or their amount in the injected sample can be obtained from the chromatographic peak area (or height). Peak areas (or peak heights) in the chromatogram are dependent on the concentration or the amount of analyte, and quantitation is done using calibration curves with standards, or by other procedures. Depending on the detection technique and the analyte properties, some HPLC analyses can provide results even for ultralow traces of a compound (below ng/mL level). The versatility and high sensitivity of HPLC has contributed to its success and widespread use. An exceptionally large number of methods using HPLC quantitation procedures have been published. Further discussion on quantitative procedures used in HPLC is given in Section 3.4 and 18.2.

Nonanalytical applications of analytical HPLC

HPLC with various detection techniques is not only an analytical tool for the determination of chemical species in various matrices, but also it can be useful in estimating physical parameters that are otherwise difficult to be measured by other approaches [15]. For example, HPLC can be used for the evaluation of molecular descriptors such as acidity/basicity constant, hydrophobicity, hydrophilicity, dipole moment, molecular polarizability, and other parameters [16]. Solubility data in various solvents, thermodynamic values for the interphase partition, molecular interactions, or structural molecular features [17] are only a few aspects that can be studied using HPLC, under various separation types [18]. Quantitative relationships between molecular descriptors and HPLC retention are often used in the literature for the characterization of the separation and to predict the chromatographic behavior of other compounds [19]. Due to the similarities between some natural systems and some HPLC partition processes, the term of biomimetic liquid chromatography has been suggested in the literature for complex types of distribution of organic species between mobile phase and stationary phase with multiple functionalities [20–23]. *Biomimetic HPLC* is considered to mimic the lipid environment of a membrane and they can be used in the

assessment of interactions between molecules and biological membranes or for the estimation of permeability through cell membranes [24].

1.2 Main types of HPLC

Criteria for the classification of HPLC procedures

HPLC is comprised of several similar techniques having in common the use of a liquid mobile phase passing a stationary phase with the result of a "high performance" separation of a mixture of compounds. Various versions of HPLC have specific differences and also different applications. For this reason, the HPLC techniques are classified in various types, the classification being done based on a number of criteria, such as the separation principle (in some separations more than one principle of separation may have a contribution) or the scale of the utilization. Additional differences have been used to distinguish more types of HPLC. This may include the nature of the stationary phase and mobile phase and/or the type of interactions that describe the energetics of the process. It should be noted that the classification based on types of molecular interactions indirectly includes differences in the nature of stationary and mobile phase. For example, ion exchange chromatography is practiced specifically on an ion exchange stationary phase and not on a reversed phase or a bioaffinity one. However, the type of molecular interaction alone was not viewed as sufficient for the differentiation of some types of HPLC. Based on the nature of the stationary phase and mobile phase, several types of HPLC can be differentiated.

Other HPLC characteristics are also used for differentiating the HPLC types. One such characteristic is related to the composition of the mobile phase, which can be kept constant during the separation or can be modified. The HPLC performed at a constant composition of the mobile phase is known as *isocratic HPLC*, while that performed with a mobile phase that changes continuously in composition during the separation according to a linear or nonlinear elution program is known as *gradient HPLC* (a step change in mobile phase composition can also be used). Gradient HPLC allows a change in the polarity and/or in the pH of the mobile phase during the separation and significantly increases the versatility of HPLC. When a sample contains solutes with very different properties and when a constant composition of the mobile phase (isocratic conditions) is used, the solutes may leave the chromatographic column at very different times. This may be seen as an advantage for the separation, but when the retention time of some of the solutes become unacceptably long, the change in the solvent composition (by using gradients) is necessary to speed up the separation.

The differences in the size of the particles used in the chromatographic column offer one more criterion for HPLC classification. The size of the particles that fills the chromatographic column affects the peak width and therefore the separation. In common HPLC, the size of the particles in the chromatographic column is indicated by an average diameter, different columns having the particles of values between 1.7 and 10 μm. The HPLC techniques that use in the chromatographic column (or cartridge) very small particles, (e.g., with a diameter below 2.5 μm), are usually indicated as *Ultra Performance Liquid Chromatography* or *UPLC*. The very small particles require additional modifications in the UPLC technique, such as significantly higher operation pressure for the mobile phase. The use of small particles in the columns can lead not only to better separations, but also to faster ones, and is one of the modern developments of HPLC. Another development in HPLC is the use of monolithic columns made of a single piece of a solid porous material.

Temperature can be another criterion to differentiate HPLC processes. Based on this

parameter, the HPLC types are classified as (1) *Low temperature* (below freezing point of water, down to −10°C), (2) *Usual range temperature* (20−60°C), and (3) *High temperature* (up to 250°C). Most separations are performed at usual temperatures. Low temperature techniques are applied especially for certain chiral separations. High-temperature separations can be used for a number of applications taking advantage of the modification of solvents properties as temperature increases. Water, for example, shows a decrease in polarity at temperatures between 100 and 250°C and can be used as solvent in RP-HPLC. This particular mode of separation has been denoted as superheated water, pressurized water, or subcritical water chromatography (as the temperatures used are lower than the critical temperature of water at 374°C) [25]. The use of water as a mobile phase can provide an environmentally friendly "green" method of chromatographic analysis.

A different classification, important for practical purposes, is based on the scale of the HPLC equipment. Three general types of chromatography can be distinguished in this way: (1) *Analytical HPLC*, (2) *Semipreparative HPLC*, and (3) *Preparative (Large scale) HPLC*. Each of these types covers in fact a range of dimensions [3]. For example, analytical HPLC can be further differentiated based on the dimensions of the HPLC column in the following subtypes: conventional, narrowbore, microbore, micro-LC-capillary, and nano-LC-capillary [26−28]. In this book, most discussions will refer to conventional, narrowbore, and microbore analytical HPLC. Analytical HPLC performed using microcapillary and nanoscale columns (still filled with a bed of particles) are less used in practice and require specialized equipment [29]. In case of preparative HPLC, the primary goal of separation is to collect fraction of pure compounds with high yields. This technique uses larger chromatographic columns and stationary phases with bigger particles compared to analytical HPLC [30].

A classification of HPLC types based on the nature of stationary and mobile phase

A variety of HPLC types were differentiated in the literature, some of these types being rather similar and others having significant differences. The differentiation was based on various criteria such as the nature of the stationary phase, the nature of the mobile phase, the type of interactions assumed to lead to the separation, but also the range of concentration of specific solvents in the mobile phase (e.g., of water), etc. Multiple types of interactions are usually involved in HPLC separations, but in many cases it is possible to indicate the dominant one. A common classification of the main types of HPLC is given in this section. Because different HPLC types have different characteristics and different applications, it is important to understand their differences and select the appropriate HPLC type for solving a specific separation/analysis problem.

(1) *Reversed-phase HPLC* (or *RP-HPLC*) is the most common HPLC technique, and a very large number of compounds can be separated by RP-HPLC. This type of chromatography is performed on a nonpolar stationary phase with a polar mobile phase containing water. A wide variety of nonpolar stationary phases is available, and RP-HPLC is very likely the most common type of chromatography used in practice. The stationary phase for RP-HPLC can be obtained, for example, by chemically bonding long hydrocarbon chains on a solid surface such as silica. The most common chain bound to silica is C18 (it contains 18 carbon atoms), which has a high hydrophobic character. The bonded phase hydrophobicity may vary depending on the nature of the substituent. For example, C18

bonded phase has a higher hydrophobicity than C8 bonded phases. Polymeric materials are also used as RP-HPLC stationary phase. The mobile phase in RP-HPLC is typically a mixture of an organic solvent (CH_3CN, CH_3OH, isopropanol, etc.) and water, with a range of content in the organic solvent. Small amounts of buffers can also be added to the mobile phase in RP-HPLC.

The main equilibrium in RP-HPLC is partition, and the main type of molecular interactions are indicated as hydrophobic interactions. These hydrophobic interactions (sometimes called hydrophobic forces) are caused by the energies resulting from the reduction of the disturbance of the structure of water/polar solvents caused by compounds with nonpolar moieties. The new hydrogen bonds and polar interactions formed in the water/polar solvents when nonpolar molecules are eliminated are a source of energy. The so-called "solvophobic effect" is produced by the energy of "cavity reduction around the analyte" in water/polar solvent when the analyte leaves the mobile phase and is placed in the nonpolar stationary phase. However, depending on the structure of the molecule of the analyte, other types of interactions including polar interactions and even ionic interactions may play some role in RP-HPLC separations. In RP-HPLC, the separation is typically considered to be based on the partition of the analyte between the stationary phase (viewed as an immobilized hydrophobic liquid) and the mobile phase. Nevertheless, some experimental results in RP-HPLC can also be explained by adsorption equilibrium. The exceptional utility of RP-HPLC is based on the fact that most organic compounds have at least some hydrophobic moiety in their structure, and this technique can be used for their separation.

(2) *Ion-pair chromatography* (*IPC*) is applied in particular to ionic or strongly polar compounds. This type of chromatography is very similar to RP-HPLC, with the difference of having a special mobile phase (ion pair RP). In the mobile phase of ion-pair chromatography, a reagent is added, which interacts with the ions of the analytes and forms less polar compounds that can be separated based on hydrophobic interactions with the stationary phase. For example, acids that are polar and have small hydrophobic moieties can be coupled with basic reagents with a large hydrophobic moiety that produces "ion pairs" amenable to be separated by RP-HPLC.

(3) *Hydrophobic interaction chromatography* (*HIC*) is a type of RP-HPLC, sometimes indicated as a milder RP-HPLC, applied to the separations of proteins and other biopolymers. The technique is based on interactions between nonpolar moieties of a protein with solvent-accessible nonpolar groups (hydrophobic patches) on the surface of a hydrophilic stationary phase (e.g., hydrophobic ligands coupled on cross-linked agarose). The promotion of the hydrophobic effect by addition of salts (such as ammonium sulfate) in the mobile phase drives the adsorption of hydrophobic areas from the protein to the hydrophobic areas on the stationary phase. The reduction of the salting out effect by decreasing the concentration of salts in solution leads to the desorption of the protein from the solid support.

(4) *Nonaqueous reversed-phase chromatography* (*NARP*) is an RP-HPLC type utilized for the separation of very hydrophobic molecules such as triglycerides. In this type of chromatography, the stationary phase is nonpolar (similar to RP), while the mobile phase, although less nonpolar than the stationary phase, is nonaqueous (usually a mixture of less polar and more polar organic solvents) and capable of dissolving the strongly hydrophobic molecules.

(5) *Hydrophilic interaction liquid chromatography* (*HILIC*) is a type of HPLC applied for polar, weakly acidic, or basic compounds. In this type of HPLC, the stationary phase is polar and the mobile phase is less polar than the stationary phase. HILIC is the "reverse" of RP-HPLC. For HILIC, the polar stationary phase is typically made by chemically bonding on a solid support molecular fragments with a polar end group (diol, amino, special zwitterionic, etc.). The chromatography performed on bare silica supported with free silanol (→Si−OH) groups can also be considered as HILIC, depending on the mobile phase. The mobile phase in HILIC is less polar than the stationary phase and contains water soluble solvent such as CH_3OH or CH_3CN, and also a certain proportion of water. The separation is based on the difference in polarity between the molecules. Ion−polar interactions and even hydrophobic interactions may also play a role in separation. The type adsorption of partition for HILIC is difficult to assess. Viewed as having the separation equilibrium based on the interaction of a solid surface with the molecules from a liquid, HILIC can be considered adsorption chromatography. However, a (polar) bonded phase may be seen as a stationary liquid phase, and in this case, HILIC is a type of partition chromatography. When the separation is done on zwitterionic phases, HILIC chromatography is sometimes indicated as ZIC.

HILIC separations can also be performed on an ion exchange stationary phase with the mobile phase containing a high proportion of an organic solvent. This type of separation is sometimes indicated as eHILIC or ERLIC (from electrostatic repulsion hydrophilic interaction chromatography). This technique can be cationic eHILIC or anionic eHILIC, depending on the nature of the ion exchange stationary phase. In this type of chromatography, the ionic stationary phase repels the similar ionic groups of the analyte and allows HILIC type interactions with the neutral polar molecules of the analyte.

(6) *Normal phase chromatography* (*NPC* or *NP-HPLC*) is a chromatographic type that uses a polar stationary phase and a nonpolar mobile phase for the separation of polar compounds. The nonpolar mobile phases used in this type of chromatography are solvents such as hexane, CH_2Cl_2, tetrahydrofuran, etc., that are not water soluble. In normal phase chromatography, the most nonpolar compounds elute first and the most polar compounds elute last. Normal phase chromatography does not have a major difference from HILIC except the absence of water in the mobile phase. Because NPC was identified as a separate type for much longer time than HILIC, it is common in the literature to identify HILIC as a subtype of normal phase chromatography, and not the other way around. A polar organic normal phase is sometimes mentioned as a type of chromatography when the nonaqueous solvent contains polar additives such as trifluoroacetic acid.

(7) *Aqueous normal phase chromatography* (*ANPC* or *ANP*) [31,32] is a technique performed on a special stationary phase (silica hydride) and the mobile phase covers the range including the types used in reversed-phase chromatography and those used in normal phase chromatography. The mobile phases for ANP are based on an organic solvent (such as methanol or acetonitrile) with a certain amount of water such that the mobile phase can be both "aqueous" (water is present) and "normal" (less polar than the stationary phase). Polar solutes are most strongly retained in ANP, with retention decreasing as the amount of water in the mobile phase increases.

(8) *Cation exchange chromatography* is a type of HPLC used for the separation of cations (inorganic or organic). In this HPLC type, the retention is based on the attraction between ions in a solution and the opposite charged sites bound to the stationary phase. In ion exchange chromatography (IEC or IC), the ionic species are retained on the column based on coulombic interactions. In cation exchange chromatography, the ionic compound consisting of the cationic species M^+ in solution is retained by ionic groups covalently bonded to a stationary support of the type $R-X^-$. The ion exchange material (e.g., an organic polymer with ionic groups) is not electrically charged, and therefore the initial form of the cation exchange already has an ionically retained cation in the form $R-X^-C^+$. The separation is achieved when different molecules in solution have different acidic or basic strength. For example, for a cation exchange material, one species (e.g., C^+) that is bound to the $R-X^-$ substrate is replaced by a stronger cationic species (e.g., M^+) such that M^+ is retained from the solution, while C^+ passes into the mobile phase. Two different cations from solution, M_1^+ and M_2^+, can be separated based on their retention strength.

(9) *Anion exchange chromatography* is a type of HPLC used for the separation of anions (inorganic or organic). This HPLC is similar in principle with cation exchange type, but the anionic species B^- from solution are retained by covalently bonded ionic groups of the type $R-Y^+$. Similarly to cation exchange stationary phases, an anion exchange is initially in the form $R-Y^+A^-$. For an anion exchange material, the anion A^- previously bound is replaced on the resin by the anion B^-, and two different anions B_1^- and B_2^- are separated based on their different retention strengths. The mobile phase in ion exchange chromatography usually consists of buffer solutions.

(10) *Ion exchange on amphoteric or on zwitterionic phases* is a type of IEC very similar in principle with the cation exchange or anion exchange IEC. The stationary phase for this type of IEC contains groups that have an amphoteric character or in the case of zwitterionic phases, both anionic and cationic groups. The mobile phase in these types of chromatography also consists of buffer solutions.

(11) *Ion exclusion chromatography* is an HPLC technique in which an ion exchange resin is used for the separation of neutral species between them and from ionic species. In this technique, ionic compounds from the solution are rejected by the selected resin (through the so called Donnan effect), and they are eluted as nonretained compounds. Nonionic or weakly ionic compounds penetrate the pores of the resin and are retained selectively as they partition between the liquid inside the resin and the mobile phase (Donnan effect or Gibbs–Donnan effect describes the distribution of ions in solution in two compartments separated by a semipermeable membrane).

(12) *Ligand exchange chromatography* is a type of chromatography in which the stationary phase is a cation exchange resin loaded with a metal ion (e.g., of a transitional metal) that is able to form coordinative bonds with the molecules from the mobile phase. The elution is done with a mobile phase able to displace the analyte from the bond with the metal, and the separation is based on the differences in the strength of the interaction (of coordinative type) of these solutes with the bonded metal ion.

(13) *Immobilized metal affinity chromatography* is closely related to ligand exchange

chromatography and uses a resin containing chelating groups that can form complexes with metals such as Cu^{2+}, Ni^{2+}, Zn^{2+}, etc. The metal ions loaded on the resin still have coordinative capability for other electron donor molecules such as proteins. The retained analytes can be eluted by destabilizing the complex with the metal, e.g., by pH changes or addition of a displacing agent such ammonia in the mobile phase.

(14) *Ion-moderated chromatography* is an HPLC technique similar to ligand exchange chromatography with the difference that the stationary phase loaded with the metal ion (e.g., Ca^{2+}, Na^{+}, K^{+}, Ag^{+}, or even H^{+}) does not form coordinative bonds with the analyte, the interactions being based mainly on polarity.

(15) *Gel filtration chromatography* (GFC) is a type of size exclusion chromatography (SEC) in which the molecules are separated based on their size (more correctly, their hydrodynamic volume). In gel filtration, an aqueous (mostly aqueous) solution is used to transport the sample through the column and is applied to molecules that are soluble in water and polar solvents. Size exclusion chromatography uses porous particles with a variety of pore sizes to separate molecules. Molecules that are smaller than the pore size of the stationary phase enter the porous particles during the separation and flow through the intricate channels of the stationary phase. Small molecules have a long path through the column and therefore a long transit time. Some very large molecules cannot enter the pores at all and elute without retention (total exclusion). Molecules of medium size enter only some larger pores and not the small ones, and are only partly retained, eluting faster than small molecules and slower than the very large ones. The separation of small molecules between themselves is not typically achieved, and the technique is utilized mainly for the separation of macromolecules and of macromolecules from small molecules. GFC is sometimes indicated as aqueous SEC.

(16) *Gel permeation chromatography* (GPC) is another type of SEC, the only difference from gel filtration being the mobile phase, which in this case is an organic solvent. The technique is used mainly for the separation of hydrophobic macromolecules (such as solutions of certain synthetic polymers). GPC is sometimes indicated as nonaqueous SEC.

(17) *Displacement chromatography* is a chromatographic technique where all the molecules of a sample are initially retained on a chromatographic column (loading phase). After the sample is loaded, a "displacement" reagent dissolved in the mobile phase is passed through the column and elutes the specific retained molecule. The method is more frequently applied as a preparative chromatographic technique than as an HPLC analytical method.

(18) *Affinity chromatography* is a liquid chromatographic technique typically used for protein and other biomolecules separation when commonly indicated as *bioaffinity chromatography*. It can be practiced on a variety of specifically made stationary phases that allow selective retention of the analytes based on affinity interactions.

(19) *Chiral chromatography on chiral stationary phases* is a type of HPLC used to separate chiral compounds. Specific applications require the separation of chiral compounds, although regular chromatography is much more common than chiral chromatography. Chiral chromatography still has numerous applications, particularly in the analysis of pharmaceutical compounds. The technique typically requires chiral stationary phases containing chiral selector groups.

(20) *Chiral chromatography on achiral stationary phases* is also possible for some chiral solutes, by using chiral modifiers in the mobile phase, although the stationary phase is not chiral. This type of HPLC separates the diastereoisomers formed between the analytes and the chiral modifier from the mobile phase.

(21) *Multimode HPLC* is a type of chromatography in which the stationary phase contains by purpose more than one type of functionality, for example, some with bonded nonpolar groups (e.g., C18), and some with ionic groups (e.g., SO_3^-). This type of stationary phases is able to participate to multiple interactions with solutes having different molecular characteristics, thus contributing to their retention on the stationary phase [33]. This type of character can also be encountered unintentionally on columns made using as a stationary phase a silica support covered with silanol groups, and also with hydrophobic groups (such as C18). In most cases, the presence of two main types of interactions (e.g., polar and hydrophobic) is not desirable, but in some instances dual properties of a stationary phase can be used to the advantage of the separation.

TABLE 1.2.1 Separation principle and main types of HPLC.

Separation interactions	Types of HPLC
Hydrophobic interactions	**(1)** Reversed phase (RP) **(2)** Ion pair **(3)** HIC **(4)** Nonaqueous reversed phase (NARP)
Difference in polarity	**(5)** HILIC **(6)** Normal phase (NPC) **(7)** Aqueous normal phase (ANP)
Ion interaction	**(8)** Cation exchange **(9)** Anion exchange **(10)** Ion exchange on amphoteric and zwitterionic phases **(11)** Ion exclusion **(12)** Ligand exchange **(13)** Immobilized metal affinity **(14)** Ion moderated
Size exclusion	**(15)** Gel filtration **(16)** Gel permeation
Displacement	**(17)** Displacement
Bioaffinity	**(18)** Bioaffinity (not always HPLC)
Chiral	**(19)** Chiral stationary phase **(20)** Chiral mobile phase
Various principles together	**(21)** Multimode

Relation between the type of HPLC, equilibrium type, and molecular interactions

The identification of equilibrium type (e.g., partition, adsorption, ionic, size exclusion) for each type of HPLC is not a straightforward subject [34]. It is possible that more than one such equilibrium takes place in a specific HPLC type, and in some cases it is difficult to decide based on experimental data which equilibrium type is involved in the separation. Also, more than one type of interactions is present in most HPLC separations. However, an association between different types of HPLC and different separation interactions can be noticed. The relation between the HPLC main groups and the equilibrium type or molecular interaction types should not be viewed as rigid. The main separation principles and the corresponding HPLC types are summarized in Table 1.2.1.

1.3 Flow of a typical HPLC analysis

General aspects

The practical steps for an HPLC analysis are conducted based on a number of decisions

regarding sample collection (procedure, quantity, number of replicates), sample preparation (choice of cleanup, concentration, and/or derivatization), type of HPLC as well as type of required type of information (qualitative or quantitative measurements). The analytical chromatography can be considered the core of the process, and it includes the identification and measurement of the analytes. The choice of HPLC as the analytical step is done for numerous types of samples. This includes small molecules with medium and low volatility, as well as larger molecules including a wide range of synthetic and biopolymers. HPLC has the capability of separating complex mixtures and performs accurate quantitation with extreme sensitivity.

Selection of the type of HPLC for a particular application

An important part of the information step in chromatographic analysis is the choice of the type of HPLC that should be used. This is done based on the nature of sample, instruments availability, as well as other factors such as cost and time of analysis. Once the HPLC technique is selected as the core analytical procedure, further decisions should be made regarding the type of HPLC. The selection of an HPLC type for the analysis of a particular set of samples is not always simple. However, there are some general rules that may be used as guidance. This choice is determined primarily by the nature of the sample with its analytes and matrix. Reversed-phase chromatography, for example, is commonly used for a wide range of compounds, including various organic molecules that have some hydrophobic moiety. More polar molecules are typically analyzed using HILIC, ion pair chromatography or in some instances even RP-HPLC can still be used for the separation. Ions (inorganic or small organic) are typically analyzed by IC. The separations of large molecules based on their molecular weight (in fact hydrodynamic volume) are performed by size exclusion. Bioaffinity chromatography is widely utilized for the separation of biological macromolecules. Details regarding selection of a type of HPLC are given in Section 18.1.

The purpose of analysis is another determining factor in the selection of HPLC type. In this choice, it is important to know if the analysis is performed for the separation by molecular weight, for specific identification and quantitation of components, or for separation and quantitation of enantiomers. Other factors also influence the choice of HPLC, such as availability of equipment, requirements regarding analysis time, the number of samples to be analyzed, availability of specific materials required for the analysis (columns, solvents, etc.), restrictions regarding safety (e.g., the nature and volume of solvents to be disposed), level of training of the operator, etc. In this section, only a general guidance regarding the selection of the HPLC type is provided, and this selection is based solely on the nature of sample.

The selection of a particular type of chromatography for a specific analysis is a complex process, the previous discussion being only a schematic guide which is far from being comprehensive. Numerous sample/analyte details may determine the final choice of a specific chromatographic separation. This book discusses various aspects of separation in analytical HPLC and provides guidance regarding choices of type of HPLC, specific columns, and mobile phases to be used for a successful analysis.

Sample collection and sample preparation for HPLC

Sample collection is a very important step for the success of any chemical analysis. The subject is discussed in various books and papers; however, this subject being outside of the purpose of this book, the reader should refer to the

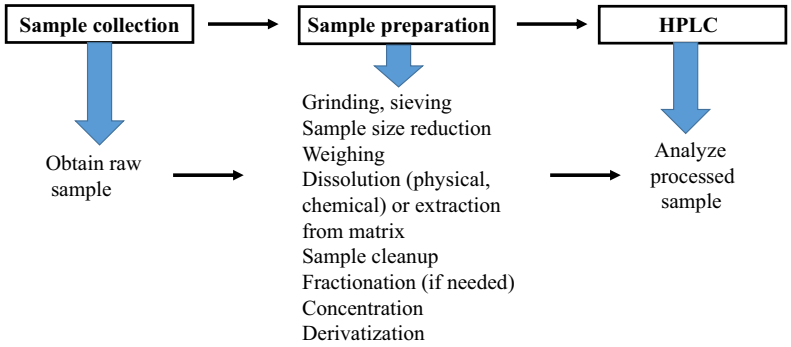

FIGURE 1.3.1 Diagram indicating the place of a sample preparation in chromatographic analysis involving dissolution or extraction from matrix, cleanup, fractionation, concentration, and derivatization.

dedicated literature (e.g., Refs. [1,35]). After sample collection, the analysis proceeds with the sample preparation, in accord with the selected analysis type. Again, numerous procedures are described in the literature for sample preparation [1]. Sample preparation may target the matrix of the sample, the analytes, or both. One common operation in sample preparation is the dissolution of the sample if the sample is solid. Then, the matrix is usually modified during a cleanup, fractionation, and concentration of the sample. The proper processing of the sample may have considerable importance for the success of the HPLC analysis. A sample that contains a "dirty" matrix, having numerous other solutes that can impede the separation or destroy the chromatographic column, must be avoided as much as possible. Also, the sample preparation may have considerable contribution to increasing the analytes concentration. The increase in the concentration of the analytes is very important in particular when traces of specific compounds must be quantitated, as necessary in many practical applications. This concentration can be done by a variety of procedures such as solid phase extraction (SPE), liquid−liquid extraction, etc. [1]. The analytes can also be modified by chemical reactions (derivatization, etc.) in order to obtain better properties for the chromatographic analysis. The process of sample preparation and its place in chromatographic analysis is schematically shown in Fig. 1.3.1.

Sample preparation is usually described for each HPLC analytical procedure when applied for a practical analysis and covers a large part of published literature on HPLC. Several books describing the general principles and various aspects of sample preparation for chromatography also have been published (e.g., Refs. [36,37]).

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CHAPTER

2

Overview of HPLC instrumentation and its use

2.1 Description of main components of HPLC instrumentation

General comments

The HPLC analytical instrument has the role to physically separate totally or partially the components of a sample in the form of a solution, and generate an electrical signal recorded as a chromatogram for the separated components. For this purpose, the instruments allow the injection of a measured small volume of sample in a liquid mobile phase. This mobile phase flows through a chromatographic column where the separation takes place, and further through a detector (or detectors) capable of generating an electrical signal. The signal is most frequently used for quantitative measurements of analytes, although special detectors may provide also qualitative information. Modern HPLC systems are controlled using complex computer software. This process can be achieved using a large number of models of HPLC systems. The construction of an HPLC instrument may vary from one manufacturer to another and depends on the intended function and size of the HPLC process. Also, modern HPLC instrumentation can be rather sophisticated, and the instruments are in continuous development. The description of HPLC instrumentation is not the main goal of this book, and more information on the subject can be obtained from various sources such as instrument manuals, dedicated books (e.g., Refs. [1–8]), peer reviewed papers, or from information from the internet.

Description of a typical HPLC instrument

The basic configuration of an HPLC instrument includes the following modules: (1) a solvent supply system (solvent containers and degasser for generating the mobile phase), (2) a high pressure pumping system, (3) an injector, frequently included in an autosampler, (4) a thermostatted column holder, (5) a chromatographic column (possibly with a guard column or precolumn), (6) one or more detectors, and (7) software for the control of instrument parameters, data acquisition, and processing. Depending on the purpose of HPLC, other instrument capabilities can be included, such as a cooling system for the autosampler/injector, a fraction collector, column switching for back-flow or multi column use, etc. The diagram of a simple type of construction for an HPLC is shown in Fig. 2.1.1 [8].

Some details on each of the components of an HPLC system are further discussed in this section.

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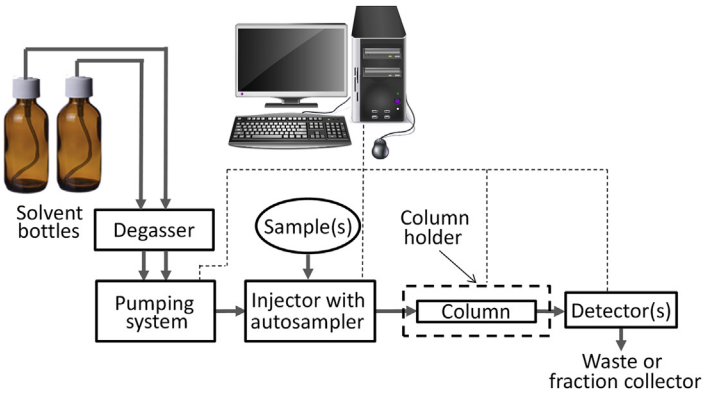

FIGURE 2.1.1 Schematic diagram of a simple configuration for an HPLC system.

Solvent supply system

The role of solvent supply module is to provide the mobile phase necessary for the HPLC separation. Usually, the solvent is contained in (inert) glass bottles and a first step in using this solvent is the degassing. The degassing can be performed by two procedures, a preliminary degassing and an in-line degassing. The preliminary degassing is optional and is done before the mobile phase inclusion in the HPLC flow. The preliminary degassing is done by sparging the solvents with an inert gas (N_2, He) at a low flow rate, or by ultrasonication, when the content of the dissolved O_2 in solvents is reduced.

The second type of degassing in an HPLC system is performed in an in-line degasser device. The principle of this device is to pass the mobile phase through a piece of special polymeric tubing placed in a vacuum chamber. The tubing material (membrane) has selective permeability to gasses, and the (mild) vacuum created by a small pump reduces the content of the gasses from the solvent (e.g., Ref. [9]). The degassers, although popular in HPLC equipment, also may pose problems in specific applications. The polymeric tubing may absorb selectively specific components from the solvents and may be a source of contamination when changing from one solvent to another. Also, it should be noted that large bubbles coming from the solvent reservoir cannot be eliminated by the degasser apparatus, and these bubbles make their way into the pumps affecting their function. The degasser can be a separate unit or can be imbedded in the pumping system.

The degassing is necessary because even very pure solvents may have dissolved in them small quantities of oxygen. This oxygen may be released in the form of very small bubbles in the HPLC system when a drop in pressure occurs (e.g., between the chromatographic column and the detector), or when a solvent with high solubility for oxygen (e.g., water) is mixed with another solvent with low solubility for this gas. When this mixing is done before the high pressure pump, the gas bubbles may lead to pressure fluctuations of the liquid delivered by the pump. The pressure fluctuation should be low and in modern HPLC systems it is typically below 0.1% of the nominal pressure. When the gasses are not removed from the solvents, even if the pumps are working properly, pressure fluctuations of 4%–6% of the nominal pressure can be noticed. Dissolved gasses in the mobile phase may also influence the injection volume when small sample volumes (e.g., 1–2 μL) are injected. Also, the reading of the detectors can be perturbed by dissolved gasses. For example, oxygen may affect the reading of electrochemical detectors, the fluorescence intensity of certain

compounds, and the UV absorption at very low wavelength range.

During degassing, in particular for premixed solvents, attention must be paid to avoid changes of solvent composition due to preferential evaporation of the volatile component. For example, when ammonia or volatile acids are used as additive in mobile phase to adjust the pH or to facilitate a specific detection, sparging is not recommended since drastic changes in the pH may occur in time by the volatilization of these components from the solution. Even with common solvent mixtures, some composition changes may occur during sparging. For example, degassing with He for premixed mobile phase consisting in 50% water and 50% organic solvent (v/v) leads to a loss of 0.005% for methanol, 0.006% for acetonitrile, or 0.05% for tetrahydrofuran [10].

The transfer of the solvent(s) from the solvent containers (bottles) to the pumping system is done through low pressure tubing. The tubes used for passing the mobile phase through the system need to fulfill mainly the requirement of being inert to the utilized solvents and to stand low pressures up to about 50 psi (1 psi = 6.89476 kPa = 6.89476 10^{-2} bar = 6.80460 10^{-2} atm; 1 bar = 14.5037738 psi). Fluorocarbon polymers such as Teflon are common materials used for this type of tubing, but polypropylene is also used. The solvent supply system of an HPLC has one or more reservoirs for the solvents used as mobile phase. For HPLC performed in isocratic conditions and using a pure or a premixed solvent, only one reservoir is necessary. However, it is common in HPLC to use gradient separations, or to use an isocratic separation but to generate a mixture of solvents using the pumps. In this case, two (or more) solvents that are mixed with the pumping system in variable proportions are required. The reservoirs must be clean and inert to the solvents they contain. The solvents from the reservoirs must be free of particles, and they are either purchased as HPLC grade or/and filtered through 0.45 μm filters before use. The filter selected for the filtration must be inert to the solvent. In the case of solvent mixtures containing a buffer, the general rule indicates that the buffer solution is made in water, then preferably filtered, and only after that mixed with the organic solvent (assuming correct concentrations and no precipitation after the organic solvent addition, due to lower solubility of the buffer in the mixture of solvents). The tubing transferring the liquid to the pump(s) typically has a frit at its mouth.

A special type of solvent delivery system can be used in ion chromatography. This system is known as eluent generator (Thermo Scientific/Dionex).

Pumping systems

The main pumping system consists of pump(s) able to deliver a constant flow of solvent through the injector, chromatographic column, and through the detector(s). The pumps must be able to generate a high pressure, which is needed mainly to overcome the resistance to flow of the chromatographic column. This flow is characterized by the *volumetric flow rate U*. In conventional HPLC systems, the pumps are usually capable of delivering U between 0.1 mL/min and 5 mL/min (or 10 mL/min for some instruments) and generate up to 6000 psi (about 400 bar). New developments in using very fine particles in the chromatographic column require higher pressure and sometimes capability to produce flows at less than 0.1 mL/min. These instruments indicated as UPLC or U-HPLC can generate up to 19,000 psi (about 1300 bar) at 1.0 mL/min [11] (lower maximum pressures are allowed at higher flow rates). For the HPLC systems used for other purposes than analytical, pumping parameters can vary significantly. The flow from the pumps (volumetric flow rate U) must be constant, without fluctuations or only with very small ones. This requirement is necessary mainly for the detectors, where the signal may fluctuate when the flow rate varies.

Most high pressure pumps used in analytical HPLC are reciprocating pumps. A single piston reciprocating pump consists of a cylinder with a reciprocating plunger in it, and two valves mounted in the head of the cylinder. The liquid enters the cylinder through an inlet (suction) valve and is pushed through a discharge valve. During the suction, the plunger retracts and the inlet valve opens causing the admission of fluid into the cylinder, while the discharge valve is closed. In the forward stroke, the plunger pushes the liquid out through the discharge valve, while the inlet valve is closed. However, the fluid flow from a single piston reciprocating pump (and therefore the pressure in the system) has a pulsating profile. When the piston is moved by a circular motion of a driving cam, the flow rate has a half sinusoid shape as shown in Fig. 2.1.2.

This type of flow is not suitable for HPLC. Dual piston pumps consisting of two reciprocating pumps that alternate the forward stoke are able to generate flow with only one zero flow point per cycle. However, with this setup, the flow is still fluctuating. The use of specially shaped driving cams or of stepper-driven motors allows the generation of an almost continuous flow of liquid. The dual piston pumps may be connected in parallel or in series. An accumulator-piston design with the pumps in series is shown schematically in Fig. 2.1.3. This type of system also requires two pistons but only three valves in order to achieve the task of generating a continuous flow.

Modern systems are able to deliver flow with a precision of about 0.07% relative standard deviation (RSD%) and a flow accuracy of less than 1% from the nominal value. Because the pumps must deliver flow at high or very high pressure, their construction requires special materials such as inert steel body, sapphire or ceramic pistons, high precision valves that do not have any leaks, special polymeric seals, etc. For ion chromatography, the whole pumping system is typically made of strong polymeric materials such as polyetheretherketone (PEEK).

In addition to the pumps and valves specially designed, the flow without fluctuations from the pumping system can be achieved using a

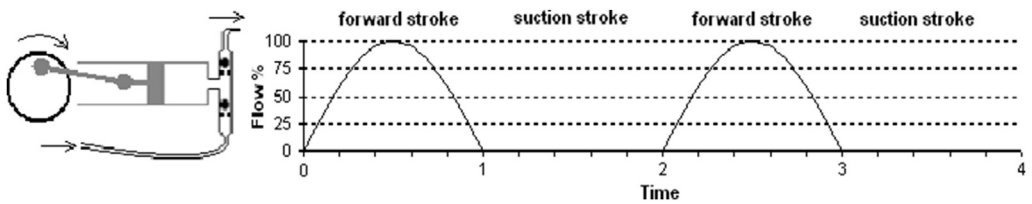

FIGURE 2.1.2 Flow from a single piston reciprocating pump when the piston is moved by a circular motion of a driving cam.

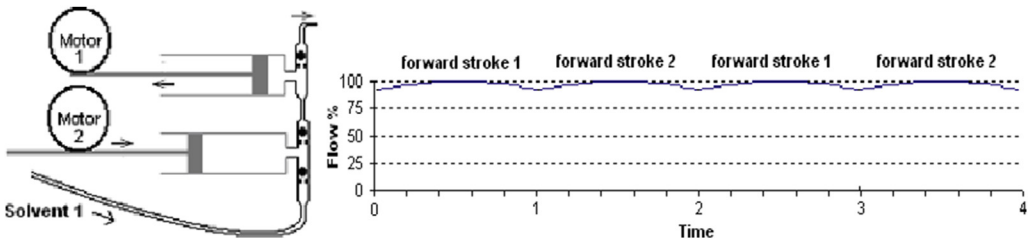

FIGURE 2.1.3 Flow from a dual accumulator-piston reciprocating pump when the pistons are moved by stepper-driven motors that allows low pulsation.

pressure pulsation damper. Pressure dampening is done, for example, by passing the fluid through a cell with a diaphragm wall that compensates the pressure variation. The pulsation with dampening can be reduced to less than 2% variation in pressure. Various models of dampers are available, and most of them have a volume of around 500 μL, in order to assure a small delay volume in the delivered fluid. A typical delay volume for an analytical HPLC system is around 700 μL. The pumping equipment of modern HPLC frequently has the capability to detect any leakage, communicating to the controlling computer of the HPLC to stop the operation when a leak is detected.

A dual piston pump can handle only one solvent and can be applied for isocratic separations that use a pure or a premixed solvent. However, since in HPLC it is frequently necessary to use gradient separation, instruments that handle more than one solvent were developed. This type of instrument is also used to generate a solvent mixture of a desired composition, even when this composition is not changed during the separation (isocratic conditions). There are three basic procedures to physically achieve the mixing of solvents: (1) low pressure mixing, where the solvents necessary for the gradient are premixed with a low pressure pump connected in front of the high pressure pump, (2) high pressure mixing that uses usually two high pressure pumps with each one dedicated to one solvent and with the mixing the flows in a low volume mixer, and (3) hybrid mixing that uses a high pressure pump with two or three proportioning inlet valves.

In low pressure mixing, two or more (usually four) solvents can be blended at the desired composition by using a low pressure pump with several proportioning inlet valves controlled by a computer. The valves open repeatedly for a short period of time (typically less than one second), the duration of time the valve is opened being proportional with the desired mobile phase composition. The mixed solvents are delivered further to one (dual piston) high pressure pump. Low pressure mixing has the advantage of using a single high pressure pump (that is typically expensive), and has more flexibility in choosing a variety of solvents (in systems with four proportioning valves). However, the changes in the mobile phase composition when using a low pressure mixing system are taking place more gradually than for the other systems (the change in composition is not instantaneous). Also, low pressure mixing may be prone to the formation of small bubbles of gas in the mixed solvent, when the solubility of oxygen, for example, is higher in one solvent than in the mixture. These bubbles may enter the high pressure pump and generate flow fluctuations.

In high pressure mixing, two high pressure (dual piston) pumps are used, and the ratio of solvents in the mobile phase is controlled by the flow rate of the high pressure pumps. The mixing of the two solvents A and B can be achieved either in a specially designed mixer (typically with volume of less than 500 μL) or in a mixing T (with virtually zero volume). High pressure mixing is considered to provide a more precise control of the composition of the mobile phase (with a typical composition precision with less than 0.15% RSD% at 1 mL/min flow), and does not have the problem of formation of bubbles caused by the difference in solubility of oxygen in the mixed solvent compared to that in one of the components. However, the cost of high pressure pumps is a disadvantage for this type of mixing. Most modern HPLC systems with high pressure mixing have two pumps and the capability of switching between two solvent pairs (A1, B1 and A2, B2).

Since instruments with low pressure mixing and instruments with high pressure mixing are both common in laboratories, when transferring an established analytical technique from one instrument to another, attention should be paid to the type of instrument. In both types of chromatographic systems, there is a specific volume

passing the system from the point at which the mobile phase solvents are mixed until they reach the head of the chromatographic column. This volume is known as *dwell volume* V_D. The dwell volume is typically different in low pressure mixing systems (2−4 mL) and in high pressure mixing systems (1−3 mL). Special instruments, such those used in microscale HPLC, may have smaller dwell volumes (less than 300 μL). For certain applications using gradient separations on common HPLC systems, differences can be seen when working with one type of instrument or with the other, although the gradient program is the same. This is in particular caused by the differences in the dwell volume from one system to another.

Hybrid mixing uses a system of two reciprocating high pressure pumps with proportioning inlet (suction) valves. Low pressure mixing systems with four proportioning valves and one dual piston high pressure pump, as well as high pressure mixing systems with two high pressure pumps (each one dual piston), are much more common than hybrid systems.

In order to be able to modify the composition of the mobile phase in gradient HPLC, the solvents that are blended in specified proportions should be perfectly miscible. Particular care must be paid to the solubility differences of certain additives present in one solvent when another solvent is added. For example, it is common in HPLC to use buffer solutions. These solutions can be easily made in water by adding mixtures of salts and acids or salts and bases. When a water solution containing these types of additives is mixed with an organic solvent (such as CH_3OH or CH_3CN), the solubility of the additives in the mixed organic/aqueous solution is drastically diminished. The formation of precipitates following the mixing must be carefully avoided and only buffers at low concentration of salts (typically less than 100 μmol/L) should be used when organic solvents are to be added to the aqueous buffer.

During the separation, the composition of the mobile phase can be kept constant for some intervals of time and modified for other periods of time. The modern instruments are commonly controlled using a computer with a dedicated program that assists in generating a specific gradient using a gradient time table. Based on the gradient time table, the computer controls the pumping system that physically generates the desired mobile phase composition by mixing in the correct proportion of the solvents from the solvent supply system. The gradient starts when the sample is injected. After the gradient ends, the HPLC chromatograph is made ready for the next injection. The total run time of the chromatogram, starting with the moment of injection until the end of the chromatographic run, is sometimes referred to as *total cycle time*. In a gradient separation, the dwell volume V_D of the system creates a dwell time t_D. Because of the dwell time, there is a delay between the change of composition at the point of solvent mixing (set in the time table) and the change in composition at the head of chromatographic column. Therefore, when attempting to modify the retention time of a peak by using a "stronger" solvent, this change should be done in the gradient time table ahead of the peak retention time.

The modification in concentration between two changing points of the gradient can be linear. For a gradient starting at time t_1 with the concentration C_1 of component A and ending at time t_2 with the concentration C_2, at an intermediate point at time t the concentration of component A in a binary system can be obtained using the following expression:

$$C(t) = C_1 + \frac{(t - t_1)}{(t_2 - t_1)}(C_2 - C_1) \qquad (2.1.1)$$

In most HPLC separations, the mobile phase is changed from one content in an organic modifier to another one. The change in the concentration of the organic modifier in a period of time is

indicated as *gradient slope*. For a linear change in concentration, the gradient slope can be defined by the following expression:

$$\Delta = \frac{(C_2 - C_1)}{(t_2 - t_1)} \qquad (2.1.2)$$

Some HPLC pumping systems allow both a linear change in the gradient and a nonlinear modification of the concentration (e.g., Ref. [12]). This change can be achieved using a variation in concentration as a function of time given by the following expression:

$$C(t) = C_1 + \left(\frac{t - t_1}{t_2 - t_1}\right)^n (C_2 - C_1) \qquad (2.1.3)$$

where n is larger than 1 for curves of increase that "holds water," and is between 0 and 1 for "does not hold water" type of curve. A different type of nonlinear curve can be obtained using a variation in concentration with a function given by the following expression:

$$C(t) = C_2 - \left(\frac{t_2 - t}{t_2 - t_1}\right)^n (C_2 - C_1) \qquad (2.1.4)$$

When n is larger than 1, the curve of increase "does not hold water," and when n is between 0 and 1, the curve of increase "holds water." In some chromatographic systems, there are used both types of gradient variation and a "type of curve" can be selected from the software controlling the HPLC instrument. As an example, for the instruments manufactured by Waters (Waters Corp., Milford, USA), Eq. 2.1.4 is used for curves 2 ($n = 8$), 3 ($n = 5$), 4 ($n = 3$), and 5 ($n = 2$), and Eq. 2.1.3 for curves 6 ($n = 1$), 7 ($n = 2$), 8 ($n = 3$), 9 ($n = 5$), and 10 ($n = 8$). These curves are shown in Fig. 2.1.4 for $t_1 = 0, C_1 = 0$, and $t_2 = 5, C_2 = 100$.

Much less frequently than reciprocating pumps, syringe pumps are sometimes used in HPLC. Only the recent developments in UPLC made the syringe pumps more attractive. UPLC requires lower flow rates (of the order of 0.2–0.5 mL/min) and uses less solvent compared to conventional HPLC [13]. In syringe pumps, a cylinder is loaded with the mobile phase that is delivered at a specified flow rate by the movement of a piston. The flow from a syringe high pressure pump can be virtually

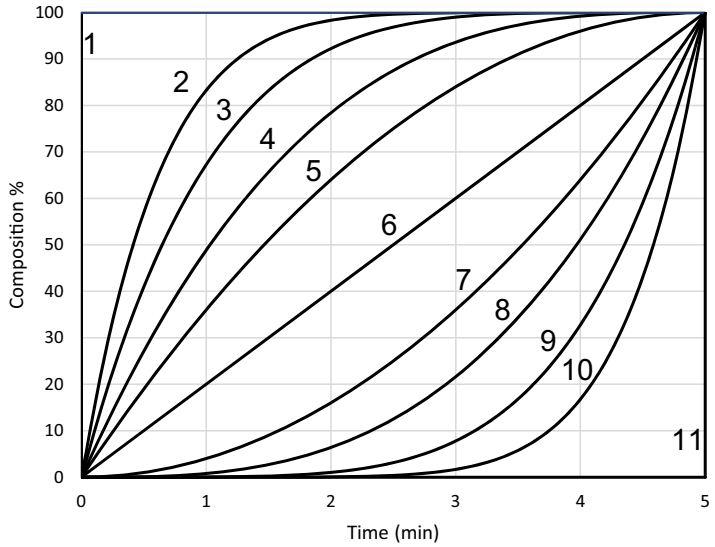

FIGURE 2.1.4 Gradient variation curves using both Eqs. 2.1.3 and 2.1.4 used in Waters instruments.

fluctuation free as compared to that from a reciprocating pump. The price of a syringe pump can also be lower. Pumping system for micro- and nano-HPLC uses specific procedures such as splitters of an initial higher flow [14].

Tubing and connectors

After the high pressure pumps, the tubing utilized for connecting different modules of the HPLC has special requirements [15]. These tubes that have a small internal diameter must be inert and also must withstand the high pressure generated by the pumps. Typical materials for the tubing are stainless steel (316 stainless steel) and PEEK. Stainless steel is inert in most solvents, while PEEK may become very stiff after using solvents such as tetrahydrofuran or dimethylsulfoxide. Also, stainless steel tubing can be used even at very high pressure, while some restrictions to pressure are applied to the PEEK tubing (as function of internal diameter). However, for IC chromatography, PEEK is the material of choice for tubes and connectors. Tubes of several internal diameters (i.d.) are available, such as 0.12 mm i.d. (0.005 in), 0.17 mm i.d. (0.007 in), 0.25 mm i.d. (0.010 in), 0.50 mm i.d (0.020 in), etc. (for both stainless steel tubing and PEEK tubing, a color code is available to designate the i.d.). The choice of the tubing after the injector starts to play a role in the shape of the sample plug. Tubing with 0.12 mm i.d. (0.005 in) is in general recommended to connect the injector with the chromatographic column. This tubing has a volume of about 0.13 μL/cm such that a sample of 5 μL will spread over about 38 cm, diminishing the effects of sample plug shape modifications. The tubing and the void volumes after the chromatographic column also play a role in potential peak broadening.

Another factor contributing to dilution and modifications in sample plug shape are the "void spaces" in fittings that connect the injector and the chromatographic column. Loss of resolution by peak broadening due to large void spaces and also due to turbulent flow either along the tubing or in fittings must be avoided. The fittings typically use a nut that connects to a port and a ferule used to secure the end of the tubing in the fitting port. Common connector parts, correct fitting, and incorrect fitting of tubing with formation of a void volume are shown in Fig. 2.1.5.

Injectors and autosamplers

The role of the injector is to add in the mobile phase a small, precisely measured volume of a solution containing the sample. The injection must be done reproducibly and accurately. Reproducibility of injection is of particular importance, and modern injectors typically show less than 0.5% RSD% in the injected volume. The accuracy errors in injection volume are important mainly when comparing different instruments since for the same instrument the use of standards for quantitation may compensate small variations from a nominal volume. However, for a specific method, it is recommended to keep the same injection volume when injecting different samples in order to avoid accuracy problems. One common type of injector uses a loop of a precise volume which is filled first with the sample and then connected to the flow circuit using a switching valve. This system allows only the injection of a fixed volume of sample, equal with the loop volume. However,

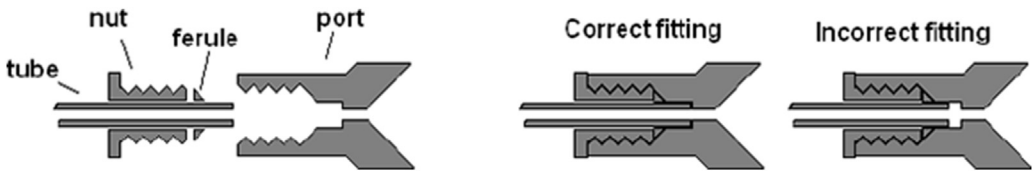

FIGURE 2.1.5 Tubing connection to a port, correct fitting, and incorrect fitting with a void space.

the volume of sample solution to be injected in different analyses may need to be varied. For example, for a very diluted sample or for detection that is not very sensitive, a larger volume of sample may be necessary in order to assure a good measurement.

Samples that generate a high detector signal need to be injected at lower volumes. Conventional HPLC systems have injectors capable to inject between 1 μL up to 100 μL sample solution depending on the instrument model, typical volumes being between 1 and 20 μL. Special systems have the capability to inject larger volume (up to 1000 μL in special systems) or very low volume (e.g., 0.1 μL). Injection of different sample volumes can be achieved using a larger loop that is only partially filled with sample (partial loop injection). The loop is typically filled initially with the mobile phase, and then the sample is introduced in the loop, occupying only a small portion of its volume. The placing of the loop in the mobile phase main circuit is then done in a similar manner as for fixed volume loops. The sample volume is typically controlled using a computer. For UPLC, the injection volumes can be between 20 and 3000 nL, which is smaller than for common analytical HPLC, and for semipreparative or preparative HPLC the volumes can be much larger. These systems may need specially designed injectors.

Injector systems with automation capability are common (computer controlled autosamplers). In autosamplers, the samples are typically placed in a tray containing capped vials with septum (e.g., 2 mL vials) each one containing a solution of the sample. From a large number of samples in different vials (or well plate), these automatic systems have the capability to select the desired sample vial from the tray, and to repeat the injection at a specified time or upon receiving an electrical signal from the computer. For injection, a needle of the injector penetrates the vial septum, draws the desired volume of sample, and loads it in the injector loop. The sample solution is subsequently placed in the mobile phase flow as a solution plug.

Since in modern HPLC systems the autosampler capability allows the injection of a number of samples (e.g., 100, 108, etc.) one after the other, the samples toward the end of the queue may stay in the autosampler for a rather long time. For improving sample stability in time, it is common that HPLC autosamplers also have the capability of cooling. This may also help in reducing evaporation when the sample solvent is volatile and the septum of the sample vial has been already punctured.

The sample is ideally introduced in the mobile phase flow as a zone (plug) with the concentration of the sample following a perfectly rectangular profile. The force pushing the fluid through the tube is given by the cross-section area of the tube multiplied by the pressure drop Δp, or $\pi r_t^2\,\Delta p$ (where r_t is the tube radius). In a laminar flow, the layer adjacent to the wall is held virtually stationary by adhesion, and the inner fluid layers slide over the ones closer to the wall creating a shear force. This force is given by the following expression:

$$F_\eta = \eta A \frac{\mathrm{d}u}{\mathrm{d}r} \tag{2.1.5}$$

where η is the liquid viscosity, u is the fluid linear velocity, $\mathrm{d}r$ is the infinitesimal distance between the layers, and A is the area of contact between the layers of the fluid [16]. The two forces (the force pushing the fluid through the tube and the shear force) being equal in a steady flow, at a distance r from the center of the tube and taking $A = 2\pi rL$ (L is the length of contact between layers), the following relation should be satisfied:

$$\pi r^2 \Delta p = -2\pi rL\eta \frac{\mathrm{d}u}{\mathrm{d}r} \quad or \quad \mathrm{d}u = -\frac{\Delta p}{2L\eta} r\mathrm{d}r \tag{2.1.6}$$

For a tube with radius r_t, by integration, Eq. 2.1.6 gives the fluid velocity at any point at distance r from the tube center:

$$u(r) = \frac{\Delta p}{4L\eta}\left(r_t^2 - r^2\right) \qquad (2.1.7)$$

Eq. 2.1.7 shows that due to the friction with the tubing walls of a viscous fluid, downstream of injector (in a laminar flow), the shape of the sample plug is changing and generates a parabolic profile. This process, as well as the diffusion and other convection effects, contributes to deviations of the chromatographic peak from the ideal Gaussian shape, introducing tailing and asymmetry (see Section 3.1).

Two important parameters must be selected by the operator regarding injection: (1) the nature of the solvent for the sample and (2) injection volume. Besides the obvious requirement that the solvent for the sample should dissolve it completely, this solvent must also be soluble in the mobile phase. The injection volume is selected depending on a number of factors including the type of instrumentation (HPLC, UPLC, detector type, etc.), the sensitivity of the detector, the loading capacity of the column (maximum amount of sample that still allows separation) (see Section 3.4), the effect of sample solvent on peak shape (see Section 7.6), etc. There are problems with both too small volumes and with too large volumes of injection. Too small volumes may lead to problems with injection reproducibility, sensitivity of the detector, or sample loss in the chromatographic column, but offer better peak shape sometimes resulting in better separation. Too large volumes may affect the peak shape (broadened, with flat top, asymmetrical) that affects separation. A large volume of sample does not necessarily mean a large amount of analyte (e.g., when the sample is very diluted), but when the large volume is also associated with too much analyte, this can be associated with an overload of the column (problems with the separation) and of the detector (leading to a nonlinear response). Further discussion on injection volume will be made in connection with the nature of sample solvent in Section 7.6.

In autosamplers, since several samples are injected one after the other, it is possible to see carryover effects. Carryover effects represent the contamination of a sample with small amounts of components from the previous sample that remained in the autosampler after an injection. This problem is typically solved using a needle wash. Some autoinjectors have the capability of mixing the sample with specific reagents from different vials, in case derivatization is necessary before the separation and detection of analytes. Also, cooling and heating capability is frequently present in modern autoinjectors.

Usually, the injection operation is unselective. However, special on-line sample preparation methods require more special injection techniques. In case of a multidimensional HPLC, for example, a fraction from eluted sample from a first column can be transferred by an interface to become an injected sample to a second column. Injection devices normally do not affect retention parameters unless operation problems occur (e.g., Refs. [17–19]). Also, in most HPLC applications, one injection is done for each chromatographic run. However, multiple injections in a single run (MISER) are possible for specific analyses, such that a larger number of samples can be analyzed within a specific interval of time, and with a lower solvent usage [20].

Column holders

The column in modern HPLC systems is usually placed in a column holder that may also play the role of a column heater/cooler. Common column holders have the dimensions capable to accommodate one, two, or even more chromatographic columns. The column is kept in the column holder at a specific temperature that can be set, usually at a value from 5°C (9°F) to 65°C (149°F), different instrument vendors offering column holders capable of achieving a specific temperature range. For some separations, the column may be kept at ambient temperature,

while in some other studies the column temperature is an important parameter to consider. Most column holders are also equipped with a leak detector. Most modern HPLC instruments utilize thermoelectric Peltier technology in order to heat or cool the separation column. Peltier effect is a temperature difference created by applying a voltage between two electrodes connected to a sample of semiconductor material.

For HPLC working at elevated temperature (indicated as high temperature HPLC), the mobile phase needs to be preheated before entering the column in order to achieve maximum efficiency. For such special technique, the column heater temperature can be maintained at a specified temperature up to 200°C [21] (see also special HPLC setups). Others custom-made column holders are available for specific applications [22].

Chromatographic columns

The chromatographic column is designed for performing the separation in HPLC. Its role and properties are related to various subjects discussed in this book, and in the present section is given only a simplified overview of the subject (see Chapter 8). The column typically consists of a tube made from metal (stainless steel) or plastic (e.g., polyetheretherketone, PEEK) that is filled with a stationary phase. At the two ends inside the column are special frits that keep the stationary phase from moving, and outside are fittings that allow the connection with high pressure tubing. The physical dimensions of common analytical chromatographic columns vary, and values for length (internal) L can be between 30 and 250 mm (common length 50, 100, 150, 250 mm), and internal diameters d can be between 1 and 10 mm (common diameters 2.1, 3.0, or 4.6 mm). Other dimensions are possible, particularly when the column is designed for special tasks. The newer columns have the tendency of being shorter and narrower, as the solid particles that form the stationary phase are made smaller. Special cartridges (microfluidic chips) are also available as containers for the stationary phase. Based on the internal diameter of the analytical column, they are sometimes classified in the literature as (1) standard (3.0−4.6 mm i.d.), (2) minibore (2.0−3.0 mm i.d.), (3) microbore (0.5−2.0 mm i.d.), (4) capillary (0.2−0.5 mm i.d.), and (5) nanoscale (0.05−0.2 mm i.d.). Larger columns are used for semipreparative and preparative purposes. Capillary columns represent a special type as they are made from a silica capillary that contains the stationary phase. Capillary columns on a chip were also experimentally made. The empty volume of the column can be easily calculated as the volume of a cylinder $V = (\pi/4)d^2L$, and for analytical columns, V ranges between 0.02 and 20 mL.

The nature of the stationary phase is selected based on the type of chromatography that is utilized for the separation (normal phase, reversed phase, ion exchange, size exclusion, etc.). A large assortment of types of stationary phases (column packings) is available. The stationary phase usually consists of small solid particles with special properties. Besides small particles, porous polymeric materials, and also monolithic materials can be used as stationary phase. When the stationary phase is made from small particles, the particles should have specific physical and chemical characteristics to serve as a stationary phase. The surface area of particles is one of the most important physical characteristics, being directly related to the retention in the column of the compounds to be separated. The effect on separation of the particle size is also important. Particle size influences in particular the *eddy diffusion*, which appears when local small streams of liquid follow different channels in the column. The effect is common within porous particles. This generates a broadening of the chromatographic bands (discussed in Section 3.1), which is not a desired feature. When the stationary phase is made from small porous particles, these particles are frequently obtained from an inert substrate material (such as silica)

that is covered with the active phase. The particles can be of three main types: porous, superficially porous (core-shell), and pellicular. Porous particles are still the most common type of stationary phase used in HPLC. They are made from particles usually of 1.7−5 μm diameter with a specific porous structure (e.g., of silica) and with the surface of this structure covered with an active constituent. This constituent can be physically or chemically bonded on the solid inert support, the bonded phases being the most common type. The column dimension and the diameter of the particles used as stationary phase determine if the separation is considered HPLC or UPLC and the adequate instrumentation is selected. The use of UPLC is necessary for columns with smaller diameter d and with smaller particles with diameter d_p of 1.7 or 1.8 μm. Since reverse-phase (RP-HPLC) is the most utilized technique, the largest variety of columns is of RP type. These columns have a hydrophobic active phase, for example, with octadecyl groups (C18) or with octyl groups (C8) bonded on silica. For other types of chromatography, the stationary phase may be made in various forms. For example, ion exchange HPLC can use particles from a substrate inert material that are covered with the active phase but also various types of ion exchange polymers. Size exclusion chromatography typically uses perfusion particles made from silica or special types of polymers. These particles contain very large pores (400−800 nm) connected with a network of smaller pores (30−100 nm). The structural rigidity of these particles is not as good as that of common porous particles made from silica, and restrictions to the maximum pressure that can be used with the columns made with these particles are typically indicated by manufacturer. At higher pressures than recommended, the stationary phase may "collapse" and the column can be damaged.

The chemical properties of the particles forming the stationary phase include (1) chemical nature of the active surface, (2) chemical stability, (3) surface reactivity, (4) density and distribution of surface reactive centers, etc. The subject of the nature of stationary phases used in various analyses is fully discussed further in this book (see Chapter 8). In some systems, more than one chromatographic column is necessary for achieving the desired separation. In size exclusion chromatography, for example, two to four columns may be connected in series. In other types of separation, the use of more than one column is less common. The nature of the columns used in series may be the same or may be different. More than one column is also used in multidimensional HPLC, where a portion of the initial separation is further submitted for a second separation in a different column (usually selected as "orthogonal").

Some chromatographic columns require a specific temperature for performing a good separation, and for this purpose special column holders are used. Common column holders have the capability to control the column temperature in a range from about 10°C below ambient to 60−70°C. Higher temperatures can be achieved with special ovens used in high temperature HPLC. In addition to heating the column, the column holders typically are able to heat the solvent entering the column, since peak shape distortions may be noticed when the column and the entering solvent have different temperatures.

In order to protect the stationary phase from the analytical HPLC column, it is common to use small pore frits (e.g., with 0.45 μm pores) as well as guard columns (cartridges) in the path of the mobile phase before the column. The frits are usually made from stainless steel or titanium and filter the mobile phase. When the mobile phase is acidic, the metallic surface of the frit can adsorb acidic solutes which influence their recovery or lead to unexpected peak tailing. The covering of metal frit surface with hybrid organic/inorganic materials mitigates analyte-to-metal interactions and improves the recovery of acidic analytes [23]. For column protection, more important than frits are the guard columns. The guard (cartridge) columns are selected to

match the stationary phase of the analytical column (same active material), but their length is much shorter (e.g., a few mm), and in some cases their stationary phase has larger particle size. In the analyzed samples, there are sometimes components that are very strongly retained by a specific stationary phase. These components do not elute and tend to accumulate at the head of the column, deteriorating its performance in time. The use of a guard column allows selective retention of these components without affecting the efficiency of the column, retention time, backpressure, or the level of analytes. Guard columns are changed from time to time, while the analytical columns have longer service life (see Section 8.3).

General comments regarding detectors

In HPLC, the mobile phase and the eluted analytes pass through a detector capable of performing measurements. These are based on the fact that the molecules of the sample have physicochemical properties different from that of the mobile phase. The measured properties are determined by the nature of the compound to be analyzed and that of the mobile phase. The choice of a specific property for detection depends on factors such as the extent of difference between that of the analytes and of the mobile phase, sensitivity of the detector to the specific property, availability of the detector, etc. The selection of a specific detector is also correlated to the separation conditions used for the analysis. Some detectors have specific requirements for the nature of mobile phase, selection of isocratic or gradient separation, selection of a specific temperature for the column and mobile phase, etc. [24].

The detector response (output) is typically dependent on the instantaneous concentration (or amount) of the detected species, and for this reason the quantitation is based on the area of the chromatographic peak (see Section 3.4). The output appears as an electrical signal recorded in the form of a chromatogram. In addition to the signal, all detectors are affected by the "noise," which is the random oscillation of the detector (electric) output. Other disturbances in the detector signal may include long-term noise and drift. The long-term noise appears as a fluctuation of the signal with a wider frequency. The drift is a continuous variation (increase or decrease) of the signal for a period of time comparable with the length of the chromatogram. Drift may be caused not by the detector itself, but by changes, for example, in the composition of the mobile phase during gradient elution.

The qualities of the detectors should include the following: (1) capability to provide quantitative or both qualitative and quantitative information, (2) good selectivity, (3) good sensitivity, (4) reproducible response, (5) large dynamic range, (6) linearity in a wide range of concentrations of sample, (7) low baseline noise and no baseline drift, (8) capability to make detection in a small volume of sample, (9) capability to not contribute to peak broadening, (10) stability to changes in flow and environmental parameters, and (11) high frequency of data collection.

Some detectors have the capability to generate only quantitative information, while others offer both qualitative and quantitative information, such as the MS detectors. Although the response of the detectors used in HPLC is typically not diagnostic for the chemical nature of the analyte, depending on the analyte and sample characteristics, the identification of compounds is possible using liquid chromatography−mass spectrometry (LC-MS), liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS), and in some cases even other detectors.

The selectivity of the detector is related to the response to only a specific type of analyte. However, some detectors are designed to respond to all analytes (such as the RI detector, or ELSD) and are indicated as *universal* detectors. When the HPLC separation is good, the universal detectors are very useful since they account for all the components. Selective detectors respond to only a class of compounds or they can be set to detect only specific compounds. The detector selectivity adds one more capability for detecting separately certain analytes, and this can be a

significant advantage when the HPLC separation is not possible (or is incomplete).

Detector sensitivity is a very important factor in HPLC analysis. This sensitivity depends on several factors such as analyte properties, sample matrix, mobile phase properties and also on detector settings (e.g., electronic amplification of the signal), and detector manufacturer. For these reasons, in analytical methods using HPLC, parameters such as limit of detection (LOD) and limit of quantitation (LOQ) are reported (see Section 3.4). They characterize globally the sensitivity of the method and consider a number of factors, including the detector sensitivity. The range of sensitivities of various detectors are indicated in Table 2.1.1.

Depending on the injected volume of the sample, the concentration of the analyte may vary in the injected solution. A typical injection volume of 10 μL would require 100 times higher concentrations per mL compared to the values indicated in Table 2.1.1.

Reproducibility of the response to the same injected sample is an important characteristic of any detector. Reproducibility of a detector is mainly related to the quality of detector construction. Since HPLC is mainly used for quantitative analysis, the reproducibility of the detector response is a very important parameter.

The dynamic range of a detector is related to the range where the detector has a response dependent on the analyte amount or concentration. The detector may not have a linear response for the entire dynamic range, but even in the range where the response is not linear, a positive dependence should exist between the signal versus the amount of analyte. The dynamic range of a detector can be independent of the nature of the analyte (for universal detectors), but also can be highly dependent on the analyte nature. The dynamic ranges of several types of detectors are given in Table 2.1.1.

Detector linearity indicates the range in which the dependence of the detector signal depends linearly on the amount or of concentration of the analyte. Similar to the detector dynamic range, the linear range of a detector can be either independent of the nature of the analyte or highly dependent on this nature. The typical linear ranges for various detector types are given in Table 2.1.1.

The low baseline noise and no baseline drift are properties typically related to the quality of the electronic parts of detector, or the quality of other detector components (e.g., life time of the UV lamp, dirty flow cell) or due to the air bubbles trapped inside the flow cell.

The capability to make detection in a small volume of sample is usually related to the

TABLE 2.1.1 Sensitivity (in amount) for various types of HPLC detectors and their typical dynamic and linear ranges.

Type of detector	Minimum mass injected (typical)	Minimum mass injected (extreme)	Dynamic range of concentrations	Linear range concentrations
UV-Vis spectrometry	0.1–1 ng	0.01 ng	10^{+5}	10^{+4}
Fluorescence spectrometry	1–10 pg	10 fg	10^{+4}	10^{+3}
Refractive index	100 ng–1 μg	10 ng	10^{+4}	10^{+3}
Electrochemical amperometric	0.1–1 ng	100 fg	10^{+4}	10^{+3}
Electrochemical conductometric	0.5–1 ng	0.5 ng	10^{+4}	10^{+3}
Mass spectrometry	1 pg–1 ng	10 fg	10^{+4}	10^{+3}
Evaporative light scattering	5 μg	0.5 μg	10^{+3}	10^{+2}
FT-IR spectrometry	1 μg	0.5 μg	10^{+3}	10^{+3}

volume of a flow-through cell or other parts in the instrument construction. For UV-Vis detectors, for example, instrument manufacturers may offer cells of different dimensions, with longer path of the beam of light in the flow-through cell for achieving higher sensitivity of the instrument, but with slightly larger cell volume, or cells with shorter path for the light beam, but also with smaller volumes. Microcells are also available for certain instruments that are designated for micro-HPLC systems. Special cells are also necessary when using capillary HPLC columns and low flow rates.

The capability to not contribute to peak broadening is also related the HPLC construction. The absence of dead volumes, small flow-through cells, laminar flow within the detector, and adjustment of the total volume of mobile phase in the detector in accordance with the range of injection volume for the sample are some of the steps toward achieving little or no contribution to peak broadening [25].

The stability of the detector response to changes in flow of mobile phase may be an intrinsic characteristic. For some detectors such as UV-Vis, the response is more stable to changes in flow rate since this detector responds to instantaneous analyte concentration. For other detectors, the stability cannot be achieved without a constant flow since they are responsive to the amount of analyte reaching the detector and not to the instantaneous analyte concentration. For example, for MS detection, the response is dependent on the amount of analyte in the source and therefore is dependent on the flow rate. In addition, the dependence of response on flow rate in MS is not linear, and higher flows beyond an optimal point may be in some cases detrimental to sensitivity. Other detection techniques, such as RI are also flow dependent. In RI, the dependence is related to baseline stability. The stability of a detector to changes in flow must be known before modifying the flow rate.

Most modern detectors used in HPLC do not generate a continuous signal based on measured physico-chemical property. The signal generated by the detector is typically obtained at short time intervals, and a measurement frequency (sometimes indicated as sampling frequency) is characteristic for each detector. It is possible in some instruments that such frequency can be set by the operator, but in other detectors this frequency is fixed. It is important for the measuring frequency of the signal to be high enough for obtaining an accurate representation of chromatographic peak. A sampling frequency of about five points per second could be sufficient for peaks with 4–5 s width (or larger). However, for narrow peaks, the sampling frequency must be higher, such as 20 or 50 Hz. The measurement frequency must be high in particular for narrow chromatographic peaks. The curve representing the peak shape is generated by connecting each measurement point, and sparse points do not account properly for this shape and introduce errors, in particular regarding peak areas.

It is common in modern HPLC systems that they have more than one detector available. For example, UV-Vis and fluorescence detectors are frequently coupled in series, although not necessarily used simultaneously. Since the flow through a detector may pose some backpressure, when using detectors coupled in series, it should be verified that the flow-cell of the detector up-stream can handle the backpressure generated by the second detector. For the detector down-stream, it should be verified that undesirable peak broadening does not occur because of the up-stream detector. The coupling of detectors in parallel is also possible, but care must be taken to assure that appropriate flow goes through all the detectors. Since different detectors may pose different backpressures to the flow, the risk exists that most (or even all) of the flow goes to a single detector. A short discussion about the main detection techniques in HPLC is further presented.

UV-Vis spectrometric detectors

This type of detectors are basically in-line UV-Vis spectrometers equipped with a flow through cell. They can be classified as fixed wavelength, variable wavelength, and photodiode array UV-Vis detectors. In these instruments, a beam of monochromatic light (more correctly a beam of light with a narrow wavelength range) is sent through the eluent flowing through a cell with two quartz windows or lenses of small volume (e.g., 1 μL for a microcell, 5 μL for a semi-micro, and 14 μL for a standard cell, with path length of 5–10 mm depending on the cell, some cells having a conical shape). The typical source of light in a UV-Vis detector is a low-pressure arc discharge deuterium lamp with light energy in the range 190–600 nm. Some instruments may have an additional tungsten filament lamp for extending the visible range. The monochromator consisting in a movable diffraction grating (or prism) for light dispersion that can be rotated allows the selection of light of a set-up wavelength through an exit slit. The monochromatic beam is split (using a beam splitter), one part going through the cell with the sample and to the detector, and the other to a reference detector. The baseline is obtained from the reading for the eluate when no solute is emerging from the column [26].

Two related quantities, transmittance T and absorbance A, are measurable for the light passing through the solution. Transmittance is defined as follows:

$$T = I_I/I_0 \tag{2.1.8}$$

In Eq. 2.1.8, I_o is the intensity of the radiant energy incoming to the sample and I_1 is the intensity of the emerging light (T can also be expressed as percent). As expected, T is a function of the wavelength λ (or of frequency $\nu = c_{light}/\lambda$, where c_{light} is the speed of light) of the radiation that is absorbed. Absorbance A_λ is defined by the logarithm in base 10 of the inverse of transmittance as follows:

$$A_\lambda = \log\left(\frac{1}{T}\right) = \log\left(\frac{I_0}{I_I}\right) \tag{2.1.9}$$

Absorbance is related to the molar concentration C_X (also noted interchangeably as $[X]$) of the absorbing species X by Lambert–Beer law:

$$A_\lambda = \varepsilon_\lambda [X] L \tag{2.1.10}$$

where ε_λ is the molar absorption (absorbance) coefficient at the specific wavelength λ and L is the path length of the light through the sample. For quantitation purposes, the absorbance is commonly used, because it is proportional with the concentration. The quantitation can be done using calibration curves or standard addition method [27]. The absorbance of the liquid eluting from the separation system is typically measured at specific (small) time intervals (not continuously), generating points that form the chromatogram. The peaks in the chromatogram indicate an increase in the absorbance A_λ when an absorbing species elutes from the chromatographic column. For variable wavelength detectors, the wavelength can be selected (by rotating the grating) and is usually set where a strong absorption of light by the analyte occurs (possibly at the maximum). This type of detector is one of the most commonly used in HPLC. Older detectors were made to measure the absorbance at a fixed wavelength such as 254 nm. This wavelength corresponds to the maximum emission (253.7 nm) of a mercury lamp that has been used as a UV source in simpler spectrophotometers. The area under the chromatographic peak of the analyte being proportional with the analyte amount injected into the column is used for quantitation with the help of a (linear) calibration curve or a response factor between peak area and the analyte concentration. The peak height can also be used for quantitation

assuming equal peak widths for all concentrations. Some UV-Vis detectors have the capability to capture the absorbance for a whole spectrum range (UV-Vis diode array detectors or DAD). If the entire UV-Vis spectrum is measured in different points across a chromatographic peak, this can be used for the evaluation of peak purity and also can be a guide for qualitative identification of the analyte, although UV-Vis spectra are very seldom sufficient for compound identification. The UV region of spectrum starts at about 190 nm, and modern UV-Vis instruments have a working range between 190 and 600 nm. However, the common range of practical utility in UV spectrophotometric measurements starts at about 205 nm or higher. At lower values than this wavelength, a strong light absorption usually takes place because the solvents used as mobile phase start absorbing. The wavelength cut-off of various solvents can be found in tables (e.g., Ref. [28]). Depending on the nature of the analyzed material, the detection limit of the UV-Vis detection in HPLC can be 0.1−1.0 ng, with a linear range of five orders of magnitude. With an appropriate solvent that does not absorb in the range of UV-Vis measurement, the use of elution gradient can be applied for separation.

Fluorescence and chemiluminescence detectors

Fluorescence (FL) is the process of emission of light by a molecule after absorbing an initial radiation (excitation light). A molecule M goes from a lower energetic state (commonly ground state) to an excited state M^* by absorbing energy. The emission process may take place by the molecule bouncing back to the initial state without the change in the wavelength of the absorbed light. In this case, the process is difficult to use for analytical applications. However, it is possible that part of the energy of the excited molecule M^* is lost by nonradiative processes such as collisions with other molecules. In this case, the electron may go to another excited electronic state with lower energy and then, emitting a photon, reaches the ground state. It is also possible that no intermediate electronic state is present, but the molecule acquires a lower vibrational energetic level and jumps to the ground state by emitting a photon of lower energy than the absorbed one. In both these cases, the fluorescence radiation has a lower frequency than the excitation radiation.

Fluorescence by emission of radiation at higher frequency than the absorbed one is also possible (anti-Stokes radiation), but it is uncommon. The average lifetime of an excited state of a molecule M^* undisturbed by collisions is about 10^{-8} s, and fluorescence can take place within this length of time. For some special compounds, the molecules can remain for a longer time in a metastable excited state. In this case fluorescence can be observed long after the initial radiation is interrupted. This type of fluorescence is commonly called phosphorescence. Fluorescence is less frequently observed than expected because it is very common that only nonradiative loss of energy takes place. The theory of fluorescence emission shows that the intensity of fluorescence F_{int} at the emission wavelength λ_2 can be expressed as a function of the intensity I_0 of the excitation radiant energy with wavelength λ_1 incoming into the sample, by the following expression:

$$F_{int,\lambda_2} = I_{0,\lambda_1}\left[1 - \exp\left(-\varepsilon_{\lambda_2}[X]L\right)\right]\Phi \qquad (2.1.11)$$

In Eq. 2.1.11, Φ is the (quantum) fluorescence yield of the process, the other parameters being the same as defined for UV-Vis. For low concentrations, $1-\exp(-\varepsilon_{\lambda 2}[X]L) \approx \varepsilon_{\lambda 2}[X]L$, and the intensity of fluorescence $F_{int,\lambda 2}$ is related to the concentration $[X]$ by the approximation relation:

$$F_{int,\lambda_2} = I_{0,\lambda_1}\varepsilon_{\lambda_2}[X]L\Phi \qquad (2.1.12)$$

In reality, only a part of emitted fluorescence is measured in the analytical instrument and the intensity of this measured fluorescence $F'_{int,\lambda 2}$ is given by the following expression:

$$F'_{int,\lambda_2} = a\, I_{0,\lambda_1}[X] \qquad (2.1.13)$$

where a is a constant coefficient that incorporates all the constants including the losses due to partial fluorescence measurement. Measurement of fluorescence intensity (usually at the maximum of the emission band) is the base of quantitation of the fluorescent species. In practice, the fluorescence intensity is measured using sensitive light detectors (FLD) that generate an electrical signal of intensity depending on a calibration constant for the instrument. The output is given in luminescence (or light) units (LU) that are arbitrary units proportional with the fluorescence intensity, but specific for the measuring instrument [29].

Similar to UV-Vis detection in HPLC, the fluorescence is measured in a flow-through cell that is connected to the flow of the eluent incoming from the chromatographic column. Modern fluorescence detectors may have the capability of recording a tridimensional emission spectrum of the analyte by stopping the mobile phase flow at a chosen retention time (selected for the analyte) and performing a scan of the entire UV range used for excitation and for the entire emission band. In this way, the tridimensional fluorescence spectrum is displayed as a dependence of fluorescence intensity on emission wavelength and excitation wavelength. It is common in modern fluorescence HPLC detectors that the excitation beam is generated by a high power lamp that flashes at a specific number of times per second (e.g., 296 times in an Agilent 1200 Ser. detector), such that the signal is modulated in time. Also, the systems typically have a reference detector that measures the excitation light and corrects the flash lamp fluctuations. The detection in fluorescence methods encounters several difficulties because of nonlinearity of fluorescence due to self-absorption effects, difficulty in discriminating between overlapping broad spectra of interfering molecules, quenching produced by oxygen dissolved with the solvent, etc. Because the intensity of fluorescence increases linearly with the intensity of the initial radiation, laser-induced fluorescence (LIF) detection is a successful technique applied in HPLC. For HPLC, lasers are a convenient excitation source because they have intense light focused into a small volume, they are highly monochromatic, and the associated Raman light has a well-defined wavelength that can be avoided with the monochromator used for observing fluorescence. However, LIF is still affected by background interference commonly arising from the Raman effect in the blank (molecular scattering) or from low level of solid impurities in the solvent producing Rayleigh light scattering. The use of fluorescence detection (FLD) in HPLC is common. Detectors with constant excitation wavelength and variable absorption or with variable wavelength excitation and absorption are commercially available. Depending on the nature of the analyzed material, the detection limit using FLD can be as sensitive as 10^{-2}–10^{-3} ng/mL, with a linear range of four orders of magnitude. When appropriately selected, the use of elution gradient can be applied for separation without interfering with the fluorescence. Different factors related to the mobile phase influence fluorescence such as pH, solvent nature, temperature (as much as 2%), presence of impurities, as well as the flow rate.

Chemiluminescence (CL), another technique used for HPLC detection, is the emission of light as a result of a chemical reaction. Certain compounds achieve excited energy states in specific chemical reactions and emit light following a transition to ground state. The wavelength of the light emitted by a molecule in chemiluminescence is the same as in its fluorescence, the energy levels of the molecules being the same. The difference comes from a different excitation process. If the energy of the chemical reaction is lower than required for attaining the excited state, the chemiluminescence does not occur. Also, the deactivation of the excited molecule

by nonradiative processes such as collisions with other molecules takes place for chemiluminescence similarly to fluorescence. The chemiluminescence intensity follows the same law as fluorescence with the difference that quantum yield Φ from fluorescence must be replaced with a different quantum yield Φ_{CL}, which is defined as the proportion of analyte molecules that emit a photon during chemiluminescence. The Φ_{CL} increases with the efficiency of the chemical reaction producing the excitation (such as the oxidation process). Higher energies required by molecules to achieve the excited state diminish Φ_{CL}. In analytical uses of chemiluminescence, one more factor that must be taken into account is the time frame of the light emission. Certain chemiluminescent systems, although with very good Φ_{CL}, may emit the light for a period of 40–50 min. Much shorter times can be achieved using a catalyst. Because no excitation light is needed in chemiluminescence, the interfering light from Raman effect or light scattering by trace particles is nonexistent. In addition, the development of detectors virtually able to detect single photons makes the technique highly sensitive. Concentrations as low as a few hundred amol/mL of material were detected using chemiluminescence for certain analytes [30]. However, the luminescent molecules are not very common and usually they are obtained by postcolumn derivatization with proper reagents [31].

Refractive index detectors

Refractive index detection (RI or RID) is another common technique in HPLC. Due to the modification of the refractive index of a solution as a function of the concentration of the solute, RI can be used for the quantitation of a variety of analytes. This detector is based on the deviation of the direction of a light beam when passing under an angle from one medium to a medium with a different refractive index. This deviation depends on the difference in the refractive index between the two media. The change in the location of the beam on the (photoelectric) detector is made to reduce the detector output proportional to the deviation of the beam from the reference position. This output is electronically modified to provide a signal proportional to the concentration of the solute in the sample cell.

The refractive index depends on the wavelength of the incident beam, and the most accurate RI measurements are done with monochromatic light (usually 589 nm, the sodium D line). With optical corrections, white light can still be used for the measurements. RID is a universal detector that can be utilized without the need for chromophore groups, fluorescence bearing groups, or other specific properties in the molecule of the analyte. In many cases, the sensitivity of RI detection is, however, not as good as that of other types of detection. Also, it is not possible to use elution with gradient for the mobile phase, since this is associated with large variations in the refractive index of the mobile phase. Refractive index is sensitive to temperature changes, and a constant temperature must be maintained during measurements. The response of the detector is given in arbitrary RI units, depending on the detector settings, but proportional with the concentration of the analyte.

Electrochemical detectors

Various types of electrochemical analytical techniques can be adapted for HPLC detection. Among these are amperometric, coulometric, potentiometric, and conductometric techniques. The techniques more commonly applied in HPLC are the conductometric, amperometric, and to a lesser extent coulometric procedures [32–34]. These techniques can have very high sensitivity, and the price of the detectors is relatively low. In ion chromatography, conductometric measurements are the most common. In amperometric techniques, the current intensity is measured in an electrochemical cell when a specific potential E is applied between two electrodes. Usually, only the reaction at one

electrode is of interest, and a cell can be composed of a working electrode coupled with a nonpolarizable electrode (one that does not modify its potential upon passing of a current). This is known as the reference electrode. More frequently, a three-electrode cell arrangement is used. In this arrangement, the current is passed between a working electrode (made, for example, from glassy carbon) and an auxiliary electrode, while the potential of the working electrode is measured relative to a separate reference electrode. The two types of cells are shown in Figs. 2.1.6A and 2.1.6B.

Any overall cell reaction comprises two independent half-reactions, and the cell potential can be broken into two individual half-cell potentials. The half-reaction of interest that takes place at the working electrode surface can be either an oxidation or a reduction. A simple reduction reaction for an oxidized compound *Ox* (notation not indicating the charge $n+$) to generate the compound *Red* is written as follows:

$$Ox + n\,e^- \rightleftarrows Red \qquad (2.1.14)$$

The electrode potential E^0 for this half-reaction is reported to the potential of a reference standard hydrogen electrode (NHE), which is taken as zero. Experimental measurements are commonly done with a saturated calomel electrode (Hg/Hg_2Cl_2 or SCE) or with an Ag/AgCl reference electrode. The potential of an SCE electrode versus NHE is +0.242 V, and the potential of an Ag/AgCl electrode is +0.197 V versus NHE. If a compound accepts electrons from a standard hydrogen electrode, it is defined as having a positive redox potential, and a compound donating electrons to the hydrogen electrode is defined as having a negative redox potential. A high positive E^0 indicates an oxidant and a low negative E^0 indicates a reducing compound. The reaction takes place spontaneously in a cell with a positive resulting potential.

Considering a reversible reduction that has a very rapid electron transfer, and assuming that both *Ox* and *Red* are soluble species, the molar concentrations C_{Ox} and C_{Red} at the electrode surface ($x = 0$) are governed by Nernst equation:

$$E = E^0 + \frac{RT}{nF}\ln\frac{C_{Ox}(x\,=\,0)}{C_{Red}(x\,=\,0)} \qquad (2.1.15)$$

where E^0 is the standard electrochemical potential of the half-cell, R is the gas constant (in SI units $R = 8.31451$ J deg^{-1} mol^{-1}), T is the temperature (in K deg.), n is the number of electrons involved in the electrochemical reaction, and F is Faraday constant ($F = 96485.332123$ C mol^{-1}). Tables of standard electrochemical potentials in specific media are available in the literature [35] and the half-reactions are expressed as reductions. (Potentiometric measurements based on Eq. 2.1.15 are also used for the pH measurements where the ratio $C_{Red}/C_{Ox} = [H^+]$).

In amperometric measurements, the current intensity is measured in an electrochemical cell when a specific potential is applied between two

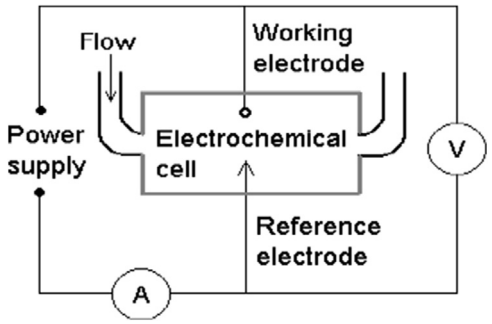

FIGURE 2.1.6A Schematics of a two-electrode flow-cell.

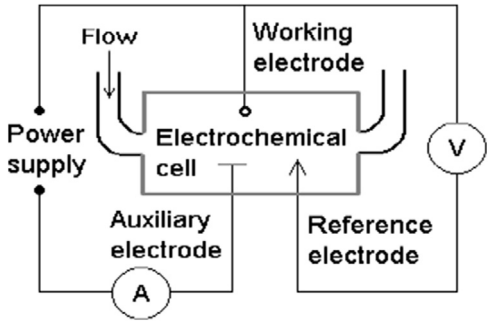

FIGURE 2.1.6B Schematics of a three-electrode flow-cell.

electrodes. For the very rapid electron transfer at the electrode surface, the rate v of the electrochemical reaction (expressed in $mol^{-1}s^{-1}cm^{-2}$) is proportional with the current intensity I and inversely proportional with the electrode area A_{el}:

$$v = \frac{I}{nFA_{el}} \tag{2.1.16}$$

This rate is determined by the mass transfer in solution and is given by the rate at which the electroactive species are brought to the surface of the electrode. In the absence of convection (for bulk solutions) and of migration under the influence of the electric field, diffusion is the only mechanism for mass transfer, and the rate $v_{mass\ transfer}$ (flux) can be approximated by the following expression (equivalent with Fick's first law):

$$v_{mass\ transfer} = m_{Ox}\left[C^*_{Ox} - C_{Ox}(x=0)\right] \tag{2.1.17}$$

where m_{Ox} is a proportionality coefficient called mass transfer coefficient, and C^*_{Ox} is the concentration of Ox in the bulk solution. The largest rate of mass transfer for Ox occurs when $C_{Ox}(x=0)=0$. The value of the current in these conditions is called the limiting current I_{lim}, and its value is given by the following expression:

$$I_{lim} = nFA_{el}m_{Ox}C^*_{Ox} \tag{2.1.18}$$

The expression for $C_{Ox}(x=0)$ can be written now using Eqs. 2.1.16–2.1.18 as follows:

$$C_{Ox}(x=0) = \frac{I_{lim} - I}{nm_{Ox}FA_{el}} \tag{2.1.19}$$

When the reducing species *Red* is absent in the bulk solution, $C_{Red}{}^* = 0$, and using a relation similar to Eq. 2.1.19 for the reduced species, the expression for C_{Red} can be written as follows:

$$C_{Red}(x=0) = \frac{I}{nm_{Red}FA_{el}} \tag{2.1.20}$$

With Eqs. 2.1.19 and 2.1.20, Nernst formula 2.1.15 can be written as follows:

$$E = E^0 + \frac{RT}{nF}ln\frac{m_{Ox}}{m_{Red}} + \frac{RT}{nF}ln\frac{I_{lim} - I}{I} \tag{2.1.21}$$

Eq. 2.1.21 represents the relation between the potential E and the current intensity I for a reversible redox reaction with very rapid electron transfer at the electrode surface.

For the current intensity equal to half of the limiting current, $I = 1/2\ I_{lim}$, the last term in Eq. 2.1.21 is null, and the corresponding potential indicated as $E_{1/2}$ is independent of the concentrations of the oxidant or reduced species and it is specific for a *Red-Ox* system. This $E_{1/2}$ potential is known as "half wave potential" and for a diffusion controlled process (static), a reversible reduction reaction with both *Ox* and *Red* species soluble and only with the oxidant initially present in the solution, the variation of the current intensity I as a function of the working electrode potential E follows an equation of the form:

$$E = E_{1/2} + \frac{RT}{nF}ln\frac{I_{lim} - I}{I} \tag{2.1.22}$$

The graph of Eq. 2.1.22 for a hypothetical reduction with $E_{1/2} = 0.5$ V is shown in Fig. 2.1.7.

For the case of the electrochemically active species flowing over the surface of an electrode, which is the case of electrochemical detection in HPLC, the current potential dependence is determined by a convective diffusion process (not only by diffusion). This makes the limiting current

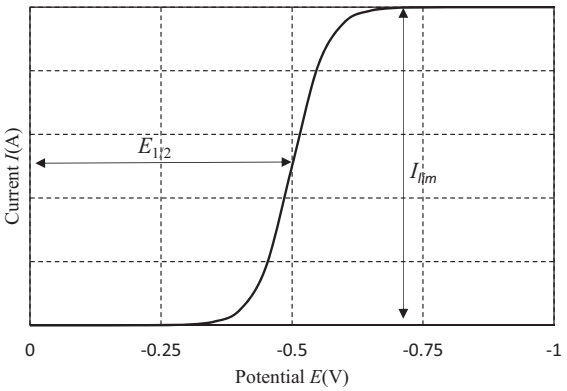

FIGURE 2.1.7 The current–potential curve of a Nernstian reaction involving two soluble species and only with the oxidant present initially. In this example, $E_{1/2} = -0.5$ V.

intensity for a Nernstian process dependent on the mobile phase flow rate and on channel and electrode geometry. For a rectangular channel flow electrode in steady state laminar flow with the working electrode at one wall, the limiting current intensity is given by the following relation:

$$I_{lim} = 1.467\, n\, F\, C^{*}\left(A_{el}\, D\, b^{-1}\right)^{2/3} U^{1/3} \quad (2.1.23)$$

where C^{*} is the bulk concentration of the analyte, A_{el} is the electrode area, D is the diffusion coefficient of the analyte, b is the channel height, and U is the volumetric flow rate [36–38]. For different channel and electrode shapes, the expression for the current intensity is different [39,40]. In amperometric detection, the current passing through the cell is measured at a fixed potential E, commonly chosen higher in absolute value than $E_{1/2}$ specific for the analyte. In these conditions, the desired electrochemical process takes place, but also all other species present in solution and having $E_{1/2}$ lower (in absolute value) than the chosen E value can become electrochemically active species. This may include even the solvent if the working potential E is very high. For eliminating this type of interference, compounds with low electrochemical potentials (in absolute value) are preferred for electrochemical detection. In HPLC, amperometric detection is frequently used for oxidation reactions. The quantitation can be done by calibration of the measured current I_{lim} versus different concentrations of analyte while maintaining strictly controlled flow conditions. Also, instead of a constant oxidation potential, a pulsed amperometric detection (PAD) can be used, alternating the oxidation analytical potential with a reducing pulse used for cleaning the electrode (depositions on the electrode may modify the nature of its surface and therefore the cell potential). The application of different working potentials is done at specific time intervals, and the measurement is made only when the active species are oxidized.

In coulometric detection, the amount of electricity (in coulombs) is measured during the electrochemical process. Potentiometric measurement can be applied for example in the case of ion concentration gradients across a semipermeable membrane. In this case, the measured potential E is given by an expression of the following form:

$$E = Const. - \frac{RT}{nF}\ln C_X \quad (2.1.24)$$

The concentration C_X can be obtained by measuring the potential E (following Eq. 2.1.24) and using previously generated calibration curves.

The conductivity (conductometry) detectors are also used for the measurement of concentrations of electrolytes in aqueous solutions. The molar concentration of an analyte that produces solution conductivity can be obtained from the measured electrical resistance of the solution. The dependence of concentration of the resistance is given by the following formula:

$$C = Const_{cell}\frac{1}{\Lambda_m}\frac{1}{Res} \quad (2.1.25)$$

where $Const_{cell}$ is a constant depending of the measuring cell, Res is the electrical resistance measured for the sample with the instrument (from Ohm's law $Res = I/V$ and at constant voltage V the intensity I measurement allows the calculation of Res), and Λ_m is the equivalent conductivity for the ionic species. Although Λ_m can be taken for practical purposes as constant, it varies with the concentration following Kohlrausch's law:

$$\Lambda_m = \Lambda_m^0 - \Theta\sqrt{C} \quad (2.1.26)$$

where Θ is a constant and Λ_m^0 is the limiting molar conductivity specific for each ion. Molar conductivity is temperature dependent. Values for limiting molar conductivities can be found tabulated [41].

In ion chromatography, the mobile phase frequently contains acids, bases, or salts (such as carbonates) that may interfere with the

electrochemical detection. Bases such as KOH or acids such as methanesulfonic acid (MSA) can be generated electrochemically (see Section 13.5). Eluent suppressors for ion chromatography are frequently necessary, in particular when the detection procedure is based on conductivity measurements. For this reason, before reaching the detector in IC, it is common to use a *suppressor*, which can reduce the conductivity caused by the eluent components by virtually eliminating the ions belonging to the mobile phase, and increasing the conductivity due to the analytes. Two general types of suppressors are commonly used for IC, one being based on the addition in the flow of an ion exchange cartridge (e.g., commercially available from Metrohm or Altech Co.) that eliminates the conductive ions of the mobile phase, and the other is based on semipermeable membranes. Special devices are commercially available that use a pair of ion exchange cartridges used alternatively in the eluent flow. One cartridge is used for suppressing the eluent ions, while the other is regenerated, such that no interruption in the operation is incurred.

Various models of suppressors based on semipermeable membrane technology are also available (e.g., self-regenerating suppressor SRS, micromembrane suppressor MMS, capillary electrolytic suppressor CES, or Atlas electrolytic suppressor AES, which are commercially available from Thermo Scientific/Dionex [42]). In a separation where the eluent is, for example, a solution of NaOH or KOH, the suppressor which is a resin or a semipermeable membrane, provides immobilized (or not permeable) $R-SO_3H$ groups. In this case, the reaction of the NaOH from the eluent through the suppressor is described by the following scheme:

$$NaOH + R-SO_3H \rightarrow R-SO_3Na + H_2O \quad (2.1.27)$$

At the same time, an analyte in the form of a Na salt (e.g., a chloride) passing through the suppressor undergoes the following change:

$$NaX + R-SO_3H \rightarrow R-SO_3Na + HX \quad (2.1.28)$$

As a result of suppression, the conductivity caused by the NaOH in the mobile phase is eliminated being changed into H_2O, while the conductivity caused by the analyte NaX is not modified being changed into HX which is also dissociated. Aqueous buffers containing $NaHCO_3$ and Na_2CO_3 are also frequently used as eluents in IC. The conductivity caused by such buffers is eliminated using a chemical suppressor by transforming the alkaline carbonates into H_2CO_3 that decomposes into H_2O and CO_2 and do not contribute to conductivity. Other anions (e.g., F^-, Cl^-, SO_4^{2-}) generate strong acids that are easily detected based on conductivity. Various other suppression techniques are used in practice ([43]), including electrolytic suppressors used in anion LC.

In the case of a cation analysis, suppression of conductivity from the mobile phase can be achieved with a resin in OH^- form. For example, H_2SO_4 or MSA can be used as an additive to the mobile phase and are retained by the anion exchange resin (in OH^- form) with the formation of H_2O in the solution. At the same time, a salt (e.g., of an alkaline ion such as Na^+ or K^+) passing through the resin can generate a strong base (NaOH or KOH) with high electric conductivity. Sulfuric acid or MSA is also used in the mobile phase for semipermeable membranes ion suppression. Semipermeable membrane for cation exchange separations may use a flow of tetrabutyl-ammonium hydroxide as a reagent on the other side of the membrane. The reagent interacts with the mobile phase acids. The OH^- ions of the reagent in the semipermeable membrane replace the SO_4^{2-} or $CH_3SO_3^-$ ions from the mobile phase. In this way, water is formed with the H^+ of H_2SO_4 or CH_3SO_3H. Other ions (e.g., Na^+, K^+, etc.) generate hydroxides that have high conductivity (NaOH or KOH are highly dissociated). Conductometric detection does not have selectivity, any ion present in the measuring flow-cell of the detector generating a signal. For this reason, the separation by the

HPLC should assure the elimination of interferences [44].

Mass spectrometric detectors

LC-MS and LC-MS/MS are two techniques that are becoming more and more common [45–49]. This was possible mainly due to the progress made in developing electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) ion sources for LC-MS able to convert efficiently the dissolved analyte from the mobile phase of the LC into gas phase ions that are further analyzed with the mass analyzer. The MS with these ion sources can be easily connected to the HPLC instrument, although the flow rate of the HPLC has some limitation, with common flow rates ranging between 0.3 and 0.6 mL/min. Lower flow rates can also be utilized but higher flow rates than 1 mL/min must be avoided. The optimization of the MS response must be performed in accordance with a specific flow rate since the sensitivity of the MS detector is influenced by the flow rate. Also, the temperatures and gas flows in specific MS ion sources should be set in accordance with a specific flow rate. LC-MS and LC-MS/MS can provide exceptional sensitivity and selectivity compared to other detection techniques. The capability to easily measure ng/mL levels of compounds in the sample, to differentiate between molecules with different mass and fragmentation patterns, as well as the potential identification capability, makes LC-MS and LC-MS/MS invaluable techniques. The good resolving power required for the separation in other detection techniques can become less necessary with MS detection when coeluting compounds can be differentiated by their MS signal.

For liquid samples delivered from an HPLC instrument, the MS detectors have the following main components: (1) an *ion source* that generates ions from the sample components, (2) a *mass analyzer*, and (3) an *ion detection device*. For MS/MS systems, additional components can be included such as an ion mobility analyzer. The MS (and MS/MS) detectors are utilized mainly on-line with the HPLC, but off-line setups are also possible. For the analysis of the effluent from a microcolumn separation, for example, the mass spectrometer can be utilized for off-line detection [50].

(1) The most common *ion sources* in LC-MS and LC-MS/MS are based on ESI and to a lesser extent APCI. In the ESI source, the effluent is introduced through a capillary at 3–5 kV potential toward an opposite plate. The spray is changed into small droplets and some of the solvent is vaporized using a current of a heated gas. The charged heated droplets lose most (but not all) of the solvent. Due to ionic repulsion, the droplets generate individual ions, most of them still solvated. In APCI source, the effluent from the HPLC column is sent through a capillary heated and having added a flow of gas, but not under an electrical potential. The jet of molecules of solvent and analyte flow further near a needle charged at a high voltage (3–5 kV) in order to generate ions. The generation of ions in ESI and APCI is schematically shown in Figs. 2.1.8 and 2.1.9.

The APCI type ionization is usually applied when the mobile phase used in the separation is low in water content or has no water. In such conditions, the ionization efficiency is low even in APCI which is less frequently used as compared to ESI.

In the electric field of the source of the MS are produced either positive or negative ions depending on the MS settings. The positive ions are usually of the type MH^+ where M are the molecules of the analyte and H^+ ions are coming from the water present in the eluent which in many LC methods using MS detection also contain a low level of a volatile acid, usually HCOOH. For negative mode of operation, molecules of the type MH can be ionized to generate M^- negative ions. Ions with more than one charge can also be formed for specific type of analytes such as peptides. Also, adducts between analyte molecule and different ions

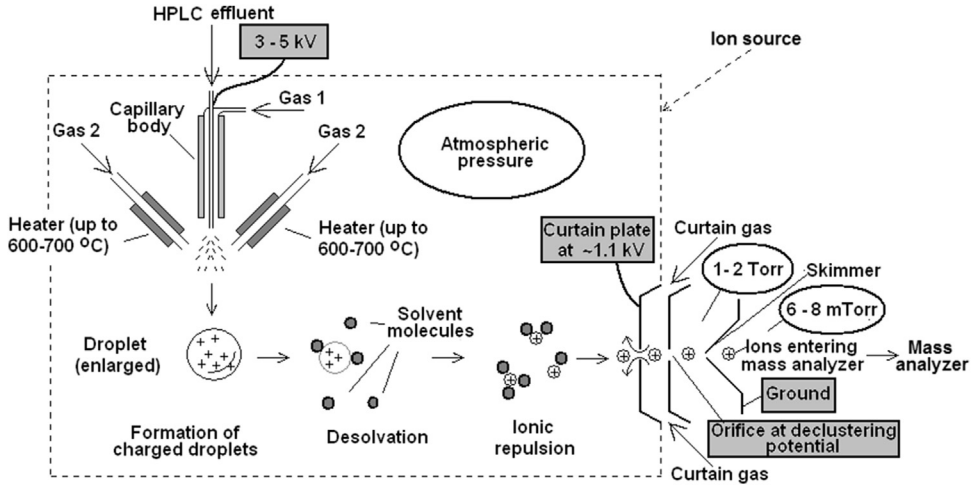

FIGURE 2.1.8 Schematic diagram of an electrospray ionization (ESI) inlet for an LC/MS instrument (positive ion mode). The decrease in electrical potential and in pressure is also indicated on the diagram (based on an AB-Sciex source).

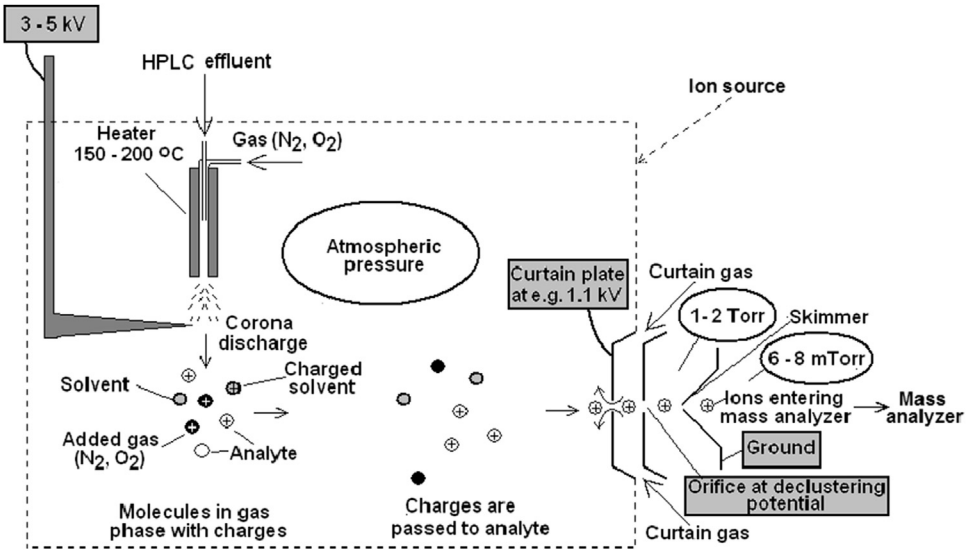

FIGURE 2.1.9 Schematic drawing of an atmospheric pressure chemical ionization (APCI) source inlet for an LC/MS instrument (positive ion mode). The decrease in electrical potential and in pressure is also indicated on the diagram (based on an AB-Sciex source).

reaching the MS interface are sometimes observed in LC-MS [51]. They are formed by ion-dipole, ion-induced dipole, hydrogen bonds, and other van der Waals interactions. In the adduct formation, the molecules of the solvent *Solv* are frequently involved. In some cases, the adducts are stable enough to be seen in the mass spectrum instead of the analyte molecular ion. Besides adducts with solvent molecules, other adducts can be formed

between the analyte M and other species in the eluted material. For example, in positive ionization mode, ions such as $[M\text{-}Na]^+$, $[M\text{-}K]^+$, $[M\text{-}NH_4]^+$, $[M{-}H_2O{-}Na]^+$, $[M\text{-}Solv\text{-}Na]^+$, and $[M\text{-}2Solv\text{-}Na]^+$ can be seen. Negative ionization is less favorable to adduct formation, but still possible with ions of the following types: $[2M]^-$, $[3M]^-$, $[2M\text{-}Na]^-$, etc.

Due to the high concentration of the solvent (although it is less ionized than the analyte), it is common that some of its molecules are also ionized to form ions $[Solv\text{-}H]^+$ (or in case of a *Solv*H molecules to form by negative ionization $[Solv]^-$). Further ionization of the analyte can be described as a proton transfer from the ionized solvent to the molecule of the analyte.

$$M + [Solv{-}H]^+ \rightarrow MH^+ + Solv \qquad (2.1.29)$$

or in case of negative ionization:

$$MH + [Solv]^- \rightarrow M^- + SolvH \qquad (2.1.30)$$

The proton transfer reactions show that the solvent has an important role in the ionization process of the analytes in LC-MS. The gas-phase basicity (or acidity) of the solvent molecules $\Delta G^0_{GPB}(Solv)$ (or $\Delta G^0_{GPA}(Solv\text{-}H)$) are important values regarding the formation of ionized solvent and the proton transfer reactions (see Section 6.2). The generation of ions is a complex process, and more than one type of reaction may participate in the formation of analyte ions. The proton transfer process as a source of analyte ions can have an important contribution to the ionization efficiency of the analyte and therefore is related to the sensitivity of mass spectral analysis. For this reason, the sensitivity of the MS detectors depends on the nature of the mobile phase. Also the charge transfer reactions may explain why some molecules analyzed in negative mode show good results regarding ionization when a low concentration of an acid such as HCOOH is present in the mobile phase. The added acid generated negative ions and through charge transfer the analyte is also ionized.

The ions formed in the mass spectrometer source are further separated and analyzed in the mass spectrometer. Depending on the specific properties of each molecule and the exact conditions of ionization (ionization voltage, temperature, etc.), in both ESI and APCI sources mainly the molecular ions are formed. Further fragmentations of the analytes for MS/MS applications are achieved in a collision cell.

The proton transfer reactions described between the molecules of the analyte and those of the solvent may also take place between the molecules of the analyte and those of a component of the sample matrix, in case that this component is not well separated from the analyte by the chromatographic process. For an analyte M (or MH) and a matrix component *Matrix*H (or *Matrix*), the transfer can be described by reactions of the type:

$$M + [Matrix{-}H]^+ \leftrightarrow MH^+ + Matrix \qquad (2.1.31)$$

or in case of negative ionization:

$$MH + [Matrix]^- \leftrightarrow M^- + MatrixH \qquad (2.1.32)$$

These types of reactions being reversible may favor the formation of the ions of the matrix and not of the analyte, and can contribute significantly to the change in ion concentration of the analyte in the LC-MS source. For this reason, even if the matrix compound has a different m/z from the analyte, the response of the detector can be influenced when the chromatographic separation is not very good. In case of large concentration of a matrix constituent, if the apex of the chromatographic peak of the analyte is different but not far from that of the matrix constituent, they elute close to each other and even the tail of matrix constituent may interfere in sensitivity.

Other procedures besides ESI and APCI are used to form ions from the analyte molecule from the HPLC effluent. One of these is the use of an intense beam of UV light for the analyte ionization instead of a corona discharge. This technique is known as atmospheric pressure

photoionization. Also, in an effort to generate for the analytes in the LC eluent mass spectra that are similar to EI+ mass spectra (searchable in standard mass spectral libraries such as NIST8), special instrumentation is being developed such as LC-MS with supersonic molecular beam and electron ionization capability [52–54].

Older techniques to interface an LC with a mass spectrometer include the following: (1) particle beam (PB), which consists of an aerosol generator from the LC flow (at 0.1–1 mL/min) followed by a desolvation chamber and a separator that directs the aerosols through a series of apertures separating the volatile compounds including the solvent from the solid aerosols, (2) continuous flow fast atomic bombardment (FAB), where the effluent is introduced directly into a vacuum region (with a flow rate of 5–10 μL/min) mixed with a matrix material such as glycerol, and the ionization is achieved using a beam of ions at 5–8 keV [55], and (3) thermospray [56], etc.

The ions generated in the source are further separated by the mass analyzer and measured using a detector. The separation power of the mass analyzer is characterized by its resolution expressed by the following expression:

$$R = \frac{M}{\Delta M} \tag{2.1.33}$$

where ΔM is the closest spacing of two peaks (in mass units) of equal intensity with the valley between them less than a specified fraction of the peak height (e.g., 50%), and M is the mass of the second peak. Sometimes ΔM is replaced with FWHM (full width at half maximum) characterizing a width of a single peak for a specific mass M of an ion. Based on their resolution, mass spectrometers are usually classified as "low resolution" and "high resolution" (sometimes "medium resolution" and "ultrahigh resolution" are recognized). The low resolution typically discriminate ions with a difference in mass of 0.2 m.u. ($R \approx 1000$ for $M = 200$), while high resolution instruments can discriminate ions with a difference in mass of 0.0001 m.u. ($R \approx 2{,}000{,}000$ for $M = 200$).

(2) Several types of *mass analyzers* are utilized, such as *quadrupole*, *ion trap*, *time-of-flight* (TOF), and other ion separation techniques such as magnetic sector, Wien filter, ion cyclotron resonance, etc.

The *quadrupole* type mass spectrometer is a common mass analyzer which separates the ions by passing them along the central axis of four parallel equidistant rods that have a fixed voltage (DC) and an alternating (RF) voltage applied to them. The field strengths (voltage) can be set such that only ions of one selected mass can pass through the quadrupole, while all other ions are deflected to strike the rods. By varying (with a precise rate) the strength and frequencies of the electric fields, different masses can be filtered through the quadrupole. A schematic diagram of a quadrupole mass analyzer is shown in Fig. 2.1.10.

With the quadrupole instruments, a low-resolution type spectrum is obtained. For an $m/z = 200$, for example, the minimum mass difference that can be separated could be around

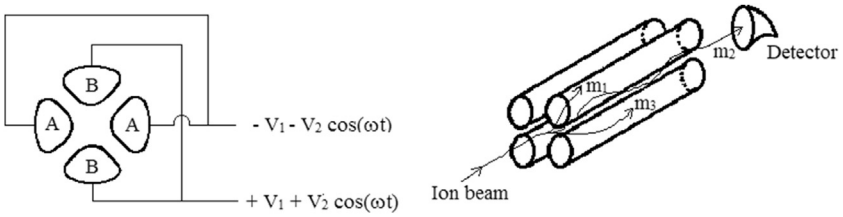

FIGURE 2.1.10 Schematic diagram of a quadrupole mass analyzer showing m_2 passing through the quadrupole and masses m_1 and m_3 eliminated.

0.2 mass units (resolution 1000). The mass range for the quadrupoles can go as high as 2000 Da, but common commercial quadrupole instruments have a mass range between 2 and 1100.

After the separation based on their m/z, the ions are detected using different procedures.

The *ion trap* mass spectrometer consists of a hyperbolic cross-section center ring electrode (a doughnut) and two hyperbolic cross-section endcap electrodes. The ion trap works in cycles. A cycle starts with the application of a low RF amplitude and fixed frequency to the ring electrode (no DC), while the endcaps are grounded. A pulse of electrons is injected into the ion trap to ionize and fragment the gaseous sample coming from the transfer line. The ring electrode at low RF amplitude traps all the ions formed during the ionization pulse. The RF amplitude is then increased, and ions of increasing mass (in fact m/z) are sequentially ejected from the trap and detected. A key parameter to the operation is the gas pressure inside the trap (usually He), which must be maintained at 10^{-3} mbar. This gas forces the ions toward the middle of the trap and provides a better sensitivity. A small AC voltage of fixed frequency and amplitude is also applied to the endcaps during the analysis part of the cycle. Then the process is repeated. The ion traps generate low-resolution mass spectra but can have very good sensitivity. Some problems have been reported regarding the similarity between the spectra generated by ion traps and other types of mass spectrometers when the concentration of a certain compound is high. For high concentration, some ion traps do not generate typical EI+ spectra. A special type of ion trap, used for generating high resolution separations, is the Orbitrap (resolution 2,000,000 for $M = 200$). Orbitrap mass analyzers are a special type of ion traps consisting of an outer barrel-like electrode and a coaxial inner spindle-like electrode that traps ions in an orbital motion around the spindle and converts the resulting frequency signal to a mass spectrum using the Fourier transform. This type of instrument achieves high resolution (e.g., $M/\Delta M \approx 60{,}000$ for $m/z = 400$ or even up to 100.000) (e.g., Ref. [57]).

The *TOF* instruments separate the ions of different m/z ratio based on their different energies. In an electric field with the potential difference U, the energy of the particles having the charge z is $E = zU$ and their kinetic energy is $E = {}^1/_2\ mv^2$. Therefore, the following expression can be written for the particle:

$$zU = \frac{1}{2}mv^2 \tag{2.1.34}$$

In a field-free TOF tube of length L, the $t = L/v$ "time of flight" for a particle is therefore obtained from the following expression:

$$t = \frac{L}{\sqrt{2U}}\sqrt{m/z} \tag{2.1.35}$$

Eq. 2.1.35 indicates that in a TOF instrument the ions will reach the detector at a time proportional to the square root of their m/z ratio.

In a reflection TOF, a constant electrostatic field is used to reflect the ion beam toward the detector. The more energetic ions penetrate deeper into the path within the reflection lenses (reflectron) and take a longer time to reach the detector. Less energetic ions of the same m/z penetrate a shorter distance into the reflectron and take a shorter time to reach the detector. A schematic diagram of a TOF is indicated in Fig. 2.1.11.

TOF spectrometers work on scans using a pulsed ion source. The number of acquired spectra per second for a TOF instrument can be

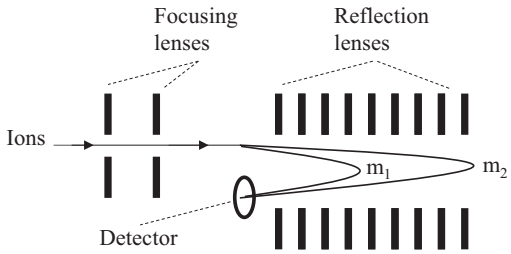

FIGURE 2.1.11 Schematic diagram of a reflectron TOF.

as high as 500. Depending on their construction, some TOF instruments generate low resolution spectra and other high resolution ($R \approx 60{,}000$). Some TOF instruments have wider mass range than quadrupole instruments.

Several other types of mass analyzers have been developed and are utilized. Magnetic sector instruments, for example, were used for the ion separation. In a magnetic sector instrument, the ions from the ion source are sent through a nonhomogeneous magnetic field where they are deflected on different arc trajectories based on their m/z ratio and focused using an electric field followed by detection. Magnetic sectors mass detectors can separate ions within a wide range of masses and can achieve high resolution with excellent sensitivity. The cost of magnetic sector instruments is however higher than for other types of mass detectors. A number of other techniques for the separation of ions are known, such as Fourier transform ion cyclotron resonance (FT-ICR-MS) capable of 1,000,000 resolution.

(3) After the separation by m/z, the *detection of ions* can be done using different procedures. Two classes of detectors are known: point ion detectors, which detect the arrival of all ions sequentially at one point, and array detectors, which detect all ions simultaneously along a surface. These detectors record the number (abundance) of individual ions at each m/z. Several point ion detectors are available, such as Faraday cup, electron multiplier, and scintillator. The array detectors are commonly made of separate point detectors (of miniature dimensions) clustered together in the area exposed to the incoming ions. The array detectors have the advantage of collecting simultaneously the signal for a series of ions, but their dynamic range and sensitivity are usually lower. The signal from the detector is further processed (amplified and analyzed) by an electronic data system. Problems such as mass/time calibration, linearity of the detector response, etc., are usually transparent when using modern mass spectrometers, but they are very important in their construction. More details about the ion sources, ion optics, detectors, or data processing systems of different mass spectrometers can be found in the literature dedicated to mass spectrometry (e.g., Ref. [58]).

A simplified schematics of an LC-MS system (based on a QDa analyzer from Waters) is shown in Fig. 2.1.12.

The typical source in LC-MS generates mainly molecular ions and this provides only limited selectivity and identification capability for the instrument. This type of instrument has the capability to detect very low level of many

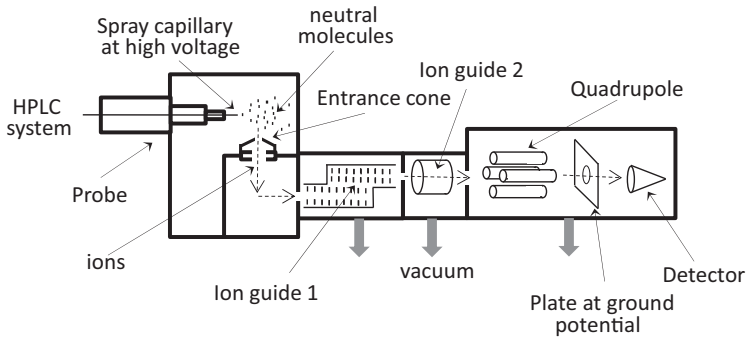

FIGURE 2.1.12 Schematic diagram of an LC-MS system (based on a QDa analyzer from Waters) with a Z type shape of ion path from the capillary to the entrance of the mass analyzer.

compounds and provides molecular weight information for the analytes. However, the absence of molecular fragments formation from the molecular ion in LC-MS limits the identification capability of this technique.

In addition to single mass spectrometers, tandem mass spectrometers are powerful analytical instruments. In tandem mass spectrometry, also known as MS/MS, multiple steps of mass spectrometry separations are used, with some form of fragmentation occurring in between the MS stages. The fragmentation of the precursor ions (parent ions) separated by the first mass analyzer is typically produced in a collision cell where product ions (daughter ions) are formed.

Multiple stages of mass analysis separation can be accomplished with individual mass spectrometer elements separated in space or using a single mass spectrometer with the MS steps separated in time. In tandem mass spectrometry in space which are the more common ones, the separation elements are physically separated. These elements can be sectors, transmission quadrupole, or TOF. When using multiple quadrupoles, they can act as both mass analyzers and collision chambers. For tandem mass spectrometry in time, the separation is accomplished with ions trapped in the same place, with multiple separation steps taking place over time. A common tandem mass spectrometer type is the triple quadrupole. In a triple quadrupole, the first quadrupole (Q1) has the role of separating the (precursor or parent) ions generated in the ion source. These molecular ions are selected for further interactions in the collision cell (Q2) where they can be fragmented. For this purpose, a gas (such as N_2 or Ar) is introduced in the cell, and a specific voltage is applied to the Q2 quadrupole (or hexapole in some instruments). Depending on the collision gas pressure and on the voltage applied to the collision cell, the precursor ions undergo different degrees of fragmentation (fragmentation by collisionally activated dissociation CAD). The fragmentation strongly depends on the structural characteristics of the analyte. The third quadrupole Q3 is used for the separation of the resulting product ions following fragmentation in the collision cell. Following Q3, the ions are detected by procedures similar to those used in LC-MS.

Several utilization alternatives are common for MS/MS analyzers, such as (1) product ion scan, when the whole range of ions generated by fragmentation of the precursor ions (parent ions) is analyzed, (2) precursor ion scan, when only one ion is selected for the detector by Q3, while Q1 is scanning the whole range of ions produced by the source, (3) neutral loss scan, when the instrument scans for a specific mass difference between the ions from Q1 and Q3, and (4) multiple reaction monitoring (or MRM) where a specific precursor ion is selected by Q1 and a specific fragment (product ion) is detected by Q3 (more than one pair of ions can be analyzed by MRM at the same time). Qualitative information from LC-MS/MS can be generated based on fragmentation of the parent ions. The fragmentation in LC-MS/MS is characteristic for each molecule. However, the nature of fragment ions in LC-MS/MS is typically used for the confirmation of a specific analyte, and less for compound identification as it is done in GC-MS. There are several reasons for this. The pattern of fragments obtained in LC-MS/MS is typically less complex than the one obtained in GC-MS with EI+ ionization at 70 eV. Also, the fragmentation strongly depends on the conditions in the collision cell (collision gas nature and pressure, collision energy, collision cell construction). Since a universally standardized set of conditions for obtaining fragments in LC-MS/MS is not available, the fragmentation is not standard. For these reasons, large libraries with LC-MS/MS spectra to be used for any compound identification are not available. New successful developments regarding the use of LC-MS/MS for qualitative analysis are in progress for specific classes of compounds (e.g., peptides).

The MS or tandem MS/MS is a highly selective and sensitive detector, but besides its benefits also it has some disadvantages. The MS detection in HPLC does not have the benefit of generating mass spectra with standard fragmentation as in GC-MS, and therefore, unknown compound identification based on searches in mass spectra libraries is limited (few search capabilities are reported [59]). Also, when monitoring only specific analytes (e.g., in MRM mode), these can be either separated or not separated by the HPLC column, and some coeluting compounds may not be detected. While this can be a big advantage since the HPLC separation may be difficult, at the same time matrix interference may occur. For this reason, recoveries must be evaluated in LC-MS and LC-MS/MS in the presence of all possible matrix constituents. Other limitations are also known for LC-MS and LC-MS/MS detectors. One such limitation is related to the requirement that all the components in the mobile phase must be volatile. This imposes that the buffers that can be used in the mobile phase must be volatile, and typically formic acid or ammonium formate are used for both enhancing ionization (HCOOH for positive, but also for negative ionization, and $HCOONH_4$ or CH_3COONH_4 mainly for negative ionization) as well as for obtaining a desired pH. The formation of fine solid particles in the ionization chamber of the MS leads to a decrease in ionization efficiency and also generates a very unstable signal in the MS instrument (high background noise). For this reason, salts such as KH_2PO_4 or K_2HPO_4 cannot be used to obtain a buffer solution to be used in LC-MS. Also, the presence of an organic phase in the mobile phase is almost always necessary. For this reason, totally aqueous mobile phase is less frequently used in LC-MS. Another problem with LC-MS using ESI or APCI sources is related to the problem of ion suppression when the amount of ions generated in the source is high. These high levels of ions can be caused by a (relatively) high level of analyte, which leads to a rather narrow dynamic range of the detector. Another source of high level of ions may result from unseparated components of the matrix that can be ionized, resulting in the reduction of the signal of the analyte.

Not only triple quadrupoles can be used in MS/MS instrumentation. Other types of MS/MS include ion-trap + quadrupole, quadrupole + time of flight (Q-TOF), and also orbitrap mass analyzers. TOF mass spectrometric detectors (MS-TOF) can also be associated with an additional electro separation based on ion mobility (IM or ion mobility spectroscopy IMS). In this technique, the ions of the analyte move in an electrical field against a carrier buffer gas. Since collisions with gas molecules have higher probability for larger molecules than for the smaller ones, a separation based on size is obtained. The separation in IM occurs at a time scale of milliseconds (in the range 10^{-1} to 10^{-3} s), while in TOF mass spectrometers the detection occurs on a microseconds scale making the MS-TOF a suitable detection procedure for IMS. Using a gating mechanism at specified intervals of time, a burst of ions is introduced into a drift chamber. The ions are moved in one direction in a linear electric field gradient through the drift chamber against a flow of a buffer gas. The buffer gas can be He, N_2, or other common gases and can have ambient pressure or reduced pressure (a few torr). After the ion mobility drift tube, the mass spectrometer is used as a detector. Before the detector, it is also possible to include a collision cell that produces fragmentation (product/daughter ions) useful for further analyte differentiation. In this way, molecular species can be separated based on their mass, charge, size, and shape, depending on the interactions with the buffer gas (e.g., Ref. [60]). Commercial instruments combining HPLC with IMS-MS are available from various vendors (e.g., Ref. [61]). Other instrument developments are continuously made in the field of LC-MS/MS. Examples are coupling an ion trap with a collision cell and a quadrupole and

coupling a triple quadrupole with a fourth ion analyzer such as an Orbitrap.

The typical response $\mathscr{S}$ for the mass spectrometer detector is related to the instantaneous mass q of the analyte entering the detector by the expression (a and b are parameters depending on detector and detector settings):

$$\mathscr{S} = a + bq \tag{2.1.36}$$

In certain applications, the dependence between the response $\mathscr{S}$ and quantity q becomes nonlinear, and several different response functions, such as quadratic, are used in the calibration process [62]. The LC-MS/MS is frequently used for highly selective and sensitive quantitative analysis on known analytes. Quantitative information using the MRM mode is characterized by exceptional sensitivity (as low as fmol/mL concentration in the analyzed solution).

Evaporative light-scattering detectors

One other detector utilized in HPLC, in particular for compounds that do not have good light absorbance in UV are not fluorescent and may be difficult to ionize, is the evaporative light-scattering detector (ELSD) (e.g., Refs. [63,64]). ELSD uses the formation of particles that do not evaporate and can scatter light, while the mobile phase forms a gas by evaporation. In this technique, the eluent is injected in the form of a spray from a nebulizer into a drift tube where also a nebulizer gas is introduced. The drift tube is heated and the solvent is evaporated, while nonvolatile molecules form a fine mist. This mist passes through a cell that is illuminated with a beam of light and the scattered light from the mist is recorded. The gas generated from the solvent does not influence light scattering. The schematic diagram of an ELSD is shown in Fig. 2.1.13.

The intensity of the scattered light is dependent on the analyte concentration (within a certain range of concentrations since the linearity is not followed for a wide range). This detector has the advantage over the RI of being useable with gradient elution. However, the presence of any salts or nonvolatile materials in the mobile phase disturbs the measurements. Also, changes in the temperature of the eluent do not affect ELSD, while with RI detectors a careful temperature control must be applied since the refractive index varies with temperature. ELSD can be more sensitive than RI in specific applications.

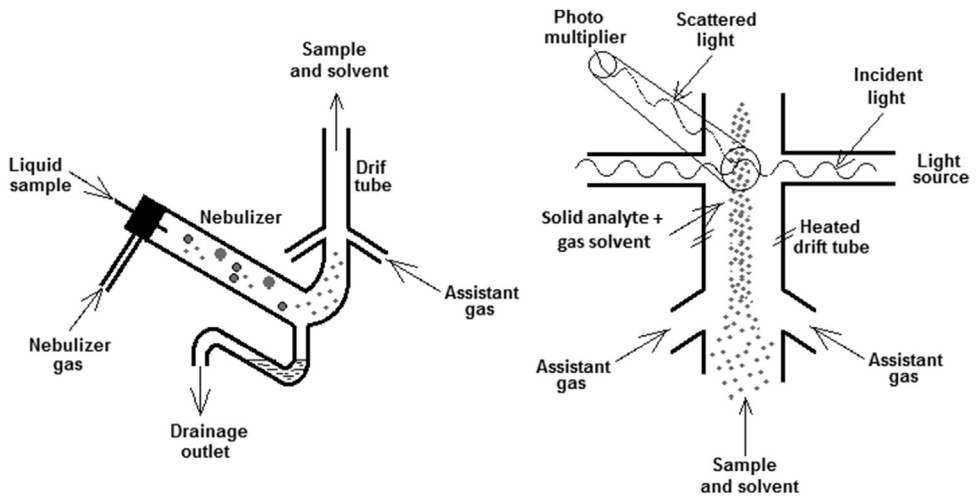

FIGURE 2.1.13 Schematic diagram of an ELSD setup.

Modifications of ELSD were attempted to further improve its sensitivity, such as by adding a saturated stream of solvent to the mist of the analyte in order to grow the particles by nucleation and detect them better (the technique is known as CNLSD) or to use lasers as light source (LLSD). A multiangle light scattering detector is also available for HPLC.

Light scattering detector does not have for all analytes a linear response to analyte concentration, in particular when the concentration is relatively high when changes can be seen in the particle size generated by the detector. For this reason, in some analyses, the calibration is performed using a log/log dependence. Using the notations C for the analyte concentration and y the peak area in the chromatogram, this dependence can be written as follows [65,66]:

$$\log C = a + b \log y \qquad (2.1.37)$$

An alternative to light scattering detection is a corona charged aerosol detector (CAD or cCAD) [67]. This detector is also based on nebulization of column effluent (e.g., with N_2) and drying of resulted droplets to remove the mobile phase components, producing analyte particles. A secondary stream of N_2 is made positively charged by passing it by a high-voltage platinum corona wire, which is sent in the stream of analyte particles to generate charged aerosols particles. The charged particles obtained by the interaction of the aerosols with the charged nitrogen gas are detected, generating an electric signal with the intensity dependent on the amount of analyte eluted from the column. The typical response of a CAD detector follows a dependence as indicated by Eq. 2.1.37. The CAD is more sensitive than ELSD and has a wider dynamic range [68]. Similarly to ELSD, one of the drawbacks of CAD is its inability to accurately quantify volatile compounds. Because CAD can only measure analytes that form particles, it cannot detect compounds that are volatile or do not readily form particles. In this situation, detection can be achieved by other specific detectors, such as chemiluminescent nitrogen detector (CLND) [69]. CAD detection is reported to be possible in a wide range of concentrations.

Other types of detectors

Less common detectors than those previously discussed can also be used in HPLC. For example, in case that the target compounds contain at least one nitrogen atom, the CLND can be employed. The principle of this detector relies on the combustion of the column effluent in a high temperature furnace that converts the N-containing compounds into NO. The dried gas stream is passed into a chamber where it reacts with O_3, a reaction that is associated with chemiluminescence (measured by a photomultiplier). This detector has a high sensitivity, but is not compatible with acetonitrile in the mobile phase [70]. Other known detection techniques include Fourier transform infrared spectrometry [71,72], nuclear magnetic resonance [73], inductively coupled plasma-mass spectrometry (ICP-MS) [74], circular dichroism (CD), optical rotatory dispersion, polarimetry, radioactivity, plasmon resonance [75], multiangle light scattering applied directly to solution of polymers used, e.g., in connection with size exclusion chromatography [76,77], etc.

Selection of a detector in HPLC

The selection of a specific detector is a very important step for an HPLC analysis, and for this reason, several factors must be considered. These include (1) availability of instrumentation, (2) the purpose of analysis, (3) the chemical nature of the analytes and of the matrix, (4) detector performance including its sensitivity, (5) the required quality of the results, (6) type of elution in the selected method, (7) the properties of the mobile phase in the selected method, (8) detector reliability, and (9) special characteristics of the detector. The choice of a specific detector is not

always possible because of availability limitations. For this reason, it is necessary in some instances to change the analytical method in order to use the detector which is available. There are also cases, when a recommended method can be modified to use a better detector in order to increase sensitivity or selectivity. Also, since more than one detector can be present in an HPLC system, the detection can be done in more than one way, which may offer some advantages. Several criteria for detector selection are further presented.

(1) Availability of instrumentation is a straightforward requirement. When several instrumental setups are available, besides other factors, the instrumental cost, cost per analysis, cost of instrument maintenance, and expertise available in the laboratory in using the specific detector should be considered.
(2) The purpose of analysis is essential in the instrument selection, in general. This influences the selection of pumps, injectors, etc., but one important part affected by the purpose of analysis is the selection of the detector. All detectors are designed to allow quantitative measurements, but some are not designed for providing any qualitative information, such as the refractive index (RI) detectors. Others detectors provide some information related to the qualitative nature of the analytes, but this information is not diagnostic, or sufficient for providing positive identification of the analyte. In this group can be included the ultra violet (UV) detectors (in particular those that can generate an absorption spectrum (e.g., DAD detectors). Mass spectrometric (MS) detectors can provide information that can be used for compound identification, but even these detectors have limitations. When only a quantitative analysis is necessary and the nature of the analyte is known, the peak identification for the known compound can be done based on the retention time alone (obtained from running standards), the only concern being an efficient chromatographic separation with no interference. Interferences may be detected using the comparison of UV spectra of the peak of pure compound with that of the corresponding peak from the sample. However, mass spectrometric detection (MS or even more reliable MS/MS) is capable of selecting only the measurement for the analyte. By setting, the MS detection for a specific mass (usually molecular ion) is an important procedure for selecting only the desired compound for measurement. For MS/MS detection, in particular when this is performed in MRM mode, the precursor ion and the product ion can be selected such that the possibilities for interferences are much reduced.

For qualitative analysis of a sample with unknown composition, even HPLC in tandem with MS is not always able to provide decisive information in structural identification. The mass spectra in LC, when using a single mass spectrometer as a detector, do not offer sufficient information about the fragments of an analyzed molecule, typically indicating only the molecular ion. Better information is obtained with the use of MS/MS instruments, but the fragmentation provided can vary considerably depending on the instrument and acquisition method. Although some library searches are available (e.g., SmileMS) as well as some mass spectral libraries for LC, the qualitative information on unknown compounds is not always conclusive. As an alternative to searching based on molecular fragmentation, the nature of an unknown compound can also be obtained from its precise molecular mass. Instruments generating high resolution mass spectra (see Section 2.3) are often equipped with search programs (e.g., MassFrontier) that help in identifying the molecular composition and

potential structures. For qualitative analysis of samples containing a mixture of compounds with a suspected structure that requires only confirmation, techniques such as mass spectrometry MS or MS/MS must be used, working in specific modes (e.g., MRM).

(3) The chemical nature of the analytes and of the matrix of the sample are important factors in the selection of the method of analysis. All these are included in method selection, which can be previously reported in the literature, or planned to be utilized for the analysis. Various aspects of this selection are further discussed in this book. The method selection includes the sample preparation before analysis, the separation, and also of the detection technique. The physical and chemical characteristics of the analytes, as well as their difference from other sample components and from the mobile phase, must be carefully evaluated in order to select the property best used for detection. For example, the presence or absence of chromophore groups typically determines the use of UV/visible absorption detectors (fixed wavelength, variable wavelength, or diode array detector DAD), which are among the most common detectors used in HPLC. For compounds that can fluoresce, it is common to select this property for detection. In some cases, e.g., in the analysis of carbohydrates, the absorption in UV is very low (except for very low wavelengths) and the compounds are not fluorescent. In such cases, other properties such as change in refractive index of eluting solution, electrochemical properties, or formation of ions capable to be analyzed by mass spectroscopy can be selected. Also techniques that do not require a specific molecular structure such as evaporative light scattering can be used. The derivatization is frequently applied during sample preparation for modifying the initial analyte properties such that it can be amenable for a specific detection. The detection technique is always described for an analytical method when this is reported in the literature.

(4) Based on the properties of the analytes, a detector with specific capabilities must be selected, such that it is sensitive to the specific analyte property that allows measurement. Some detectors are responding to most analytes, and are indicated as universal detectors [78]. Other detectors are specific for a class of compounds, and some have relatively limited applicability (e.g., radioactivity detectors, or CD detectors). Refractive index detectors (RI) are typically universal detectors. Other detectors used for compounds that do not contain chromophores or fluorophores are electrochemical (e.g., amperometric), evaporative light scattering (ELSD), corona-charged aerosol (CAD or cCAD), etc. MS detectors are also universal, but their sensitivity is also dependent on the nature of the analyte.

The selection of the settings of the detectors is also important in attaining a required sensitivity, and the settings depend on the nature of the analyte and its concentration in the sample. This may include the choice of wavelength of absorption for UV, the choice of excitation and emission wavelength for FLD, or the choice of several parameter settings and masses to be monitored for MS.

Detector sensitivity is another factor important for selection. This sensitivity selection should be related to the concentration of the analyte in the analyzed sample. The detectors used in LC can have very different sensitivities, which depend on the type of detector and can be different toward different analytes, depending on the analyte nature. For example, the sensitivity of RI detectors is typically much lower than that of fluorescence (FL) detectors for a fluorescent compound, but in the absence of fluorescence, the RI detector can be utilized,

while the FLD is useless. Detector settings, model, and manufacturer play an important role regarding the detector sensitivity.

(5) The required quality of the results is a factor determining the needed precision and accuracy a detector is capable to attain. This needed precision and accuracy is also dependent on the nature of the analyte, the level of the analyte in the sample, and on the methods used for sample preparation. Depending on the purpose of analysis, the selection of a detector or of a detector setting must be done in such a way to cover the analysis needs and be capable of achieving a required LOQ. The sensitivity of various types of detectors can vary significantly. When a method has been already selected, e.g., from the literature, the choice of the detector is indicated in the method. However, it is common that a better sensitivity is necessary when adopting a method from literature. In such case, a different selection of a detector can be attempted. Also, when a new method of analysis is developed, the detector should be selected based on detector capability.

(6) The type of elution also plays a key role in the selection of a detector, and in many cases the choice is made the other way around, the available detector determining the choice of isocratic or gradient elution. Several techniques are not applicable for gradient elution or are not responding very well to the change of the composition of mobile phase. Refractive index detector, for example, must be used only with isocratic elution. Other detectors such as ELSD or CAD can be used with gradient elution, but the solvents must be volatile (should not contain any nonvolatile additives) and the change in solvent composition may also generate some drift in the baseline. Even MS and MS/MS detectors may show differences in sensitivity at one solvent composition or the other, and the choice of gradient versus isocratic elution is sometimes influenced by this difference. Detectors such as UV can be used with gradient separation without any problem (as long as the mobile phase does not have absorption by itself).

(7) The properties of the mobile phase also contribute to the decision regarding the choice of a specific detector. Mobile phase composition plays a crucial role in most separations, and the subject of mobile phase will be presented in detail in Chapter 7. A specific mobile phase may determine the type of detector that can be used, and in some instances, the method is developed particularly to be used with a specific detector.

Physical and chemical properties of the mobile phase must be considered in relation to the requirements of a specific detector. For example, when detection is done in UV, the cut-off wavelength of the solvent must be considered, such that the solvent is "transparent" at the measuring wavelength. For the detection using ELSD or CAD, the mobile phase should be totally volatile, and in the case of the need of salt buffers in the mobile phase, these detectors are not useable. In LC/MS (and LC-MS/MS), the mobile phase composition influences the detection, and the presence of nonvolatile salts in the mobile phase is not recommended. Since LC/MS and LC-MS/MS detection may offer particular benefits to the analysis (very good sensitivity, qualitative information), it is common that the selection of the mobile phase is done such that it is suitable for the MS detector, and not the other way around (choose the detector to accommodate the mobile phase). Also, the sensitivity of the detection may be drastically influenced by the mobile phase composition in MS and MS/MS. When a method has been developed for another type of detection (e.g., using nonvolatile buffers) and must be applied with MS detection, for example, for enhancing sensitivity or for obtaining some qualitative information on the

analytes, it is common to modify the mobile phase to be suitable for the new detection technique.

(8) Detector reliability is another factor to be considered in its selection. Some detectors such as those based on UV absorption are typically extremely stable and reliable. Other detectors may be more prone to problems, which also may depend on instrument age, manufacturer, environmental conditions, etc. For example, electrochemical detectors may show some drift or unreproducible results depending on the cleanliness status of the measuring electrode. In some instances, a choice between sensitivity and stability of the detector must be made. For this reason, other characteristics also must be considered in detector selection, such as stability of the signal, frequency of the measurement per unit time (that assures an accurate evaluation of the peak shape), resistance to acids and bases in the mobile phase, capability to be used in series with other detectors, acceptable backpressure in case of connection in series with another detector, etc.

(9) Some detectors require special maintenance and more effort for establishing the proper operating conditions, such as the MS or MS/MS detectors. Other detectors are very simple to operate and require virtually no adjustments. The advantages of such detectors must be weighed versus disadvantages, such as loss of sensitivity or lack of qualitative information.

Because of the importance of the detector selection in an HPLC method, the subject will continue to be discussed in this book.

Other devices that can be part of the HPLC system

Various analysis needs may require the increase in the complexity of the HPLC instrumentation. These may require auxiliary devices such as systems for column switching. Another device sometimes used in HPLC is a flow splitter. This device is necessary when the flow from a conventional HPLC pump is either too high to be used with a capillary LC column or with a specific detector (such as a mass spectrometer). A flow setting below a specific limit at a conventional HPLC pump may lead to undesirable fluctuations or to a difficult to control gradient program. The flow splitter allows the selection of a desired fraction from the total flow from the pump by using a waste outlet with controllable backpressure. Some HPLC techniques require the addition to the eluent flow from the chromatographic column a reagent or an additional solvent. This can be done by including a "mixing T" to the tubing connecting the column and the detector, and connecting the mixing T, besides the detector, to an additional pump. The flow from this additional pump may contain, for example, a derivatization reagent that requires time for interacting with the analytes. In such cases, a reactor type device that allows heating of the effluent may be necessary. Simply, a longer tubing may be sufficient, that allows a longer time of contact between the analytes and reagent before reaching the detector. These types of experimental setups are described in various special methods of analysis [79].

After some HPLC separations, some fractions of interest may need to be collected as the effluent exits the detectors. This collection can be done using a fraction collector. Automated systems for fraction collection are available. They direct the flow emerging from the last detector to specified vials either at a given time or upon receiving a signal from the detector (indicating an eluting peak).

More complex or special HPLC setups

More complex HPLC setups can be designed for various purposes that are not limited to the

basic system made from (1) solvent supply, (2) pump, (3) injector, (4) column, and (5) detector. Among such setups that require two different chromatographic columns each one with its own solvent supply and pumping system include those that allow the separation of a heartcut (segment of time during the chromatographic run) from the eluate of the first column to be sent for further separation to a second column. This type of setup is usually indicated as *bidimensional* (or two-dimensional) and the columns used in such systems are selected to be very different and preform different types of separation being indicated as *orthogonal* [80,81].

Other systems are designed, for example, for sample enrichment in analytes. In this use, the flow with the sample is sent to a column where the analytes are strongly retained for the specific solvent composition and, possibly, the matrix components are less retained. The flow to this column may carry a large volume of a diluted sample. The analytes are accumulated usually at the head of the column and the flow is sent to waste. After enough analytes are accumulated, the flow in the column is reversed (with a switching valve), and the solvent is changed such that the analytes are eluted from the head of the first column and sent to a different column where the separation takes place. The flow from this second column is sent to the detectors.

One special type of HPLC setup is that designed for high temperature HPLC. For those systems, the internal temperature of the column can be set at a specific point (e.g., up to 200°C) through an external heating and cooling source fixed on the surface of the column wall. An example of a temperature programming unit consists of a column tightly enclosed in an aluminum block. For higher temperature programs, the system includes an eluent preheating unit, the column heating unit, and a mobile phase cooling unit. When a temperature gradient is applied, the temperature of the preheating unit and the temperature of the column are increased simultaneously [82]. The system assures acceptable data variation, with the relative standard deviation of the measured retention times being below 5% [83]. Fast temperature gradients can be applied in capillary HPLC using, for example, fused silica column coated with a this nickel layer connected to a power source and a temperature control system [84].

Instrument control and data processing unit

Part of modern HPLC (UPLC) instrumentation is a computer with dedicated programs for instrument control and data processing. Instrument manufacturers developed sophisticated such programs that are necessary for the control of HPLC pumps, the autosamplers, the temperatures of column compartment, and the parameters for the detectors. Also the programs include capabilities for data processing, peak detection, peak integration, and other data manipulation such as data storing and recovery, peak purity verification, data smoothing, calibrations using linear or nonlinear curves, etc. Some programs also incorporate libraries of spectra that allow qualitative identification of the compound from each peak. Dedicated literature describes in detail the capabilities of such programs (e.g., instrument manuals).

Selection of the HPLC system and transition from HPLC to UPLC

The selection of an HPLC system is frequently limited by the instrument availability. The main differences between instruments can be considered the type of pumping (low pressure mixing or high pressure mixing) and the type of detector. When possible, the selection of the instrument should be done based on the requirements of the analysis (as previously discussed for pumping system selection and detector selection). The instruments can be identified

as HPLC and UPLC, although the UPLC instruments can be used in UPLC mode as well as HPLC mode. The UPLC instruments are characterized by the capability of pumps to deliver higher pressures, a higher precision of the injection system that can inject correctly as low as 0.1 μL sample, and detectors with small dead volumes (e.g., small volume cells for UV absorption measurements). Most UPLC systems can be used as simple HPLC, without taking advantage of certain UPLC features. For this reason, the transition from HPLC to UPLC on an instrument capable to be used as UPLC can be a gradual process. The key element that decides the separation of UPLC use from HPLC use is the chromatographic column. The columns with small and very small particles (e.g., 1.6−1.8 μm diameter) require higher pressures from the pumps. For this reason, the use of columns with small particles requires a UPLC instrument. Such columns provide a better resolution, but sometimes do not have large loading capability. This implies that small injection volumes are required and they should be very precise. For small injection volumes, the detectors must be sensitive, should involve small dead volumes, and should have high sampling frequency. The increased use of mass spectrometry detectors usually involves lower flow rates (0.1−0.6 mL/min) and the precision of gradient composition of the mobile phase must be precise. In addition to the previously listed features, attention must be given in UPLC to the use of small i.d. tubing (between 0.065 mm i.d (0.0025 in) and 0.12 mm i.d. (0.005 in)) and correct fittings that do not produce void volumes.

The transition from HPLC use to UPLC use generally provides better sensitivity and shorter run times for the separations. Depending on the detector and the nature of the analytes, the use of UPLC can routinely allow the measurement of analytes concentrations as low as a few pg/mL, and produce chromatograms with run times of 2−3 min.

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CHAPTER

3

Parameters for the characterization of HPLC separation

3.1 Parameters describing the chromatographic peak

General aspects

Analytical techniques are characterized by specificity/selectivity, reproducibility and repeatability, accuracy, range of linearity between the quantity of analyte and the response of the analytical instrument, limit of detection (LOD), limit of quantitation (LOQ), recovery yield of the sample processing, robustness, ruggedness, and stability (e.g., Ref. [1]). In order to achieve optimum method characteristics, the HPLC must be properly conducted and controlled. A number of parameters are used in HPLC for this purpose. This chapter is focused on parameters used to characterize the HPLC separation. Some of these parameters are related to the quality of the chromatographic separation and other are related to the physical characteristics of the HPLC system. Some parameters can be obtained by inspecting the chromatograms and other by knowing the conditions in which the chromatogram was generated.

Flow rate of the mobile phase

Before presenting the parameters characterizing the chromatographic peak, it is necessary to discuss the flow of the mobile phase that plays an important role for peak characteristics. The flow of the mobile phase in the chromatographic column can be described by the *volumetric flow rate* U and by the *linear flow rate* u. The volumetric flow rate U is the volume of fluid that flows per unit time (expressed e.g., in mL/min) through the chromatographic column. This parameter is set in the HPLC instrument by the user (see Section 2.1).

The linear flow rate u is the velocity of a point in the fluid passing through the column (expressed as length per time), and can be considered the velocity of an unretained molecule in the chromatographic column. The linear flow rate depends on the volumetric flow rate by an expression of the form:

$$u = \frac{U}{A_c} \tag{3.1.1}$$

where A_c is the area of the channel in which the flow takes place. It should be noticed that A_c is not the surface area of the circular cross-section of the empty column since the column is filled with the stationary phase. For a column with the inner diameter d, surface area of the empty column is $\pi d^2/4$ and the A_c is given by the following expression:

$$A_c = \varepsilon^* \pi d^2/4 \tag{3.1.2}$$

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where ε^* is a constant depending on column packing porosity. The relation between U and u can therefore be written as follows:

$$U = \frac{\varepsilon^* \pi d^2}{4} u \tag{3.1.3}$$

For a chromatographic column containing particles with about 5 μm diameter, the column packing porosity ε^* can be taken with the approximate value $\varepsilon^* \approx 0.7$. The value for ε^* may vary depending on the stationary phase particle size and structure such that the values of ε^* can be somewhat different from $\varepsilon^* \approx 0.7$. For smaller particle size, the value of ε^* is smaller than 0.7 [2].

Retention time

The peak *retention time* $t_R(X)$ is the time (usually measured in min) from the injection of the sample into the chromatographic system to the time of elution of the compound X. The time is taken at the maximum (the apex) of the chromatographic peak. The retention time $t_R(X)$ is an important chromatographic characteristic for a molecular species X, since it has a constant value as long as the other parameters of the chromatographic separation are kept constant. In this way after establishing the retention time for a specific compound, for example using a standard, the retention time can be used for the compound identification (assuming no changes in the chromatographic conditions and no interference from other compounds).

The retention time $t_R(X)$ can be separated into two components: the time analyte X spends in the mobile phase moving through the column known as *dead time* or *void time* t_0, and the time the analyte is retained on the stationary phase $t'_R(X)$ known as *reduced retention time*. In this way, the retention time is given by the following expression:

$$t_R(X) = t'_R(X) + t_0 \tag{3.1.4}$$

Retention time in a chromatogram is determined by the properties of the compound X, the retention capability of the column, the nature of the mobile phase, but also by the flow rate of the mobile phase. The dead time t_0 is not dependent on the compound X. It depends on column construction and on the flow rate of the mobile phase. This is expressed by the following formula:

$$t_0 = \frac{L}{u} \tag{3.1.5}$$

where L is the length of the chromatographic column (a very small fraction from the value of t_0 represents the time taken for the sample to flow through the tubing from the injector to the column and to the detector). From Eq. 3.1.5 and Eq. 3.1.3, t_0 can also be expressed as a function of U as follows:

$$t_0 = \frac{\varepsilon^* \pi d^2 L}{4U} \tag{3.1.6}$$

The value for t_0 can be obtained experimentally [3]. For example, an approximation of t_0 can be obtained by measuring the time for the elution of a compound that is virtually not retained (very slightly retained, since it can be difficult to find a compound that is not retained at all on a chromatographic column). For example, the solvent used for injecting the sample (when it is different from the mobile phase) can be such a compound, and the retention time of this solvent peak can be taken as dead time. Similarly, the retention time of a deuterated solvent analogous of a mobile phase component can be used for measuring t_0. Several other procedures for the estimation of t_0 are known [4,5]. One procedure uses the minor disturbances in the background signal created by the sample injection. For RP-HPLC, the use of uracil or of inorganic salts that are assumed not to be retained on a hydrophobic column is also a common procedure for t_0 estimation. Another more elaborate procedure involves the use of a plot of retention times for a homologous series of compounds

that are retained less and less as the number of carbon atoms is decreasing and extrapolated to zero. A similar approach based on linear free energy relationships and the measurement of the elution volume of a series of alkylbenzene standards from toluene to heptadecylbenzene was used for estimating the void volume in HILIC type HPLC for different mobile phase compositions [6,7]. As indicated, a small delay is also caused by the time spent by the analyte in the tubing. However, for a length of 20 cm for tubing with 0.12 mm i.d. (0.005 in), at a flow rate of 1 mL/min, the tubing volume contributes with about 0.16 s delay which is usually neglected.

Run time

The time for the whole chromatographic separation is indicated as the *run time*. The total time necessary for completing a chromatographic separation is taken slightly longer than the retention time of the last peak in the chromatogram. This time is sometimes referred to as total run time or length of the chromatogram. In practice when multiple samples are analyzed, the total run time is an important parameter since its value is related to the number of samples analyzed within a certain length of time.

Retention volume

For a specific molecular species X, the *retention volume* $V_R(X)$ is defined as the volume of mobile phase flowing from the time of injection until the corresponding retention time $t_R(X)$ of a molecular species. The values for V_R and t_R are related by the simple formula:

$$V_R(X) = U\, t_R(X) \tag{3.1.7}$$

The retention volume corresponding to the dead time t_0 is known as *dead volume* V_0, or void volume. This volume corresponds to the volume of liquid in the column (and in the transfer lines from the injector to the column and from the column to the detector). The chromatographic column has a "volume not occupied by the stationary phase," which is the space between the stationary phase particles and inside their pores. The not-retained molecule has to travel through the tubing from the injector to the column (which is very small), through the volume not occupied by the stationary phase, and through the tubing from the column to the detector (also very small), which accounts for the t_0 and for the dead volume V_0. Corresponding to the reduced retention time t'_R, a *reduced retention volume* V'_R can be defined by the following formula:

$$V'_R = V_R - V_0 \tag{3.1.8}$$

The dead volume V_0 of a chromatographic column can be found by multiplying the dead time with the volumetric flow ($V_0 = t_0 U$), but also by direct measurements.

The value of V_0 can be considered proportional with the volume of the empty column V_e, the proportionality constant ε^* depending on the dimension (and shape) of the stationary phase particles, and also on the way they are packed (common approximation $\varepsilon^* = 0.7$). For a column of length L and inner diameter d, the empty column volume is $V_e = (\pi/4)d^{\,2}L$. However, for the column being filled with the stationary phase, only a fraction of this volume will give the dead volume. This can be expressed with the use of column packing porosity ε^*. The packing porosity ε^* can be calculated from the ratio between the empty space volume of the column after packing with stationary phase (V_{pore}) and its geometrical volume (the volume of an empty column, i.e., $(\pi/4)d^2L$, L being the length column). The value of V_{pore} represents

the volume of open space from packed column being accessed by the mobile phase and the sum between external porosity (interstitial voids) and internal porosity (the volume of particle pores). A practical procedure to determine V_{pore} is pycnometry, by which the weight of the packed column is measured separately by filling with two solvents of different density (e.g., dichloromethane and tetrahydrofuran [2]). The volume V_{pore} is calculated from the ratio $(m_{solv1} - m_{solv2})/(\rho_{solv1} - \rho_{solv2})$, where m_{solv1} and m_{solv2} are the mass of packed column + solvent 1 (with density ρ_{solv1}) and column + solvent 2 (with density ρ_{solv2}), respectively. Since the column i.d. and the column length are typically expressed in mm, while the volume is usually expressed in mL (cm^3), a factor of 10^{-3} must also be included in the calculation when these units are used. Including the packing porosity, the column dead (void) volume is $V_0 = \varepsilon^*(\pi/4)d^2L$. Table 3.1.1 gives some typical values for the empty volume $V_e = (\pi/4)d^2L$ and dead volume ($V_0 = \varepsilon^* V_e$) for an HPLC column, depending on its dimensions. At $U = 1$ mL/min, the void time t_0 is numerically equal to the void volume V_0 as given in Table 3.1.1. Precise void volume of a column must be experimentally measured with an unretained compound.

The estimation of the void volume of the column is important for the calculation of several parameters in chromatography (see further retention factor). Also, during gradient operation, the void volume must be considered for evaluating when a certain mobile phase concentration is reaching the end of the column (dwell time is typically much smaller than void volume).

Migration rate

While the linear flow rate u in the chromatographic column is the migration rate of an unretained molecule, the velocity at which the species X moves through the column is indicated by a parameter $u_R(X)$ known as *migration rate* of species X. The value of $u_R(X)$ is a constant only when the mobile phase composition does not change during the separation (isocratic conditions).

The migration rate multiplied with retention time equals the length of the column for both retained and not retained compounds, and the following expression can be written:

$$u_R(X)\, t_R(X) = u\, t_0 = L \qquad (3.1.9)$$

If during the separation all the molecules of compound X would be all the time in the mobile phase, then $u_R(X)$ is equal to u. However, some of the molecules are intermittently retained and when retained they do not move, such that

TABLE 3.1.1 Typical values for the void volume of packed HPLC columns.

Dimensions (i.d × length in mm)	Empty volume mL	Void volume V_0 mL	Dimensions (i.d × length in mm)	Empty volume mL	Void volume V_0 mL
2.1 × 100	0.35	0.24	4.6 × 250	4.15	2.90
2.1 × 150	0.52	0.37	4.6 × 300	4.99	3.49
2.1 × 250	0.87	0.61	10.0 × 100	7.85	5.50
2.1 × 300	1.04	0.73	10.0 × 150	11.78	8.25
4.6 × 100	1.66	1.16	10.0 × 250	19.63	13.75
4.6 × 150	2.49	1.75	10.0 × 300	23.56	16.49

only a fraction of molecules of compound X that are present in mobile phase are moving. The value of $u_R(X)$ is determined by this fraction. Assuming that during the separation process the number of molecules of compound X that are in the mobile phase is $\nu_{mo}(X)$ and in the stationary phase is $\nu_{st}(X)$, then $u_R(X)$ will be given by the following expression:

$$u_R(X) = \frac{\nu_{mo}(X)}{\nu_{mo}(X) + \nu_{st}(X)}\, u \qquad (3.1.10)$$

In Eq. 3.1.10, the following notation can be used:

$$\nu(X) = \frac{\nu_{mo}(X)}{\nu_{mo}(X) + \nu_{st}(X)} \qquad (3.1.11)$$

From Eq. 3.1.10 and Eq. 3.1.11, $u_R(X)$ can be written in the following form:

$$u_R(X) = \nu(X)\, u \qquad (3.1.12)$$

Eqs. 3.1.9 and 3.1.12 indicate that for the retention time the following formula is valid:

$$t_R(X) = \frac{1}{\nu(X)} t_0 \qquad (3.1.13)$$

Relation Eqs. 3.1.13 shows that for a solute present only in the mobile phase, $\nu(X) = 1$ and $t_R(X) = t_0$, and for a solute completely retained on the stationary phase $\nu(X) = 0$ and $t_R(X) = \infty$. The typical situation is in between these two limits and, for example, if in a separation, 25% of the molecules of an analyte are present in the mobile phase, $t_R(X) = 4\ t_0$. A similar relation with Eq. 3.1.13 is valid between the retention volume $V_R(X)$ and the dead volume V_0.

Equilibrium constant and phase ratio in HPLC separations

During the chromatographic process, the molecules that are separated can be considered as being in a continuous equilibrium between the mobile phase and the stationary phase. For a unique molecular species X, this equilibrium can be written as follows (see also Eq. 4.1.18):

$$X_{mo} \leftrightarrow X_{st} \qquad (3.1.14)$$

and can be considered governed by an *equilibrium constant* $K(X)$, defined as follows:

$$K(X) = \frac{C(X)_{st}}{C(X)_{mo}} \qquad (3.1.15)$$

where $C(X)_{mo}$ is the molar concentration of species X in the mobile phase and represents the amount (in moles) of X in the volume V_0 of the mobile phase in the chromatographic column. This amount is proportional with the fraction ν of molecules in the volume V_0 of mobile phase. Similarly, the concentration in the stationary phase $C(X)_{st}$ can be considered as representing the amount in moles of X from the stationary phase (proportional with $1 - \nu$) in the volume V_{st} of the stationary phase. As a result, the equilibrium constant $K(X)$ can be written in the following form:

$$K(X) = \frac{(1 - \nu(X))/V_{st}}{\nu(X)/V_0} = \frac{1 - \nu(X)}{\nu(X)}\, \frac{V_0}{V_{st}} \qquad (3.1.16)$$

As shown in formula 3.1.16, $K(X)$ depends on the ratio between the fraction of molecules of compound X that are present in the stationary phase (equal with $1 - \nu(X)$) and the fraction of molecules that is present in the mobile phase (equal with $\nu(X)$), as well as the ratio of the volumes V_0 and V_{st}. Since the ratio $(1 - \nu(X))/\nu(X)$ is kept constant only in isocratic conditions in a chromatographic separation, it becomes obvious that $K(X)$ is a constant only for isocratic chromatographic separations (unchanged composition of the mobile phase). The dependence of $K(X)$ on the value for $\nu(X)$ in formula 3.1.16 can be easily replaced with a dependence on

retention time. This can be achieved considering formula 3.1.13, such that the value for $K(X)$ can be written in the following form:

$$K(X) = \frac{t_R(X) - t_0}{t_0} \frac{V_0}{V_{st}} \quad (3.1.17)$$

The ratio V_0/V_{st} in formula 3.1.17 depends on the chromatographic column construction. Its inverse is indicated as phase ratio Ψ:

$$\Psi = \frac{V_{st}}{V_0} \quad (3.1.18)$$

Phase ratio is an important chromatographic parameter. For its evaluation, the necessary value V_0 has been previously described, and for the estimation of stationary phase volume V_{st}, a number of procedures have been reported in the literature [8,9]. However, the correct evaluation of V_{st} is not a simple task, since there is no sharp boundary between the mobile phase and the stationary phase such that the volume is difficult to assess. In addition, the separation boundary depends on the mobile phase composition. Other procedures to evaluate Ψ, not using the values for V_0 and V_{st}, have been reported [10]. Values for Ψ for C8 and C18 columns vary between 0.15 and 0.55 depending on column construction [11].

Retention factor

The ratio $(t_R(X) - t_0)/t_0$ from formula 3.1.17 is an important parameter used for the description of chromatographic process and it is indicated as *retention factor* (indicated in the past as capacity factor). The retention factor will be noted in this book $k'(X)$, although the IUPAC recommendation is to use for retention factor the notation $k(X)$. However, the older notation k' was preferred because it differentiates better the retention factor from other constant also noted with k (e.g., the reaction rate constant). The expression for k' (X) is the following:

$$k'(X) = \frac{t_R(X) - t_0}{t_0} = \frac{t'_R(X)}{t_0} \quad (3.1.19)$$

Based on formula 3.1.17 for $K(X)$, and formula 3.1.18 for Ψ, the expression for retention factor is the following:

$$k'(X) = K(X)\Psi \quad (3.1.20)$$

Eq. 3.1.20 indicates that retention factor depends on two parameters governing the separation in HPLC. One is the equilibrium constant $K(X)$ which is determined by the nature of analyte X and the nature of stationary and mobile phases in the chromatographic column. The other parameter is phase ratio Ψ that depends on the characteristics of the stationary phase, but also on other factors.

The retention factor k' (X) has the advantage of being dimensionless and independent of the flow rate of the mobile phase or the dimensions of the column, and for this reason, it is a very common and useful parameter for peak characterization. From Eq. 3.1.19, for example, it can be seen that reduced retention time $t'_R(X)$ is related to the retention factor by the following formula:

$$t'_R(X) = k'(X)t_0 = K(X)\Psi t_0 \quad (3.1.21)$$

Eq. 3.1.21 can be written in the following form:

$$t_R(X) = t_0 + k'(X)t_0 \quad (3.1.22)$$

The retention factor k' can be used to establish a relation between t_R and t'_R. The ratio of Eqs. 3.1.21 and 3.1.22 leads to the following formula (compound X not specified):

$$\frac{t'_R}{t_R} = \frac{k'}{k' + 1} \quad (3.1.23)$$

Eq. 3.1.22 shows that retention time in HPLC depends on $k'(X)$ and therefore on the equilibrium constant for the analyte $K(X)$, the chromatographic column construction (phase ratio Ψ), and t_0. The retention factor k' is frequently expressed in logarithmic form in base 10 or in base e (either $\log_{10} k' = \log k'$, or $\log_e k' = \ln k'$).

Similar formulas to Eq. 3.1.21, Eq. 3.1.22, and Eq. 3.1.23 relating t_R, t'_R, t_0, and k' can be

established for retention volumes V_R, V'_R, V_0, and a relation similar to Eq. 3.1.21 can be written in the following form:

$$V'_R(X) = K(X)V_{st} = K(X)\Psi V_0 \qquad (3.1.24)$$

The retention factors k' should have values between 1.1 and 10, obtained by selecting the column and the mobile phase for a set of analytes. These values for k' are necessary for performing the separation at acceptable retention times. From the void volume of a column given by expression $V_0 = \varepsilon^*(\pi/4)d^{\,2}L$ where $\varepsilon^* \approx 0.7$, for a flow rate $U = 1$ mL/min, it can be evaluated that for a column with dimensions (i.d × length) of 4.6 × 150 mm, the dead time $t_0 \approx 1.75$ min, and for a compound with $k' = 10$ the result is $t_R \approx 19.25$ min. Once the column is selected, the k' value in the desired range can be adjusted by modifying the mobile phase composition. Retention factors $k'(X)$ below 1 indicates poor retention of component X. Low values for $k'(X)$ generate short retention times. Since many compounds from the sample matrix may also be poorly retained, a low $k'(X)$ for the analyte poses the risk of interference from matrix constituents. If the separation of the analytes is still good, lower k' values typically indicate lower t_R for the peaks of interest, which is desirable. However, it is preferable to achieve low t_R values by using columns with smaller dimensions, small particles in the chromatographic column, special particles (such as core-shell), elevated column temperature, etc., to speed up the separation that shortens t_0 while affecting k' as little as possible. Generally, retention factors exceeding a value of 10 indicate strong retention. The corresponding peaks elute after a longer time and are typically wide. Retention factors up to 20 are sometimes necessary, mainly when very complex samples are studied, but such values for k' indicate excessively long run times for the chromatogram.

Since the retention factor k' depends on equilibrium constant $K(X)$ (see Eq. 3.1.20) and the equilibrium constants depend on temperature, k' also depends on temperature. This dependence is further discussed in Section 4.6 (see Eq. 4.6.2).

General equation of solute retention

Formula 3.1.21 describing the retention time in a chromatographic column can also be derived from the basic principle of mass conservation. For this purpose, an infinitesimal cross-section with thickness dx and volume dV in the chromatographic column of length L will be considered. Taking an initial concentration C_{mo} of the solute in the mobile phase, the change in the amount of solute after passing the infinitesimal volume dV of mobile phase is given by the following:

$$\frac{\partial C_{mo}}{\partial x} dx dV \qquad (3.1.25)$$

This total change is caused by the distribution of the solute between the mobile and stationary phases. The solute concentration C in the mobile phase will change by the following amount:

$$-\frac{V_0}{L}\frac{\partial C_{mo}}{\partial V} dx dV \qquad (3.1.26)$$

The change of the amount of solute in the stationary phase will take place by the amount:

$$-\frac{V_{st}}{L}\frac{\partial C_{st}}{\partial V} dx dV \qquad (3.1.27)$$

From the principle of mass conservation, the following relation must be satisfied (Vault equation):

$$\frac{\partial C_{mo}}{\partial x} dx dV = -\frac{V_0}{L}\frac{\partial C_{mo}}{\partial V} dx dV - \frac{V_{st}}{L}\frac{\partial C_{st}}{\partial V} dx dV \qquad (3.1.28)$$

In Eq. 3.1.28, the value for C_{st} can be substituted using Eq. 3.1.15, and the result can be written in the following form:

$$\frac{\partial C_{mo}}{\partial x} + \frac{\partial C_{mo}}{\partial V}\left(\frac{V_0}{L} + K\frac{V_{st}}{L}\right) = 0 \qquad (3.1.29)$$

The differential Eq. 3.1.29 in C_{mo} has the general solution in the form of an arbitrary function $\varphi(z)$ [3]:

$$C_{mo} = \varphi(z) \quad where \quad z = V - \frac{x}{L(V_0 + KV_{st})} \tag{3.1.30}$$

The formula of function φ is determined by the initial conditions for Eq. 3.1.29. When the sample is introduced in the column as a narrow plug, and the movement of this plug is studied without considering other effects, the expression for φ should be given by the delta function $\delta(z)$:

$$\delta(z) = 0 \quad if \quad z \neq 0, \quad and \quad \int_{-\infty}^{+\infty} \delta(z)\mathrm{d}z = 1 \tag{3.1.31}$$

Eq. 3.1.31 indicates that $\varphi(z) = \delta(z)$ is nonzero only when $z = 0$ and therefore when:

$$V - \frac{x}{L}(V_0 + KV_{st}) = 0 \tag{3.1.32}$$

The solute emerges at the end of the column at $x = L$ and $V = V_R$. Then, Eq. 3.1.32 takes the following form:

$$V_R = V_0 + KV_{st} \tag{3.1.33}$$

Eq. 3.1.33 is equivalent with Eq. 3.1.24 ($V_{st} = \Psi V_0$) that was generated on an empirical basis, and expressed in retention times which is equivalent with Eq. 3.1.21. This indicates that previous formulas obtained for retention time and retention volumes are in fact justified by mass conservation principle.

Characteristics of an ideal peak shape in chromatography

The sample is injected in the HPLC system as an extremely narrow band (plug), but during the chromatographic run, this band is broadened. Further discussion does not account for the width of the sample "plug" injected in the chromatographic column. The peak broadening is an important (negative) effect taking place during the chromatographic process, and its study is necessary. Broadening is generated, for example, by the ordinary diffusion in time of the analyte molecules, such that the ideal shape of the chromatographic peaks can be described by a Gaussian bell curve. The transport of the band of the analyte across the HPLC system is supposed to not affect directly the peak broadening, which in reality is not the case. A number of other effects contribute to peak broadening but, at first, the present discussion will be limited to diffusion. Being a diffusion process, peak broadening can be studied based on Fick's laws. Fick's second law (e.g., Ref. [12]) for diffusion in one direction (longitudinal diffusion in the direction of x) has the expression:

$$\frac{dC}{dt} = D\frac{\partial^2 C}{\partial x^2} \tag{3.1.34}$$

In Eq. 3.1.34, t is time, C is concentration expressed in units of mass per units of length, and D is the diffusion coefficient of the diffusing species in a specific solvent, and at a specific temperature, expressed typically in $\mathrm{cm^2 s^{-1}}$ (for a specific compound X, the concentration is indicated as C_X, and the diffusion coefficient as $D_{X,A}$ where A indicates the solvent). Fick's law can be directly applicable, for example, to the diffusion in a tube or a rectangular channel where the concentration C varies only along the channel length and is the same across the channel. On the basis of the assumption that for $t = 0$ (initial condition), the concentration is described by a given function $C(x,0)$, the solution of Eq. 3.1.34 can be written as follows (e.g., Ref. [13]):

$$C(x,t) = \frac{1}{\sqrt{\pi D t}} \int_{-\infty}^{+\infty} C(\eta, 0) \exp\left[\frac{-(\eta - x)^2}{4Dt}\right] d\eta \tag{3.1.35}$$

With the assumption that D is constant, and the whole amount q_{inj} of material was initially injected (at $t = 0$) and is contained in one point

at $x = 0$, upon integration, Eq. 3.1.35 leads to the following expression (e.g., Ref. [14]):

$$C(x,t) = \frac{q_{inj}}{2\sqrt{\pi Dt}} \exp\left(\frac{-x^2}{4Dt}\right) \qquad (3.1.36)$$

In Eq. 3.1.36, t is the time of diffusion, x is the distance from the initial point of application of the amount q_{inj}, and C is a mass per length (since D is expressed in cm^2/s, C is expressed in mass/cm). Variable $C(x,t)$ can be considered equivalent with an instantaneous concentration. A common notation is introduced in Eq. 3.1.36:

$$\sigma_L^2 = 2Dt \qquad (3.1.37)$$

(σ_L is expressed in *length* since D is expressed in cm^2/s). With this notation, the formula for $C(x,t)$ (where $t = \text{const.}$ and C depends on t through σ_L) can be written as follows:

$$C(x,t) = \frac{q_{inj}}{\sqrt{2\pi\sigma_L^2}} \exp\left(\frac{-x^2}{2\sigma_L^2}\right) \qquad (3.1.38)$$

Eq. 3.1.38 characterizes a typical Gaussian bell curve (as a function of x while t is fixed but part of the value of σ_L), is called a normal probability density function, and is used to describe a random process. For example, the observational random errors in an experiment are assumed to follow such a normal distribution. The parameter σ_L^2 (also noted simply σ^2) is called the variance, and σ (or σ_L) is called standard deviation. The parameter σ_L describes the width of the Gaussian curve, larger σ_L leading to wider bell shapes as seen in Fig. 3.1.1 where the ratio $C(x)/q_{inj}$ as a function of x is plotted.

The graphs show the Gaussian bell shape of the concentration distribution. The whole amount q_{inj} of material was initially contained at $x = 0$. The apex of the Gaussian curve is obtained for $x = 0$ and $C_{max}/q_{inj} = (2\pi\ \sigma_L\ ^2)^{-1/2}$. The value for migration distance x corresponding to a concentration C, with $0 < C < C_{max}$,

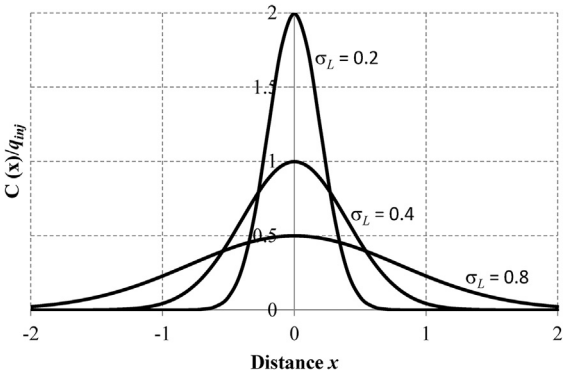

FIGURE 3.1.1 The variation of $C(x)/q_{inj}$ as a function of distance x for three values of σ_L, namely $\sigma_L = 0.2$ (corresponding for example to $D = 10^{-2}$ and $t = 2$), $\sigma_L = 0.4$, and $\sigma_L = 0.8$.

can be obtained based on Eq. 3.1.38, using the following formula:

$$x = \pm\sqrt{-2\sigma_L^2 \ln\left[\left(C/q_{inj}\right)\sigma_L\sqrt{2\pi}\right]} \qquad (3.1.39)$$

From Eq. 3.1.39, the bell width $W = 2|x|$ is obtained as an increasing function of σ_L. Of particular importance in chromatography are the width at half height W_h and the width at the inflection point W_i of the Gaussian curve. Taking $C = 0.5$, $C_{max} = 0.5\ m\ (2\pi\ \sigma_L^2)^{-1/2}$ in Eq. 3.1.39, the following result is obtained for the Gaussian width (σ_L is sometimes indicated simply as σ):

$$W_h = 2(2\ln 2)^{1/2}\sigma_L \qquad (3.1.40)$$

From the second derivative (as a function of x) of Eq. 3.1.38 (that gives the inflection of Gaussian curve), the value for W_i is obtained as follows:

$$W_i = 2\sigma_L \qquad (3.1.41)$$

The value for W_i for a given compound in a specific mobile phase can be obtained from Eq. 3.1.37 using the value for D and the diffusion time. As an example, for the diffusion of aniline in water at 25°C, $D = 1.05\ 10^{-5}\ cm^2/s$. For a diffusion time of 5 min, $W_i \approx 1.6$ mm.

Up to this point, the diffusion process was considered for a "static material" that diffuses

in one dimension and formula 3.1.38 gives the concentration C as a function of distance x from the point of application. However, in a chromatographic process, the compound of amount q_{inj} not only diffuses (along the length x), but also advances (is eluted) along the chromatographic column due to the movement of the mobile phase. At the same time as the analyte is diffusing, it is also moved with the distance ξ. The expression for the concentration C, as a function of the distance x from the point of application, after the diffusion zone was eluted with the distance ξ, is the following:

$$C(x) = \frac{q_{inj}}{\sqrt{2\pi\sigma_L^2}} \exp\left(\frac{-(x-\xi)^2}{2\sigma_L^2}\right) \qquad (3.1.42)$$

Eq. 3.1.42 can be used for the understanding of peak broadening in a chromatographic process. For a separation where the mobile phase has a linear flow rate u, the distance from the origin to the center of the moving zone is $\xi = u\, t_R$. Therefore, from Eq. 3.1.37 for a given ξ, the resulting σ_L^2 is given by the following expression:

$$\sigma_L^2 = 2D\frac{\xi}{u} = 2Dt_R \qquad (3.1.43)$$

Formula 3.1.43 shows that at larger values for the distance ξ (equivalent to larger t_R), the value of σ_L^2 is larger. Eq. 3.1.42 for C as a function of x (from the application point) is in this case the following:

$$C(x) = \frac{q_{inj}}{2\sqrt{\pi D t_R}} \exp\left(\frac{-(x-u\,t_R)^2}{4Dt_R}\right) \qquad (3.1.44)$$

The same Gaussian curves shown in Fig. 3.1.1, but including the movement with the distance ξ (due to the moving of the diffusing band as the mobile phase flows), are shown in Fig. 3.1.2.

For a chromatographic process, the concentration $C(x)$ in Eq. 3.1.42 can be replaced with the peak height $h(x)$, as generated by the response to instant concentration of a chromatographic detector. The variation of h as a function of distance x from start is described in this case by the following equation:

$$h(x) = \frac{A_{peak}}{\sqrt{2\pi\sigma_L^2}} \exp\left(\frac{-(x-\xi)^2}{2\sigma_L^2}\right) \qquad (3.1.45)$$

In Eq. 3.1.45, A_{peak} is the total peak area (used for quantitation in HPLC), ξ is the distance to the middle of the moving zone (and the maximum of the Gaussian curve), and σ_L determines the extent of peak broadening.

The broadening in units of length of the chromatographic peak previously discussed should now be converted into a broadening in units of time. In the chromatographic processes, the measured parameter is the retention time and not the length of the path in the column, and for this reason, σ_L (function of distance) should be replaced with a σ_t a function of *time* (time broadening). This time broadening is given by the following formula:

$$\sigma_t = \frac{\sigma_L}{u}\frac{t_R}{t_0} = \frac{\sigma_L}{L}t_R \qquad (3.1.46)$$

With this replacement, Eq. 3.1.44 will become

$$C(t) = \frac{q_{inj}}{\sqrt{2\pi\sigma_t^2}} \exp\left(\frac{-(t-t_R)^2}{2\sigma_t^2}\right) \qquad (3.1.47)$$

In Eq. 3.1.47, $C(t)$ is expressed in units of mass per time. By replacing σ_L with σ_t, Eq. 3.1.47 will give the variation of $h(t)$ (as peak height per time) by the following formula:

$$h(t) = \frac{A_{peak}}{\sqrt{2\pi\sigma_t^2}} \exp\left(\frac{-(t-t_R)^2}{2\sigma_t^2}\right) \qquad (3.1.48)$$

The maximum instantaneous concentration along the chromatographic peak is obtained for $t = t_R$. At this point, the concentration (measured by the detector) is given by the following formula:

$$C_{max} = \frac{q_{inj}}{\sigma_t\sqrt{2\pi}} \qquad (3.1.49)$$

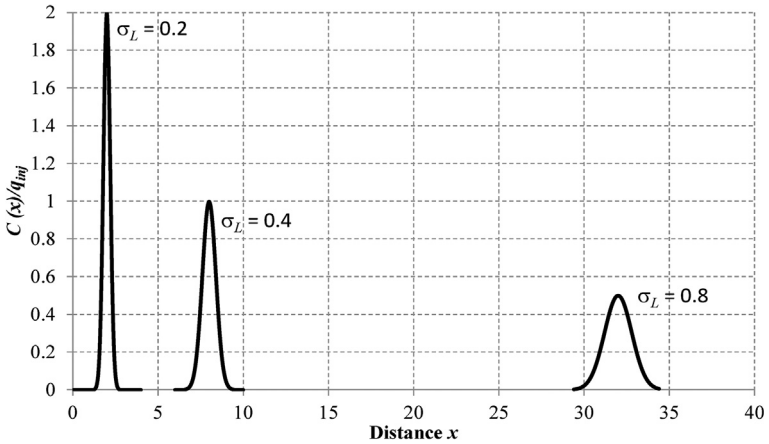

FIGURE 3.1.2 Peak broadening (in units of length) of an eluting analyte along a chromatographic column with the same σ_L values as those shown in Fig. 3.1.1: $\sigma_L = 0.2$, $\sigma_L = 0.4$, and $\sigma_L = 0.8$. These values for σ_L can be obtained, for example, for $D = 0.01$ and $\xi/u = 2$, 4, and 32, respectively (arbitrary units).

The maximum (instantaneous) concentration C_{max} in the effluent passing the detector in chromatography is translated into a maximum height h_{max} of the chromatographic peak, the height being also dependent on instrument sensitivity.

Similar to peak broadening as a function of distance x (expressed in units of length), a peak broadening in units of time can be measured. Time is typically the x-axes of a chromatogram, and broadening must be described in time units. For example, half height width W_h and the width at the inflection point W_i of the Gaussian curve are needed in units of time. Also, a width at the baseline $W_b = 2\,W_h$ is commonly measured in time units. The peak width W_b is measured between the points of intersection of baseline with the tangents to the curve at the inflexion points to the Gaussian curve representing the chromatographic peak. Fig. 3.1.3 shows the measurements of t_R, W_i, W_h, and W_b on a (model) chromatographic peak having the x-axis expressed as time.

The calculation of σ_t from Eqs. 3.1.40 and 3.1.41 generates the following formulas (where σ_t, W_h, W_i, and W_b are in time units):

$$\sigma_t = (8 \ln 2)^{-\frac{1}{2}} W_h \approx 0.5\, W_i = 0.25\, W_b \quad (3.1.50)$$

Broadening of the chromatographic peaks is not caused only by diffusion. Other processes with random character also contribute to peak broadening. These processes also produce a Gaussian bell curve distribution of retention times of individual molecules around the apex of the chromatographic peak, and the theory previously developed for diffusion remains valid with the difference that formula 3.1.43 for ${\sigma_L}^2$ (and consequently σ_t^2) is not anymore valid. At the same time, Eqs. 3.1.47 and 3.1.50 remain valid with the condition that the correct σ (σ_L or σ_t) values are used. The variance σ^2 combining all the random processes contributing to peak broadening can in this case be written as follows:

$$\sigma^2 = \sum_n \sigma_n^2 \quad (3.1.51)$$

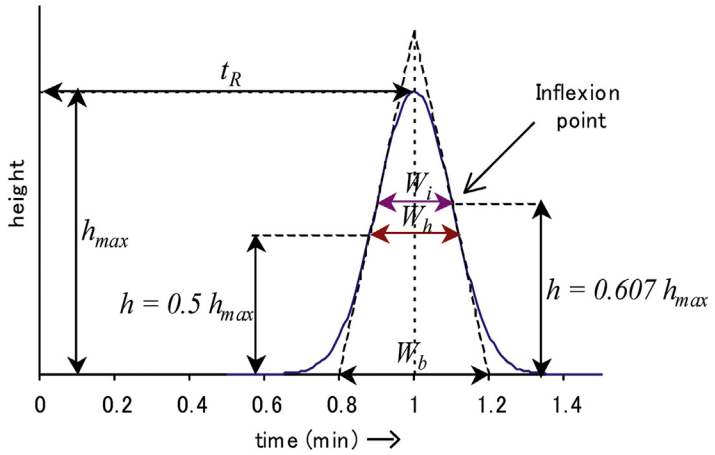

FIGURE 3.1.3 Measurements of retention time t_R and peak broadening W_i, W_h and W_b on a (model) chromatographic peak with time as x-axes.

where σ_n^2 are the variances generated by each of these random independent processes. All the broadening effects should also be included in σ_t from relation 3.1.50 that relates σ_t to the peak broadenings W_h, W_i, and W_b (expressed in time units). For example based on Eq. 3.1.50, the expression for W_b can be written as follows:

$$W_b = 4\sigma_t = 4\sqrt{\sum_n \sigma_n^2} \tag{3.1.52}$$

Efficiency of a chromatographic column

The value of σ_L (space broadening) is related to another parameter used to characterize zone spreading, namely the *height equivalent to a theoretical plate H* (HETP), which is defined as follows:

$$H = \frac{\sigma_L^2}{L} \tag{3.1.53}$$

This parameter is very useful in chromatography for the characterization of peak broadening per unit length of the column. Expressed as a function of time, formula 3.1.53 can be written in the following form:

$$H = \frac{\sigma_t^2 L}{t_R^2} \tag{3.1.54}$$

In addition to H, the peak broadening characterization in a column can be obtained using the *theoretical plate number N*. For a column of length L, N is defined as follows:

$$N = \frac{L}{H} \tag{3.1.55}$$

The theoretical plate number N can be expressed as a function of length and σ_L^2 by a simple substitution of Eqs. 3.1.53 in 3.1.55 to give the following expression:

$$N = \frac{L^2}{\sigma_L^2} = \frac{L^2}{2Dt_R} \tag{3.1.56}$$

The value of σ_L^2 in Eq. 3.1.56 is expressed in length can be further related to peak broadening in time W_b. For this purpose, σ_L will be replaced with σ_t using Eq. 3.1.46, and L will be expressed as function of time as $L = t_0\ u$. With these two substitutions in Eq. 3.1.56, the following formula for N is obtained:

$$N = \frac{t_R^2}{\sigma_t^2} = 16\frac{t_R^2}{W_b^2} \tag{3.1.57}$$

Similar relations can be established between N and W_i or W_h (time units) with the exchange of coefficient 16 with 4 or 5.5452, respectively. The value of σ_t can also be translated in a volume

$\sigma_V = \sigma_t\ U$ and for a column with diameter $d = 2.1$ mm and $L = 50$ mm values $\sigma_V^2 < 20\ \mu L^2$ are considered as providing good efficiency.

In addition to the theoretical plate number N, an effective plate number $\boldsymbol{n}$ is defined by using t'_R in Eq. 3.1.57 instead of t_R. The formula for $\boldsymbol{n}$ will be

$$\boldsymbol{n} = 16\frac{t'^2_R}{W_b^2} \tag{3.1.58}$$

From Eq. 3.1.57 and 3.1.58, the relation between $\boldsymbol{n}$ and N can be written as follows:

$$\boldsymbol{n} = N\frac{t'^2_R}{t_R^2} \tag{3.1.59}$$

The value for $\boldsymbol{n}$ is smaller than that for N since $t'_R < t_R$. Since t_R (and t'_R) as well as W_b are chromatographic parameters dependent on the eluting compound (index X was omitted in previous formulas), the vale for N and $\boldsymbol{n}$ are also compound dependent. Both Eq. 3.1.57 or 3.1.58 can be used to measure the theoretical plate number or effective plate number based on experimental data obtained with a given column. This measurement is useful in practice to select columns (higher N gives lower peak broadening) and also to assess the loss in performance of a column after a certain period of usage when the N values start to decrease. Because N is related to the important characteristic of peak broadening, it is common to indicate it as a parameter to characterize the efficiency of a column. The values for N for HPLC columns can be given either for a specific column or reported as efficiency per meter (N/m). Both N and $\boldsymbol{n}$ are used for the characterization of *column efficiency*, and $\boldsymbol{n}$ is sometimes named simply *efficiency*.

For modern HPLC analytical columns the efficiency per meter N/m can be between 20,000 and 150,000. In HPLC, depending on the nature of the column, it can vary (per m) between 40,000 and 120,000 (or even higher), and for core-shell columns can be as high as 300,000 (common column length L is between 50 and 250 mm) (see Section 8.1). The values for N (per unit length of the column) are influenced by physical properties of the stationary phase such as dimension of stationary phase particles, homogeneity of the particles dimensions, and structure of particles.

Factors contributing to peak broadening and van Deemter equation

As indicated by Eq. 3.1.51, besides the longitudinal diffusion, a number of effects are contributing to peak broadening. These effects include (1) longitudinal diffusion (already discussed, producing the variance σ_L), (2) eddy diffusion, (3) lateral movement of molecules due to convection, (4) the differences between individual molecules in the mass transfer rate in and out the stationary phase, and (5) presence of random spots of stagnant mobile phase in the porous material of the column (mass transfer in and out mobile phase). The inclusion of the contribution of all these factors to the value of plate height H (HEPT) requires the replacement of Eq. 3.1.53 with a new formula of the following form:

$$\begin{aligned} H &= \frac{\sigma_L^2 + \sigma_E^2 + \sigma_C^2 + \sigma_T^2 + \sigma_S^2}{L} \\ &= H_L + H_E + H_C + H_T + H_S \end{aligned} \tag{3.1.60}$$

Each term in Eq. 3.1.60 can be evaluated and also experimental procedures were developed for their measurement [15]. The value of H_L can be obtained in theory from Eqs. 3.1.37 and 3.1.53 but since the diffusion medium is not homogeneous and the packing material is "obstructing" to a certain extent the diffusion, the value for H_L should include a correction factor γ such that the corrected formula becomes the following:

$$H_L = \gamma\frac{2Dt}{L} = \gamma\frac{2D}{u} \tag{3.1.61}$$

The value for γ depends on the column packing and is typically around 0.625. The value for diffusion coefficient D of the diffusing species in a specific solvent depends on both the nature of the analyte and of the solvent. During the gradient

HPLC, the value of D may change for a given solute. This change may contribute to a (small) variation of H_L across a peak that elutes in gradient conditions, contributing to deviations from a Gaussian peak shape. The values for the diffusion coefficient D (in cm^2s^{-1}) are reported in the literature for various solutes and solvents [16].

Eddy diffusion is caused by the fact that in a packed material the flow occurs through a tortuous channel system with various path lengths. The molecules of the same solute may randomly take different paths. This path will depend on the average diameter of a particle, and using the notation d_p for an average value for the particle diameter in the stationary phase, the contribution to the plate height of the eddy diffusion can be written in the following form:

$$H_E = \Lambda d_p \tag{3.1.62}$$

The coefficient Λ depends on how irregular the particle shape is and also on other packing characteristics.

Another random process contributing to peak broadening is the lateral movement of material due to convection, which is stronger at higher linear flow rates u of the mobile phase. Convection depends on column packing (through a parameter Γ) and increases when the particle diameter d_p increases, decreasing when the diffusion coefficient D increases. The contribution to the plate height for convection is given by the following expression:

$$H_C = \frac{\Gamma d_p^2}{D} u \tag{3.1.63}$$

The rate of transfer of solute into and out of the stationary phase is controlled by the rate of diffusion in the liquid stationary phase or by the adsorption–desorption kinetics in the case of adsorption processes. It can be shown using a random walk model that, for a distribution process, the contribution to the plate height H_T due to this effect can be expressed by the following formula:

$$H_T = \Theta \frac{k'}{(1+k')^2} \frac{d_f^2}{D_s} u \tag{3.1.64}$$

In formula 3.1.64, Θ is a proportionality constant (estimated as $\Theta = 1/30$), d_f is the depth of the stationary phase on the solid support, D_s is the diffusion coefficient of the analyte in the stationary phase, and k' is the retention factor for the solute.

The contribution to the plate height from the mass transfer in the stagnant mobile phase in the porous material is given by an expression similar to 3.1.64 where the parameter Θ is about the same, but d_f should be replaced by d_p (diameter of the particle which is related to the depth of the pores), and D_s should be replace by D the diffusion coefficient in the mobile phase. This effect, known as mobile phase mass transfer contribution, gives the following increase H_S to the theoretical plate height:

$$H_S = \Theta \frac{k'}{(1+k')^2} \frac{d_p^2}{D} u \tag{3.1.65}$$

The combination of all the above contribution leads to the following formula for the plate height:

$$H = \Lambda d_p + \gamma \frac{2D}{u} + \left[\Gamma \frac{d_p^2}{D} + \Theta \frac{k'}{(1+k')^2} \left(\frac{d_f^2}{D_s} + \frac{d_p^2}{D} \right) \right] u \tag{3.1.66}$$

Eq. 3.1.66 gives a general formula describing the dependence of H (HEPT) on the linear flow rate u and on various parameters related to the stationary phase and mobile phase. This

equation is known as van Deemter equation [17] and can be written in the following form:

$$H = A + \frac{B}{u} + Cu \qquad (3.1.67)$$

The expression for the coefficients A, B, and C in Eq. 3.1.67 can be obtained by comparison with the equivalent Eq. 3.1.66. An example of a plot for the van Deemter equation for $A = 4\ \mu m$, $B = 500\ \mu m^2/s$, and $C = 0.0005\ s$ is given in Fig. 3.1.4.

An equivalent formula with 3.1.67 in which the diffusion coefficient D and d_p the average value for the particle diameter are separated from the constants A, B, and C can be written as follows:

$$H = A' d_p + B' \frac{D}{u} + C' \frac{d_p^2}{D} u \qquad (3.1.68)$$

(the value of d_f is small and can be considered as dependent on d_p). This formula shows the explicit contributions of the diffusion coefficient D and of the dimension of the particles of the stationary phase d_p. For a chromatographic column of length L, with $N = L/H$, Eq. 3.1.68 can be used to evaluate the dependence of column plate number on particle diameter d_p.

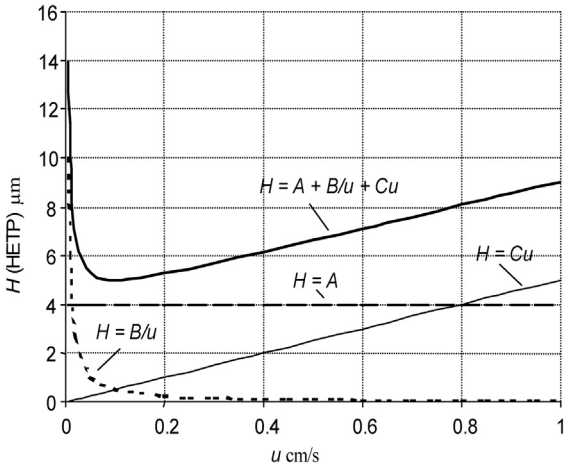

FIGURE 3.1.4 The plot of van Deemter equation and of its components for $A = 4\ \mu m$, $B = 500\ \mu m^2/s$, and $C = 0.0005\ s$.

The shape of dependence of H on u given by van Deemter equation indicates that a minimum value for H is obtained at a specific u value that can be obtained from the condition $dH/du = 0$, leading to the following expression:

$$u_{opt} = \frac{D}{d_p}\sqrt{\frac{B'}{C'}} \qquad (3.1.69)$$

For the optimum volumetric flow rate, Eq. 3.1.69 leads to the following expression:

$$U_{opt} = \frac{\pi \varepsilon^* d^2 D}{4 d_p}\sqrt{\frac{B'}{C'}} \qquad (3.1.70)$$

Eq. 3.1.70 indicates that U_{opt} for a chromatographic column is larger when the column diameter d is larger, the diffusion coefficient D is larger, and when the particle diameter d_p is smaller. Other column construction characteristics are included in parameters ε^*, B', and C'. Including the formula for u_{opt} in Eq. 3.1.68, the expression for H_{min} for a chromatographic column is the following:

$$H_{min} = d_p(\Lambda + 2\sqrt{B'A'}) \qquad (3.1.71)$$

It can be roughly estimated that $\Lambda + 2\sqrt{B'A'} \approx 2\ to\ 3$, and $H_{min} \approx (2{-}3)\ d_p$. A too slow flow or an increase in flow rate beyond u_{opt} leads to a decrease of column performance due to the increase in H. Faster flows than u_{opt} (and U_{opt}) may be sometimes needed for generating shorter run times, but they should not exceed too much U_{opt} in order to not decrease too much the theoretical plate number N. Nevertheless, some ultrafast HPLC separations at high flow rates are reported in the literature. For example, the enantiomers of the herbicide haloxyfop are separated in 3 s on a short 2 cm column containing teicoplanin as chiral selector using 8 mL/min flow rate [18].

Various experimental techniques were developed for evaluating the different contributions to band broadening such as the pulse response or the peak parking [19]. In peak parking

technique, for example, an injection is performed in the HPLC system and before the elution of the compound the flow is stopped for a specific period of time, followed by the elution of the compound. The broadening of the peak depends on the "parking" time, and from the peak broadening, various parameters of the diffusion in static condition are evaluated. These experimental attempts to verify Eq. 3.1.67 showed that some deviations from the theoretical model occur. The main explanation for this effect is that eddy diffusion and mobile phase mass transfer are not totally independent effects. The differences in the intraparticle velocity for the local streams affect the mobile phase mass transfer, and in return, the eddy diffusion. The coupling of the two processes is captured in a different expression for H, given by Knox equation [20]. Knox equation is typically written using "reduced" dimensionless parameters defined as follows:

$$h = \frac{H}{d_p} \quad and \quad v = \frac{u\,d_p}{D} = \frac{4Ud_p}{\pi\varepsilon^* D d^2} \tag{3.1.72}$$

with these notations, Knox equation is written in the following form:

$$h = A\,v^{1/3} + B''\frac{1}{v} + C''v \tag{3.1.73}$$

Knox curves have, as expected, similar features with van Deemter curves, but at higher flow rates the increase of h (and therefore of H) is less pronounced. Even Knox equation does not account for all the effects contributing to peak broadening. For example, it was shown that the mobile phase viscosity also influences some of the processes previously considered [21], and this aspect is not included (in explicit form) in Eq. 3.1.67 or Eq. 3.1.73. The implicit dependence of H on the mobile phase viscosity results from the fact that the diffusion coefficient D is dependent on liquid phase viscosity.

Various other studies were reported for the evaluation of plate height H (or its reduced form h) (e.g., Ref. [22]). Similar to the deduction of van Deemter equation the results can be obtained as solutions of a differential mass balance equation that accounts for the processes of analyte diffusion and convection in the analytical column having the following form:

$$\frac{\partial C_{mo}}{\partial t} + \frac{1-\varepsilon^*}{\varepsilon^*}\frac{\partial C_{st}}{\partial t} + u\frac{\partial C_{mo}}{\partial x} = D\frac{\partial^2 C_{mo}}{\partial x^2} \tag{3.1.74}$$

In Eq. 3.1.74, t is time, x is the distance of migration in the chromatographic column, and the other parameters as previously defined (ε^* the column packing porosity, C_{mo} the concentration of the analyte in the mobile phase, C_{st} the concentration in stationary phase, u linear velocity of the mobile phase, D diffusion coefficient of the analyte). Various solutions of Eq. 3.1.74 are described in the literature [23,24]. One such solution generates a value for H similar to Eq. 3.1.66. The formula for H was also developed for columns with core-shell particles [25] and for monolithic columns [26].

Besides the effects previously discussed to produce peak broadening, in practice, the chromatographic peaks can also be broadened by other causes. Such causes may include the presence of void volumes in the chromatographic system before the sample reaches the stationary phase. Also, larger injected sample volumes lead to peaks broadening such that the ideal theoretical plates number decreases compared to its optimum value obtained for a very narrow injection. Besides peak broadening, an experimental peak compression is also possible, but this compression does not affect the five effects (longitudinal diffusion, eddy diffusion, etc.) previously discussed. The peak compression may compensate for effects such as large injection volumes and void volumes in the HPLC system. This is achieved by the accumulation of the analytes at the front end of the column, before the elution process starts by using a sample solvent and an initial mobile phase that does not elute the sample from the column head (see Section 7.6).

The variation of theoretical plate number N as a function of dead time t_0 can be obtained by replacing in van Deemter equation the values

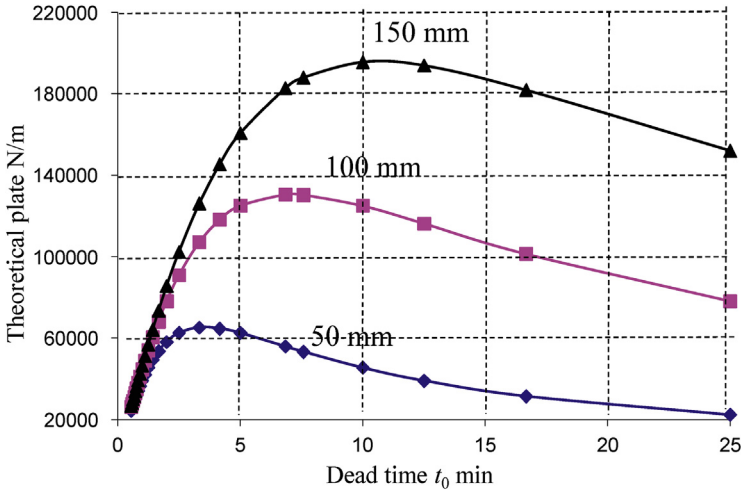

FIGURE 3.1.5 Dependence of the number of theoretical plates N/m for a column as a function of the dead time t_0 for three columns 50, 100 and 150 mm long with irregular particles of 5 μm.

$u = L/t_0$ and $H = L/N$. With these substitutions, van Deemter equation can be written in the following form:

$$N = \frac{L^2 t_0}{ALt_0 + Bt_0^2 + CL^2} \tag{3.1.75}$$

Such a dependence is shown in Fig. 3.1.5 for three columns 50, 100, and 150 mm long with irregular particles of 5 μm.

As shown in Fig. 3.1.5, the optimum number of theoretical plates is obtained at different dead times and therefore at different flow rates [27]. These optimums can be unacceptable for a practical separation. For example, for the 150 mm column length the optimum value for N is reached at about 12.5 min and a compound with retention factor $k' = 5$ will elute at about 50 min. This range of retention time is usually unacceptable. For this reason, a higher flow rate is preferable even if the number of theoretical plates is diminished. However, the flow rate in a chromatographic column cannot be increased indefinitely, being limited by the increase in the pressure drop across the column Δp_{max}. The difference Δp between the pressure at the column inlet and that at the outlet of the column (filled with porous particles) when the linear flow is u is given by the following expression (Darcy equation):

$$\Delta p = \frac{\eta u \phi_r L}{d_p^2} = \frac{\eta \phi_r L^2}{d_p^2 t_0} \tag{3.1.76}$$

where η is the mobile phase viscosity, L is column length, d_p is the diameter of the particles in the bed, and ϕ_r is the column flow resistance factor. This type of variation of Δp as function of the dead time t_0 (for $\eta = 2$ mPa/s and $\phi_r = 1$ mm^2) is shown in Fig. 3.1.6 for the same three columns considered for Fig. 3.1.5.

By setting a limiting backpressure for example to about 300 bar (30,000 kPa), it is possible to calculate the limit of how low the dead time can be set. For obtaining information on N at this limit of dead time (back pressure) for different columns (different length or different stationary phases), it is necessary to generate graphics displaying the variation of N for the points where each column reaches the limiting backpressure. These graphs are indicated as kinetic plots. One type of kinetic plot is Poppe plot [28]. This is a plot of $\log(t_0/N)$ as a function of $\log N$ where t_0 is selected for a specific Δp

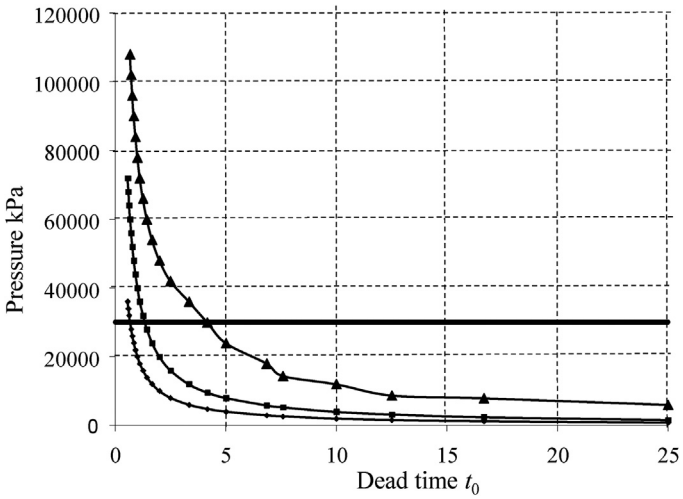

FIGURE 3.1.6 Variation of back pressure (pressure drop across the column) at different dead times t_0 for the three columns 50, 100, and 150 mm long with irregular particles of 5 μm. A horizontal line is shown for 300 bar.

value (maximum acceptable for the evaluated system). Other similar types of kinetic plots were reported in the literature [29]. Since the maximum acceptable column backpressure Δp for a common silica-based column can be set relatively high (e.g., 300 bar or for UPLC 500−600 bar), the kinetic plots do not have a high utility. However, in size exclusion chromatography, the maximum acceptable pressure is typically lower since this technique uses stationary phases that can collapse at higher pressures. For this reason, kinetic plots in SEC are more frequently useful.

Application of van Deemter equation

Van Deemter equation can be practically utilized for determining the optimum flow rate in a column in order to achieve the maximum performance regarding the theoretical plate number N, and also to verify the performance of a column when new or after a number of injections during its utilization. For the verification of the performance of a column, a "test mixture" of compounds can be used in specified conditions. An example for such a test mixture used for the performance evaluation of reverse phase columns contains 6 μg/mL uracil, 10 μg/mL toluene, 25 μg/mL fluorene, and 40 μg/mL fluoranthene. The separation is recommended in isocratic conditions, 30% water and 70% acetonitrile. The separation should be repeated at several flow rates (e.g., between 0.4 and 2.0 mL/min with a step increase of 0.2 mL/min), and the parameters t_0, t_R, and W_b are measured on the chromatograms (t_0 is typically taken from the retention time of uracil that is assumed not retained on the column). From the length of the column, the linear flow rate is calculated using $u = L/t_0$, the theoretical plate number N is calculated using Eq. 3.1.57, and H (HETP) is calculated using Eq. 3.1.55. The graph representing H as a function of u can be obtained. Having three different compounds in the test mixture, the values obtained for N and H will be slightly different, but they should not differ considerably. The results of such an experiment are shown as an example in Fig. 3.1.7 for a Zorbax Eclipse XDB-C8 column, 3 μm particle size, 150 mm length, and 4.6 mm i.d. The column was kept at 25°C. The peak detection was done using UV absorption at 254 nm. The resulting chromatograms are shown in Fig. 3.1.7 (only

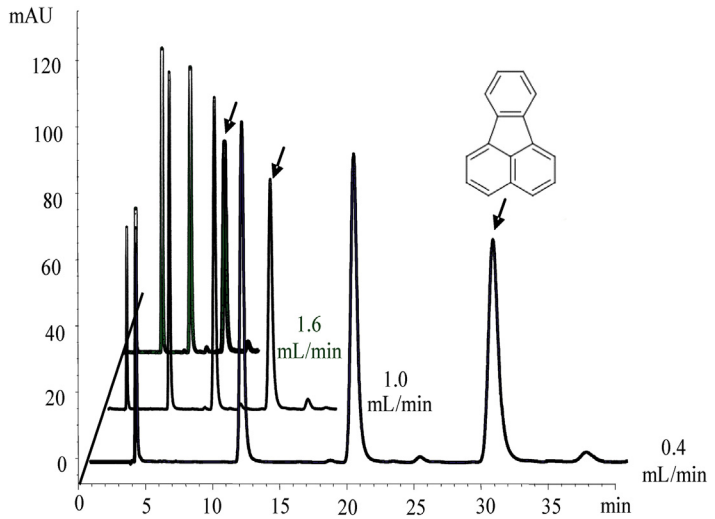

FIGURE 3.1.7 Example of chromatograms obtained at different flow rates from a test mixture for the verification of a Zorbax Eclipse XDB-C8 new column performance in isocratic conditions, using van Deemter equation (see text for conditions).

three traces shown from seven measured). The experiment was performed for the new column and also after 800 injections of biological samples.

Following the calculation of u from the retention time of uracil for different volumetric flow rates U and calculation of H from the broadening W_b of fluoranthene peak, the results were plotted and the generated graph is shown in Fig. 3.1.8.

The results shown in Fig. 3.1.8 indicate that an optimum value for u is around 6.75 cm/min. Assuming for the column $\varepsilon^* \approx 0.7$, Eq. 3.1.3 gives an optimum $U \approx 0.8$ mL/min (the i.d. of the column is $d = 0.46$ cm). Some degradation of column performance after 800 injections of samples is also noticed, although the H value for the old column is still relatively close to the initial value. Optimization of flow rate based on van Deemter equation in order to obtain the best N values is not the only optimization of practical use. In practice, short run times are also desirable. For this reason, it is common that the flow rate in a column is selected higher than indicated by van Deemter equation, as long as the separation is still good, and the column back pressure acceptable. The flow rate is

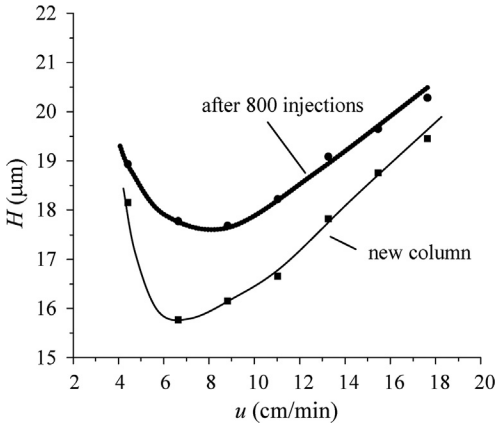

FIGURE 3.1.8 Plot of H as a function of u for a Zorbax Eclipse XDB-C8 column (see text for conditions).

typically limited by the column backpressure (or HPLC maximum pressure) and not by the deviation from maximum column efficiency. However, column selection and flow rate optimization can be combined for obtaining the optimum separation results [30].

Using $H = 15.5$ μm and $L = 150$ mm in Eq. 3.1.55, the resulting value for the theoretical plate number N is 9677 and the effective plate number

n can be obtained from Eq. 3.1.59 and (for fluoranthene) is about 7354. The column efficiency n per meter for the column is about 50,000 (indicating a good column). A higher n would be generated with an earlier eluting peak such as toluene. The effective plate height n or the theoretical plate height N is typically indicated by the manufacturer for commercially available chromatographic columns (as measured for a standard compound which is usually toluene or ethylbenzene for C18 columns). The value for N/m varies depending on the type of column. For C18 type columns, N/m varies between 60,000 and 120,000 for columns with 5 μm particles, but it is higher (up to 300,000) for columns with diameter d_p smaller than 2 μm, and also it is higher than 200,000 for columns with core-shell particles.

An estimation of N for common porous particles columns can be obtained using the following expression:

$$N = \frac{1000\,L}{Ct.\,d_p} \tag{3.1.77}$$

In Eq. 3.1.77, the constant $Ct.$ is taken between 2 and 3.5 depending on other characteristics of column construction.

Peak asymmetry

Theoretically, chromatographic peaks should have a Gaussian shape. However, some analytes are present in the mobile phase in more than one form, such as molecular and ionic (e.g., for acids or for amines). These different species may interact differently with the stationary phase, in particular when the retention does not occur based on a unique mechanism. This different retention generates asymmetrical peaks. The peak asymmetry may also contribute various minor interactions that cannot affect at the same time all the molecules of the analyte, column contaminations, column overload with sample, etc. Two parameters are used for the characterization of peak asymmetry. One is the *asymmetry parameter* $As(X)$ and the other is *tailing factor* $TF(X)$, both dependent on the nature of the analyte X. The asymmetry $As(X)$ is defined as the ratio of the rear r versus front f segments cut on the chromatographic peak by a parallel to the baseline at 10% peak height (some other choices are possible), and separated by the perpendicular from the apex, and it is given by the following formula:

$$As(X) = \frac{r}{f} \tag{3.1.78}$$

The rear r and front f segments cuts at 10% of the height are shown on an asymmetrical peak in Fig. 3.1.9.

The peak tailing is defined by the following formula:

$$TF(X) = \frac{f' + r'}{2r'} \tag{3.1.79}$$

where f' and r' are measured in the same way as f and r, but at 5% of the peak height. Peak asymmetry may be very different from compound to compound, since the interaction with the stationary phase may involve different types of interactions depending on the compound nature.

Another parameter used for the description of peak asymmetry is the skew. This parameter takes into consideration that the Gaussian shape of a chromatographic peak (with variance σ_t^2) is due to the random spreading of the injection plug in the chromatographic column, but a modification is necessary for accounting for other broadening effects. This modification is based on an exponential decay of the form $h(t) = h(t_0)\exp(-t/\tau)$ and depends on a parameter τ which is the mean lifetime (time at which the initial value is reduced by the factor $1/e$). Skew is defined by an expression depending on the ratio σ_t/τ. Other functions besides Gaussian are also used for modeling the shape of chromatographic peaks [31], and peak characteristics can be calculated from these shapes. Deviation from Gaussian shape of the front of the chromatographic peak is typically indicated as *fronting*.

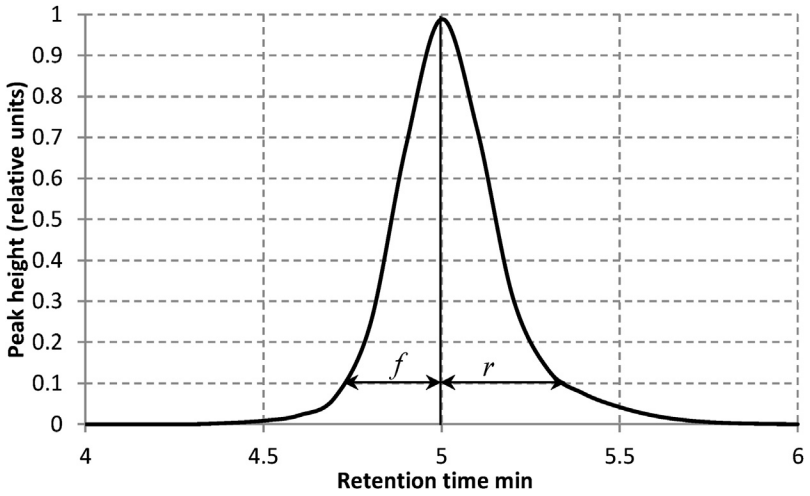

FIGURE 3.1.9 Front f and rear r in an asymmetrical chromatographic peak.

Statistical moments for the description of peak characteristics

Some of the characteristics of chromatographic peaks can be obtained using statistical methods. The Gaussian shape of the peak can be seen as generated by a population of points with random normal (Gaussian) distribution. The estimation of population parameters such as mean, variance, etc., can be done in statistics by calculating the *population moments*. For a discrete distribution of measurements, the momentum results from a sum. For a continuous distribution described by a Gaussian function, the momentum will be given by an integral. Zero momentum for the chromatographic peak is given by the integral:

$$\Omega_0 = \int_0^\infty h(t)\mathrm{d}t \tag{3.1.80}$$

where $h(t)$ is given by Eq. 3.1.45. It can be seen that Ω_0 is the area A_{peak} of the chromatographic peak since

$$\int_0^\infty \frac{1}{\sqrt{2\pi\sigma_t^2}} \exp\left(\frac{-(t-t_R)^2}{2\sigma_t^2}\right)\mathrm{d}t = 1 \tag{3.1.81}$$

Peak area is proportional with the amount of analyte and is the main parameter used for quantitation in HPLC.

The first momentum is given by the following expression:

$$\Omega_1 = \frac{1}{\Omega_0}\int_0^\infty th(t)\mathrm{d}t \tag{3.1.82}$$

It can be shown that the first momentum is in fact the retention time t_R ($\Omega_1 = t_R$). The first momentum gives the expected value of a random variable, and assuming that no spreading of the chromatographic peak would occur, the expected value of time where the peak would be formed is t_R.

Higher moments can be defined by the following formula:

$$\Omega_n = \frac{1}{\Omega_0}\int_0^\infty (t-\Omega_1)^n h(t)\mathrm{d}t \tag{3.1.83}$$

For example, the second momentum gives the variance and has the following formula:

$$\Omega_2 = \frac{1}{\Omega_0}\int_0^\infty (t-t_R)^2 h(t)\mathrm{d}t \tag{3.1.84}$$

Second momentum gives the value for σ_t^2 ($\Omega_2 = \sigma_t^2$). Also, it can be seen from Eq. 3.1.57 that the theoretical plate number N can be obtained from the following formula:

$$N = \frac{t_R^2}{\sigma_t^2} = \frac{\Omega_1^2}{\Omega_2} \tag{3.1.85}$$

The third momentum is zero for a perfect Gaussian peak (as well as all higher momentum values). However, for a real peak that deviates from the Gaussian shape being asymmetrical, the third momentum Ω_3 describes this peak asymmetry also known as *skew*. A positive value for Ω_3 indicates tailing. The formula for the skew is the following:

$$\Omega_3 = \frac{1}{\Omega_0}\int_0^\infty t^3 h(t)\mathrm{d}t - 3\Omega_0\Omega_2 + 2\Omega_0^3 \tag{3.1.86}$$

The fourth momentum (Ω_4) describes yet another property of peaks deviating from the perfect Gaussian shape indicating the extent of vertical flattening known as *excess* [32]. A positive value for Ω_4 indicates sharpening of the peak.

Peak characterization using exponentially modified Gaussian shape

The Gaussian shape of a chromatographic peak is due mainly to the random spreading of the injection plug in the chromatographic column. However, the dead volumes along the chromatographic system and the viscous flow also contribute to the shape of the chromatographic peak [33]. It was suggested that a better equation indicated as exponentially modified Gaussian (EMG) describes more accurately the true chromatographic peak shape [34–39]. In this modified function, a Gaussian function with the variance σ_t^2 is combined with an exponential decay depending on a parameter τ. A Gaussian, an exponential decay, and the combined curve are pictured in Fig. 3.1.10.

The EMG function describing the peak height has the following expression:

$$h(t) = A_{peak}\frac{\sigma_t}{\tau\sqrt{2\pi}}\exp\left[\frac{1}{2}\left(\frac{\sigma_t}{\tau}\right)^2 - \frac{t-t_R}{\tau}\right]\cdot \int_{-\infty}^{z}\exp\left(-\frac{\xi^2}{2}\right)\mathrm{d}\xi \tag{3.1.87}$$

In Eq. 3.1.87, the parameter z has the following expression:

$$z = \frac{t-t_R}{\sigma_t} - \frac{\sigma_t}{t} \tag{3.1.88}$$

In Eq. 3.1.86, A_{peak} is the peak area, σ_t is the standard deviation of the Gaussian peak, and τ is the time constant of exponential axis. Unfortunately, the calculation of $h(t)$ for any t along the chromatogram using the EMG function given

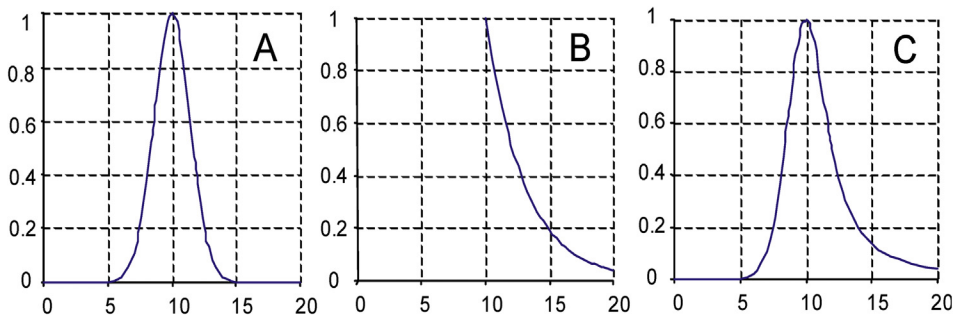

FIGURE 3.1.10 A Gaussian curve (A), an exponential decay (B), and the resulting combined curve (C).

by Eq. 3.1.86 is difficult. The expression from Eq. 3.1.86 can be obtained in terms of error function:

$$\mathrm{erf}(z) = \frac{2}{\sqrt{\pi}} \int_0^z \exp(-\xi)^2 \mathrm{d}\xi \qquad (3.1.89)$$

The values for error function allow an easier calculation, since erf (z) is tabulated and available in computer program packages [37].

From formula 3.1.86, different desired parameters for the chromatographic peak are obtained using the statistical moments (also calculated using computer packages), as indicated in the following formulas:

$$\Omega_1 = t_R + \tau \quad \Omega_2 = \sigma^2 + \tau^2 \quad \Omega_3 = 2\tau^3 \quad \Omega_4 = 3\sigma^4 + 6\sigma^2\tau^2 + 9\tau^4 \qquad (3.1.90)$$

From these expressions are calculated the following values:

$$skew = \frac{\Omega_3}{(\Omega_2)^{3/2}} \quad excess = \frac{\Omega_4}{(\Omega_2)^2} - 3 \quad N = \frac{(\Omega_1)^2}{\Omega_2} \qquad (3.1.91)$$

In practice, the calculation of these moments depends on the sampling rate of detecting system and needs baseline correction [40]. The use of EMG for the deconvolution (using appropriate computer programs) of the chromatographic peaks, in particular of those that are not well resolved, has been proven very efficient [39,41,42].

3.2 Parameters describing the separation

General aspects

In addition to various parameters used for peak characterization, a number of parameters are used for describing how good a peak separation is. In HPLC practice, the use of selective detectors such as MS may differentiate compounds based on their different mass (or mass and fragmentation in MS/MS detection) even if they are poorly separated chromatographically. In this section only, the parameters related to the chromatographic separation are presented.

Selectivity (separation factor)

Selectivity (or *separation factor*) is another empirical parameter used for column characterization and is related to the separation of two compounds X and Y. The notation for selectivity is usually α, and this parameter is defined by the following ratio:

$$\alpha(X,Y) = \frac{t'_R(Y)}{t'_R(X)} \qquad (3.2.1)$$

where $t'_R(Y) > t'_R(X)$. Parameter α indicates the ratio of the distances in time between the apexes of two chromatographic peaks (for compounds X and Y). The selectivity (separation factor) is usually of interest for compounds that give adjacent peaks, since peaks that are well distanced do not pose separation problems. Using Eq. 3.1.21, it can be easily noticed that α can also be expressed by the following formula:

$$\alpha(X,Y) = \frac{k'(Y)}{k'(X)} = \frac{K(Y)}{K(X)} \qquad (3.2.2)$$

In any chromatographic separation, larger α values are desirable for a better separation. However, the value of α alone cannot describe how good the separation of two compounds is. Even when α is large, peak broadening can be so large that the separation can be poor. The values for α are solute dependent, but also depend on the nature of the stationary phase and of the mobile phase. The chemical nature of the stationary phase is one of the two main factors influencing the separation for a given

set of analytes, the second main factor being the choice of mobile phase. For this reason, the choice of a chromatographic column is frequently based on its selectivity α toward the analytes being separated. A value for $\alpha > 1.2$ is typically necessary for an acceptable separation (provided the peaks are not very broad).

Resolution

Regardless how far apart the apexes of two chromatographic peaks are (as described by α), if the peaks are broad, their separation can be compromised. A parameter that truly characterizes peak separation is the *resolution* R. This parameter is defined by the following formula:

$$R(X,Y) = \frac{2[t_R(Y) - t_R(X)]}{W_b(X) + W_b(Y)} \tag{3.2.3}$$

The difference in the retention times $t_R(Y) - t_R(X) = \Delta t_R$ can be replaced with $t'_R(Y) - t'_R(X)$. The values for Eq. 3.2.3 can be obtained from the chromatogram as illustrated in Fig. 3.2.1. A good peak separation is typically considered acceptable when $R > 1.0$ and good when $R > 1.5$ when the two peaks are separated at the baseline ($2\,\Delta t_R > W_b(X) + W_b(Y)$).

The widths at the baseline of the two peaks can be different (as also shown in Fig. 3.2.1), but as an approximation it is possible to take $W_b(X) = W_b(Y) = W_b$. With these assumptions, the formula for R can be written in the following form:

$$R = \frac{\Delta t_R}{W_b} \tag{3.2.4}$$

The difference in retention time between two analytes Δt_R can be written as a function of selectivity α in the following form:

$$\Delta t_R = (\alpha - 1) t'_R(Y) \tag{3.2.5}$$

The expression for R with this substitution becomes the following:

$$R = (\alpha - 1) t'_R(Y)/W_b \tag{3.2.6}$$

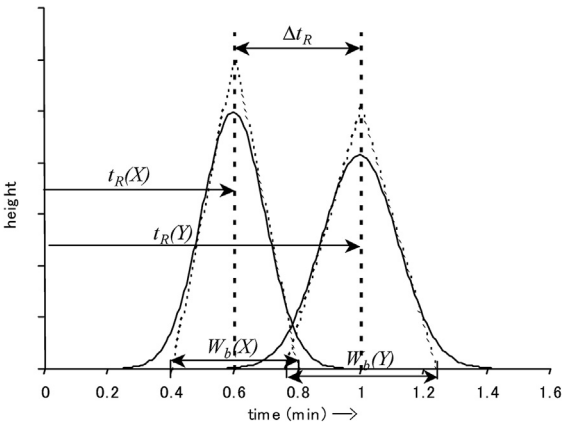

FIGURE 3.2.1 An idealized chromatogram showing the measurable parameters used for the calculation of resolution R.

The value for $t'_R(Y)$ can be replaced with $t_R(Y)$ using Eq. 3.1.23 to obtain the following formula:

$$R = (\alpha - 1)\frac{k'(Y)}{1 + k'(Y)}\frac{t_R(Y)}{W_b} \tag{3.2.7}$$

From Eq. 3.1.57, the value for $t_R(Y)/W_b$ can be expressed as a function of N, and the value for R will become the following:

$$R = \frac{1}{4}(\alpha - 1)\frac{k'(Y)}{1 + k'(Y)}N^{1/2} \tag{3.2.8}$$

Eq. 3.2.7 indicates the resolution relative to the "previous peak." Similar formula for R can be obtained relative to the "next peak" by using the following substitution:

$$\Delta t_R = \frac{(\alpha - 1)}{\alpha} t'_R(X) \tag{3.2.9}$$

In this case, the resulting formula for R for the "next" peak is the following:

$$R = \frac{1}{4}\frac{(\alpha - 1)}{\alpha}\frac{k'(X)}{1 + k'(X)}N^{1/2} \tag{3.2.10}$$

Both relations 3.2.8 and 3.2.10 are approximations and the two values should be close to each other. Both expressions are obtained by taking the peak width at the baseline as equal for the

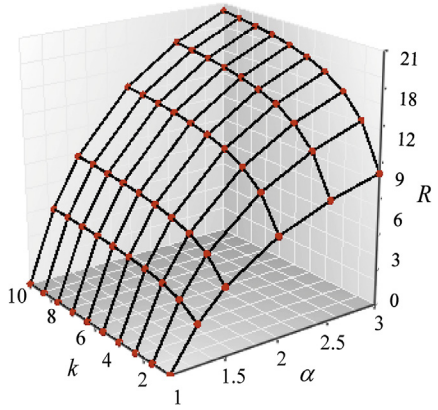

FIGURE 3.2.2 Graph showing the variation of R as a function of α and k' assuming a chromatographic column with $N = 18{,}000$.

two peaks, and the theoretical plate number N as measured for one compound (Y in Eq. 3.2.8) or for the other (X in Eq. 3.2.10). The value for R is most sensitive to the parameter α, which is critical for obtaining a good separation. Larger k' values are also useful, but as k' increases, its importance for the increase in R is diminished. This is exemplified in the diagram from Fig. 3.2.2, for a chromatographic column with $N = 18{,}000$.

From the dependence of R on α and k', it is simple to obtain the formula for the dependence of R on $K(X)$, $K(Y)$, and Ψ. Using in Eq. 3.2.5 simple substitutions for α (Eq. 3.2.1) and for k' (Eq. 3.1.20), the following formula for R is obtained:

$$R(X,Y) = \frac{1}{4}\left[\frac{K(X)}{K(Y)} - 1\right] \frac{K(Y)}{1/\Psi + K(Y)} N(Y)^{1/2} \tag{3.2.11}$$

Eq. 3.2.11 shows that a large α, (large $K(X)/K(Y)$) a large Ψ, and a large N are desirable for obtaining a large R. Both Ψ and N depend mainly on physical properties of the chromatographic column, while K also depends on the nature of the separated compounds (besides the nature of stationary phase).

The requirement for the value of resolution R to be higher than 1.0 in order to have a good separation is translated for the theoretical plate number N in the requirement to satisfy the following relation:

$$N > 16 \frac{(1+k')^2}{k'^2} \frac{\alpha^2}{(\alpha-1)^2} \tag{3.2.12}$$

In many practical applications, the separation factor α between an analyte and other components of a specific matrix may be too close to unity. The increase in the number of theoretical plates N of the column can be helpful for enhancing separation in these cases.

Peak capacity

The efficiency of an HPLC separation can be characterized by a parameter known as peak capacity P [43]. This parameter gives the number of peaks in a chromatogram that can be separated from one another with a resolution $R = 1$ within a specified windows of time defined by the longest retention time $t_{R,max}$. The theory of a maximum peak capacity is based on the fact that an ideal peak (Gaussian shape) has the peak width $W_b = 4\sigma_t$ (see Eq. 3.1.50). As the peak width changes with the retention time, the peak capacity P can be defined by the following formula:

$$P = 1 + \int_{t_0}^{t_{R,\,max}} \frac{dt}{4\sigma_t} \tag{3.2.13}$$

Eq. 3.2.13 can be interpreted as the length (in time) of a chromatogram $\left(\int_{t_0}^{t_{R,max}} dt\right)$ divided by one peak width ($W_b = 4\sigma_t$). The value of σ_t can be related using Eq. 3.1.57 ($\sigma_t = t_R/\sqrt{N}$)

and 3.1.19 ($t_R = t_0(k'+1)$) to the theoretical plate number by the following formula:

$$\sigma_t = \frac{t_0}{\sqrt{N}}(k'_e + 1) \tag{3.2.14}$$

where k'_e is the retention factor k' at the point of elution. For isocratic separations, introducing Eq. 3.2.14 in the integral given by Eq. 3.2.13 and performing the integration, the resulting value for the peak capacity P is given by the following formula:

$$P = 1 + \frac{\sqrt{N}}{4}\ln\left(\frac{t_{R,max}}{t_0}\right) \tag{3.2.15}$$

It should be specified that Eq. 3.2.15 giving the number of peaks in a chromatogram that can be separated from one another is valid only for isocratic separations. Eq. 3.2.15 indicates that in a longer chromatogram for a column with a higher number of theoretical plates, a larger number of compounds can be in theory separated.

Peak capacity can also be calculated as a sum of the resolution of all consecutive pairs of peaks X, Y ($X = i - 1$, $Y = i$) in the chromatogram with $(n + 1)$ peaks, starting with the peak of the unretained compounds ($i = 1$). This value of capacity is obtained with the following formula:

$$P = 1 + \sum_{i=1}^{n} R(i-1, i) \tag{3.2.16}$$

This formula can be adapted to different types of separations and has specific forms, for example, for size exclusion chromatography (see Section 15.1). For the gradient elution, this parameter is discussed in Section 3.5.

3.3 Summary of chromatographic peak and separation characteristics

General comments

A summary of peak characteristics can be obtained by processing the peaks in a chromatogram either manually or more commonly by using the capability of data processing programs from the computer that controls the HPLC. A summary of such characteristics is given in Table 3.3.1.

3.4 Parameters related to quantitation in HPLC

General comments

The main utilization of HPLC, regardless of its type, is in quantitative analysis. Quantitation involves both a separation of the analytes as well as their detection. Detector characteristics such as selectivity, sensitivity, response range, and linearity are key factors in performing the quantitation. Some aspects regarding the characterization of detection sensitivity are further discussed related to the LOD and LOQ in HPLC analysis. Also, several practical procedures for performing the quantitation of an analyte are presented in this section.

Quantitation parameters from the chromatographic peak

The integration of the expression for the variation of concentration of the analyte as a function of time $C(t)$ as given by expression 3.1.47 for t between $-\infty$ and $+\infty$ leads to the following result:

$$\int_{-\infty}^{+\infty} C(t)dt = q_{inj} \tag{3.4.1}$$

Expression 3.4.1 is based on the fact that the following relation is true:

$$\frac{1}{\sqrt{2\pi\sigma_t^2}}\int_{-\infty}^{+\infty} \exp\left(\frac{-(t-t_R)^2}{2\sigma_t^2}\right)dt = 1 \tag{3.4.2}$$

At the same time, integration of the expression for the variation of peak height as a function

TABLE 3.3.1 Typical peak and separation characteristics obtained using the capability of data processing program from the computer that controls the HPLC.

No.	Parameter	Notation	Formula	Common range
1.	Retention time	t_R	3.1.4	1–30 min (longer times for complex chromatograms)
2.	Void (dead) time	t_0	3.1.5	1–3 min
3.	Retention factor	k'	3.1.19	2–10
4.	Peak height	h	3.1.45	Depends on detector settings
5.	Peak area	A	3.1.45	Depends on detector settings
6.	Peak width at half height	W_h	3.1.50	0.05–1 min
7.	Peak width at baseline	W_b	3.1.50	0.1–2 min
8.	Peak start	t_{start}	–	Depends on t_R and W_b
9.	Peak end	t_{end}	–	Depends on t_R and W_b
10.	Symmetry (at 10% height)	*As*	3.1.78	1–1.3
11.	Tailing	*TF*	3.1.79	1–1.3
12.	Skew	*Skew*	3.1.91	1–1.3
13.	Excess	*Excess*	3.1.91	1–1.3
14.	Noise at peak baseline			Depends on detector settings
15.	Signal to noise ratio	$\mathscr{S}/\mathscr{N}$	–	Depends on detector settings, compound nature and concentration, etc.
16.	Integration type		–	Base to base, base to shoulder, etc.
17.	Data points per peak		–	Depends on the rate of detector measurements of signal
18.	Theoretical plate number (plates/column)	N	3.1.57	4000–40,000
19.	Theoretical plate number (plates/meter)	N	3.1.57	30,000–300,000
20.	Efficiency (plates/column)	$\boldsymbol{n}$	3.1.58	4000–40,000
21.	Efficiency (plates/meter)	$\boldsymbol{n}$	3.1.58	30,000–300,000
22.	Foley–Dorsey plates/column[a]			N corrected for asymmetry (lower than N)
23.	Foley–Dorsey plates/meter			N corrected for asymmetry (lower than N)
24.	Selectivity	α	3.2.1	1.5–10 (depending on separation)
25.	Resolution to previous peak:	R	3.2.7	1.5–15 (depending on separation)
26.	Resolution to next peak	R	3.2.10	1.5–15 (depending on separation)
27.	Peak capacity	P	3.2.15	1.6

[a] *Note: The plate number can be corrected for peak asymmetry by Foley–Dorsey formula:* $N = 41.7(t_R/W_{0.1})^2(f/r+1.25)$ *where* $W_{0.1}$ *is the peak width at 10% of its height,* f *is front, and* r *is rear of the peak, as shown in Fig. 3.1.9.*

of time $h(t)$ as given by expression 3.1.48 for t between $-\infty$ and $+\infty$ leads to the following result:

$$\int_{-\infty}^{+\infty} h(t)dt = A_{peak} \tag{3.4.3}$$

where A_{peak} *is* the total peak area (A_{peak} for the compound X, as area units, Ar.u.) under the curve representing the function $h(t)$. The comparison of expressions 3.4.1 and 3.4.3 indicates that the peak area in a chromatogram is proportional with the mass of the analyte. In other words, the following expression can be written:

$$q_{inj} = \frac{1}{\beta} \cdot A_{peak} \tag{3.4.4}$$

In chromatographic instruments, peak area A_{peak} is obtained using instrumental detection procedures. The proportionality constant $1/\beta$ depends on the detector sensitivity and has the corresponding units to change the area in mass (e.g., μg/Ar.u.). The value of $1/\beta$ is truly a constant only assuming that the detector response is linear to the instantaneous analyte concentration or mass passing the detector (see Section 2.2). Area measurement is typically performed using dedicated programs for data processing with numerical procedures used for area integration expression [44].

Since the injections in HPLC are performed using a given volume of the solution of the sample, the mass of injected material X is $q_{inj} = C_X V_{inj}$ where C_X is the concentration of X in the injected sample and V_{inj} is the injection volume (and it is known). As a result, the following expression can be used in HPLC for quantitation (measurement of an unknown C_X):

$$C_X = \frac{A_{peak}}{\beta\, V_{inj}} \tag{3.4.5}$$

A common quantitation procedure to determine $1/\beta$ is the use of calibration curves between peak area A_{peak} and the concentration C_X of an analyte. Various practical procedures are used for this purpose as further described. In practice, deviations from rel. 3.4.5 may sometimes be encountered. For example, the relation between sample concentration and peak area can be of the following form:

$$C_X = a + \frac{A_{peak}}{\beta\, V_{inj}} \tag{3.4.6}$$

Other types of dependence besides the linear one are sometimes encountered in practice due to nonlinear detector response to the analyte concentration (see Section 18.2).

Besides the peak area, peak height can also be used for quantitation. For a peak with a Gaussian shape, the peak area can be approximated using the following expression:

$$A_{peak} = 0.5\, h_{max} W_b \tag{3.4.7}$$

This formula indicates the proportionality between the peak area and the peak height. This observation shows that the peak height is also proportional with the initial concentration C_X of the solute in the injected sample. In addition, it shows that for a given area A_{peak}, it is advantageous to have a small W_b (a narrow peak) in order to obtain a larger h_{max}. Peak detection is determined by h_{max} (see rel. 3.4.29 for the definition of LOD).

From expression 3.1.48, it can be seen that $C(t)$ in a chromatogram is maximum when $t = t_R$. Because $q_{inj} = C_X\ V_{inj}$, expression 3.1.49 can be written in the following form:

$$C_{max} = \frac{C_X V_{inj}}{\sigma_t \sqrt{2\pi}} \tag{3.4.8}$$

The value C_{max} is the concentration of the analyte at the maximum of the peak (apex) in the chromatogram, and $C_{max} = ct\ h_{max}$, where the proportionality constant ct depends on the

detector response factor and the peak shape. The quantitation using peak height also requires the use of calibration curves. Although the measurement of the concentration of the injected sample seems to be equally possible using the peak area or the peak height, there are some differences between the two procedures. formula 3.4.8 has been developed based on the assumption that the peak has an ideal Gaussian shape. This is not the case in all practical situations. Even if the peak shape deviates from Gaussian, expression 3.4.5 remains valid, and the peak area in the chromatograms remains proportional with the amount of sample injected in the HPLC system. For peaks with a shape different from Gaussian, more variability regarding the proportionality between the peak height and the amount of the injected sample is typically seen.

The maximum concentration C_{max} for the apex of a chromatographic peak is an important parameter since it determines the maximum signal generated by the detector (h_{max}) and therefore is related to the detection limit of an analytical method. The ratio C_X to C_{max} is indicated as *dilution* $\mathfrak{D}$ and C_{max} depends on C_X based on the following formula:

$$C_{max} = \frac{C_X}{\mathfrak{D}} \tag{3.4.9}$$

Expression 3.4.9 shows that the maximum (instantaneous) concentration of the analyte passing the detector is directly proportional with the initial concentration injected in the HPLC instrument and inversely proportional with dilution $\mathfrak{D}$. It is desirable to obtain in a separation as high C_{max} as possible and therefore to have a small $\mathfrak{D}$. The value for $\mathfrak{D}$ can be obtained by combining expressions 3.4.8 and 3.1.46. By taking $\sigma_L = L/\sqrt{N}$, the formula for $\mathfrak{D}$ can be written as follows:

$$\mathfrak{D} = \frac{\sqrt{2\pi}}{V_{inj}\sqrt{N}} t_R \tag{3.4.10}$$

Expressions 3.4.9 and 3.4.10 indicate that a larger instantaneous concentration of analyte C_{max} at the apex of the chromatographic peak can be obtained when using large injection volumes (that puts more material in the chromatographic system for a given concentration C_X), columns with high number N of theoretical plates, and fast elution of the compound (short t_R). Such choices lead to larger detector signals.

Using a rough approximation for the peak shape as an isosceles triangle, it can be assumed that two peaks with the same area (same sample amount) have their height inversely proportional with the peak width (Area $= {}^1/_2$ height $\cdot$ base). A peak x times narrower is x times higher (has x times higher signal), which corresponds to x times better LOQ and LOD (see rel. 3.4.29 and 3.4.31). The reduction of peak broadening is also improving the integration accuracy of the chromatographic peak areas. Electronic peak detection for modern HPLC instruments is typically done based on the change in the slope of the chromatographic trace (traces and tangent to the trace at the start of the peak are illustrated in Fig. 3.4.1). The slope (tangent at the trace) is increasing much faster along a narrow peak than for a broad peak such that the peak detection and its (automatic) integration is done more accurately for narrow peaks.

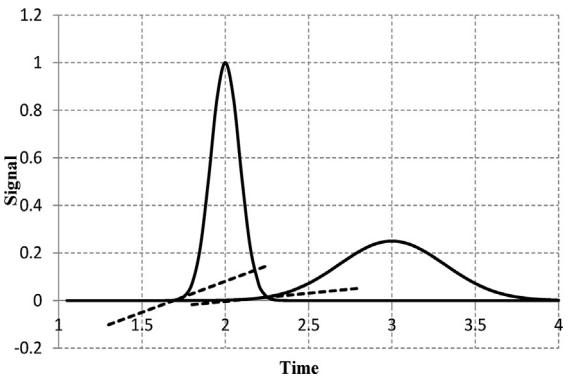

FIGURE 3.4.1 Difference in the slope of the trace at the start of a narrow and a broad peak.

Sample volume and amount injected in the chromatographic column

The injected sample in an HPLC system is characterized by its volume usually in the range of 1−25 μL for standard analytical HPLC. However, other injection volumes are used. For certain UPLC applications or for micro-HPLC, volumes in the range of 20−500 nL are common. Large volume injections up to 1 mL can also be used in special applications [45,46]. The injection volume must be precise and the injection must be reproducible. The injection volume V_{inj} is directly related to the amount of analyte $q_X = C_X\ V_{inj}$ delivered to the HPLC system (C_X being the sample concentration and V_{inj} the injected volume). Since the area A_{peak} of the chromatographic peak (detector response) is proportional with the volume of the injected sample V_{inj} (see expression 3.4.5), a larger sample injected volume will generate a larger signal (peak area). This indicates that for samples of lower concentration, a larger injection volume may be desirable. However, a large injection volume may affect the chromatographic peak width [47]. Generally, the sample loading capacity of the column or the saturation capacity of the stationary phase [48] is indicated by the maximum amount of sample that can be injected into a column that leads to a theoretical plate number equal to half of the maximum plate count [49].

In developing the theory about peak broadening, the value of W_b was not considering any broadening due to injection volume. In such ideal conditions, when an extremely small (narrow) injection is made, keeping a constant value of q_X, the ideal peak broadening is given by expression 3.1.57, which can be written in the following form:

$$W_b = \frac{4t_R}{N_0^{1/2}} \tag{3.4.11}$$

where the notation N_0 indicates the theoretical plate number in ideal conditions, without any peak enlargement because of the injection volume of the sample. For the time length W_b in the chromatogram, the volume of mobile phase passing through the column during a peak in the chromatogram is given by the following expression:

$$V_{0,peak} = W_b U \tag{3.4.12}$$

The volume $V_{0,peak}$ is further indicated as "ideal peak volume." For a sample volume V_{inj}, the peak volume increases and the new volume can be approximated using the following expression [50]:

$$V_{peak} = \left(1.333\ V_{inj}^2 + V_{0,peak}^2\right)^{1/2} \tag{3.4.13}$$

In expression 3.4.13, the coefficient 1.333 is an empirical value to account for additional broadening during the injection. From expression 3.4.13, the new value for N obtained due to the larger sample volume can be calculated using formula 3.4.11:

$$N = \frac{16\ (t_R U)^2}{1.333\ V_{inj}^2 + V_{0,peak}^2} \tag{3.4.14}$$

Expression 3.4.14 allows the evaluation of the decrease in the column efficiency (as measured by the theoretical plate number) as a function of the volume of the injected sample. The value of N is changed from N_0 by the following formula:

$$N = N_0 \frac{V_{0,peak}^2}{1.333\ V_{inj}^2 + V_{0,peak}^2} \tag{3.4.15}$$

Formula 3.4.15 allows to estimate that the loss of efficiency less than 10% corresponds to a value for the injected volume $V_{inj} < 0.3\ V_{0,peak}$, and for a 1% loss of efficiency a value $V_{inj} < 0.1\ V_{0,peak}$.

An estimation of $V_{0,peak}$ is now necessary for an estimation of an optimum injection volume that would affect only to a small extent the peak width. For this purpose, expression 3.4.12 will be utilized and W_b must be estimated. This

can be done using estimations for t_R in expression 3.4.11. The value for t_R is obtained from $t_R = t_0\ (1 + k')$ with an estimation for t_0. The value for t_0 can be obtained from expression 3.1.6 as $t_0 = (\varepsilon^* \pi\, d^2 L)/4U$. Putting together all these estimations, the result is expressed by the following formula:

$$V_{0,peak} \approx \varepsilon^* \pi d^2 L(1+k')/N^{1/2} \qquad (3.4.16)$$

For a sample injection that produces a loss of efficiency less than 10% ($V_{inj} < 0.3\ V_{0,peak}$) with $\varepsilon^* \approx 0.7$, the injection volume should be selected such that (d in mm, L in mm):

$$V_{inj} < 0.6 \cdot d^2 L(1+k')/N^{1/2} \qquad (3.4.17)$$

For a column with particles dimensions d_p, the empirical formula 3.1.77 can be used for the estimation of N. This estimation and considering the value of $Ct = 3.4$ in Eq. 3.1.77 leads to the following approximate formula for V_{inj} (d_p in μm):

$$V_{inj} < 0.035 \cdot d^2 d_p^{1/2}\, L^{1/2}(1+k') \qquad (3.4.18)$$

A few examples of column dimensions and properties with the maximum allowed injection volume required to achieve less than 10% change in the optimum column efficiency (as measured by N) and assuming $k' = 1$ are given in Table 3.4.1.

TABLE 3.4.1 Examples of maximum injection volume of sample in a column with the condition of not decreasing efficiency with more than 10% (assuming $k' = 1$), calculated by Eq. 3.4.17.

Length L (mm)	Diameter i.d d (mm)	Efficiency N	Max V_{inj} μL
150	4.6	9000	40.1
150	3.0	9000	17.1
100	4.6	10,000	25.4
100	2.1	10,000	5.3
50	2.1	8000	3.0

Although the increased injection volume may produce a reduction of column efficiency, larger volumes than recommended are sometimes used when the resolution is still satisfactory and an increase in analysis sensitivity is necessary for the measurement of compounds present in traces that do not provide a good detector signal. Columns with higher value for N are more sensitive to the volume of the injected sample since the peaks from these columns are narrower. In practice, when the sensitivity of the detector is not high enough, larger injection volumes than described in Table 3.4.1 are utilized.

The amount of material injected in the chromatographic column is an additional parameter that affects the separation. Excessive amount of sample in the chromatographic column leads to stationary phase overload. In such case, the stationary phase becomes saturated with a specific analyte, and the retention cannot take place within the narrow region occupied by the zone in the column containing the sample. The mobile phase carries the unretained solute on a "fresh" portion of the column where the analyte is retained, but this effect is associated with an apparent lowering of k' values (shorter retention times), increase in peak width, and tailing. The amount (in μg) that can be loaded in an analytical chromatographic column can be roughly estimated using the following expression:

$$q_{inj} = x\, V_0(1+k')N^{-1/2} = \frac{x\left(\varepsilon^* \pi\, d^2 L\right)}{4\, N^{1/2}}(1+k') \qquad (3.4.19)$$

In expression 3.4.19, x is a constant in mg depending on the nature of the stationary phase, V_0 is the dead volume of the column (in mL), k' is the retention factor, N is the number of theoretical plates of the column, d is column diameter, and L (in mm) is the column length. For conventional analytical columns, x can be between 0.02 and 0.2 mg but the value may vary considerably depending on the nature of stationary phase.

Larger columns (with larger V_0) may accommodate more mass of analyte.

Excessive amount of sample in the chromatographic column leads to stationary phase overload. In such case, the stationary phase becomes saturated with a specific analyte, and the retention cannot take place within the narrow region occupied by the zone in the column containing the sample. The mobile phase carries the unretained solute on a "fresh" portion of the column where the analyte is retained (depending on its specific equilibrium constant K), but this is associated with an apparent lowering of k' values (shorter retention times) and tailing. This effect is suggested in Fig. 3.4.2 where the shapes of overloaded peaks are indicated.

The maximum amount q_{inj} in a sample is, however, very frequently limited by the maximum level of analyte required for a reliable response form the detector. In Table 2.1.1, the minimum amount of analyte injected for a response above LOD are listed for different detectors (see Eq. 3.4.29). The table also indicates the corresponding dynamic range. The estimated maximum amount of analyte that can be loaded for the detectors are listed in Table 3.4.2. This level is however compound dependent and can vary significantly from the values indicated in this table.

The comparison of maximum material to be loaded on the chromatographic column and that of acceptable for a detector shows that for some detectors the column capability to accommodate a specific mass significantly exceeds the maximum load for the detector (e.g., fluorescence and MS detectors), while for other detectors, the limiting amount of mass to be loaded is the column capability. In such cases, the use of larger columns is recommended.

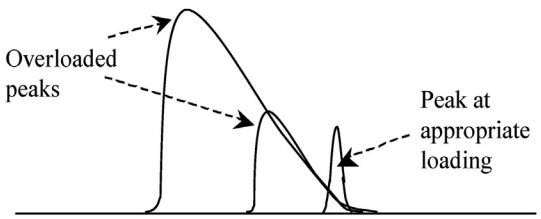

FIGURE 3.4.2 Shortening of the retention time and tailing typical for column overloading.

TABLE 3.4.2 Estimation of maximum levels of analyte that can be loaded for various detectors to obtain a reliable response (the level may be very different from compound to compound).

Type of detector	Max. Estimated mass injected (typical)
UV-Vis spectrometry	20.0 μg
Fluorescence spectrometry	0.05 μg
Refractive index	500.0 μg
Electrochemical amperometric	1.0 μg
Electrochemical conductometric	1.0 μg
Mass spectrometry	0.2 μg
Evaporative light scattering	250.0 μg
FT-IR spectrometry	100.0 μg

For very diluted samples, larger injection volumes are sometimes necessary in order to have good detection. Also, it is not uncommon that the injected samples contain a low level of the analyte of interest, but a large level of other solutes that form the sample matrix. In such cases, if the matrix reduction is not possible by sample preparation, the amount of sample injected in the chromatographic column may need to exceed up to 10 to 20 times the column loading capacity regarding the matrix amount. Even in these conditions, the sample concentration may remain low for the analytes but at acceptable levels for detection. The early overloading compounds may or may not have any effect on the peak shape of low level analytes eluting after the matrix peak. Large peaks however may have long tails that can interfere with the

separation, and the conditions must be selected such that the peaks of interest elute beyond this tail. Late overloading peaks may contribute to the shortening of the retention time of the peaks that elute earlier and have a low (in acceptable range) concentration. These types of interference from one compound to another are difficult to predict, and specific details are given in individual method descriptions.

Limit of detection in HPLC

Since HPLC main use is related to quantitative analysis, it is useful to describe two parameters related to the sensitivity of an HPLC method of analysis. The intensity of the detector response is a main factor regarding sensitivity in HPLC, but separation also plays a role by generating narrow peaks for which signal to noise ($\mathcal{S}/\mathcal{N}$) level is high. Sensitivity is usually described by two parameters. One such parameter is *limit of detection* (or LOD) that indicates the concentration of an analyte below which the detection is not possible. The detection limit can be discussed first in terms of signal and then transformed in terms of amount or concentration of the analyte using the calibration function. The signal y for an analytical measurement is in fact the difference between the signal for a sample y_s and that of a blank y_b, both affected by random errors. The frequency function which describes well the *relative frequency of occurrence of random errors* in large sets of measurements is given by the Gauss formula:

$$f(x) = \frac{1}{(2\pi\sigma^2)^{1/2}} \exp\left[-\left(x - \underline{\mu}\right)^2 / 2\sigma^2 \right] \tag{3.4.20}$$

This frequency function $f(x)$ (known as *Gaussian density function*) characterizes an ideal set of measurements indicated in statistics as a *population*. The *population* is characterized by the mean μ of the *population* and the standard deviation σ of the *population* which are obtained as the limit of the values for the mean of the measurements and their standard deviation when the number of measurements tends to ∞. Formula 3.4.20 shows that the point of maximum frequency is obtained for the mean (when $x = \mu$), the distribution of positive and negative errors is symmetrical, and as the magnitude of the deviation σ from the mean μ increases, an exponential decrease in the frequency takes place. The errors with the relative frequency of occurrence given by rel. 3.4.20 have a so-called *normal distribution* $N(\mu,\sigma)$. Besides Gaussian density functions, other frequency functions are known (e.g., Student frequency function).

The signals for the blank (or the noise) are assumed to have a mean $\underline{\mu}_b$ and the signals from the sample to have a mean $\underline{\mu}_s$. The standard deviations for both the blank and the sample can be considered equal to the same value $\sigma_y = \sigma$. A decision must be made of how high must be the signal of the sample y_s in order to be differentiated from the blank y_b. Since both signals are affected by random errors, this decision must be associated with a specific probability of confidence.

A specific value of the signal considered as not generated by the noise is indicated as the decision limit L where the signal from the sample is noticeable and its value is higher with $k\sigma$ than the noise (with mean signal $\underline{\mu}_b$). This can be expressed by the following formula:

$$L = \underline{\mu}_b + k\sigma \tag{3.4.21}$$

Assuming that the errors in the signal of the blank and sample have a normal distribution, the probability to obtain signals from the blank higher than the decision limit L is given by the following expression:

$$\alpha = \int_L^{\infty} f(y_b)\,\mathrm{d}y_b \tag{3.4.22}$$

In formula 3.4.22, the function f is given by formula 3.4.20 (Gaussian density function) that

has $\underline{\mu} = \underline{\mu}_b$ (σ, the standard deviation is the same for both the blank and the sample).

The probability to consider a noise as being signal from the analyte for a value higher than L is given by $P = \alpha$. For $k = 2.33$, the resulting value for the one-sided normal distribution gives $\alpha = 0.01$ (or 1% if expressed in percent). Therefore, if the signal is higher than $\underline{\mu}_b + 2.33\ \sigma$, the probability of false positives (signal from the background to be considered analyte) is 1%.

A signal y with $\underline{\mu}_s = L$ has, however, the problem of possibly generating false negatives. The probability of false negatives is given by the following expression:

$$\beta = \int_{-\infty}^{L} f(y_s)\,dy_s \qquad (3.4.23)$$

In expression 3.4.23, $f(y_s)$ is a Gaussian with $\underline{\mu} = \underline{\mu}_s$. This has the probability $P = .5$ (or 50%) because the normal curve is symmetrical around the mean. Therefore, the possibility of false negatives is very high, and 50% of the true positives will be considered noise. This situation is depicted in Fig. 3.4.3.

A higher signal (higher values for $\underline{\mu}_s$) and maintaining $L = \underline{\mu}_s$ will continue to diminish the chances for false positives since the area α will become smaller and smaller as the second Gaussian moves to higher values of y. However, maintaining $L = \underline{\mu}_s$ does not modify the probability of false negatives. For a chosen probability $P = 0.01$ of obtaining a false negative, the corresponding value of the signal can be calculated. This value is noted by D, should be smaller than $\underline{\mu}_s$, and has the expression:

$$D = \underline{\mu}_s - k''\sigma \qquad (3.4.24)$$

For the probability $\beta = 0.01$, the resulting value for the one-sided normal distribution is $k'' = 2.33$.

For $L = D$, the subtraction of rel. 3.4.24 from rel. 3.4.21 leads to the following expression:

$$\underline{\mu}_s = \underline{\mu}_b + (k + k'')\sigma \qquad (3.4.25)$$

The overlapping of frequency functions for the two probabilities is shown in Fig. 3.4.4.

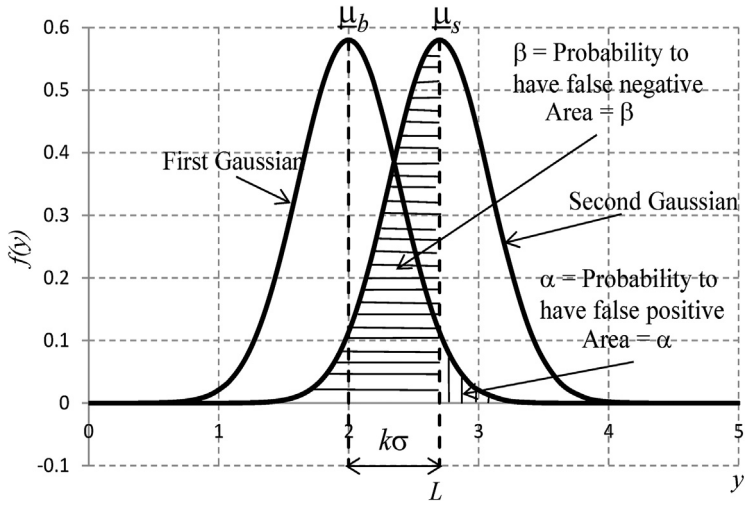

FIGURE 3.4.3 Graph showing on the horizontal axis the signal y, the values $\underline{\mu}_b$ and $\underline{\mu}_s$ as well as the decision limit $L = \underline{\mu}_s$. The vertical axis gives the frequencies $f(y)$ of occurrence for the value of a measurement, where f is given by rel 3.4.20 and describes a normal distribution.

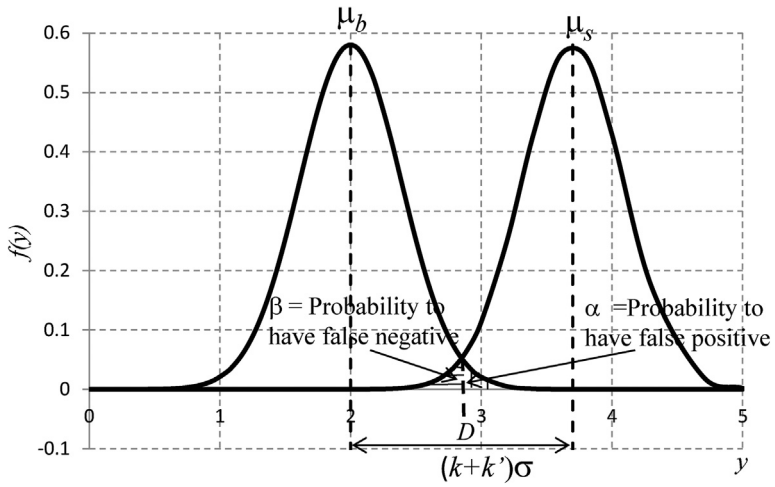

FIGURE 3.4.4 Graph showing on the horizontal axis the signal y, the values μ_b and μ_s as well as the decision limit $D = \underline{\mu}_s - k''\,\sigma$ The vertical axis gives the frequencies $f(y)$ of occurrence for the value of a measurement where f is given by rel 3.4.20 and describes a normal distribution.

The result of these considerations is that in order to have a probability of 1% for a false negative signal, and a probability of 1% for a false positive signal requires $k + k'' = 4.66$, and in this case, it can be written by the following formula:

$$\underline{\mu}_s = \underline{\mu}_b + 4.66\sigma \qquad (3.4.26)$$

If the signal is considered $\mathscr{S} = \underline{\mu}_s - \underline{\mu}_b$ and the noise is taken as $\mathscr{N} = \sigma$, formula 3.4.26 is equivalent with the following formula:

$$\frac{\mathscr{S}}{\mathscr{N}} \approx 4.66 \qquad (3.4.27)$$

For the detection to be possible in analytical practice, a value of $\frac{\mathscr{S}}{\mathscr{N}} \approx 3.33$ that is slightly smaller than 4.66 was considered to be sufficient (with lower probability than 1% for false negatives, and higher than 1% to give false positives). The value $\frac{\mathscr{S}}{\mathscr{N}} = 3.33$ has been adopted in analytical practice for the definition of *detection limit or limit of detection*, LOD [51,52]. The estimation of $\frac{\mathscr{S}}{\mathscr{N}} = 3.33$ in a set of chromatographic measurements can be done directly on the plot of chromatograms (typically using the capabilities of the data processing software of the chromatographic instrument). The concentration (or amount) generating $\frac{\mathscr{S}}{\mathscr{N}} = 3.33$ is taken as LOD (although $\mathscr{S}$ and $\mathscr{N}$ are not concentrations, their ratio can be considered as proportional with concentrations). As previously indicated, the theory supports the selection of the value $\frac{\mathscr{S}}{\mathscr{N}} \approx 3.33$ for LOD, although in chromatographic practice this definition may generate scattered results. The noise is typically measured as the variation in detector response at the baseline (difference between the minimum and the maximum of the baseline signal), and the signal $\mathscr{S}$ for a peak is measured as the difference from the maximum of the peak and the average value of the baseline. The simplest way to check the baseline noise $\mathscr{N}$ is to measure the variation of the detector response for a chromatogram without making an injection [53]. The measure of the variation of the detector response in any part of the chromatogram where no extraneous peaks are present is also common. The place where the noise is measured still remains arbitrary, in spite of some recommendations that the noise should cover a specific range of time, wider

than the chromatographic peak of the analyte and on both sides of the peak. In addition to that, the noise can be different from one chromatogram to another for the same method of analysis. A common way to reduce baseline noise is to increase the detector time constant. An optimum value for the detection time constant is about 1/10 of the peak width of the narrowest peak of interest from chromatogram [54].

A better procedure for LOD evaluation is based on repeated measurements and the use of the approximations $\underline{\mu}_s - \underline{\mu}_b \approx m$ and $\sigma = s$ (s is also indicated as SD). The choice $\frac{\mathscr{I}}{\mathscr{N}} = 3.33$ is equivalent with following the expression:

$$m = 3.33s \tag{3.4.28}$$

Expression 3.4.28 indicates that for detection to be possible, a concentration (or level) at least equal to $3s$ for a set of measurements (at low concentration) is necessary. Based on expression 3.4.28, it results that for a set of measurements at low analyte concentration the definition of LOD should be based on the following expression [55–57]:

$$\text{LOD} = 3.33s \tag{3.4.29}$$

The convention of taking LOD equal with 3.33 versus 4.66 increases the probability P of obtaining a false positive and decreases the probability P of obtaining a false negative (in Fig. 3.4.4 a smaller coefficient for σ moves the point D toward lower values).

Various other formulas were developed for the evaluation of LOD. For example, for a dependence of the form $y = a + bC$, when the average signal of the blanc $\underline{\mu}_b$ and the standard deviation of the signal s are known, the LOD for the concentration of the analyte can be obtained from the following expression ($\underline{\mu}_s$ is the average signal of the analyte):

$$\text{LOD} = \left(\underline{\mu}_s - a\right)/b = 1/b\left(\underline{\mu}_b - a + 3.33\sigma\right) \tag{3.4.30}$$

The measurement of signal and noise in a chromatogram is schematically shown in Fig. 3.4.5 showing two peaks with idealized triangular shape.

The signal from the first peak is larger than 3.33 times the noise and the other lower than 3.33 times the noise. As a result, the first peak

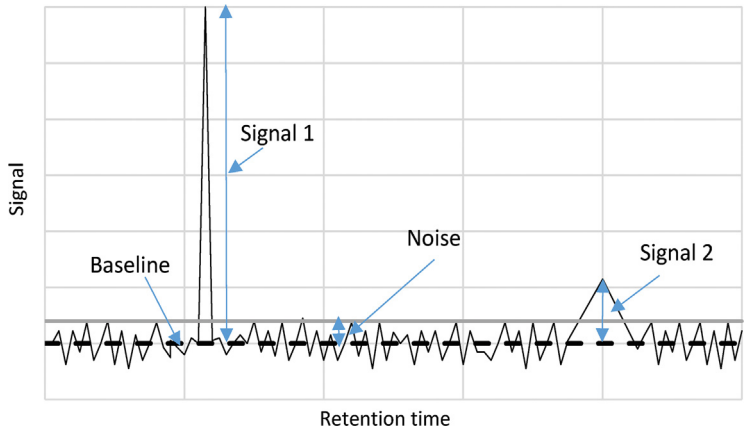

FIGURE 3.4.5 Schematic chromatogram with two peaks with idealized triangular shape and same area.

is above the LOD, while the other one is below LOD and as a result cannot be used for detection. The area of the first and second peak is however taken to be equal. This indicates that in a chromatographic separation the peak shape is a very important parameter. Although the analyte amount present in the two peaks is the same (the peaks having the same area), a narrow peak with a small peak width W_b, and as a result with a small H (HEPT), allows the detection, while a broad peak may not provide a sufficiently high $\mathscr{S}/\mathscr{N}$ to be used for detection. Besides the increase of detector sensitivity, improvement in separation plays an important role in the use of HPLC for quantitative analysis.

Limit of quantitation

An additional parameter indicated as *limit of quantitation* LOQ has been introduced, with the goal of decreasing the probability of false positives. The definition of LOQ is the following:

$$\mathrm{LOQ} = 10s \tag{3.4.31}$$

LOQ indicates the minimum amount (or concentration) that can be quantitated by a specific method. The choice of $10s$ for LOQ decreases the probability P for obtaining a false positive but increases the probability P of obtaining a false negative. To prevent the problem of generating false negatives, the measurements with results below $10s$ are not labeled as "analyte absent" but "analyte below LOQ."

Although it is common for an HPLC method to establish LOD and LOQ using standards, the matrix remaining in the processed sample after sample preparation step may still have some effect on an analytical procedure sensitivity (LOD and LOQ values). For this reason, for some procedures instead of reporting LOD and LOQ obtained with standards, a (practical) PLOD and PLOQ are reported. In these cases, for obtaining the standard deviation s or the signal to noise $\frac{\mathscr{S}}{\mathscr{N}}$, standards containing the residual matrix are used.

3.5 Parameters characterizing the gradient separation

General comments

The characterization of gradient separations using the same parameters as for the isocratic separation (see Sections 3.1 and 3.2) encounters the problem of variability along the chromatographic run of key parameters such as retention factor k', selectivity α, and resolution R. In isocratic conditions, the retention factor $k'(X)$ and the equilibrium constant $K(X)$ are constants. In gradient separations, the mobile phase composition changes, and the migration rate of species X changes during the chromatographic run. For this reason, it is not possible to define anymore a constant $u_R(X)$ (see rel. 3.1.12). In gradient, the migration rate of compound X becomes a function of time $u_R(X,t)$ where t varies in the interval $t_0 < t < t_R(X)$. In such conditions, from formulas 3.1.12 and 3.1.16, the equilibrium constant $K(X)$ can be written as follows:

$$K(X,t) = \frac{u - u_R(X,t)}{u_R(X,t)} \frac{1}{\Psi} \tag{3.5.1}$$

The result is that both $K(X,t)$ and $k'(X,t)$ (that depends on K) are changing during the chromatographic run when the separation is performed using gradient, and with those the retention time, the selectivity, the resolution, etc., are also changing.

In reversed-phase HPLC, for example, the change in mobile phase composition consists usually in changing the proportion of an organic component (such as acetonitrile or methanol) and that of water (or aqueous component). The volumetric fraction of organic component ϕ in the mobile phase in a binary mixture with water is given by the following formula:

$$\phi = \frac{V_{org}}{V_{org} + V_w} \tag{3.5.2}$$

where V_{org} indicates the volume of the organic component and V_w the volume of water (the

values of ϕ varies between 0 and 1 and can be indicated as proportion by multiplication with 100).

Retention factor in gradient separations

Further discussion is focused on reversed phase HPLC, although the same considerations can be applied for other HPLC types (e.g., HILIC). For the retention factor $k'(X)$ in the mobile phase consisting of a mixture of an organic solvent with water, an empirical formula has been established as depending on a retention factor $k'_w(X)$, where $k'_w(X)$ refers to retention of compound X in pure water as mobile phase. This formula can be written as follows:

$$\log k'(X) = \log k'_w(X) - S(X)\phi \qquad (3.5.3)$$

The value for $k'_w(X)$ is not usually known and is obtained from extrapolation to 100% from the values of $k'(X)$ as the content of water increases. Formula 3.5.3 is only as an approximation and different values for $S(X)$ are reported in the literature and for RP-HPLC some are indicated in Table 7.1.1. In RP-HPLC, $S(X)$ is positive such that log k' (X) decreases when the organic content of mobile phase increases. In HILIC, log k' (X) decreases when the water content ϕ_w increases (volumetric fraction of water ϕ_w is defined by a formula similar to Eq. 3.5 2 in which the numerator is V_w). The parameter $S(X)$ is specific for a solute X, a solvent mixture, and a column, but does not depend on ϕ. For a linear gradient performed for a period of time equal to t_{grad}, the composition of the mobile phase changes following the expression:

$$\phi(t) = \phi_0 + \frac{\Delta\phi}{t_{grad}}t \qquad (3.5.4)$$

where $\Delta\phi/t_{grad}$ is the gradient slope given by expression 2.1.2. With $\phi(t)$ given by expression 3.5.4, the expression for the retention factor $k'(X,t)$ as given by expression 3.5.3 becomes the following:

$$\log k'(X,t) = \log k'_w(X) - S(X)\left(\phi_0 + \frac{\Delta\phi}{t_{grad}}t\right) \qquad (3.5.5)$$

Expression 3.5.5 shows that in linear gradient conditions, the capacity factor $k'(X,t)$ depends across the chromatogram not only on the nature of the compound X, but also on the instantaneous mobile phase composition. The variation of $k'(X,t)$ during a gradient run time for two compounds having different $k'_w(X)$ values and the same S is illustrated in Fig. 3.5.1. As the values for k' approach zero ($k'(X,t) \approx 0$), the compound is not anymore retained and moves along the chromatographic column at the linear flow rate of the mobile phase u and it is eluted rapidly.

The variation of k' for the two compounds shown in Fig. 3.5.1 indicates, for example, a significant difference in the migration rate of the two compounds X and Y at lower ϕ values, while as the ϕ increases above 0.7, the two compounds migrate at rate close to that of the mobile phase, but the compounds are already separated.

The gradient slope $\Delta\phi/t_{grad}$ is typically included in another parameter indicated as *gradient steepness* defined by the following formula:

$$b = \frac{S\Delta\phi}{t_{grad}}t_0 \qquad (3.5.6)$$

With the use of gradient steepness, expression 3.5.5 is written in the following form:

$$\log k'(X,t) = \log k'_w(X) - S(X)\phi_0 - b\frac{t}{t_0} \qquad (3.5.7)$$

Because a compound has different k' values during the chromatographic run, it is not possible to use k' in gradient separations for the calculation of a number of parameters such as

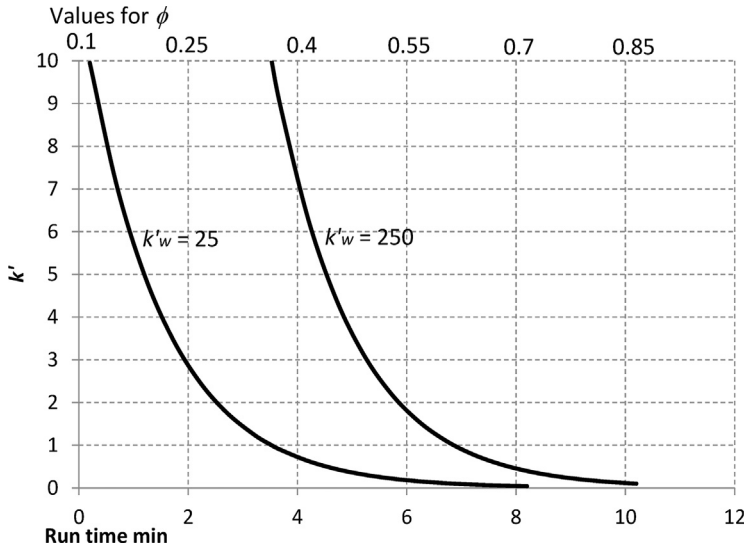

FIGURE 3.5.1 Variation of $k'(X,t)$ during a linear gradient in RP-HPLC for two compounds with $k'_w(X) = 25$ and $k'_w(Y\underline{\mu}_s = 250$, $S = 4$, $\phi_0 = 0.1$, $\Delta\phi = 0.6$, $t_0 = 0.2$ min, and $t_{grad} = 10$ min.

t_R, α, or R, that are typically used for the characterization of the chromatographic process. Since k' changes as the compound migrates across the chromatographic column, it was useful to define an effective value for k'. This *effective value* is the gradient retention factor k^*, and can be obtained using various approximations such as averages of $k'(X,t)$ at different points during the gradient.

A common procedure to obtain an expression for retention factor k^* in gradient separations starts with the assumption that the analyte X is present in the chromatographic column in a very narrow zone, moving along the chromatographic column with the instantaneous linear velocity $u_x(X)$ when it reached distance x from the column head. The expression for instantaneous linear velocity u_x can be obtained with the following formula:

$$u_x = \frac{dx}{dt} \tag{3.5.8}$$

The time t spent by compound X in the column after traveling the distance x can be obtained from formula 3.1.22 and can be written as follows:

$$t_x(X) = t_0 + k'[(\phi(t)] \cdot t_0 \tag{3.5.9}$$

where t_0 is the dead time for the column and k' $[\phi(t)]$ is the retention factor, which depends on the compound X, the stationary phase, and the mobile phase composition ϕ. During the gradient, the mobile phase composition ϕ (t) changes with time which determines the changes in k'. For $k'(\phi)$ taken as the instantaneous capacity factor and u_0 as the linear flow rate of the mobile phase expression 3.5.9 is equivalent with the following formula:

$$\frac{1}{u_x(X)} = \frac{1 + k'(\phi)}{u_0} \tag{3.5.10}$$

For further calculations, in rel. 3.5.8, the following substitution is convenient to be made:

$$t = z + \frac{x}{u_0} \tag{3.5.11}$$

Making in rel. 3.5.8 two substitutions, one with expression 3.5.11 and the other with rel. 3.5.10 for $u_x(X)$, the following expression for dt can be written:

$$dt = \frac{1 + k'(\phi(z))}{k'(\phi(z))} dz = \left(1 + \frac{1}{k'(\phi(z))}\right) dz \tag{3.5.12}$$

Making the assumption that the solute is injected at $x = 0$ and $t = 0$, expression 3.5.12 can be integrated for the interval from $z = 0$ to $z = t_R(X) - t_0$ (where $t_R(X)$ is the peak retention time). In those conditions, the result of the integration can be written as follows:

$$t_0 = \int_0^{t_R(X)-t_0} \frac{1}{k'(\phi(z))} dz \qquad (3.5.13)$$

In formula 3.5.13, in order to resolve the integral from the right term, an explicit formula for k' must be known. For a linear gradient, this type of dependence is offered by expression 3.5.7 that can be written in the following form:

$$\log k'(X,t) = \log k'_0(X) - \frac{b}{t_0} t \qquad (3.5.14)$$

where the following notation was used:

$$\log k'_0(X) = \log k'_w(X) - S(X)\phi_0 \qquad (3.5.15)$$

As the result, the final integration in expression 3.5.13 leads to the following formula for the retention time in gradient elution:

$$t_R(X) - t_0 = \frac{t_0}{b}\log\left(2.303 k'_0(X) b + 1\right) \qquad (3.5.16)$$

In the process of obtaining expression 3.5.16 for t_R, it was assumed that the gradient starts changing at the head of the chromatographic column at $t = 0$, or in other words that the dwell volume $V_D = 0$, and as a result the dwell time $t_D = V_D/U = 0$. However, the dwell volume in common HPLC should not be disregarded. Including the dwell time of the system, the correct expression for $t_R(X)$ will become the following:

$$t_R(X) = t_0 + \frac{t_0}{b}\log\left(2.303 k'_0(X) b + 1\right) + t_D \qquad (3.5.17)$$

Expression 3.5.17 is the equivalent for gradient separations of expression 3.1.22 for isocratic separations. For a gradient separation described by the gradient steepness b, a capacity factor $k'_g(X,b)$ can be defined using the following expression:

$$k'_g(X,b) = \frac{1}{b}\log\left(2.303 k'_0(X) b + 1\right) \qquad (3.5.18)$$

The *effective* gradient retention factor k^* usually applied for the characterization of gradient separations, taken as an "average" value, corresponds to a peak that would migrate half way through the chromatographic column if isocratic conditions would have $k' = k'_g(X,b)$. For this reason, the value for k^* is usually taken the double of $k'_g(X,b)$, with the following formula:

$$k^*(X) = 2k'_g(X,b) \qquad (3.5.19)$$

Other parameters for the characterization of chromatograms in gradient separations

The theory of parameters describing the gradient elution has been the subject of numerous developments that attempted either to improve the approximations used for obtaining previous relations or to extend the results for different gradient profiles (e.g., gradient starting at $t \neq 0$ or multiple gradient slopes). Expressions other than 3.5.19 that approximate a k^* for gradient separations are reported in the literature [58–60].

The parameter $k^*(X)$ can be used for defining other effective parameters used for the characterization of gradient separations. Among these are the effective selectivity α^* and effective resolution R^*. These parameters are defined by the following formulas:

$$\alpha^* = \frac{k^*(X)}{k^*(Y)} = \frac{k'_g(X,b)}{k'_g(Y,b)} \qquad (3.5.20)$$

$$R^* = \frac{1}{4}\left(\frac{\alpha^* - 1}{\alpha^*}\right)\left(\frac{k^*}{1+k^*}\right)N^{1/2} \qquad (3.5.21)$$

The number of theoretical plates N is also compound dependent, and rel. 3.1.21 would be

more correctly written as dependent on N^*. However, $N^* \approx N$ can be taken with good approximation.

The peaks undergo a "narrowing" effect in gradient separations due to the acceleration of elution as the concentration of stronger eluent increases. If we use a similar dependence for the peak width in isocratic elution given by Eq. 3.4.11, this parameter can be written for the gradient elution as follows:

$$W_b^g = 4t_0\left(1+k'_g\right)N_g^{-1/2} \tag{3.5.22}$$

In expression 3.5.22, a modified theoretical plate number N_g for gradient conditions is used. By combining Eq. 3.4.11 and 3.5.22, a "compressing factor" G induced by the gradient elution in comparison to isocratic elution may be defined by the following ratio:

$$G = \frac{1+k'_g}{1+k'}\sqrt{\frac{N}{N_g}} \tag{3.5.23}$$

The utility of *effective parameters* in gradient separations is lower than that those used for isocratic separation [61–63].

Peak capacity in gradient elution (P_g) is much more complicated than in case of isocratic elution, and considering the gradient steepness b and retention factor k'_g previously defined, this parameter is demonstrated for reversed-phase HPLC as having the following dependence [64]:

$$P_g = 1+\frac{\sqrt{N}}{4}\frac{1}{b+1}\ln\left[\frac{b}{b+1}\exp\left(bk'_g\right)-\frac{1}{b}\right] \tag{3.5.24}$$

In RP-HPLC, the value of b for small molecules is situated in the interval 0.03–3, while for larger molecules, such as peptides, the b values are much higher [65].

During a gradient separation, one additional parameter that changes is the viscosity of the mobile phase. This viscosity change may lead to undesirable increases in the column backpressure. The column backpressure can be calculated for a column filled with porous particle using rel. 3.1.76. By replacing the linear flow rate u with the volumetric flow rate U (see rel. 3.1.3), this expression becomes the following:

$$\Delta p = \frac{4\eta(\phi)U\phi_r L}{\pi\varepsilon^* d^2 d_p^2} \approx \frac{2500\,\eta(\phi)UL}{d^2 d_p^2}\ (psi) \tag{3.5.25}$$

where common notations were used, and η (ϕ) is the solvent viscosity dependent on composition, d is column diameter, d_p is the particle diameter, and ϕ_r is the column flow resistance factor). The maximum pressure during the separation is obtained using maximum η during the gradient (rel. 3.5.24 tends to predict lower pressure values than experimentally obtained). As will be shown in Section 7.2, the viscosity of solvent mixtures can be significantly higher than that of pure solvents, and an increase in the backpressure during the gradient is very common (see Fig. 7.2.2 indicating the variation in viscosity for three water/solvent mixtures). Gradient elution can be used for the prediction of isocratic retention data when the results for two or more gradient elution runs are known [66]. This procedure can be applied for the evaluation of log k'_w (retention factor for water as mobile phase, with no added organic component).

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CHAPTER

4

Equilibrium types in HPLC

4.1 Partition equilibrium

General comments

In Section 1.1, it was indicated that HPLC separation is based on the equilibrium established between the molecules from the mobile phase and those present in the stationary phase, and that the equilibrium can be of different types. A more detailed description of types of equilibria acting in HPLC separation is given in this chapter. The types of equilibria in HPLC separations should be considered just models since practical separation may involve more than one type. In some separations, one type of equilibrium can be considered dominant, but in other cases, it is difficult to clearly establish which equilibrium type is more important. Each equilibrium is dependent on the analyte retention, which is commonly described in chromatography by the retention factor k'. The formal dependence of k' on thermodynamic variables will be discussed in this chapter [1]. Also, the influence on the equilibrium of parameters such as pH and temperature is further presented.

Liquid–liquid partition

Liquid–liquid partition represents the equilibrium type of various separations based on two immiscible liquids. These include solvent extractions and different types of HPLC separations. In liquid–liquid partition, the component X is distributed between two nonmiscible liquid phases A and B in an equilibrium of the type:

$$X_B \leftrightarrows X_A \tag{4.1.1}$$

In HPLC, the phase B represents the mobile phase, and A, a stationary phase that can also be considered a liquid which is immobilized on a solid support (the nature of A and B is different). A typical example of a liquid immobilized on a solid support is that of water on silica. In this case, the water molecules are "bound" to the solid silica frame by hydrogen bonds to the silanol groups from the silica surface (phase A). This type of stationary phase and a mobile phase exemplified as acetonitrile (phase B) are schematically shown in Fig. 4.1.1.

HPLC is more frequently practiced using a stationary phase made of a solid support covered with a bonded phase. If this bonded phase is equated with an immobilized liquid, the formal theory of liquid–liquid partition can be successfully applied for the description of the equilibrium taking place in different types of HPLC.

In the theory regarding the equilibrium, it is assumed that no "nonequilibrium" effects take place. However, such effects exist. One nonequilibrium effect is due to the nonequilibrium solute transport through a chromatographic column of

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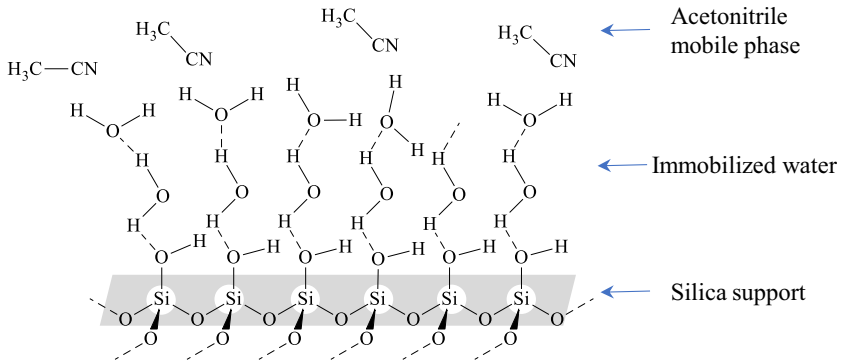

FIGURE 4.1.1 Schematic description of water immobilized on a silica surface and acetonitrile as a mobile phase.

cylindrical geometry [2], locally thermal variations of the partition process [3], or to the locally inhomogeneous portions of the stationary phase surface. Also, for a sufficiently large amount of compound X, it is possible that the equilibrium cannot be attained due to the saturation of the stationary phase. Such cases are not discussed here, and a sufficient small amount of solute is assumed to participate to the equilibrium, such that the process is close to "ideal."

The detailed energetics of the process, describing the interactions at the molecular level in this distribution equilibrium, is not taken into consideration in the present treatment. When equilibrium is attained for "distributing" the compound X between phases A and B, the difference between the chemical potentials $\mu_{X,A}$ and $\mu_{X,B}$ of the component X in each of the two phases A and B must be zero (the common expression for the chemical potential is $\mu_X = \mu_X^0 + RT \ln a_X$). This can be written in the following form:

$$\mu_{X,B}^0 + RT \ln a_{X,B} = \mu_{X,A}^0 + RT \ln a_{X,A} \quad (4.1.2)$$

In Eq. 4.1.2, $\mu^0{}_{X,A}$ and $\mu^0{}_{X,B}$ are the standard chemical potentials of compound X, $a_{X,A}$ and $a_{X,B}$ are the activities of analyte X in the two phases A and B, respectively, T is absolute temperature, and R is the gas constant ($R = 8.31451$ J/deg/mol $= 1.987$ cal/deg/mol). The rearrangement of Eq. 4.1.2 leads to the following expression:

$$\ln\left(\frac{a_{X,A}}{a_{X,B}}\right) = -\left(\mu_{X,A}^0 - \mu_{X,B}^0\right)/(RT) = -\Delta\mu_X^0/(RT) \quad (4.1.3)$$

In Eq. 4.1.3, $\Delta\mu^0{}_X$ represents the change in standard chemical potential when analyte X is transferred from the mobile phase B into the stationary phase A. Eq. 4.1.3 shows that for a constant temperature T the ratio of the activities of the analyte X in the two phases is always a constant expressed as follows:

$$\boldsymbol{K}_X = \frac{a_{X,A}}{a_{X,B}} = \exp\left(-\frac{\mu_{X,A}^0 - \mu_{X,B}^0}{RT}\right) \quad (4.1.4)$$

The constant $\boldsymbol{K}_X$ is the *thermodynamic distribution constant* (or *partition constant*) for the partition process of component X between liquid phases A and B (regardless if phase A, for example, is immobilized on a solid support).

The activities a_X can be expressed by the product between activity coefficients γ_X and the molar concentrations C_X using the following formula:

$$a_X = \gamma_X C_X \quad (4.1.5)$$

With the use of Eq. 4.1.5 for a_X, Eq. 4.1.4 can be written as follows:

$$\boldsymbol{K}_X = \frac{\gamma_{X,A} C_{X,A}}{\gamma_{X,B} C_{X,B}} = \exp\left(- \frac{\mu^0_{X,A} - \mu^0_{X,B}}{RT} \right) \tag{4.1.6}$$

The use of molar concentrations C_X instead of activities a_X is more convenient and the ratio of the concentrations of C_X in phase A and B defines the equilibrium constant K_X given by the following formula (identical to Eq. 3.1.15 where $A = st$ and $B = mo$):

$$K_X = \frac{C_{X,A}}{C_{X,B}} \tag{4.1.7}$$

The equilibrium constant K_X for the molecular species X between two phases A and B is indicated as the *distribution* or *partition constant*. With Eq. 4.1.7, the relation between K_X and $\boldsymbol{K}_X$ is the following:

$$K_X = \frac{\gamma_{X,B}}{\gamma_{X.A}} \boldsymbol{K}_X \tag{4.1.8}$$

The constant K_X is commonly used to characterize analytical partition processes. It gives the ratio of the concentrations of X in phases A and B at equilibrium, as compared to $\boldsymbol{K}_X$ that gives the ratio of activities. The value of K_i is dependent on temperature and also on the chemical nature of component X and of the two solvents A and B, although no chemical interaction is assumed in the system.

For a constant pressure and temperature, $\Delta\mu^0 = \Delta G^0$, where ΔG^0 is the variation in the standard free enthalpy (Gibbs free energy). The dependence of K_X on the variation of free enthalpy can be written in the following form:

$$K_X = \frac{\gamma_{X,B}}{\gamma_{X.A}} \exp\left(- \frac{\Delta G^0_X}{RT} \right) \tag{4.1.9}$$

In diluted systems, the activity coefficients γ are very close to 1 and as a result, $\gamma_{XB}/\gamma_{XA} \approx 1$. With the common expression of free enthalpy $\Delta G^0 = \Delta \boldsymbol{H}^0 - T\,\Delta S^0$ where $\Delta \boldsymbol{H}^0$ and ΔS^0 are the standard enthalpy and entropy changes for the transfer of the analyte from the mobile to the stationary phase (e.g., Ref. [4]), the distribution constant can be written in the following form:

$$K_X = \exp\left(- \frac{\Delta G^0_X}{RT} \right) = \exp\left(\frac{-\Delta \boldsymbol{H}^0_X + T\Delta S^0_X}{RT} \right) \tag{4.1.10}$$

The theory of liquid–liquid partition can be used to explain chromatographic processes where the stationary phase is assumed to be a liquid or a liquid-like material immobilized on an inert support. The classification of an HPLC separation as *partition chromatography* is in some cases an incomplete description of the process. For this reason, the use of some of the parameters developed for liquid–liquid partition must be done only as an approximation. In HPLC, the retention factor k' being related to the equilibrium constant by Eq. 3.1.20 ($k'_X = K_X \Psi$), the following expression can be written for k':

$$\ln k'(X) = - \frac{\Delta G^0}{RT} + \ln \Psi \tag{4.1.11}$$

Eq. 4.1.11 can also be written in the following form:

$$\log k'(X) = \frac{-\Delta \boldsymbol{H}^0 + T\Delta S^0}{2.303\, RT} + \log \Psi \tag{4.1.12}$$

Eq. 4.1.12 is known as van't Hoff equation and shows that the retention factor k' for a chromatographic separation depends on the change in enthalpy $\Delta \boldsymbol{H}^0$ and entropy ΔS^0 of the system in equilibrium when X is transferred from the phase *mo* into phase *st* (k' also depends on the column phase ratio Ψ). A larger $\Delta \boldsymbol{H}^0$ (in absolute value since $\Delta \boldsymbol{H}^0$ is negative for exothermic processes) indicates a stronger retention. For a system at constant pressure and volume, as the chromatographic conditions are supposed to

be, the enthalpy and energy are equal ($\Delta H^0 = \Delta E^0$), and for this reason, it is common when discussing the equilibria in HPLC to indicate changes in energy instead of changes in enthalpy. The contribution of changes in entropy during the separation process is typically lower than the energy component (except for size exclusion separations). Eq. 4.1.12 also describes the variation of log k' with the temperature.

The partition model suggesting that the separation process takes place between two phases has been successfully used for describing the HPLC separation. However, more elaborate models have been reported in the literature [5]. In such model, the intraparticle volume in the chromatographic column is divided into four parts: "zone 1" the layer of alkyl chains (in RP-HPLC) from stationary phase surface, "zone 2" the layer of liquid (mobile phase components) covering the stationary phase support, surrounding the alkyl chains, "zone 3" the layer of liquid enriched in the organic modifier that extends outside the stationary phase layer (with enrichment taking place because the organic component of the mobile phase has affinity for the alkyl chains), and "zone 4" the bulk of mobile phase. These zones have different contributions to the retention of compounds in the stationary phase and peak broadening. The major contributors to retention were found to be the diffusion of retained compounds into "zone 1" where the equilibrium is an adsorption process, and into "zone 2" where the equilibrium is true partition.

Dependence of retention factor on mobile phase composition in partition chromatography

The contribution to the free enthalpy change for the transfer of X from the phase *mo* into phase *st* comes from the changes suffered by the entire system, and not only from the interactions generated by the analyte with the mobile or stationary phase. These changes must also include any energetic modification in the mobile phase upon the transfer of X from the mobile phase into the stationary phase. As a result, the following formula can be written as follows:

$$\Delta G^0 = \Delta G^0_{analyte} + \Delta G^0_{eluent} \tag{4.1.13}$$

With the help of Eq. 4.1.13, the partition model of the chromatographic equilibrium provides a means to estimate the dependence of its equilibrium constant on the organic content in the mobile phase when this phase is made, for example, from a mixture of water and an organic solvent. For this purpose, it should be assumed that the free energy ΔG^0_{eluent} can be separated into the contributions of the two solvents ($\Delta G^0_{H_2O}$ and ΔG^0_{org}) as follows:

$$\Delta G^0_{eluent} = (1-x)\Delta G^0_{H_2O} + x\Delta G^0_{org} \tag{4.1.14}$$

In Eq. 4.1.14, x is the molar fraction of the organic component in the mobile phase. Molar fraction for a component Y in a solution is the number of moles n_Y of Y divided by the sum of the total number of moles for the solution components, and it is given by the formula $x_Y = n_Y \Big/ \sum_i^{total} n_i$. For a diluted solution x_Y is related to molar concentration C_Y and the average molecular weight of the solvent Mw_{mo} by the formula $x_Y \approx \gamma_Y C_Y Mw_{mo}/(1000\rho_{mo})$, where γ_Y is the activity coefficient for Y and ρ_{mo} is the density of the solvent. Including Eqs. 4.1.14 and 4.1.13 in Eq. 4.1.12, the following expression can be written:

$$\ln k'(X) = -\frac{\Delta G^0_{analyte} + (1-x)\Delta G^0_{H_2O} + x\Delta G^0_{org}}{RT} + \ln \Psi \tag{4.1.15}$$

The terms in Eq. 4.1.15 can be grouped as the free term $-(\Delta G^0_{analyte} + \Delta G^0_{H_2O})/RT$ and the coefficient for x giving the term $x(\Delta G^0_{analyte} - \Delta G^0_{H_2O})/RT$, both terms being constant at a constant

temperature. Since ln Ψ is also a constant (for a specific separation system) and because x and the volume fraction of the organic solvent in mobile phase ϕ (see Eq. 3.5.2) are proportional ($x = ct \cdot \phi$), the expression for ln $k'(X)$ (or log $k'(X)$) from Eq. 4.1.15 can be written in the following form:

$$\log k'(X) = a - b\phi \qquad (4.1.16)$$

Eq. 4.1.16 is equivalent with Eq. 3.5.3 previously discussed for gradient separations, and for RP-HPLC b is positive. The linear dependence is verified experimentally for many compounds and solvents [6], although deviations from linearity were also encountered [7]. These deviations were found in particular for compounds with more complex interaction with mobile phase (e.g., acetonitrile) and the stationary phase, but the partition equilibrium is still considered as predominant in the separation process. The variation of log k' with the concentration of the organic phase as predicted by Eq. 4.1.16 is illustrated in Fig. 4.1.2 for three compounds (benzene, toluene, and ethylbenzene) separated on a Chromolith Performance RP-18 100 × 4.6 mm, pore size 130 Å column, with the mobile phase water/methanol, at different methanol proportions.

As shown in Fig. 4.1.2, log k' depends very close to linear on ϕ (% CH_3OH) indicating a partition process. The graphs shown in Fig. 4.1.2 also indicate the decrease of log k' as the organic component increases in the mobile phase, as predicted by Eq. 4.1.16. Partition is the dominant process in most RP-HPLC separations. Deviations from the linearity expressed by Eq. 4.1.16 have been studied and explained by various retention models [8].

Distribution coefficient

The previous discussion regarding distribution of component X between the two nonmiscible liquid phases A and B is based on the assumption that the analyte X participates in the partition as a sole species and that it is not involved in any secondary chemical equilibria. Although the analytes are not supposed to react during the HPLC separation, one common case of secondary chemical equilibrium is that in

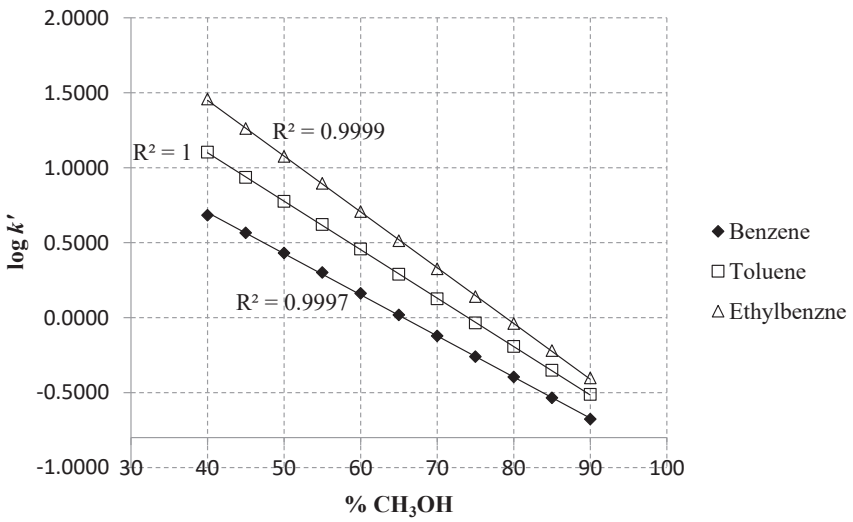

FIGURE 4.1.2 Dependence of log k' on % organic component in the mobile phase for benzene, toluene, and ethylbenzene, separated on a C18 monolithic column, with water/methanol mobile phase.

which a compound is present as several forms, but it is still identified as compound X. This type of equilibrium may include tautomerism, dimerization, ionization (electrolytic dissociation), ion-pairing, or complexation. For example, a simple acid can be present as molecular species RCOOH, and as $RCOO^-$ ion, and it is still identified as a unique compound. For such cases when the analyte X participates in other equilibria represented by species X_1, X_2, …, X_n, all being still identified as compound X, the entire partition process is described by a global parameter D_X given by the following expression:

$$D_X = \frac{C_{X_1,A} + C_{X_2,A} + \cdots C_{X_n,A}}{C_{X_1,B} + C_{X_2,B} + \cdots C_{X_n,B}} \qquad (4.1.17)$$

In Eq. 4.1.17, the numerator contains the sum of the molar concentrations of all species X in phase A, and the denominator the sum of molar concentrations of all species X in phase B. Parameter D_X is named *distribution coefficient* (*ratio*). (Note: The name coefficient and the name constant are sometimes confused, and the difference must result from the meaning of the parameter. Also, the partition and distribution names are sometimes used interchangeably.)

The concept of distribution coefficient defined for the equilibrium of species X_1, X_2, …, X_n, all still identified as compound X, between two nonmiscible liquid phases A and B can be extended to species A being an immobilized liquid (stationary phase) and B as a mobile phase. In such case, the equilibrium constant $K(X)$ defined by Eq. 3.1.15 is replaced with

$$D(X) = \frac{C_{X_1,st} + C_{X_2,st} + \cdots C_{X_n,st}}{C_{X_1,mo} + C_{X_2,mo} + \cdots C_{X_n,mo}} \qquad (4.1.18)$$

Peak shape in partition chromatography

One application of the theory of partition chromatography is related to the shape of the chromatographic peak. As shown previously, the distribution constant K_X (notations $K(X)$ or K_X used interchangeably) for a given system appears to depend only on temperature. The graph representing $C_{X,A}$ as a function of $C_{X,B}$ at a given temperature is called an isotherm and has the following equation (identical with Eq. 4.1.7):

$$C_{X,A} = K_X C_{X,B} \qquad (4.1.19)$$

The isotherm is a straight line when K_X is a true constant. In this case, at a constant temperature, the equilibrium process maintains a constant ratio between $C_{X,A}$ and $C_{X,B}$ during the separation. Eq. 4.1.19 indicates that in an ideal HPLC separation where A is the stationary phase and B the mobile phase, Eq. 4.1.19 is equivalent with the following:

$$C_{X,st} = K_X C_{X,mo} \qquad (4.1.20)$$

In some cases, K_X varies with $C_{X,st}$ and $C_{X,mo}$ and the isotherm is not anymore a straight line. When in a chromatographic separation the constant K_X varies with the concentration of the analyte, it will result that the retention time will also be modified since $t'_R(X) = K(X)\Psi t_0$ (see Eq. 3.1.22). This is going to happen across the chromatographic peak where the analyte concentration starts at zero before the peak reaches a maximum at the peak apex, and then decreases again to zero. This change in the retention time is not large, but in some cases can be large enough to modify the shape of the peak from the Gaussian shape to peaks showing fronting or tails. For example, it can be assumed that for a given analyte at the beginning of the peak $K = K^{\text{ideal}}$ (and does not depend on the concentration), but at the apex of a peak $K < K^{\text{ideal}}$. In this case the retention time t'_R for the apex will be slightly shorter than ideal and the peak will show fronting. The reverse will happen for cases when at the apex of a peak $K > K^{\text{ideal}}$, and the peak will show tailing.

Evaluation of retention factor from liquid–liquid distribution constants

Different studies on liquid–liquid extraction (e.g., Ref. [9]) showed that the partition constants $K_{X,A}$ and $K_{X,B}$ for a compound X in two systems, A solvent-1/water and B solvent-2/water, are related by the following expression:

$$\log K_{X,A} = a \log K_{X,B} + b \tag{4.1.21}$$

where a and b are constants that are characteristic for the two solvents that are utilized and can be obtained using best regression fit for a large number of compounds. Eq. 4.1.21 is very well followed for systems that are somewhat similar such as octanol/water and hexanol/water. This type of correlation becomes weaker for systems that are very different. Further studies [9–12] showed that this type of correlation can be extended from liquid–liquid extraction data to values for the retention factor k'_X for chromatographic systems. The correlation between $K_{X,A}$ and k'_X (for a specific stationary phase) is similar to Eq. 4.1.21 and has the following expression:

$$\log k'_X = a \log K_{X,A} + b \tag{4.1.22}$$

(constant b also includes in this case the value of phase ratio Ψ). The partition constant $K_{X,A}$ that is of particular interest in HPLC is the partition constant for octanol and water, typically indicated as K_{ow} (or P_{ow}).

Octanol/water partition coefficient represents the ratio of concentrations of a compound between two phases, one being octanol and the other water, and for a compound X is described by the formula (molar concentration C_X are noted interchangeably as $[X]$, and square brackets indicate molar concentrations, but for the value of K_{ow} the concentration units are irrelevant):

$$K_{X,ow} = \frac{[X]_{octanol}}{[X]_{water}} \tag{4.1.23}$$

The octanol and water are in contact to each other and the octanol is saturated with water and the water with octanol. Many compounds can be present in the form of different molecular species. These compounds are characterized by a related parameter to K_{ow}, which is the distribution coefficient D_{ow}. The distribution coefficient $D_{ow}(X)$ for a given compound X (existent in more than one form X_1, X_2, X_3,..) is defined by a formula similar to Eq. 4.1.17 (o indicates octanol and w water):

$$D_{X,ow} = \frac{[X_1]_o + [X_2]_o + [X_3]_o + \cdots}{[X_1]_w + [X_2]_w + [X_3]_w + \cdots} \tag{4.1.24}$$

For K_{ow}, and D_{ow}, extended information is available in the literature regarding their values and applications [12–14].

Since K_{ow} describes the partition of a compound X between a polar solvent (water) and a solvent with a large hydrophobic moiety (octanol), its value is expected to correlate well with the corresponding retention factor of X for reversed phase liquid chromatography that uses a C8 or C18 stationary phase. This assumption was proven correct for numerous compounds and the following relation is valid.

$$\log k'_X = a \log K_{X,ow} + b \tag{4.1.25}$$

Depending on the stationary phase and the mobile phase utilized, the coefficients a and b are obtained as best linear regression fit. As an example, the dependence of log k'_X versus log K_{ow} for 72 mono and disubstituted aromatic compounds, with k'_X values obtained for a C18 stationary phase using water/methanol 50/50 (v/v) as a mobile phase [10], is described by a linear expression of the form 4.1.25. Such a linear dependence, with $a = 0.5199$, $b = 0.3311$, and $R^2 = 0.9355$, indicating a very good correlation, is shown in Fig. 4.1.3.

A similar study was performed for a set of 12 different C18 columns and 6 different C8

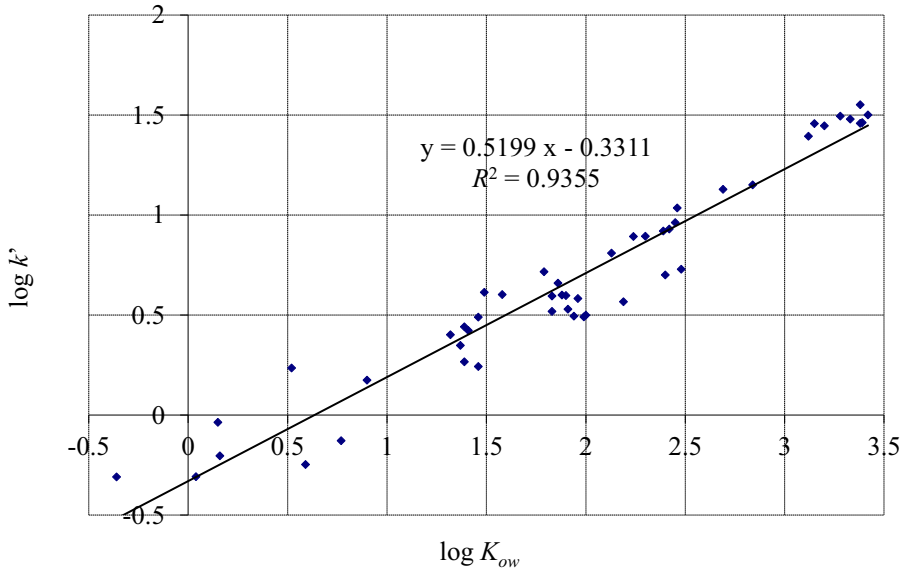

FIGURE 4.1.3 Dependence of log k'_X versus log K_{ow} for 72 mono- and disubstituted aromatic compounds with k'_X values obtained for a C18 stationary phase with water/methanol 50/50 (v/v) as a mobile phase [10].

columns by correlating retention coefficient log $k'_{X,w}$ (k'_X value extrapolated to pure water or aqueous buffer as mobile phase) with log $K_{X,ow}$ [15]. The study was performed for 25 different compounds and linear correlations with R^2 values between 0.9288 and 0.9958 were obtained, depending on the column. This good linearity indicates that for RP-HPLC the partition equilibrium is a good model for describing the separation.

4.2 Adsorption equilibrium

Liquid–solid equilibrium

In adsorption HPLC, the molecular species of the solutes from the mobile phase have the capability to be adsorbed on the surface of the solid stationary phase. An equilibrium is assumed to be established between the molecules of the solute (analyte) dissolved in the mobile phase and those adsorbed on the surface of stationary phase. Similar to the case of partition equilibrium, a small amount of solute is assumed to participate in this equilibrium. The stationary phase can be practically considered covered by a monolayer of molecules of either solute X or of mobile phase S. The distribution of X between mobile phase and stationary phase can be expressed by the displacement equilibrium:

$$X_{mo} + nS_{st} \rightleftarrows X_{st} + nS_{mo} \qquad (4.2.1)$$

The process described by Eq. 4.2.1 can be characterized by a thermodynamic distribution constant $\boldsymbol{K}_X$ given by the ratio (a indicates activities of compound X and solvent S in stationary phase or in mobile phase):

$$\boldsymbol{K}_{X,ad} = \frac{a_{X,st}}{a_{X,mo}}\left(\frac{a_{S,mo}}{a_{S,st}}\right)^n \qquad (4.2.2)$$

There are several assumptions necessary to get a constant value for $\boldsymbol{K}_{X,ad}$ (for a given temperature). These assumptions include the following: (1) the surface is uniform, i.e., all adsorption sites are equivalent, (2) the adsorbed molecules do not interact between them, (3) all adsorptions take

place by the same type of interactions, (4) only a monolayer of molecules is formed even at maximum adsorption, (5) in practice it is very common that the mobile phase consists of a mixture of two or even more miscible solvents but it is assumed that only one of the solvents (e.g., S) is adsorbed on the surface of the stationary phase and it is replaced by the analyte in an equilibrium of the type 4.2.1.

All these requirements are not always fulfilled, and therefore the assumption that $\boldsymbol{K}_{X,ad}$ is a constant should be considered only an approximation [16]. From the equality of chemical potentials μ for the components of the two sides of the equilibrium 4.2.1, the thermodynamic distribution constant can be written as follows (see Eq. 4.1.4):

$$\boldsymbol{K}_{X,ad} = \exp\left(-\frac{\mu^0_{X,mo} + n\mu^0_{S,st} - \mu^0_{X,st} - n\mu^0_{S,mo}}{RT}\right) \tag{4.2.3}$$

Replacing the activities in Eq. 4.2.2 with concentrations, equilibrium 4.2.1 is described by the constant $K_{X,ad}$ with the following form:

$$K_{X.ad} = \frac{C_{X,st}}{C_{X,mo}}\left(\frac{C_{S,mo}}{C_{S,st}}\right)^n \tag{4.2.4}$$

For all activity coefficients $\gamma \approx 1$ (see Eq. 4.1.5) and replacing activities with molar concentrations, $K_{X,ad} \approx \boldsymbol{K}_{X,ad}$ and the dependence of $K_{X,ad}$ on chemical potentials is also given by Eq. 4.2.3. This formula can be further simplified since the two values for the standard chemical potentials in the mobile phase $\mu^0{}_{X,mo}$ and $n \cdot \mu^0{}_{A,mo}$ are usually equal and cancel each other. This leads to the following relation:

$$K_{X,ad} = \exp\left(-\frac{n\mu^0_{A,st} - \mu^0_{X,st}}{RT}\right) \tag{4.2.5}$$

The equilibrium of only solute X between the stationary and mobile phases can be also written as a partition equilibrium:

$$X_{mo} \rightleftarrows X_{st} \tag{4.2.6}$$

This equilibrium is characterized by a constant K'_X, which written for $C_{X,st}$ as the number of moles of X per gram of adsorbent, and $C_{X,mo}$ as the molar concentration of X in the mobile phase has the common form:

$$K'_X = \frac{C_{X,st}}{C_{X,mo}} \tag{4.2.7}$$

If the quantity of adsorbed mobile phase per unit weight of adsorbent $C_{S,st}$ taking $C_{S,mo} = 1$ (mobile phase is made only from solvent S), and assuming that one single molecule of solvent S is replaced by one molecule X ($n = 1$), the relation between K'_X and $K_{X,ad}$ will be the following:

$$K'_X = K_{X,ad}C_{S,st} \tag{4.2.8}$$

As a result, the following expression can be written for K'_X:

$$K'_X = \exp\left(-\frac{n\mu^0_{A,st} - \mu^0_{X,st}}{RT}\right)C_{S,st} \tag{4.2.9}$$

Similar to the case of partition type equilibrium, the detailed energetics of the adsorption process is not taken into consideration in this treatment.

Dependence of retention factor on mobile phase composition in adsorption equilibrium

For the evaluation of retention coefficient k' in adsorption chromatography, it is convenient to replace in Eq. 4.2.2 the concentrations of the solvent S with molar fractions. The relation between molar fraction $x_{X,mo}$ and $C_{X,mo}$ can be written as follows:

$$C_{X,mo} = x_{Xmo}\left(C_{X,mo} + C_{X,st}\right) \tag{4.2.10}$$

In this case, Eq. 4.2.2 can be written as follows:

$$K_{X,ad} = \frac{C_{X,st}}{C_{X,mo}}\left(\frac{x_{S,mo}}{x_{S,st}}\right)^n \tag{4.2.11}$$

The assumption that the surface of stationary phase is basically covered mainly with solvent

molecules gives $x_{S,st} = 1$. With the use of Eq. 4.2.7, expression 4.2.11 can be written as follows:

$$K_{X,ad} = K'_X (x_{S,mo})^n \tag{4.2.12}$$

From Eq. 3.1.20 for retention factor, k' can be obtained by the multiplication of Eq. 4.2.7 with phase ratio Ψ. Using the notations $k'_{ad}(X) = K_{X,ad} \cdot \Psi$ and $k'(X) = K'_X \cdot \Psi$, the following formula is obtained from 4.2.12 (after taking the logarithm):

$$\log k'_{ad}(X) = \log K'_X + \log\Psi - n \log x_{S,mo} \tag{4.2.13}$$

The phase ratio Ψ in adsorption chromatography can be considered as having the same definition as indicated by Eq. 3.1.18, where V_{st} is the volume of mobile phase immobilized on the stationary phase surface. Eq. 4.2.13 is known as Soczewinski (or Snyder–Soczewinski) equation and indicates that if the chromatographic equilibrium is based on adsorption/desorption, the logarithm of retention factor should depend linearly on the logarithm of the molar fraction of solvent S in the mobile phase [17]. The molar fraction x being proportional with volume fraction ϕ, Eq. 4.2.13 can be reduced to the simplified form ($b = n$):

$$\log k'_{ad}(X) = a - b \log \phi \tag{4.2.14}$$

Eq. 4.2.14 known as Snyder–Soczewinski model shows that the logarithm of retention factor $\log k'$ for the adsorption/desorption equilibrium depends linearly on $\log \phi$ (the logarithm of volume fraction of solvent S), and not on ϕ as indicated by Eq. 4.1.16 (which is valid for the partition model). This difference, when verified experimentally, is sometimes used as a criterion to differentiate partition from adsorption equilibrium. Deviation from linearity of $\log k'$ on $\log \phi$ is due to the influence of the nature and the volume fraction of solvent S on K'_X and phase ratio Ψ, and in practice, such a dependence can have graphically a U-shape. Various studies on the dependence of k' on solvent composition for the adsorption equilibrium were reported in the literature [18,19].

Peak shape in adsorption chromatography

Similar to the case of partition chromatography, the graph representing $C_{X,st}$ as a function of $C_{X,mo}$ at a given temperature is called an isotherm. The isotherm is a straight line when K'_X is a true constant at a constant temperature. However, in some cases, K'_X varies with $C_{X,mo}$ and the isotherm is not anymore a straight line. This can be seen, for example, at large loads of the column when because of the limited surface capacity of the stationary phase, some part of the sample is in contact with the stationary phase and another part is in contact with molecules already adsorbed (when the separation takes place based on adsorption). Also, when different molecules in a mixture are competing for the stationary phase surface, the adsorption of one component may depend on the adsorption of another component. Isotherms of different shapes deviating from linear were reported in the literature (e.g., Refs. [16,20]). As indicated in Section 4.1, in an ideal system the equilibrium constant depends only on temperature and does not change during the separation (for T = constant). For a further evaluation of constancy of K'_X, it is convenient to replace the concentrations with molar fractions x since x and C are proportional (see Eq. 4.2.10). With this type of substitution, Eq. 4.2.7 becomes the following:

$$K'_X = \frac{x_{X,st}}{x_{X,mo}} \tag{4.2.15}$$

For simplification, it can also be assumed that $n = 1$ (one analyte molecule replaces one solvent molecule) and because typically $x_{X,mo}$ is very low and $(1 - x_{X,mo}) \approx 1$, Eq. 4.2.4 can be written as follows:

$$K_{X,ad} = \frac{x_{X,st}}{x_{X,mo}(1 - x_{X,st})} \tag{4.2.16}$$

Eq. 4.2.16 can be rearranged in the following form:

$$x_{X,st} = \frac{K_{X,ad} x_{X,mo}}{1 + K_{X,ad} x_{X,mo}} \qquad (4.2.17)$$

Eq. 4.2.17 is known as *Langmuir isotherm*. The representations of these isotherms for hypothetic systems with $K_{X,ad} = 0.2$, 0.8, and 1.6 on two concentration ranges for $x_{X,mo}$ (0–0.2 and 0.2–1.0) are shown in Fig. 4.2.1.

From Fig. 4.2.1, it can be seen that at low concentrations for the analyte X and for lower $K_{X,ad}$ values, the dependence of the molar fraction of the adsorbed material practically depends linearly on the concentration in the mobile phase. In other words, a true linear dependence occurs having the following expression (K'_X is constant):

$$C_{X,st} = K'_X C_{X,mo} \qquad (4.2.18)$$

This is not the case at higher concentrations or higher $K_{X,ad}$ values when the amount of adsorbed material is higher.

Not all adsorption systems are well described by a simple Langmuir isotherm, and other formulas have been developed for a better description of the adsorption process [21]. For a system where dependence between $C_{X,st}$ and $C_{X,mo}$ is linear, the value of $K_{X,ad}$ is a true constant and is not affected by the increase in concentration of the analyte. This is the case for low concentrations of X in the mobile phase and for relatively low values for $K_{X,ad}$, In such cases, $1 + K_{X,ad}\, x_{X,mo} \approx 1$ and Eq. 4.2.16 becomes basically identical with Eq. 4.1.20 ($K_{X,ad} = x_{X,st}/x_{X,mo} = C_{X,st}/C_{X,mo}$) developed for the partition process. The change in the values of $K_{X,ad}$ with the change in the concentration (molar fraction) of the analyte in mobile phase as described for adsorption equilibria has consequences in modifying the peak shape in chromatographic separations based on this type of equilibrium, as it was previously described for partition in Section 4.1 and the ideal Gaussian shape is not generated.

4.3 Equilibrium involving ions

General aspects

Most equilibria involving ions are encountered in ion chromatography. Before discussing the separation in ion chromatography, it is important to understand the retention process of ions from solution onto an ion exchange resin

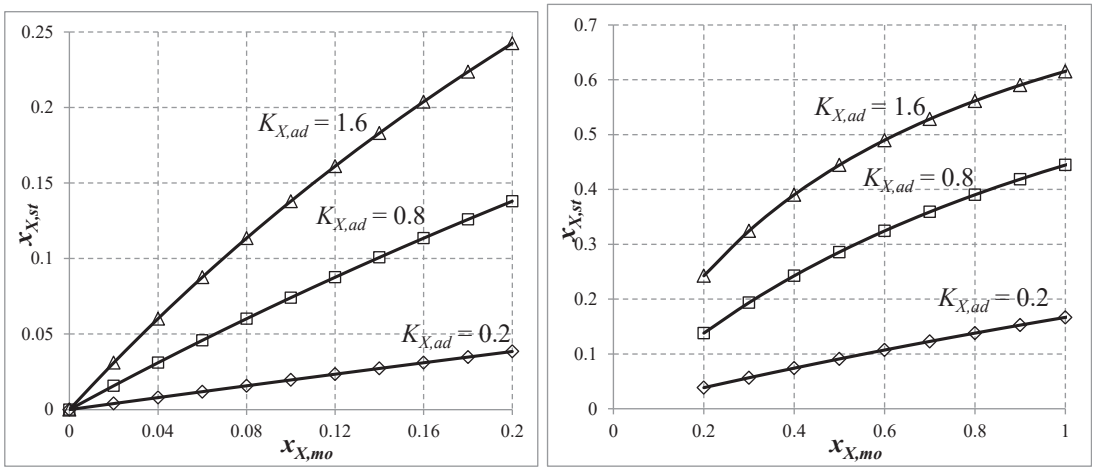

FIGURE 4.2.1 Hypothetic graphics for Langmuir isotherm $K_{X,ad} = 0.2$, 0.8, and 1.6 for two concentration ranges.

that acts as the stationary phase. The ion exchange process takes place between an ion species in the solution and another ion species present in the stationary phase being connected through ionic interactions with the stationary counterionic groups bonded to the stationary phase [22]. In the ion exchange process, equivalent amounts of ions are exchanged. However, it can be a difference between the affinity of the ions of different species for the resin acting as stationary phase and based on this difference a separation can be achieved.

Ion exchange chromatography is subdivided into cation exchange chromatography and anion exchange chromatography. In cation exchange chromatography, the ions M^+ in solution are exchanged with the cations C^+ that were initially retained by stationary phase with ionic groups of the type *res*-X^-. One example of such phase is a polystyrene/divinylbenzene polymer with sulfonic groups covalently bound on the polymer structure. In anion exchange, the anionic species B^- in solution are exchanged with the anions A^- that were initially retained by the stationary counterionic groups of the type *res*-Y^+. In this way, the electroneutrality of the system is always maintained (the ions in solution also have counterions). A special type of ion exchange may also take place on zwitterionic stationary phases (ZIC phases containing, for example, a sulfonic and an amino group in the bonded phase) when the mobile phase is a totally aqueous buffer. In this case, only one group is involved in the ion exchange process, and the phase acts either as cation or as anion exchange material.

Retention equilibrium involving ions

The study of ion exchange equilibria is usually based on a number of simplifying assumptions. One such assumption is that the maximum uptake of ions by an ion exchange resin is constant and determined by the number of functional groups on the resin matrix [23]. Another assumption is that strict stoichiometric coupling takes place between the different components involved in the ion exchange process. This implies that each ion from the resin phase is replaced by another one from the liquid phase with an equivalent charge for maintaining electroneutrality. Also, it is assumed that cations do not penetrate an anion exchange particle and anions do not penetrate a cation exchange particle. These assumptions are useful for the prediction of the ion exchange behavior of simple, dilute strong electrolyte solutions. However, when dealing with more complex systems, such as weak electrolyte solutions or solutions with high solute concentrations exceeding the resin capacity, the simplifying assumptions are not always valid. For example, the uptake of ions may exceed the total capacity of the resin in case of high concentrations.

For a given ion M^+ in solution and a resin in C^+ form, the exchange equilibrium is the following:

$$resX^-C^+ + M^+ \rightleftarrows resX^-M^+ + C^+ \qquad (4.3.1)$$

The constant for this equilibrium can be written using previous assumptions as follows:

$$K_{M,C} = \frac{[M^+]_{res}}{[M^+]_{mo}} / \frac{[C^+]_{res}}{[C^+]_{mo}} \qquad (4.3.2)$$

where the index *res* indicates resin and *mo* indicates mobile phase (square parentheses such as $[M^+]$ or $[C^+]$ indicate molar concentration and are more frequently used for indicating ion concentrations that the notation C_{M^+} or C_{C^+}). The exchange constant $K_{M,C}$ indicates the degree to which an ion M^+ is preferred in the exchange process, compared to the ion C^+. Larger constants for $K_{M,C}$ indicate higher affinity for the resin of species M^+. The difference in the values of exchange constant $K_{M,C}$ for different ions is usually explained by the Gibbs–Donnan effect [24]. The exchange constant can be expressed as a function of a distribution constant between the resin and the solution. The equilibrium

described by Eq. 4.3.2 can be viewed as equivalent with two independent equilibria:

$$C^+_{mo} \rightleftarrows resX^-C^+ \tag{4.3.3}$$

$$M^+_{mo} \rightleftarrows resX^-M^+ \tag{4.3.4}$$

The equilibria 4.3.3 and 4.3.4 are described by the constants:

$$K_{res,C} = \frac{[C^+]_{res}}{[C^+]_{mo}} \tag{4.3.5}$$

$$K_{res,M} = \frac{[M^+]_{res}}{[M^+]_{mo}} \tag{4.3.6}$$

Each solute has a distribution constant (retention constant) for a specific resin. Using Eqs. 4.3.5 and 4.3.6, the equilibrium constants Eq. 4.3.2 can be expressed in the following form:

$$K_{M,C} = K_{res,M}/K_{res,\,C} \tag{4.3.7}$$

The expressions developed for equilibrium 4.3.1 can be easily extended for a multicharge ion equilibrium taking place between the solution and the resin. This type of equilibrium has the following form:

$$z\ resX^-C^+ + M^{z+} \rightleftarrows (resX^-)_zM^{z+} + z\ C^+ \tag{4.3.8}$$

The corresponding equilibrium constant is the following:

$$K_{M,C} = \frac{[M^{z+}]_{res}}{[M^{z+}]_{mo}} \Big/ \left(\frac{[C^+]_{res}}{[C^+]_{mo}}\right)^z \tag{4.3.9}$$

Based on Eq. 4.3.2 or Eq. 4.3.9, it can be seen that the concentration of an ion in solution and the value of the equilibrium constant are important factors in the retention of an ion from solution. In practice, the ion exchange column must be initially conditioned and made in the form C^+. The ion selected as C^+ is frequently H^+ (the initial form of the exchange resin is H^+ form, and C^+ is identical with H^+).

For an efficient retention of ions M^+ in the resin when a solution containing these ions are passed through a resin bed in H^+ form is that the value of $K_{res,M}$ is high (higher than $K_{res,H}$). In this process, the H^+ ions will be released in solution. The exchange of H^+ ions with M^+ ions can continue until the equilibrium described by Eq. 4.3.9 is reached.

The change of the column into H^+ form before the separation can be accomplished by slowly flowing through it a solution of an inorganic acid. For example, a solution of 0.1 N *HCl* is passed through the stationary phase resin. This changes the resin into H^+ form. Eq. 4.3.9 (with C^+ being H^+) is applicable only to describe an equilibrium, and this is not the case during the column conditioning when the concentration $[M^{z+}]_{mo} = 0$ and $[H^+]_{mo}$ is high. However, Eq. 4.3.9 shows that $[H^+]_{res}$ will become as high as possible when $[H^+]_{mo}$ is high and since $[M^{z+}]_{mo}$ is practically zero, also $[M^{z+}]_{res}$ will tend to zero. After conditioning, the resin is thoroughly washed with water to eliminate the remaining *HCl* traces.

The same discussion for the equilibrium on a cation exchange column can be easily applied for an anion exchange column. In this case, a column in the form A^- exchanges a base B^- in the following equilibrium:

$$resY^+A^- + B^- \rightleftarrows resY^+B^- + A^- \tag{4.3.10}$$

This equilibrium is governed by the constant $K_{B,A}$ with the following expression:

$$K_{B,A} = \frac{[B^-]_{res}}{[B^-]_{mo}} \Big/ \frac{[A^-]_{res}}{[A^-]_{mo}} \tag{4.3.11}$$

For any anion, a retention constant can be defined with the following formula:

$$K_{res,B} = \frac{[B^-]_{res}}{[B^-]_{mo}} \tag{4.3.12}$$

For an efficient retention of the anion B^- is that $K_{res,B} > K_{res,A}$. In anion exchange

chromatography, the ion A^- used as the initial form of the resin is frequently OH^- (A^- is identical with OH^-), but other anions such as Cl^- are also common.

Equilibrium of ions in the presence of a complexing reagent

The use of various complexing agents in the solution interacting with an ion exchange resin is another procedure used in ion exchange HPLC for obtaining separations. The equilibrium between the complexing agent (ligand) in solution and the ions to be exchanged reduces the concentration of the free ions available for the exchange process. In this case two simultaneous equilibria take place in the mobile phase:

$$resX^-C^+ + M^+ \rightleftarrows resX^-M^+ + C^+ \quad and$$
$$L^- + M^+ \rightleftarrows LM \tag{4.3.13}$$

Assuming that the reaction with the ligand is present only in the mobile phase and is described by the equilibrium constant K_{LM} given by the following expression:

$$K_{LM} = \frac{[LM]_{mo}}{[L^-]_{mo}[M^+]_{mo}} \tag{4.3.14}$$

the concentration of M^+ in the resin is given by the following expression:

$$[M^+]_{res} = K_{res,M}\frac{[LM]_{mo}}{[L^-]_{mo}K_{LM}} \tag{4.3.15}$$

Eq. 4.3.15 shows that a higher concentration of ligand or a high complexation constant diminishes the amount of species M^+ retained in the column. However, complexation can be used to favor retention on the resin. For example, specific ions form negatively charged complexes. Assuming that an ion M^{2+} forms with a ligand L^- four combinations ML^+, ML_2, ML_3^-, and ML_4^{2-}, the negatively charged complexes and the ligand can be retained on an anion exchange resin, while the positive ions and the neutral molecules are not retained. In a mixture of ions, only some having the complexing capability with the formation of negatively charged compounds, an anion exchanger can be used for the separation of the desired species. In this case, two constants contribute to the value of the concentration of ML_4^{2-}, in the resin. One indicated as K_{comp} describes the equilibrium:

$$4\,L^- + M^{2+} \rightleftarrows ML_4^{2-} \tag{4.3.16}$$

The other constant is the distribution constant between the mobile phase and the resin for ML_4^{2-} species indicated as K_{res,ML_4}. With these two constants, the concentration of ML_4^{2-} in the resin can be obtained using the following expression:

$$[ML_4^{2-}]_{res} = K_{res,ML_4}K_{comp}[L^-]^4[M^{2+}] \tag{4.3.17}$$

Each complex ion has its specific distribution constants in the resin. The constant depends on factors such as bond strength, hydrophobic interactions, steric hindrance, etc. The adsorption of complex ions on resins is a more complicated process, because in addition to the complexation in solution, the ligand adsorbed on the resin may still participate in complex formation. The donor electrons from the ligand retained as counterion are still available for complexation and may further retain M^{2+} ions from solution. The use of ion exchange resins for the retention of metal ions as complexes in different pH conditions has been thoroughly studied and reported in the literature [25,26]. Example of complexing agents are ethylenediaminetetraacetic acid, nitrilotriacetic acid, iminodiacetic acid, citric acid, etc. [27,28].

4.4 Equilibrium in size exclusion processes

General aspects

Size exclusion chromatography (SEC) is a chromatographic technique used for the

separation of substances according to their molecular size, or more correctly, their hydrodynamic volume. Size exclusion is mainly used for the separation of different polymers and for the separation of polymers from small molecules. The technique is also known as gel permeation chromatography when the mobile phase is an organic solvent or gel filtration chromatography when the mobile phase is an aqueous (mostly aqueous) solution.

Polymeric molecules that are usually separated by SEC have various shapes, and typically, are not spherical. Also, the macromolecules during the separation may be solvated/hydrated such that the molecular shape and volume are changed from that without solvent [29]. For these reasons, the "true" size/shape of the molecule is difficult to define. The diffusional properties of the macromolecule can be selected to describe the molecular apparent size. The hydrodynamic volume of a molecule is the volume of a hypothetical hard sphere that diffuses with the same speed as the particle under examination. For SEC, the hydrodynamic volume is therefore the true parameter related to the separation.

Equilibrium between interstitial mobile phase and pore mobile phase

In SEC equilibrium for a molecule retained in the stationary phase and the one present in the mobile phase can be described by the equilibrium constant K_{SEC} which represents the ratio of the concentrations of the macromolecule (analytes) in the pore (C_{pore}) and the concentration in the solution from the interstitial volume (C_{inter}):

$$K_{SEC} = \frac{C_{pore}}{C_{inter}} \tag{4.4.1}$$

Initially, it can be assumed that all molecules are in the mobile phase at a concentration $C_{inter} > 0$. The initial concentration of macromolecules within the pores can be considered zero ($C_{pore} = 0$). The concentration gradient between interstitial volume outside the pores ($C_{inter} > 0$) and within the pore (at $C_{pore} = 0$) "pulls" macromolecules into the pores because of the tendency to equalize the chemical potentials in the interstitial volume and in the pore volume. Macromolecules outside of the pores are in an "expanded" shape. When pulled into the pore in order to equalize concentrations outside and inside the pore, the macromolecules are squeezed and their conformational entropy decreases. In other words, the macromolecules inside the pore are contracted and lose part of their conformational entropy ($\Delta S^0 < 0$). Some macromolecules cannot enter entirely the pore volume because the loss in entropy would exceed the pulling force inside the pore. A schematic representation of the equilibrium process in SEC separation is illustrated in Fig. 4.4.1.

This process indicates that the values of K_{SEC} are situated within the interval [0,1]. If $K_{SEC} = 0$, the sample fraction will elute in the void volume (total exclusion), and when $K_{SEC} = 1$, the sample fraction elutes in the total column volume ($V_{inter} + V_{pore}$). However, this process implies an ideal equilibrium of SEC where the analytes do not exhibit any attractive or repulsive interaction with column packing except for the effects caused by the imperviousness of the pore walls. In some instances, values $K_{SEC} > 1$ are seen, indicating that other interactions take place between the analyte and the stationary phase. The expression for K_{SEC} using the general Eq. 4.1.10 is the following:

$$K_{SEC} = \exp\left(\frac{-\Delta \boldsymbol{H}^0 + T\Delta S^0}{RT}\right) \tag{4.4.2}$$

where $-\Delta \boldsymbol{H}^0$ is the standard enthalpy and ΔS^0 is the entropy change for the transfer of the analyte from the mobile to the stationary phase. However, in the ideal case of no energetic interaction with column packing, the value for $\Delta \boldsymbol{H}^0$ in Eq. 4.4.2 is zero ($\Delta \boldsymbol{H}^0 = 0$), and the separation is controlled exclusively by the entropy of the

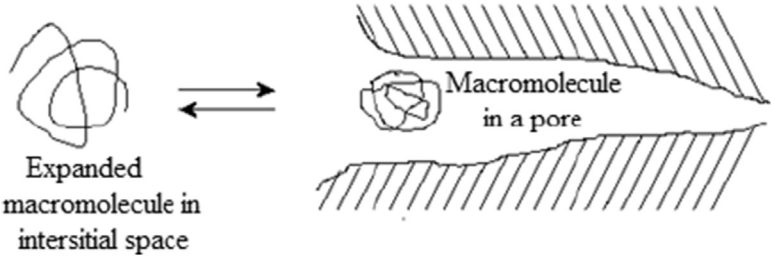

FIGURE 4.4.1 Schematic representation of the equilibrium of a coiled macromolecule between solution (interstitial space) and the pore of stationary phase (expansion of the molecule is associated with increase in entropy and shrinking is associated with decrease in entropy).

process. Therefore, the expression for a "pure" size exclusion process described by the constant K_{pure} is given by the following formula:

$$K_{pure} = \exp(\Delta S^0 / R) \tag{4.4.3}$$

Size exclusion of macromolecular analytes in the absence of any energetic interactions with the stationary phase is therefore an entropy-controlled process. Such process is usually indicated as an entropic equilibrium [30]. The loss of entropy when the molecules are trapped inside the stationary phase makes ΔS^0 to have negative values [31] and as a result $K_{pure} << 1$.

Eq. 4.4.3 indicates that temperature should not influence significantly the exclusion processes. This was proven experimentally in several eluents that are good solvents for polymers [32]. The change in entropy is more significant for larger molecules (of polymers) and less important for smaller ones. This can be understood by starting with the following expression for entropy:

$$S = k_B \ln\Omega \tag{4.4.4}$$

where k_B is Boltzmann constant and Ω is the number of possible (equally probable) micromolecular states. The number of ways in which the individual molecules can occupy the space within the pore of a stationary phase is significantly larger for a small molecule than for a large one. This indicates that the number of micromolecular states for a small molecule in the pore is considerably larger than for a large molecule. Starting with similar number of states for large and small molecules in solution, the large molecules will have a smaller S value in the stationary phase, therefore a considerable loss of entropy. At the same time, the small molecules will have only a minor loss of entropy. The result is that during the adsorption process in the pores, the large molecules will have a larger (in absolute value) negative ΔS^0.

In some cases, the separation also has an enthalpic contribution besides the entropic change for the SEC separation ($\Delta \boldsymbol{H}^0 \neq 0$). This happens in practice when some interactions between the packing and the macromolecular species are taking place, and for this reason, the SEC equilibrium constant can be defined by two terms:

$$K_{SEC} = K_{pure}\, K_{interaction} = \exp\left(\frac{\Delta S^0}{R}\right)\exp\left(\frac{-\Delta \boldsymbol{H}^0 + T\Delta S^{*0}}{RT}\right) \tag{4.4.5}$$

where $K_{interaction} = \exp\,[(-\Delta \boldsymbol{H}^0 + T\ \Delta S^{*0})/(RT)]$, $\Delta \boldsymbol{H}^0$ is the enthalpic term, and ΔS^{*0} represents the entropy variation during the interaction process. For the SEC separations where $K_{SEC} > 1$, there is always an enthalpic contribution to the separation. For K_{SEC} in [0,1] interval, it is difficult

to decide whether $\Delta H^0 = 0$ or it is only small. A more detailed discussion on thermodynamic factors affecting SEC separation can be found in the literature [33–35].

4.5 The influence of pH and of additives on retention equilibria

Preliminary information about pH

An important parameter in almost all analytical separations is the pH. The pH was initially defined for water as the solvating medium. The pH gives in logarithmic form (base 10) the activity of hydrogen ions a_{H^+} or more precisely the activity $a_{H_3O^+}$ of hydronium ions (H_3O^+). The pH expression is given by the following formula:

$$\text{pH} = -\log a_{H_3O^+} = -\log C_{H_3O^+} - \log \gamma_{H_3O^+} \tag{4.5.1}$$

In expression 4.5.1 $\gamma_{H_3O^+}$ is the activity coefficient of hydronium ions and where $C_{H_3O^+}$ is their molar concentration. The reason for the replacement of hydrogen ions H^+ with hydronium H_3O^+ or even with higher species (e.g., $H_9O_4^+$) is to account for solvation of protons, but all these ions describe in fact the same entity (and notations H^+ or H_3O^+ are frequently used interchangeably).

In water, the pH scale is between 0 and 14 at 25°C. The activity factor $\gamma_{H_3O^+}$ can be estimated with Debye–Hückel equation, written in the following form:

$$\log \gamma_{H_3O^+} = -\frac{A\sqrt{I}}{1 + a_s B\sqrt{I}} \tag{4.5.2}$$

In Eq. 4.5.2, I represents the ionic strength of the solution, a_s is an ion size parameter, and A and B are solvent and temperature-dependent parameters, respectively. Their values are tabulated from empirical data. In an infinitely diluted water solution, $\gamma_{H_3O^+} = 1$, and the second term from Eq. 4.5.1 is zero. The ionic strength in a solution depends on the concentration of species X, C_X, and their net charges, z_X, by the following formula:

$$I = \frac{1}{2}\sum_X C_X z_X^2 \tag{4.5.3}$$

The pH measurement in partially aqueous or nonaqueous media is necessary since in many separations the mobile phase is not entirely aqueous [36,37]. Measuring pH of solutions in the presence of organic solvents is however difficult due to the shift in pH readings induced by the nonaqueous solvents on the activity of H^+ ions and on the pH electrode [38]. Moreover, the conventional pH sensors are not suitable for such applications, when the aqueous electrolyte of conventional pH electrodes (e.g., KCl solution) is not miscible with organic solvents. The pH of the solvating medium influences the dissociation of acidic or basic species, and therefore, it has influence on the chromatographic retention. Due to the importance of pH in liquid chromatographic separation, these aspects are detailed in the section dedicated to the mobile phases in HPLC (Section 7.4).

Dependence of compound structure on pH

Compounds with acidic, basic, or amphoteric character may have structures that are strongly dependent on the pH of the solution (e.g., in the mobile phase). The structure of such compounds may change at different pH values. This change is caused by the interaction of the compound with the protons. Each ionization equilibrium between the protonated and deprotonated forms of the molecule can be described with its dissociation constant K_a or $pK_a = -\log K_a$. The pK_a value can be defined for both acids and bases. For simple acids HX, the dissociation takes place as follows:

$$HX \rightleftarrows H^+ + X^- \tag{4.5.4}$$

The equilibrium constant K_a for the acid known as acidity constant (usually expressed in logarithm form - $\log K_a = pK_a$) is defined by the following formula:

$$K_a = \frac{C_{H^+} \cdot C_{X^-}}{C_{HX}} \tag{4.5.5}$$

The values for K_a are frequently expressed in "log" form, with the following notation:

$$\log K_a = -pK_a \tag{4.5.6}$$

Assuming $C_{H+} = C_X^-$, expression 4.5.5 gives the following expression for C_{H+} in a solution of a simple acid:

$$[H^+] = C_{H^+} = \sqrt{K_a C_{HX}} \tag{4.5.7}$$

(Note: Both notations C_X and $[X]$ indicate molar concentration and are used interchangeably).

For simple bases BOH, the acidity constant can be obtained based on the following equilibrium:

$$BOH_2^+ \rightleftarrows BOH + H^+ \tag{4.5.8}$$

The acidity constant K_a is defined by the following formula:

$$K_a = \frac{C_{H^+} C_{BOH}}{C_{BOH_2^+}} \tag{4.5.9}$$

A base dissociation constant K_b can also be defined by the following:

$$K_b = \frac{C_{OH^-}\ C_{B^+}}{C_{BOH}} \tag{4.5.10}$$

By multiplying Eq. 4.5.9 and Eq. 4.5.10, since $C_{B^+} = C_{BOH_2^+}$ the following expression is obtained:

$$K_a K_b = C_{H^+} C_{OH^-} = K_w \tag{4.5.11}$$

The constant K_w is the water ion product and $K_w = 1.008\ 10^{-14}$ at 25 C. Acidic or basic character can be assigned to the molecule according to Brønsted's rule that indicates that an acid is a proton donor and a base a proton acceptor. As a result, a basic compound is protonated as a positive ion, while an acidic compound is protonated as a neutral molecule. An acidic compound will be deprotonated as a negative ion. Certain molecules can dissociate as both an acid or as a base and are known as amphoteric. Also, molecules can be multiprotic, having more than one ionizable group. The influence of pH on the structure of a molecule with amphoteric character is shown, as an example, for valine in Fig. 4.5.1 where the percent of a specific form of the compound in function of solution pH is indicated. At low pH, valine will act as a base and accept a proton, at pH in the range 4–8 the neutral molecule will be present, and in basic conditions valine will act as an acid and will ionize a proton.

The presence of a compound in different forms as a function of pH is very common, and this influences significantly the separation process. In this section, the influence of pH on various equilibria is discussed.

The influence of pH on partition equilibrium

Partition equilibrium of a compound X between an aqueous phase (or partially aqueous phase indicated as w) and an organic phase indicated as o is described by the partition (distribution) constant K_X. This is given by Eq. 4.1.7 replacing $A = o$ and $B = w$ in the concentration ratio of X in the two immiscible phases, at equilibrium:

$$K_X = \frac{C_{X,o}}{C_{X,w}} \tag{4.5.12}$$

In a simple partition equilibrium between two liquid nonmiscible solvents, the distribution coefficient (D_X) and the distribution constant (K_X) are identical for apolar or polar solutes X that have no acid or base functional groups in their molecule that would lead to some dissociation in the aqueous or partially aqueous medium

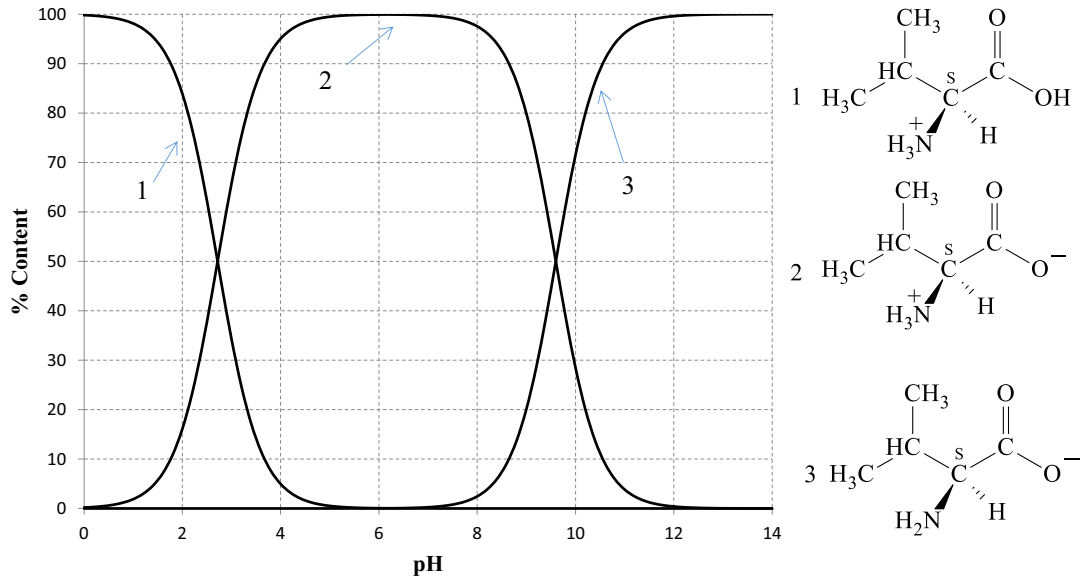

FIGURE 4.5.1 Variation in the proportion of different forms of valine in a solution at different pH values.

(indicated as w). When the solutes contain ionizable functional groups such as—OH, —SH, —COOH, $-SO_3H$, $-NH_2$, and others, it is possible for those solutes to have interactions into aqueous phase with proton or hydroxide ions leading to ionized species. In such cases, the two distribution parameters (D_i and K_i) are not identical, and the one that truly describes the partition process is the distribution coefficient D_i. In Fig. 4.5.2, a schematic example is shown, which describes the partition process for a molecular weak acid, denoted by HX.

Similar to the case of solvents is the one where the organic phase role is taken by an organic stationary phase, and the aqueous phase is a mobile phase with a certain water content (indicated as w). In the case of the acids HX, the distribution constant K (see Eq. 4.1.10) and distribution coefficient D (see Eq. 4.1.17) are given by the following relations:

$$K_{HX} = \frac{C_{HX,o}}{C_{HX,w}} \tag{4.5.13}$$

$$D_{HX} = \frac{\sum C_{HX,o}}{\sum C_{HX,w}} = \frac{C_{HX,o}}{C_{HX,w} + C_{X^-,w}} \tag{4.5.14}$$

Eqs. 4.5.13 and 4.5.14 show that higher K_{HX} or D_{HX} indicate a higher concentration of the analyte in the organic phase. This remains valid when the o phase is the stationary phase (e.g., in reversed phase chromatography). For these cases, a higher K_{HX} or D_{HX} are related to a higher retention factor k' based on Eq. 3.1.20 and Eq. 3.1.20, respectively, and therefore to longer retention times in the chromatographic process.

Since in the case of ionizable groups the distribution process is controlled by the distribution coefficient D and not by the distribution constant K, it is important to analyze the

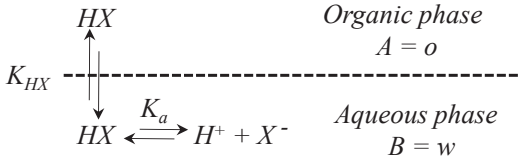

FIGURE 4.5.2 Representation of the partition process of a weak acid HX.

variation of D for several particular cases. In the case of an acid HX, the concentration of the dissociated form in Eq. 4.5.14 can be calculated from the dissociation constant (K_a) as follows:

$$C_{X^-,w} = K_a \frac{C_{HX,w}}{C_{H^+,w}} \tag{4.5.15}$$

By substituting $C_{X^-,w}$ from Eq. 4.5.15 in expression 4.5.14, the relation between the partition constant K_{HX} and distribution coefficient D_{HX} can be written as follows:

$$D_{HX} = \frac{C_{HX,o}}{C_{HX,w}\left(1 + K_a/C_{H^+,w}\right)} = K_{HX}\frac{10^{-pH}}{K_a + 10^{-pH}} \tag{4.5.16}$$

The dependence given by rel 4.5.16 is illustrated in Fig. 4.5.3 for a compound with $K_a = 10^{-4.54}$, ($pK_a = 4.54$). At low pH values, $D_{HX} \approx K_{HX}$. At high pH values, $D_{HX} \approx 0$; and at pH $= pK_a$ which is the inflection point of the sigmoid curve of dependence, $D_{HX} \approx 0.5\ K_{HX}$.

A weak basic type compound Y can be involved in an acid/base equilibrium as shown below:

$$Y + HOH \rightleftarrows YH^+ + OH^- \tag{4.5.17}$$

The above equilibrium is characterized by a basicity constant K_b ($K_b = K_w/K_a$). The same calculations as applied for the acids distribution lead to the following dependence of distribution coefficient D_Y on K_Y and pH:

$$D_Y = K_Y \frac{K_w}{K_w + K_b \cdot 10^{-pH}} \tag{4.5.18}$$

(where K_w is the ionic product of water (10^{-14} at 25°C). The plot of this dependence is shown in Fig. 4.5.4 for a base with $K_b = 10^{-4.64}$ ($pK_b = 4.64$).

Eqs. 4.5.16 and 4.5.18 (as well as the graphs from Figs. 4.5.3 and 4.5.4) indicate that for weak acidic or basic analytes, the pH plays a major role in the distribution of the analyte. Even if the distribution constant is highly favorable for the organic phase (large K values), since the parameter that truly describes the distribution process is D, its value can be very small if the pH of the aqueous phase is not selected properly. This effect can also be successfully used in a separation process. For example, the separation of a number of acids with different pK_a values can be easily separated using a gradient elution with the mobile phase changing its pH value.

Compounds with amphoteric character contain both acidic and basic functional groups

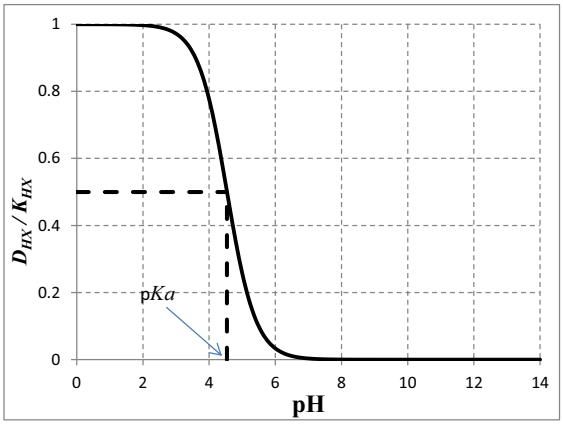

FIGURE 4.5.3 The dependence of D_{HX}/K_{HX} as a function of pH for an acid HX ($pK_a = 4.54$).

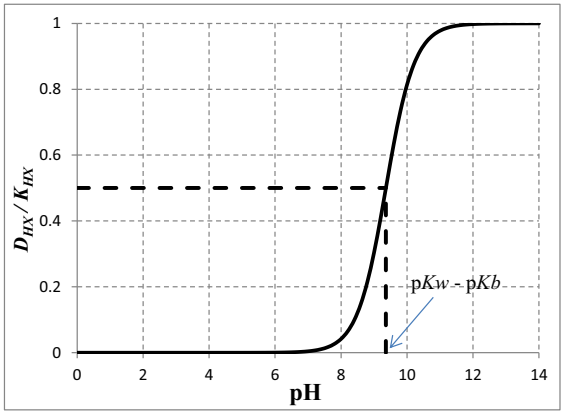

FIGURE 4.5.4 The dependence of D_Y/K_Y as a function of pH for a basic compound Y ($pK_b = 4.64$).

(for example, amino acids). If such a compound is indicated as XYH and participates in a distribution process, in the aqueous phase (mobile phase can be partly aqueous) it is part of two acid base equilibria. These equilibria and their corresponding K_a and K_b constants are listed in the following expressions:

$$XYH \rightleftarrows XY^- + H^+ \tag{4.5.19}$$

$$K_a = \frac{C_{XY^-,w} \cdot C_{H^+,w}}{C_{XYH,w}} \tag{4.5.20}$$

$$XYH + HOH \rightleftarrows XYHH^+ + OH^- \tag{4.5.21}$$

$$K_b = \frac{C_{XYHH^+,w} \cdot C_{OH^-,w}}{C_{XYH,w}} \tag{4.5.22}$$

The distribution of the neutral species between the two phases (o and w) is characterized by partition constant K_{HXY} and distribution coefficient D_{HXY}: with formulas of the type Eqs. 4.1.1 and 4.1.17 that can be written as follows:

$$K_{XYH} = \frac{C_{XYH,o}}{C_{XYH,w}} \tag{4.5.23}$$

$$D_{XYH} = \frac{C_{XYH,o}}{C_{XY^-,w} + C_{XYHH^+,w} + C_{XYH,w}} \tag{4.5.24}$$

Combining Eqs. 4.5.20, 4.5.22, and 4.5.23 with 4.5.24, the following expression for D can be obtained:

$$D_{XYH} = \frac{K_{XYH}}{1 + \frac{K_a}{C_{H^+}} + \frac{K_b \cdot C_{H^+}}{K_w}} \tag{4.5.25}$$

Eq. 4.5.25 is identical with the following expression:

$$D_{XYH} = K_{XYH} \frac{K_w \cdot 10^{-pH}}{K_a K_w + K_w \cdot 10^{-pH} + K_b \cdot 10^{-2pH}} \tag{4.5.26}$$

The graph showing the variation of D_{HXY}/K_{HXY} as a function of pH is given in Fig. 4.5.5 for a compound with $pK_a = 2.47$ and $pK_b = 4.55$ (phenylalanine).

Taking into account that $K_a \cdot K_w << 10^{-14}$, $K_w = 10^{-14}$, and $K_b >> K_w$, the limit value of D for the acidic pH domain is given by the following expression:

$$D_{XYH}^{pH \to 0} = K_{XYH} \frac{K_w}{K_b} \tag{4.5.27}$$

In the example shown in the graph from Fig. 4.5.5, $K_w << K_b$ and the value for D_{XYH} is much lower than that for K_{XYH} (practically zero).

For pH → 14 and taking into account that $K_a >> K_w$, the limit value of D_{HXY} for basic pH domain is given by the following expression:

$$D_{XYH}^{\mathrm{pH} \to 14} = K_{XYH} \frac{K_w}{K_a} \tag{4.5.28}$$

For the example shown in the graph from Fig. 4.5.5, $K_w << K_a$ and the value for D_{XYH} is again much lower than that for K_{XYH} (practically zero).

The maximum value for the ratio D_{XYH}/K_{XYH} can be obtained from the condition $\frac{\partial D_{XYH}}{\partial C_{H^+}} = 0$ which leads to the following expression for D_{HXY}:

$$D_{XYH}^{max} = K_{XYH} \frac{1}{1 + 2\left(\frac{K_a K_b}{K_w}\right)^{1/2}} \tag{4.5.29}$$

This maximum takes place for the following pH value:

$$\mathrm{pH}_{XYH}^{max} = 7 + \frac{1}{2}(pK_a - pK_b) \tag{4.5.30}$$

The result for D^{max}, for example, taken for Fig. 4.5.5 ($pK_a = 2.47$ and $pK_b = 4.55$) shows that $D^{max} \approx 1.6\ 10^{-4}\ K_{XYH}$. This result indicates that in an HPLC separation where the organic phase is a stationary phase of the type C18 or C8, much less compound will be present in the stationary phase than predicted from its K_{XYH} value, and the elution of the compound will take place very fast.

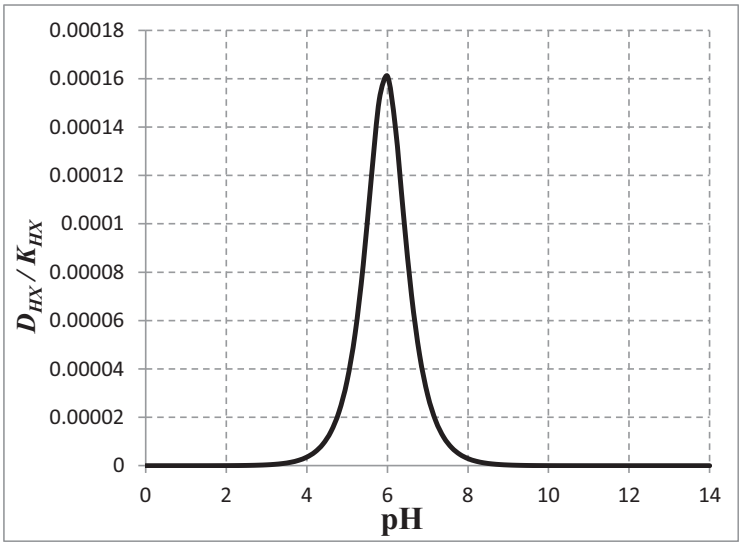

FIGURE 4.5.5 The dependence of D_{HX}/K_{HX} for an amphoteric compound with $pK_a = 2.47$ and $pK_b = 4.55$ as a function of the pH of the aqueous phase.

In the previous discussion of the influence of pH on the distribution of a molecule that has acidic, basic, or amphoteric character, it was assumed that an equilibrium of different species of compound X takes place only in the mobile phase, and that the ionized species are not at all partitioned in the stationary phase. However, the ionized species can also be considered as distributed between the aqueous and organic phase. This type of equilibrium is schematically shown in Fig. 4.5.6.

For the case of the partition process of a weak acid HX with the ion X^- also subject to the distribution, Eq. 4.5.18 will remain valid for K_{HX}, but the expression for D_{HX} should be replaced by the following formula:

$$D_{HX} = \frac{\sum C_{HX,o}}{\sum C_{HX,w}} = \frac{C_{HX,o} + C_{X^-,o}}{C_{HX,w} + C_{X^-,w}} \qquad (4.5.31)$$

and an additional equilibrium constant will be defined as follows:

$$K_{X^-} = \frac{C_{X^-,o}}{C_{X^-,w}} \qquad (4.5.32)$$

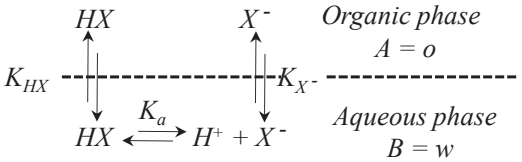

FIGURE 4.5.6 Representation of the partition process of a weak acid HX when the ion X^- is also subject to the distribution process.

By substituting in Eq. 2.5.29 the following terms: $C_{X^-,w}$ obtained from Eq. 4.5.15, $C_{X^-,o}$ obtained from Eq. 4.5.32, and $C_{HX,o}$ from Eq. 4.5.13, upon simplification with $C_{HX,w}$, the expression for D_{HX} can be written as follows:

$$D_{HX} = \frac{K_{HX} \cdot 10^{-pH} + K_{X^-} K_a}{K_a + 10^{-pH}} \qquad (4.5.33)$$

Similar expressions can be developed for a basic compound, amphoteric compound, dibasic acid, etc. [39].

Relations 4.5.16, 4.5.18, or 4.5.26 allow the evaluation of the retention factor k'_X based on Eq. 4.1.21 for a compound X when the

distribution constant K_X, the phase ratio Ψ, and the pH of the mobile phase are known for an acidic, basic, or amphoteric analyte, respectively. For an acidic species HX, this dependence can be written in the general form as follows:

$$\log k'_{HX} = a \log D_{HX} + b \qquad (4.5.34)$$

where the constant b is identical to phase ratio Ψ.

Dependence on pH of octanol/water distribution coefficient D_{ow}

The octanol/water partition constant K_{ow} (see Eq. 4.1.23) is a parameter widely used in quantitative structure–activity relationship (QSAR) and related drug designed techniques as a measure of molecular hydrophobicity. The values of this parameter for a large number of compounds are available in the literature [12,13,40], and various computer programs are available for its calculation (e.g., MarvinSketch 5.4.0.1, ChemAxon Ltd., [14], EPI Suite [41]). As shown in Section 4.1, log K_{ow} shows a good correlation with capacity factor in RP-HPLC for many organic compounds [10,11,42]. For these reasons, the values for K_{ow} represent a convenient resource for the characterization of hydrophobicity of organic compounds with application to HPLC. For compounds having ionizable groups that exist in solution as a mixture of different forms (some ionic and some neutral), a distribution coefficient D_{ow} is defined by Eq. 4.1.24. The general discussion on the dependence of distribution coefficient D on pH given in this section is equally applicable to the distribution coefficient D_{ow}, which depends on the water pH for ionizable compounds. For compounds having no ionizable groups, $K_{ow} = D_{ow}$. Variation of D_{ow} with the pH can be obtained using computing programs (e.g., MarvinSketch 5.4.0.1).

As an example, in an acidic pH, an acid may be very little or not ionized at all. At pH values where the compound is not ionized $D_{ow} = K_{ow}$, while for other pH values they are different and $D_{ow} < K_{ow}$. This is exemplified for butyric acid in Fig. 4.5.7, for butyric acid where at low

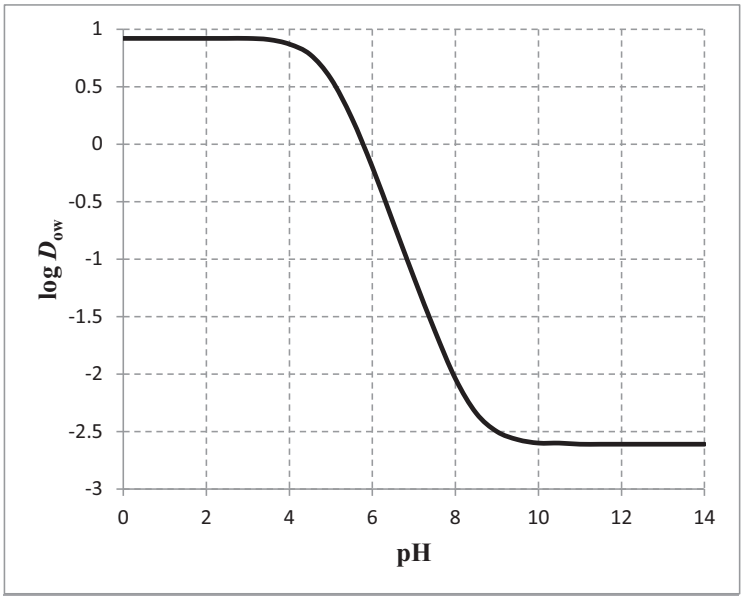

FIGURE 4.5.7 Variation of log D_{ow} with pH for butyric acid, max log $D_{ow} = 0.92$, log $K_{ow} = 0.92$.

pH (pH < 3), the maximum value for D_{ow} is 0.98 and $K_{ow} = D_{ow}$. For some amphoteric compounds, even in a pH where a neutral form would be expected, the compound is present in zwitterionic form, and always $D_{ow} < K_{ow}$. Amino acids are this type of compound, and, for example, in the case of valine $K_{ow} = 0.31$, and the dependence of D_{ow} on pH shown in Fig. 4.5.8 shows that a maximum value is $D_{ow} = -2.09$. The discrepancy is the result of assumption that valine can be a neutral molecule ($K_{ow} = 0.31$), while in reality it always contains ionic groups.

The results pictured in Figs. 4.5.7 and 4.5.8 indicate that for compounds that can be present in ionic form, in order to have information regarding their octanol/water distribution, the values for D_{ow} should be considered at a specific pH. For amino acids, peptides, and proteins that are present in zwitterionic form, the value for log D_{ow} at the isoelectric point (pI) is important for their characterization (the isoelectric point (pI) is the pH value at which the molecule carries no net electrical charge). For molecules with multiple zwitterionic points, log D_{ow} at the isoelectric point may be in between the lowest and the highest value of log D_{ow} at different pH values.

Influence on separation of additives not involved in the equilibrium

The addition of additives in the mobile phase is a subject further discussed in Chapter 7 regarding mobile phase composition. Additives may play a variety of roles regarding the separation, and for the use as IPA (ion pairing agents) or of chiral modifiers in the mobile phase. One additional influence of additives in the mobile phase is that of affecting the effective concentration (thermodynamic activity) of the analytes. For a compound X, activity a_X can be defined based on expression 4.1.5. The activity coefficient γ_X can be related to the change in the enthalpy of mixing when the compound X

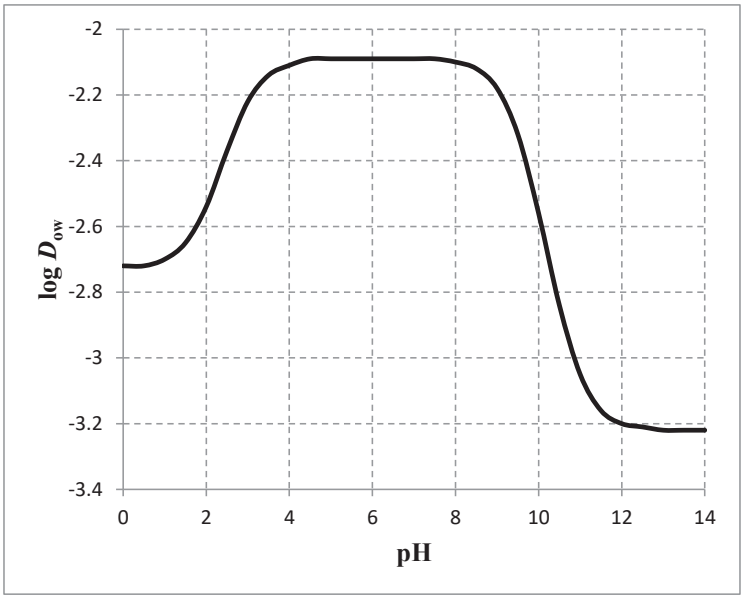

FIGURE 4.5.8 Variation of log D_{ow} with pH for valine, max log $D_{ow} = -2.09$, log $K_{ow} = 0.31$.

is placed in a solvent and does not form an ideal solution but a regular solution (solutions with mixing enthalpy different from zero) [43]. For such a regular solution, the expression for the chemical potential of compound X is given by the following expression:

$$\mu_X = \mu_X^0 + \frac{1}{n_X}\left(\boldsymbol{H}_X - \boldsymbol{H}_X^0\right) + RT \ln C_X \quad (4.5.35)$$

where n_X is the number of moles of compound X and $\boldsymbol{H}_X - \boldsymbol{H}_X^0$ is the change in enthalpy caused by the interactions during mixing. The expression 4.5.35 can be compared with the common expression for chemical potential:

$$\mu_X = \mu_X^0 + RT \ln a_X \quad (4.5.36)$$

By taking into consideration that $a_X = \gamma_X C_X$ (see expression 4.1.5), the following formula is obtained for the activity coefficient:

$$\ln \gamma_X = \frac{1}{n_X RT}\Delta \boldsymbol{H}_{mix,\,X} \quad (4.5.37)$$

The change in the enthalpy of mixing depends on the interactions of the solute with the solvent and the solvent can be a pure compound, a mixture of solvents, and also may contain additives. The nature of the solvent (solvents) as well as additives influence the interactions and these affect the activity coefficient and as a result of the effective concentration of the analyte. One type of additives that are known to strongly affect the interactions in solution is known as chaotropes.

Chaotropic salts influence on equilibria

A chaotropic agent is a molecule in water or water/organic solvent solutions that can disrupt the hydrogen bonding and solvation of ions. Chaotropic solutes interfere with intramolecular interactions such as hydrogen bonding, van der Waals forces, and hydrophobic effects and as shown by Eq. 4.5.37, they affect the activity coefficient of the analytes. Chaotropic salts that dissociate in solution can produce a shielding of ionic charges. Also since hydrogen bonding is stronger in nonpolar media, and salts increase the chemical polarity of the solvent, hydrogen bonding is affected. Chaotropic salts can be added in some mobile phases to influence the activity of the analytes and therefore the separation.

4.6 The influence of temperature on retention equilibrium

General aspects

Similar to all equilibria, the separation process is influenced by temperature. For a separation equilibrium in HPLC, the retention factor k' is dependent on temperature following Eq. 4.1.12, known as van't Hoff equation and written below:

$$\ln k'(X) = \frac{-\Delta \boldsymbol{H}^0}{R}\left(\frac{1}{T}\right) + \frac{\Delta S^0}{R} + \ln \Psi \quad (4.6.1)$$

In Eq. 4.6.1, $\Delta \boldsymbol{H}^0$ and ΔS^0 are the standard enthalpy and entropy changes, respectively, for the transfer of the analyte from the mobile to the stationary phase (Eq. 4.1.12 was developed based on distribution equilibrium, but can be used in principle for any HPLC process). Since the retention is an exothermic process, $\Delta \boldsymbol{H}^0$ has negative values. Eq. 4.6.1 indicates that for typical separations, the value of k' decreases as the temperature increases (deviations from this rule are further discussed). When the stationary phase, the analyte, and the mobile phase properties do not change with a temperature change, $\Delta \boldsymbol{H}^0$ and ΔS^0 can be considered temperature invariants. However, this assumption is only an approximation, and more correct estimations of the values for $\Delta \boldsymbol{H}^0$ and ΔS^0 at different temperatures can be made. Corrections as function of temperature T of thermodynamic functions

ΔH^0 and ΔS^0 are known (e.g., Ref. [44]). For numerous separations, the linear dependence of ln k' on $1/T$ can be experimentally verified [45]. The graphs representing ln k' as a function of $1/T$ are known as van't Hoff plots. Regarding the variation of Ψ with temperature, this is also commonly considered constant although recent studies demonstrate that in fact Ψ also varies with temperature [46–48].

The common HPLC and UPLC work is performed either at (controlled or not) room temperature or at temperatures up to 60–65°C. The effect of an increase in the temperature where separation takes place is usually a decrease in the retention factor k' as indicated by Eq. 4.6.1. This effect is usually opposite to obtaining a better separation. However, other benefits may result from a temperature increase, such as a decrease in mobile phase viscosity (see Eq. 7.2.3). This can be useful for decreasing the backpressure in the analytical column. Also, special HPLC techniques indicated as high temperature have been developed using temperatures in the range 150–200 C [49].

Evaluation of thermodynamic parameters of a separation from van't Hoff plots

Linear van't Hoff plots are those where the variation of log k' can be written in the simple form:

$$\ln k' = a' + b'\left(\frac{1}{T}\right) = \left[\frac{\Delta S^0}{R} + \ln \Psi\right] - \frac{\Delta H^0}{R}\left(\frac{1}{T}\right) \tag{4.6.2}$$

From the derivatives of expression 4.6.2 in function of $(1/T)$ the following formulas are generated for ΔH^0 and ΔS^0:

$$\Delta H^0 = -R\,b' \quad \text{and} \quad \Delta S^0 = R\,(a' - \ln \Psi) \tag{4.6.3}$$

The parameters a' and b' are obtained from the experimental plots of log k' in function of $1/T$. From Eq. 4.6.3, these parameters are further used for the evaluation of enthalpy and entropy changes associated with the retention process, for the entropy evaluation being also necessary to know the value of phase ratio Ψ [50]. As an example, ΔH^0 and ΔS^0 can be calculated from the van't Hoff plots for several pesticides with structures given in Fig. 4.6.1.

The van't Hoff plots for these pesticides were obtained for a separation performed on a C8 stationary phase and two compositions of the mobile phase, one containing 40% acetonitrile (AcCN) and 60% water and the other containing 45% acetonitrile and 55% water [51]. These plots are shown in Fig. 4.6.2.

Using the estimation $\Psi \approx 0.25$ [52] from the plots shown in Fig. 4.6.2, the values obtained for ΔH^0 and ΔS^0 are given in Table 4.6.1.

The results obtained for ΔH^0 and ΔS^0 given in Table 4.6.1 are in fair agreement with thermodynamic data reported in the literature, for example, for the interaction between two hydrocarbon side chains of a protein (−1.2 to −7.5 kJ/mol for ΔH^0 and −7 to −45 J/(K mol) for ΔS^0 at 298 K) [53]. The value of ΔH^0 reflects the degree of interaction between analyte and stationary phase, and a more negative ΔH^0 (larger absolute value) indicates a stronger interaction [54].

The correction introduced by taking into consideration that ΔH^0 and ΔS^0 depend in fact on temperature does not affect significantly the previous discussion. The variation with the temperature T for these parameters is given by the expressions (e.g., Ref. [9]):

$$\Delta H^0_T = \Delta H^0_{T_0} + \Delta C_p (T - T_0) \tag{4.6.4}$$

and:

$$\Delta S^0_T = \Delta S^0_{T_0} + \Delta C_p \ln\left(\frac{T}{T_0}\right) \tag{4.6.5}$$

where ΔC_p is the variation in heat capacity and T_0 is a reference temperature. Although C_p for solid and liquid solutes also varies with temperature to a certain extent, this variation is usually

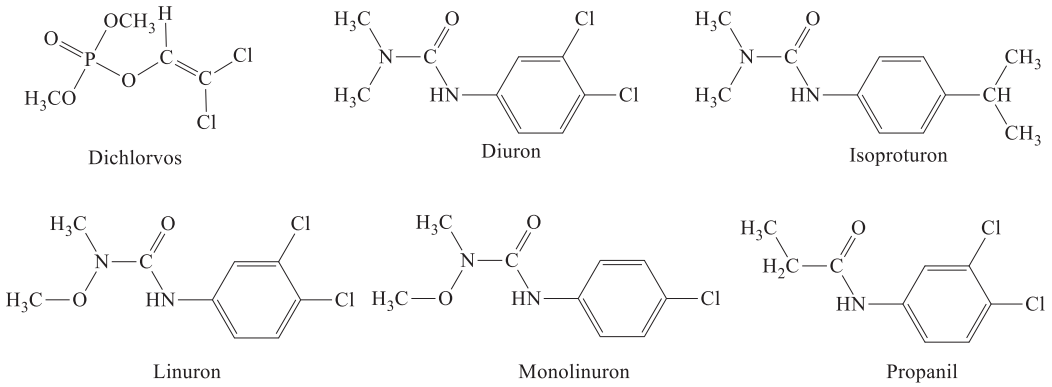

FIGURE 4.6.1 Chemical structures of several commercial pesticides.

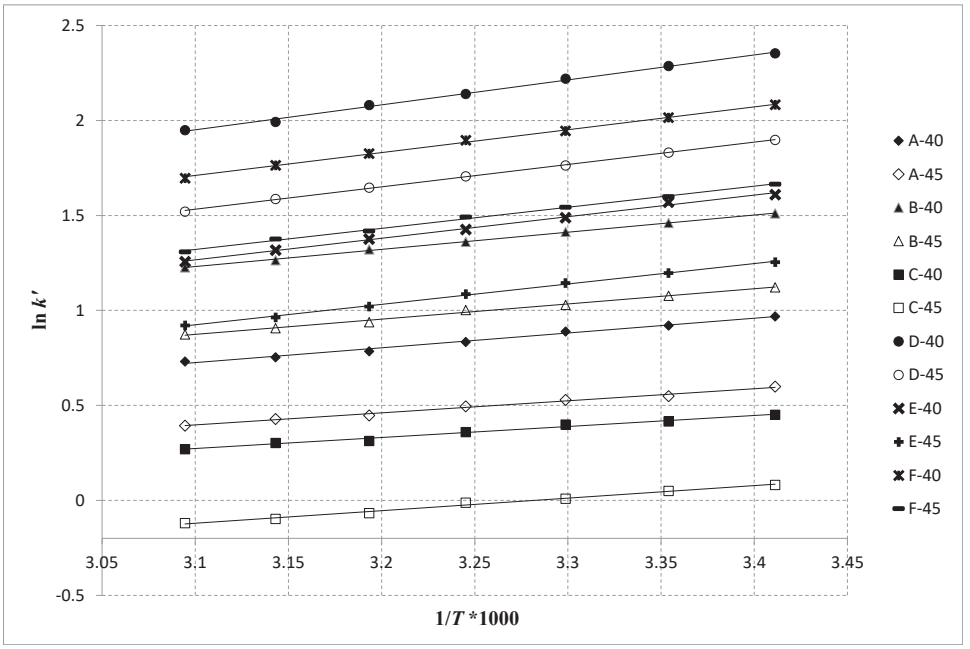

FIGURE 4.6.2 Van't Hoff plots for six pesticides A = Dichlorvos, B = Diuron, C = Isoproturon, D = Linuron, E = Monolinuron, and F = Propanil for a C8 column and mobile phase 40% acetonitrile (AcCN) and 60% water (40) and for mobile phase 45% acetonitrile (AcCN) and 55% water (45).

TABLE 4.6.1 Evaluation of thermodynamic parameters ΔH^0 and ΔS^0 for the separation of six pesticides on a C8 column, and for two mobile phase compositions [51].

Commercial name	% AcCN in water	ΔH^0 (kJ/mol)	ΔS^0 (J/mol/K)
Dichlorvos	40	−6.16	−1.51
	45	−5.16	−1.12
Diuron	40	−7.57	−1.72
	45	−6.98	−2.91
Isoproturon	40	−5.27	−2.62
	45	−4.95	−4.71
Linuron	40	−10.98	−6.34
	45	−9.95	−6.61
Monolinuron	40	−9.68	−8.01
	45	−8.96	−8.62
Propanil	40	−9.91	−4.95
	45	−9.09	−5.69

very small. With these corrections, for temperatures in a range relatively close to T_0, by taking with a good approximation $\Delta C_p \ln (T/T_0) \approx 0$, the dependence of $\ln k'$ on temperature will be described by the following formula:

$$\ln k' = \frac{\Delta S^0_{T_0}}{R} - \frac{\Delta H^0_{T_0}}{R}\left(\frac{1}{T}\right) - \Delta C_p\left(1 - \frac{T_0}{T}\right) + \ln \Psi \tag{4.6.6}$$

For a value $T \approx T_0$, or $T \neq T_0$ but $\Delta C_p \approx 0$, Eq. (4.6.6) is identical with Eq. (4.6.1) and the results of calculation of ΔH^0 and ΔS^0 remain unchanged.

The variation of Ψ with temperature has, however, a more noticeable effect on the calculation of ΔH^0 and ΔS^0 from van't Hoff plots. The expression of ΔH^0 is also obtained from the derivatives of function of log k' versus $1/T$ but taking into account that Ψ is not a constant. In this case, the following expression is obtained [46]:

$$\Delta H^0 = -R\left[b' - \frac{\partial \ln \Psi}{\partial\left(\frac{1}{T}\right)}\right] \tag{4.6.7}$$

The value for ΔS^0 obtained using formulas 4.6.7 and 4.6.2 will become the following:

$$\Delta S^0 = R\left[a' - \ln \Psi + \frac{\partial \ln \Psi}{\partial\left(\frac{1}{T}\right)}\left(\frac{1}{T}\right)\right] \tag{4.6.8}$$

Expressions (4.6.7) and (4.6.8) indicate that the assumption of the independence on temperature of phase ratio Ψ in the calculation of enthalpy and entropy for the equilibrium process in HPLC based on van't Hoff plots of log k' as a function of $1/T$ must be reconsidered. The values of ΔH^0 and ΔS^0 depend on the compound and the specific stationary and mobile phase on which the separation is performed. For such a specific system (aromatic hydrocarbons, C18 columns, and water methanol mobile phase), the differences between the calculation of ΔH^0 and ΔS^0 considering Ψ temperature constant or variable can be as high as 3−4 kJ/mol for ΔH^0 and as high as 6−7 J/mol/deg for ΔS^0 [47].

Nonlinear dependence of the retention factor on 1/T

The dependence of $\ln k'$ (or log k') on $1/T$ is not linear for every compound. Several examples of such cases were reported in the literature [45,55,56]. Nonlinear dependence of $\ln k'$ on $1/T$ is exemplified below for the drugs vincamine, epivincamine, and drotaverine. The formulas for these compounds are shown in Fig. 4.6.3.

The variation of $\ln k'$ with $1/T$ for vincamine and epivincamine is shown in Fig. 4.6.4A for the compounds being separated on a Zorbax

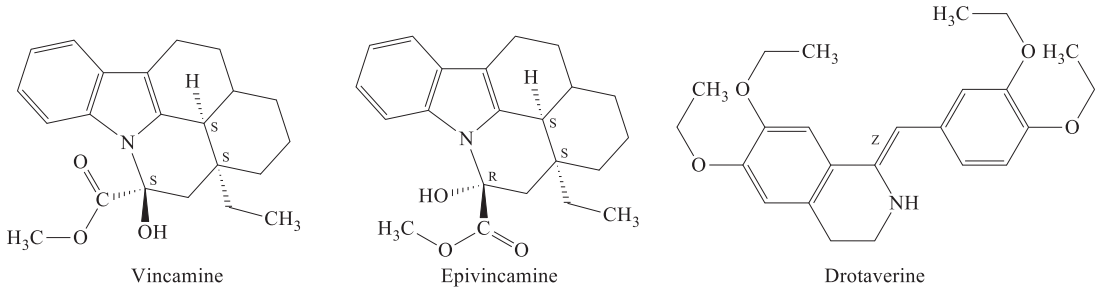

FIGURE 4.6.3 Chemical formulas for vincamine, epivincamine, and drotaverine.

XDB-C18 column 150 mm L × 4.6 mm i.d. and 3.5 μm particles, with a mobile phase consisting of a mixture of 0.2% triethylamine brought to pH = 6.0 with H_3PO_4 and acetonitrile, in the ratio 65/35 (v/v), and a flow-rate 1 mL/min. The same type of plot for drotaverine separated on the same column and a mobile phase consisting of aqueous component containing 25 mM ammonium formate brought to pH = 4.5 with formic acid and ACN in the ratio 62.5/37.5 (v/v) at a flow rate of 1 mL/min is shown in Fig. 4.6.4B.

Similar nonlinear dependences as shown in Fig. 4.6.4A and B were reported in the literature for other compounds, and their shape depends on temperature interval, type of analyte, type of buffer, the composition of the mobile phase, etc. [55].

The explanation of nonlinear dependence in van't Hoff plots can be that during the separation process more than one type of interaction takes place between the analyte *X* and the stationary and mobile phase, and these interactions are influenced differently by the temperature [57]. The different interactions may be caused by the fact that the analyte is present in more than one form in solution (e.g., tautomers [56,58]). When the presence of different forms of an analyte is not plausible, and nonlinear dependencies are followed, then the different

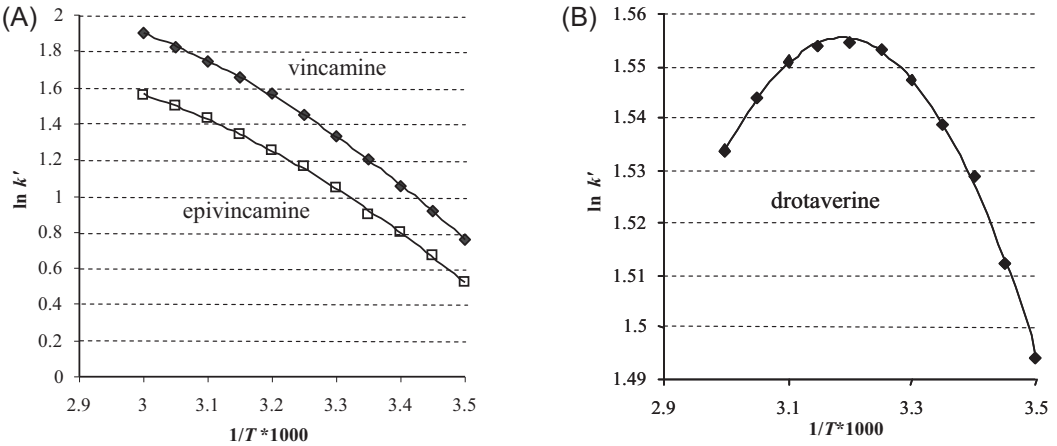

FIGURE 4.6.4 (A) Variation of ln k' with temperature for vincamine and epivincamine. (B) Variation of ln k' with temperature for drotaverine.

types of interaction between the species X and the stationary phase are the likely explanation of nonlinear behavior [45]. In such cases, a part of X molecules has one type of interaction and other part a different type. The separations in HILIC, or chiral type HPLC, are in particular known to have contributions from a combination of molecular interaction types (e.g., Ref. [59]). For these types of separations, the nonlinear van't Hoff plots are more common.

Considering, for example, only two different interaction types and for simplification considering that a partition equilibrium takes place between the stationary and mobile phase, the expression for the equilibrium constant $K(X)$ can be written in the following form:

$$K(X) = \frac{[X_1]_s + [X_2]_s}{[X_1]_m + [X_2]_m} \tag{4.6.9}$$

where indices "$_1$" and "$_2$" indicate either different molecular structures or that the unique type of molecules undergo two different separation mechanisms. Each mechanism can be assumed as described by a different equilibrium constant:

$$K_1 = \frac{[X_1]_s}{[X_1]_m} \quad \text{and} \quad K_2 = \frac{[X_2]_s}{[X_2]_m} \tag{4.6.10}$$

A formal equilibrium can be considered to exist between the molecules separated by the two different interactions, such that a constant K_{21} can be defined by the following formula:

$$K_{21} = \frac{[X_2]_m}{[X_1]_m} \tag{4.6.11}$$

From expressions 4.6.9, 4.6.10 and 4.6.11, the following formula can be written for $K(X)$:

$$K(X) = \frac{K_1 + K_2 K_{21}}{1 + K_{21}} \tag{4.6.12}$$

Each constant is related to a corresponding free enthalpy value ΔG (index "0" omitted for simplicity of writing). As a result, the expression for $K(X)$ can be written in the following form:

$$\ln K(X) = \ln \frac{\exp\left(-\frac{\Delta G_1}{RT}\right) + \exp\left(-\frac{\Delta G_2 + \Delta G_{21}}{RT}\right)}{1 + \exp\left(-\frac{\Delta G_{21}}{RT}\right)} \tag{4.6.13}$$

The expression 4.6.13 for $\ln K(X)$ can be represented in a Taylor series and will have the general form (e.g., Ref. [56]):

$$\ln K(X) = a' + b\frac{1}{T} + c\frac{1}{T^2} + \sum_{n=3}^{n} d_n \left(\frac{1}{T}\right)^n \tag{4.6.14}$$

By neglecting the terms in $(1/T)^n$ for $n > 2$, the approximation of the dependence of $\ln K(X)$ on $(1/T)$ and therefore of $\ln k'(X)$ will have a quadratic form (where $a = a' + \ln \Psi$) as follows:

$$\ln k'(X) = a + b\left(\frac{1}{T}\right) + c\left(\frac{1}{T}\right)^2 \tag{4.6.15}$$

The values for a, b, and c can be obtained by quadratic fitting the experimental dependence of $\ln k'(X)$ on $1/T$. When the values for a, b, c and of $\ln \Psi$ are known, a value for $\Delta \boldsymbol{H}(T)$ and for $\Delta S(T)$ can be obtained from the following expression:

$$\frac{\Delta S(T)}{R} + \ln \Psi - \frac{\Delta \boldsymbol{H}(T)}{R}\left(\frac{1}{T}\right) = a + b\frac{1}{T} + c\frac{1}{T^2} \tag{4.6.16}$$

The use of the first derivative in $(1/T)$ of both sides of expression (4.6.16) will generate the following expression:

$$\frac{\partial \Delta S(T)}{\partial(1/T)} - \Delta H(T) - \frac{\partial \Delta \boldsymbol{H}(T)}{\partial(1/T)}\,\frac{1}{T} = R\left(b + \frac{2c}{T}\right) \tag{4.6.17}$$

With a good approximation, it can be assumed that the following relation is valid:

$$\frac{\partial \Delta S(T)}{\partial (1/T)} - \frac{\partial \Delta \boldsymbol{H}(T)}{\partial (1/T)} \frac{1}{T} = 0 \qquad (4.6.18)$$

and the expression for $\Delta \boldsymbol{H}(T)$ will be the following:

$$\Delta \boldsymbol{H}(T) = -R\left(b + \frac{2c}{T}\right) \qquad (4.6.19)$$

By replacing the value of $\Delta \boldsymbol{H}(T)$ in Eq. 4.6.18, the expression for $\Delta S(T)$ is the following:

$$\Delta S(T) = R\left(a - \ln \Psi - \frac{c}{T^2}\right) \qquad (4.6.20)$$

Formulas 4.6.18 and 4.6.20 will replace the expressions 4.6.3 for the linear van't Hoff dependence, and they are reduced to expressions 4.6.3 when $c = 0$.

Evaluation of enthalpy–entropy compensation from van't Hoff plots

One of the applications of van't Hoff plots is related to the detection of the temperature where *enthalpy–entropy compensations* (EECs) occur in an HPLC separation for a series of compounds. The EEC process is characterized by a linear dependence between $\Delta \boldsymbol{H}_j^0$ and ΔS_j^0 at a specific temperature T_C for a series of homologue compounds when the relation between $\Delta \boldsymbol{H}_j^0$ and ΔS_j^0 (at T_C) has the following form:

$$\Delta \boldsymbol{H}_j^0 = a + b\Delta S_j^0 \; j = 1, 2, 3, ..n \quad (a \text{ and } b \text{ are constant}) \qquad (4.6.21)$$

(For a given compound in a separation, $\Delta \boldsymbol{H}_j^0$ and ΔS_j^0 are considered constant and not depending on temperature.) Eq. 4.6.21 implies that ΔG_j^0 is the same for all the compounds showing EEC effect. This is a direct consequence of the expression for the Gibbs free energy which is equivalent with the form $\Delta \boldsymbol{H}^0 = \Delta G^0 + T\Delta S^0$ and for a to be a constant ΔG^0 must be the same for $j = 1,2,3,\ldots\ n$. This effect was, for example, reported for a series of alkylphenols [60]. To determine T_C, for the series of compounds showing EEC effect, the van't Hoff plots are first obtained. Assuming that these plots are linear in case of EEC, the lines with equations $\ln k' = a' + b'(1/T)$ are intersecting at a specific temperature T_C. At that temperature, $\ln k'$ is the same for all compounds and therefore ΔG^0 is the same. In this case, the parameters a and b in Eq. 4.6.21 are constant and are given by following formula:

$$a = \Delta G^0_{T_C} \quad b = T_C \qquad (4.6.22)$$

The procedure is schematically described in Fig. 4.6.5 for four hypothetic compounds. In the hypothetic graph, $1/T_C = 2.5\ 10^{-3}$ corresponding to $T_C = 400$ K.

Based on the general definition of Gibbs free energy $\Delta G^0 = \Delta \boldsymbol{H}^0 - T\Delta S^0$, in order for ΔG^0 to be the same for several compounds with different $\Delta \boldsymbol{H}^0$ and ΔS^0 it requires that when $\Delta \boldsymbol{H}^0$ has a more negative value (indicating more interaction) for one compound, ΔS^0 must be smaller (ΔS^0 has positive values). This proves that from compound to compound a type of "compensation" takes place. This is explained in the retention process by the fact that when a higher interaction of the retained molecule with the stationary phase takes place, this stronger interaction is associated with a limitation of the movement of the molecule (in terms of the ability to rotate, vibrate, etc.) and hence with a decrease in the molecule's entropy. Since the change in enthalpy ($\Delta \boldsymbol{H}$) and the change in entropy (ΔS) have opposite signs, the result is that the value for ΔG will remain basically constant.

Specific tests were developed for proving a true physical compensation [61]. The temperature where the compensation takes place was found to be different for different separation

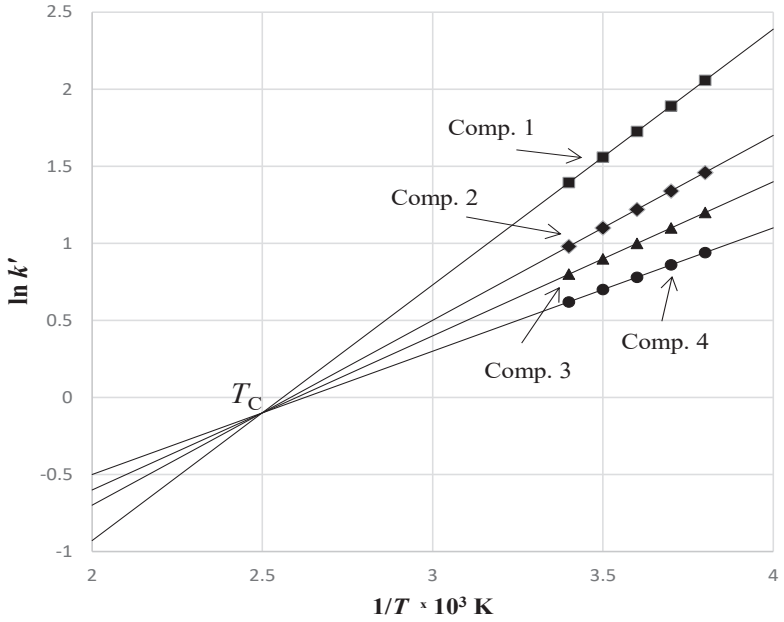

FIGURE 4.6.5 Determination of compensation temperature using van't Hoff plots. The graph shows the points of measured values for ln k', the corresponding van't Hoff lines, and T_C.

mechanisms. For example, in NP-HPLC, the compensation process takes place around 150 K, while in RP-HPLC, the compensation takes place at higher temperatures and in a range between 500 K and 1000 K [60].

High-temperature HPLC

High-temperature HPLC has been developed as an alternative procedure to accelerate the HPLC separation [49,62]. As indicated by Eq. 4.6.1, the temperature increase has as a result the decrease in retention factor k' and this can be detrimental to a separation. In some cases, however, the separation is good enough and other benefits can be obtained by working at controlled temperatures above 100 C and usually below 200 C and not higher than 250 C. One such benefit is the possibility to achieve faster separations. In some cases, the chromatograms can be up to 50 times shorter than at room temperature (25 C). Another benefit can be related to the solubility of the analytes in the mobile phase when the analytes (e.g., certain polymers) are soluble only at elevated temperature. The decrease in mobile phase viscosity and the pressure drop are also favored by increased temperatures [63]. Also, the technique may offer differences in separation that can be beneficial in some cases [62].

The technique also encounters several problems such as the lack of resistance of most silica-based HPLC columns at temperatures above 65 C. Only the development of certain silica-based temperature-resistant columns, or the use of zirconia- or titania-based columns allowed the use of higher temperatures for the separation. At high temperatures, most of silica-based columns have shorter life for usage due to the increased hydrolysis rate of stationary phase. Experiments carried out on different columns set up at 200°C showed the highest bleeding effects monitored by UV absorption

and charged aerosol detector for silica-based C-18 stationary phases and the lowest bleed for carbon clad titanium dioxide columns [64,65]. Another problem with this technique is the requirement to strictly control the temperature of the incoming solvent, of the chromatographic column, and also in the detector. Effects such as band broadening and peak splitting due to radial temperature gradient in the column, viscous fingering of the solvent, etc., are easily produced during high temperature HPLC [66].

By increasing the temperature, the dielectric constant of water changes significantly. At 25°C, the dielectric constant of water is $\varepsilon = 77.5$, and at 300°C, the value of ε is about 20. At 200°C (more accessible for high temperature HPLC than 300 C), the value of ε for water becomes comparable to that of acetonitrile ($\varepsilon = 37.5$) and methanol (value of $\varepsilon = 32.7$) at 25°C [67]. This property allows the use of pure water as mobile phase, as a need for "green" liquid chromatography. However, some practical limitations (instrumentation and stability of stationary phase), but also the thermal stability of labile compounds, or the possibility of hydrolysis catalyzed by active sites present on the stationary phase surface are weak points for this approach [68].

4.7 Reactions occurring in HPLC column

General comments

In most cases, the separated solutes by HPLC are stable during the elution process, and they do not modify their structure due to chemical reactions with the components from mobile phase or interactions with the stationary phase. However, chemical modifications of solutes are possible during an HPLC separation, for example, when the sample contains components prone to solvolysis in the mobile phase, when a derivatization procedure is applied to the sample and the reaction is not completed and continues after the injection into the column, or when tautomerism influenced by the mobile phase composition is possible for the analyzed molecules. The analysis of reactive compounds, such as aldehydes, sulfonate salts/esters, hydrazine functionalities, boronate esters/acids, sulfate acid/esters, or acyl halides, compounds commonly used in the synthesis of pharmaceutical drug substances, may pose difficulties in HPLC separations. These molecules can be involved in various chemical transformations including oxidation, hydrolysis, polymerization, condensation, elimination, substitutions, or isomerization, depending on the mobile phase composition [69,70]. An example of oxidation is the observation that by using older analytical columns, an on-column oxidation of methionine-containing peptides can be produced during the chromatographic elution, potentially catalyzed by traces of metal ions present on the stationary phase surface [71].

A typical example of changes during separation is the HPLC of certain aldehydes under reversed-phase conditions where the addition of water to the double bond C=O may lead to the formation of a geminal diol species. The effect of this transformation on separation leads to a noticeable fronting for the peaks of aldehydes even when the separation is performed at subambient temperatures [72]. Another situation of structural modification during the HPLC elution is encountered for stereo-labile chiral compounds, which can be studied by dynamic chromatography together with stopped-flow techniques [73].

The possibility of combining the conversion of compounds simultaneously with their chromatographic separation is known as dynamic chromatography. For example, by this technique, a reactant X is periodically fed into the mobile phase stream and transported through the column packed with the stationary phase. During the elution process, the reactant X is involved in a reaction to produce other species. The process can be catalyzed homogeneously

(the catalyst included in the mobile phase), or heterogeneously, by the catalytic effect of the stationary phase [74,75]. This procedure is however more related to the preparative chromatography, and the modeling of the process is rather complicated and beyond the analytical usage [76].

Dynamic HPLC on chiral stationary phases has become a well-established technique to investigate chiral molecules with internal motions that result in stereointerconversion and occur on the time scale of the chromatographic process. Kinetic parameters for the on-column interconversion processes can be estimated from the experimental peak characteristics (peak area, shape) by computer simulation or by direct calculation methods. This approach has been used in a range of temperatures and is complementary in scope to the dynamic NMR spectroscopy [77].

Enantiomerization and diastereomerization

Chiral compounds may suffer changes in their chiral configuration, and this process can take place during an HPLC separation due to the influence of the solvents in the mobile phase, as a result of the interaction with stationary phase, or both. One such change is the reverse of chiral configuration. This process is known as enantiomerization. Another different process is racemization resulting in the formation of equal level of both chiral forms (a racemate) from a pure enantiomer [78]. When two or more chiral centers (or elements) are present in a molecule and one of them is configurationally labile, epimerization may occur, which is a particular case of diastereomerization, during the elution process [79].

When a chiral configuration is changing during a separation, it is common that the peaks of the two separated enantiomers are noticeable, but besides them, an additional cluster of peaks representing the interconverted enantiomers can be observed in the chromatogram as a plateau between the two peaks [80]. The shape of the peak cluster depends on the quality of the separation and on the interconversion rate of the enantiomers. If the interconversion process is slow compared to the HPLC separation of enantiomers, which can be achieved by accelerating the separation process or by lowering the temperature, a partial separation with characteristic plateau formation or peak broadening can be recorded. A simulation of two chromatograms, without and with enantiomerization during the chiral HPLC separation, is shown in Fig. 4.7.1. There are attempts to apply deconvolution procedures in order to identify the peaks generating this plateau (e.g., Ref. [81]).

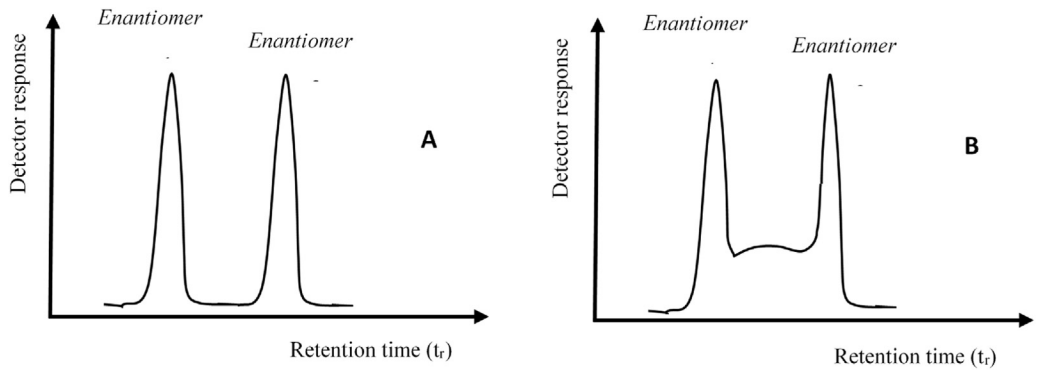

FIGURE 4.7.1 Simulation of the separation of two enantiomers, without interconversion during the chromatographic elution (A) and with enantiomerization in column, with a plateau between the two peaks (B).

Overall, the interconversion process can be written for the R enantiomer denoted as X_R and for S enantiomer X_S, as the equilibrium:

$$X_R \underset{k_{S\to R}}{\overset{k_{R\to S}}{\rightleftarrows}} X_S \qquad (4.7.1)$$

In equilibrium 4.7.1, $k_{R\to S}$ and $k_{S\to R}$ are the rate constants for $R \rightarrow S$ enantiomerization and for $S \rightarrow R$ enantiomerization, respectively. The equilibrium constant of this process, K_{chiral}, is the ratio:

$$K_{chiral} = \frac{k_{R\to S}}{k_{S\to R}} \qquad (4.7.2)$$

In a static system, the mixture of the enantiomers after interconversion equilibrium has a composition that depends on K_{chiral}. When the $k_{R\to S}$ and $k_{S\to R}$ rate constants are equal, $K_{chiral} = 1$ and the interconversion process leads to racemization. However, this is not always the case and one enantiomer may be favored to another. Also, because the chromatographic process takes place in a limited interval of time, the equilibrium may not be attained at the $t_r(X_R)$ for a specific enantiomer. In such case, assuming that the kinetic of the chiral configuration change is of the first order, the change in the concentration of X_R (for example) can be written in the following form:

$$\frac{d[X_R]}{dt} = -\, k_{R\to S}[X_R] \qquad (4.7.3)$$

The integration of Eq. 4.7.3 for the time interval 0 to t_R, leads to the following expression:

$$\ln \frac{[X_R]_{initial}}{[X_R]_{final}} = k_{R\to S} t_r(X_R) \qquad (4.7.4)$$

Eq. 4.7.4 gives the change in the concentration of X_R during the separation process. The ratio will be larger (more compound X_R will be converted) for a longer retention time and a faster reaction rate, as expected. For the evaluation of this change, the value of $k_{R\to S}$ must be known, and various studies were reported on this subject (e.g., Ref. [82]).

Various examples of chiral changes during HPLC separation were reported in the literature such as that of some chiral benzodiazepines (lorazepam, temazepam, and oxazepam) [83], of 2,3-pentadienedioic acid [82], or of atropine [84]. Some flavanones are also known to undergo ring opening under basic conditions to form the corresponding chalcones and change in chiral configuration. Examples of such flavones are naringin, narirutin, hesperidin, and neohesperidin with separation on Cyclobond I 2000 column that contains β-cyclodextrin bonded to silica gel. The diastereomerization is either accelerated or reduced by the inclusion of these flavones in the cavity of β-cyclodextrin [85].

Diastereomerization of 1,8-bis(2,2′-diphenyl-4,4′-dipyridyl)naphthalene (shown in Fig. 4.7.2) has been proved by HPLC, using a CN stationary phase, and mobile phase with hexane/ethanol (3/2, v/v), at low temperatures (−65 and −43°C). The interconversion between *syn* and *anti* rotamers of this compound is fast at ambient temperature and the energy barrier to rotation about the naphthylpyridyl axis can be evaluated [86].

In some separations, the interconversion between stereoisomers can be stopped by placing in the mobile phase an additive that forms an inclusion pair with one of the isomers. As an

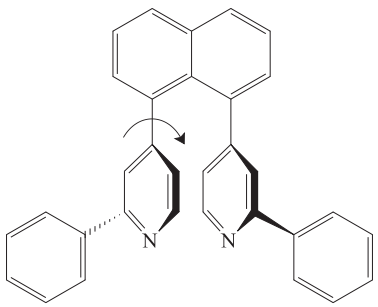

FIGURE 4.7.2 Formula of 1,8-bis(2,2′-diphenyl-4,4′-dipyridyl)naphthalene and *syn* − *anti* rotamer change [86].

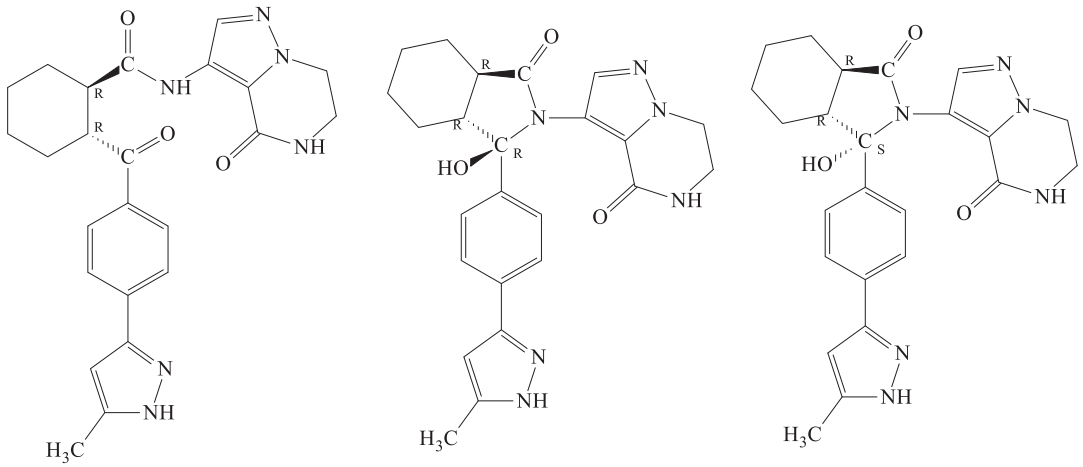

FIGURE 4.7.3 Tautomeric and diastereoisomeric structures of AZD5718 [88].

example, diazepam conformers can be separated by chiral HPLC with addition of β-cyclodextrin in the composition of the mobile phase when the plateau between the two peaks assigned to the conformers disappears [87].

Tautomerism

Tautomers are structural isomers (constitutional isomers) of chemical compounds that readily interconvert each other, by a reaction of relocating a proton (tautomerization). Common tautomeric pairs are ketone−enol and enamine−imine. The tautomer structures may be difficult to separate by HPLC. However, there are situations when the tautomers formed in the mobile phase can be completely separated and identified. This is the case of the tautomers (two are diastereoisomers) of AZD5718, (1R,2R)-2-[4-(5-methyl-1H-pyrazol-3-yl)benzoyl]-N-(4-oxo-4,5,6,7-tetrahydropyrazolo [1,5-a] pyrazin-3-yl) cyclohexane-1-carboxamide, a pharmaceutical drug for the treatment of diseases that involve chronic inflammation including coronary artery disease. The tautomeric and diastereoisomeric structures of AZD5718 are indicated in Fig. 4.7.3. The isomers are separated by reversed-phase HPLC and identified by MS all showing the same spectrum [88].

Tautomerism can modify the polarity of bonds from molecular structure that influence the interaction with the stationary phase. For example, adenine can be involved in tautomeric equilibria with modification of the partial charge on different atoms as shown in Fig. 4.7.4. A change in molecular polarity may influence the separation.

Tautomer equilibria can be influenced by the polarity of the mobile phase and involve small energy differences (between 8 and 12.5 kJ/mol) [89]. The tautomeric equilibria may be the origin of atypical retention behavior of adenine on perfluorophenylsilicagel-based stationary phase, with a dependence between log k' of adenine and mobile phase composition ϕ different than that described by Eq. 3.5.3 [90].

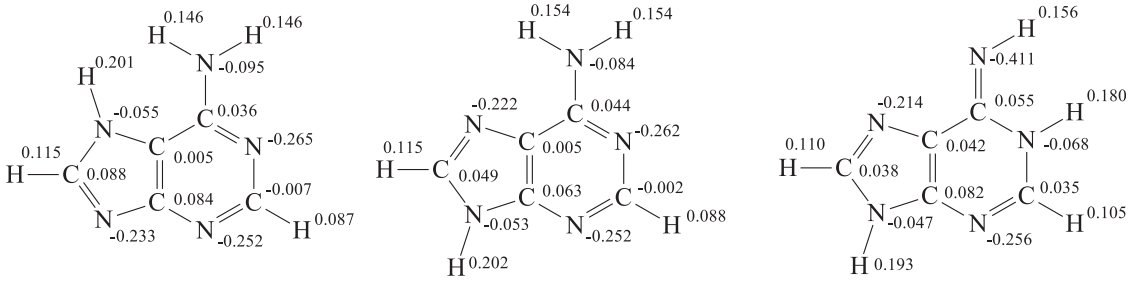

FIGURE 4.7.4 Charge simulation of adenine tautomers. *Obtained with MarvinSketch http://www.ChemAxon.com.*

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CHAPTER

5

Intermolecular interactions

5.1 Forces between molecules

General comments

The evaluation of molecular interactions is important for the understanding and even potential prediction of the behavior of the solutes in the separation process. Although this procedure is in many instances successful in predicting the directional behavior of sample components in a specific separation, the quantitative results regarding the separation are not always precise. The approaches based on molecular-level interactions provide understanding of the retention process of solutes in equilibrium between a bonded phase and the mobile phase. For example, for the adsorption equilibrium, the studies at molecular level describe the interactions of solutes with the active sites from stationary phase [1,2]. The main difficulty in these studies is caused by the complexity of the chromatographic process and by the approximations frequently used to evaluate molecular interactions and properties. Typically, alternative paths are employed for obtaining quantitative information. These paths may combine the calculations of the interaction intensity with empirical data and/or with physico-chemical or molecular parameters of the participants in the separation process (solutes, mobile phase, stationary phase). Also, statistical techniques that do not provide a cause/effect relation between a specific property and a parameter (or set of parameters) are frequently utilized for generating the desired information. For example, a common procedure for the prediction of a given chromatographic property P_r as a function "f" of several physico-chemical or molecular characteristics y_i is to express the property P_r as follows:

$$P_r = f\left(a_1 y_1 + a_2 y_2 + \ \ldots\ a_n y_n\right) \qquad (5.1.1)$$

where a_1, a_2 … a_n are coefficients calculated by multiple (linear) regression. Among the molecular characteristics, y_i are structural additive parameters (carbon number, molecular mass, parachor, molar volume, molar refractivity, polarizability), physico-chemical parameters (boiling point, dipole moment, ionization potential, formal charges in the molecule, n-octanol−water partition coefficient), topological parameters related to the molecule shape, etc. (e.g., Ref. [3]).

The study of specific interactions that may take place between the species participating in the chromatographic process remains an important tool for the understanding of the separation process, and some aspects of molecular interactions are discussed in this section. Since molecular interactions are discussed at the level of particles (ions, molecules) and not at molar level (gram quantities), the thermodynamic functions such as energy E, enthalpy $\boldsymbol{H}$, free energy A, free enthalpy G, chemical potential μ, etc., obtained for a molecule must be multiplied with Avogadro

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number $\mathcal{N} = 6.02214179 \times 10^{23}$ mol^{-1} for obtaining the corresponding molar functions. Also, for interactions at molecular level, instead of gas constant $R = 8.31451$ J deg^{-1} mol^{-1} $= 1.987$ cal deg^{-1} mol^{-1}, the Boltzmann constant $k_B = R/\mathcal{N} = 1.3806504 \cdot 10^{-23}$ J K^{-1} will be used. However, for simplicity, the notation E, H, A, G, μ, etc., will not be different for quantities at molecular level compared to those used for moles of material (bulk level). The SI units are typically used for expressing various physical parameters, although occasionally other units are indicated.

Some of the different thermodynamic functions at the molecular level may have identical values. For example, the relation between the energy E and free energy A is given by the formula $E = A - T\left(\frac{\partial A}{\partial T}\right)_V$. Assuming A not temperature dependent, the result is $E = A$, and therefore, the two thermodynamic functions can be used interchangeably. Also, at constant volume, expression $\Delta G = \Delta A + p\ \Delta V$ indicates $\Delta G = \Delta A$. The assumptions that lead to equality of different thermodynamic functions must be considered carefully when extending the findings at molecular level to bulk level. Also, a problem regarding the application of the findings at the molecular level to the bulk level is that some properties of the continuum, such as dielectric constant, should be also considered for the interaction at the molecular level. The two basic approaches of the estimation of interactions from continuum characteristics or from molecular characteristics should lead to consistent results, and this may impose corrections to each of the two approaches.

An additional problem when considering intermolecular interactions is that of a reference state. The expressions obtained for the interactions between molecules (or atoms, or ions) typically have a reference state where the distance between participating species is infinite ($r = \infty$), and all the values of energies (or free energies) must be considered as values for the change in the energy (ΔE) or free energy (ΔA). Nevertheless, the sign Δ for difference is omitted in most cases, for simplicity.

In the formulas further developed for the evaluation of the interactions, besides one to one electrostatic interactions that would be expected in a particle model, some concepts from the continuum model (bulk property) are also included. The explanation for this approach comes from the idea that the interaction energy indicates the energy value of bringing the two particles at the distance of interaction as they approach each other through the bulk medium and not through vacuum.

Charge to charge interactions

The free energy A of the interaction between two charges q_1 and q_2 separated by the distance r is described by the Coulomb law:

$$A = \frac{1}{4\pi\varepsilon_0}\frac{q_1 q_2}{\varepsilon r} = k_e \frac{z_1 z_2 e^2}{\varepsilon r} \qquad (5.1.2)$$

In expression 5.1.2, ε_0 is the vacuum permittivity ($\varepsilon_0 = 8.854 \cdot 10^{-12}$ C V^{-1} m^{-1} where V indicates volt, and C coulomb), q_1 and q_2 are the charges of particles given in terms of elementary charge ($q = z \cdot e$ where z is the numerical charge and $e = 1.602 \cdot 10^{-19}$ C), r is the distance in m, and ε is the dielectric constant of the medium relative to vacuum ($\varepsilon = 1$ for vacuum). The constant k_e is Coulomb constant ($k_e = 9.98755 \cdot 10^{+9}$ N m^2 C^{-2} where N are Newtons). Eq. 5.1.2 shows that the ionic interactions are very strong. Since all the interactions are assumed to take place at constant volume (in condensed phase), this free energy of interaction can be assumed to be equal with the free enthalpy of interaction ($\Delta G = \Delta A + p\ \Delta V$). Therefore, $A = G$ for the specified system. Also, since no temperature T appears in the expression 5.1.2, it can be concluded that $A = E$, the total energy of the system.

In Eq. 5.1.2, the dielectric constant ε is that of the bulk of the medium (and not that of the region between the charges). This value for ε brings to the particle model a bulk property, since the particles are brought at the distance of interaction through the medium with dielectric constant ε.

Taking as an example the energy between two ions with the charges +1 and −1, at the distance $r = 0.276$ nm $= 2.76$ Å (that corresponds to the distance between the centers of Na^+ and Cl^- in NaCl), the calculation gives the following value:

$$A = \frac{-\left(1.602\ 10^{-19}\right)^2}{4\pi\left(8.854\ 10^{-12}\right)\left(0.276\ 10^{-9}\right)} = -8.3573\ 10^{-19}\ \text{J} \qquad (5.1.3)$$

The value given by Eq. 5.1.3 is negative indicating the attraction of ions. For 1 mol of material, the calculation using Eq. 5.1.3 gives $A = -8.3573 \cdot 10^{-19} \cdot 6.02214179 \cdot 10^{23} = 503.29$ kJ/mol. Since 1 cal = 4.184 J, the interaction energy is 120.3 kcal/mol. The true energy in an ionic crystal should take into consideration all the interactions for the ion pairs in the crystal lattice, but just comparing the energy of two ions with that of a harmonic oscillator at temperature T ($A = k_B\ T$), it results that at room temperature the ion energy is (in absolute value) about 200 times higher than that of the harmonic oscillator made by the two particles ($k_B\ T = 1.3806504 \cdot 10^{-23} \cdot 300 \approx 4.1 \cdot 10^{-21}$ J). In a solvent where the dielectric constant ε is significantly higher than 1 (e.g., for water $\varepsilon = 77.46$), the interaction energy is diminished. The variation of the free energy of interaction for two ions ($z_1 = -1$ and $z_2 = 1$, and also $z_1 = -1$ and $z_2 = 2$) at a distance varying from less than 10 Å up to 100 Å in water is shown in Fig. 5.1.1. This variation assumes that no other interactions take place in solution and that no other ions are present. In reality, the decrease (in absolute value) of the interaction energy is much more rapid due to the screening caused by other particles present in solution (other ions and solvent molecules). Nevertheless, the interactions between ions in solution extend at distances much longer than molecular radii of small molecules.

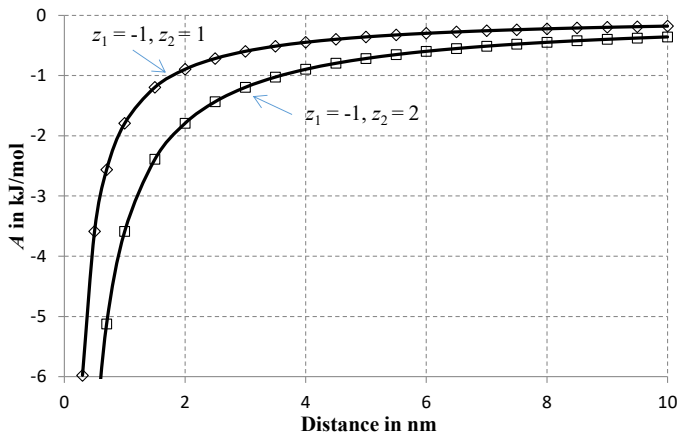


FIGURE 5.1.1 Hypothetical variation of the free energy of interaction for two ions in vacuum ($z_1 = -1$ and $z_2 = +1$, and also $z_1 = -1$ and $z_2 = +2$) at a distance varying from less than 10 Å up to 100 Å in water (negative values for the free energy A indicates attraction).

The value of the interaction force F (which is in fact a vector) between two charges can be easily obtained from Eq. 5.1.2 using the following expression:

$$F = -\frac{dE(r)}{dr} = -\frac{dA(r)}{dr} = \frac{z_1 z_2 e^2}{4\pi\varepsilon_0\varepsilon r^2} \quad (5.1.4)$$

(an attractive force is indicated by a negative sign which is obtained if z_1 and z_2 are of opposite sign). Since the force is a vector, the interacting force between charges should have a sense and a direction. These are the same as the radius vector between the charges. In other words, expression 5.1.4 should be written in vector form as follows:

$$\vec{F} = \frac{z_1 z_2 e^2}{4\pi\varepsilon_0\varepsilon r^2}\frac{\vec{r}}{r} \quad (5.1.5)$$

The expression for the force also allows the evaluation of the electrical field created by a charge q. The intensity of this field is given by the following expression:

$$\vec{\mathbb{E}} = \frac{q}{4\pi\varepsilon_0\varepsilon r^2}\frac{\vec{r}}{r} \quad (5.1.6)$$

and the field $\vec{\mathbb{E}}_1$ generated by the charge $q_1 = z_1\, e$ acting on the charge $q_2 = z_2\, e$ generates the force given by the formula 5.1.5. Similar to the force, the electrical field is a vector and its direction is the same as that of the radius vector between the charges (the vector sign is not uniformly used further in the notation).

In reality, as previously indicated for the interaction energy, in a solution containing ions, the electric field is strongly perturbed by other ions and polar particles in the surrounding solution, and the resulting energy decreases faster than $1/r^2$ as predicted by Eq. 5.1.4 [4].

Energy of an ion in a continuous medium

An ion, even if it is not interacting with other ions (or electrical charges), has a specific free energy, equal to the electrostatic work done for forming that ion. For an ion with the charge q and radius r, the increase of the charge with dq will require the energy given by the following expression:

$$dA = \frac{q\,dq}{4\pi\varepsilon_0\varepsilon r} \quad (5.1.7)$$

The total free energy for charging one ion to the final charge ze will be the following:

$$A = \int_0^{ze} \frac{q\,dq}{4\pi\varepsilon_0\varepsilon r} = \frac{(ze)^2}{8\pi\varepsilon_0\varepsilon r} \quad (5.1.8)$$

The energy given by Eq. 5.1.8 is also known as Born energy. This energy is positive indicating that the formation of an ion requires external work. In a medium with dielectric constant ε, the energy necessary to form a mole of ions will be given by the following expression:

$$A = \mathcal{N}\frac{(ze)^2}{8\pi\varepsilon_0\varepsilon r} \quad (5.1.9)$$

The Born energy given by Eq. 5.1.9 with $\varepsilon = 1$, but with a negative sign, can be considered as approximating the energy necessary to bring a mole of ions from vacuum into a solvent with a high dielectric constant ($\varepsilon >> 1$). The expression of the free energy (at constant volume) for this transfer is given by the following formula:

$$\Delta A = -\mathcal{N}\frac{(ze)^2}{8\pi\varepsilon_0 r}\left(1-\frac{1}{\varepsilon}\right) \approx -\mathcal{N}\frac{(ze)^2}{8\pi\varepsilon_0 r} \quad (5.1.10)$$

For this reason, Born energy is also indicated as solvation energy of ions (not considering the energy necessary to create the cavity in the solvent for accommodating the ions).

The change of the ionic interactions in different solvents due to the differences in the dielectric constant ε can be used to explain the difference in salt solubility in two solvents with different dielectric constants ε_1 and ε_2. In this case, using Eq. 5.1.9 for the expression of the

free energy, the change of the ionic interactions in different solvents can be written as follows:

$$\Delta A = \mathcal{N}\frac{(ze)^2}{8\pi\varepsilon_0 r}\left(\frac{1}{\varepsilon_1}-\frac{1}{\varepsilon_2}\right) \qquad (5.1.11)$$

The value of ΔA is negative when $\varepsilon_1 > \varepsilon_2$ indicating that the solubility is higher in a solvent with higher dielectric constant. Although this approach represents only a rough approximation of reality, an (approximate) linear dependence (with negative slope) was observed between the values for the solubility of monovalent ions and $1/\varepsilon$ of the solvents [5].

Ions in solutions or ionized groups connected to a stationary phase are typically encountered in ion chromatography and in ion exchange processes. Also, in other types of chromatography, ion formation and interactions play a role in the separation process. For example, in RP-HPLC performed on a silica-based stationary phase, the residual silanol groups on the stationary phase may ionize when the pH value of the mobile phase is high, and the silanol groups may bring additional types of interactions besides those involving hydrophobic forces. The comparison of interacting energies for ionic forces with the energies of other forces (polar–polar, between polarizable molecules, etc.) indicates that ionic forces are comparably strong. Also, comparing the interacting distances, the ionic forces act at longer distances. This is an additional reason to eliminate as much as possible ionic interactions in RP-HPLC that may affect in an unpredictable way the results of a separation that is assumed to be based on a unique mechanism of separation.

Polar molecules

Polarity in a molecule refers to the separation of the center of positive charges from that of negative charges, leading to an electric dipole of the molecule. The dipole of a polar molecule is characterized by the dipole moment $\vec{m}$ defined by the following formula:

$$\vec{m} = q\vec{d} \qquad (5.1.12)$$

where $\vec{d}$ is a vector with the length equal to the distance (in nm) between the two separated point charges $+q$ and $-q$ directed from the negative charge to the positive charge. The vector character of the dipole moment is frequently neglected ($\vec{m}$ is usually indicated as m). The unit of dipole moment is the debye (D) and 1 D $= 3.336\cdot10^{-30}$ C m. Besides molecular dipole moments, the value for $\vec{m}$ (m) can be calculated for chemical bonds and also for molecular fragments. Extensive tables with dipole moments are available in the literature [6]. Water, for example, has a dipole moment $m = 1.85$ D $= 6.1716\cdot10^{-30}$ C m. This value allows the calculation of the dipole moment of the OH bond. Considering that the H–O–H angle in water is $\theta = 104.5$ degrees, the OH bond dipole moment will be $m_{OH} = m_{H2O}/[2\ \cos\ (0.5\ \theta)] =$ 1.51 D.

The generation of the dipole moment can be explained by the displacement of the center of all electrons in the valence shell of the molecule, which is not located over the center of positive charges in the molecule. For example, considering the water molecule with eight valence electrons (core electrons of oxygen are not included), the distance separating the charges (of $8e$) is given by $d = m/q = 6.2\cdot10^{-30}/(8\ .\ 1.602\cdot10^{-19}) = 4.84\cdot10^{-12}$ m $\approx$ 0.005 nm (10 times smaller distance compared to 0.05 nm the Bohr radius of hydrogen). This indicates for water a very small charge separation compared to the molecule diameter. Using this model of creating the dipole moment, a very small separation in space of the charge centers is typical for most polar molecules.

More commonly the charges that generate the dipole moment can be considered as partial point charges centered at the extreme atoms in the molecule (In the literature, various notations for the partial point charges can be encountered). For a water molecule as an example, the partial charge $q = 0.41$ separated by a distance of 0.094 nm will generate the dipole moment $m = (0.41 \cdot 1.602 \cdot 10^{-19}) \cdot (0.094 \cdot 10^{-9}) = 6.1741 \cdot 10^{-30} \approx 1.85$ D.

The self-energy for a dipole can be calculated from the sum of the Born energies of the two partial charges $+q$ and $-q$ (on ions assumed to have radius $a = d/2$) minus the energy to bring the two charges together (separated by the distance $r = d = 2a$). Therefore, Born energy for a dipole is given by the following expression:

$$A = \frac{1}{4\pi\varepsilon_0\varepsilon}\left(\frac{q^2}{2a} + \frac{q^2}{2a} - \frac{q^2}{r}\right) = \frac{q^2}{8\pi\varepsilon_0\varepsilon a} = \frac{m^2}{4\pi\varepsilon_0\varepsilon d^3} \tag{5.1.13}$$

Formula 5.1.13 indicates that the self-energy for dipoles is about the same as that of an ion where the charge $z\ e$ is replaced by the partial charge q. This energy (with negative sign) can also be related to the solvation energy of a molecule with dipole moment m.

Eq. 5.1.13 also allows the understanding of differences in the solubility of a polar compound in different solvents. Similar to the ionic compounds, for polar compounds, a (approximate) linear dependence (with negative slope) was observed between the values for the solubility and $1/\varepsilon$ of the solvent. This indicates that the solubility of polar compounds parallels that of ionic compounds and they have a higher solubility in solvents with higher dielectric constant.

While the definition of the dipole moment using the concept of two point charges is simple, in reality, the charge distribution in a molecule is a more complicated concept. For this reason, it is more convenient to use for dipole moment definition the volume charge density $\rho(\vec{r})$, which is the amount of electric charge in an infinitesimal volume at a point $\vec{r}$. In this case, the dipole moment is defined by the following formula:

$$m = \int_0^V \rho\left(\vec{r}\right)\vec{r}\ \mathrm{d}V \tag{5.1.14}$$

The charge density (as well as the dipole moment) can be obtained, for example, using quantum chemical calculations [7]. For a molecule, the charge density can also be evaluated from an array of point charges q_i, using the following expression:

$$\rho\left(\vec{r}\right) = \sum_{i=1}^{n} q_i \delta\left(\vec{r} - \vec{r}_i\right) \tag{5.1.15}$$

where δ is the Dirac delta function ($\delta = 0$ for $\vec{r} \neq \vec{r}_i$ and ($\delta = 1$ for $\vec{r} = \vec{r}_i$) and $\vec{r}_i$ is the vector to the charge q_i from a reference point. With this observation, the dipole moment is given by the following formula:

$$m = \sum_{i=1}^{n} q_i \int_0^V \delta\left(\vec{r} - \vec{r}_i\right)\vec{r}\,dV = \sum_{i=1}^{n} q_i \vec{r}_i \tag{5.1.16}$$

Eq. 5.1.16 indicates that the dipole moment is the vector sum of individual dipole moments created by the point charges.

Ion to dipole interactions

The interaction between an ion and a dipole can be reduced to the interaction between a charge ze and the two partial charges $+q$ and $-q$ of the dipole. Assuming the length of the dipole is d and the distance between the charge and the middle of the dipole is r, the Coulomb energy of the interaction is given by the following expression:

$$A = \frac{ze \cdot q}{4\pi\varepsilon_0\varepsilon}\left(\frac{1}{\mathrm{AB}} - \frac{1}{\mathrm{AC}}\right) \tag{5.1.17}$$

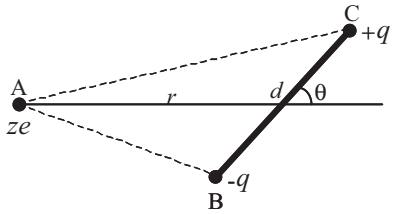


FIGURE 5.1.2 Schematic drawing of the interaction between a charge ze and a dipole generated by the partial charges q.

where the distances AB and AC are shown in Fig. 5.1.2.

From simple geometric considerations, it can be shown that the distances AB and AC can be calculated with the following formulas:

$$\mathrm{AB} = \left[(r - 0.5\,d\cos\theta)^2 + (0.5\,d\sin\theta)^2\right]^{1/2} \approx r - 0.5\,d\cos\theta \tag{5.1.18a}$$

$$\mathrm{AC} = \left[(r + 0.5\,d\cos\theta)^2 + (0.5\,d\sin\theta)^2\right]^{1/2} \approx r + 0.5\,d\cos\theta \tag{5.1.18b}$$

(the approximation in Eq. 5.1.18a and 5.1.18b being valid when $r >> d$). Replacing the expressions for AB and AC in Eq. 5.1.17, the interaction energy becomes the following:

$$A = \frac{ze\cdot q}{4\pi\varepsilon_0\varepsilon}\left(\frac{1}{r - 0.5\,d\cos\theta} - \frac{1}{\mathrm{r} + 0.5\ \mathrm{dcos}\,\theta}\right) = \frac{ze\cdot q}{4\pi\varepsilon_0\varepsilon}\left(\frac{d\cos\theta}{r^2 - 0.25\,d^2\cos^2\theta}\right) \tag{5.1.19}$$

Since $r >> d$, Eq. 5.1.19 can be reduced to the following expression:

$$A = \frac{ze\cdot m\cos\theta}{4\pi\varepsilon_0\varepsilon\, r^2} \tag{5.1.20}$$

Eq. 5.1.20 allows the calculation of the energy of interaction between a dipole and an ion. This energy is attractive if the negative partial charge of the dipole points toward a positive ion ($0° < \theta < 90$ degrees) showing a maximum for $\theta = 0°$, and is repulsive if the positive charge points toward the positive partial charge of the dipole (90 degrees $< \theta <$ 180 degrees), showing a minimum for $\theta = 180$ degrees.

The ion–dipole interaction is weaker than the ion–ion one, but when the separation distance ion–dipole is shorter than 0.2–0.4 nm, it is still significantly higher than $k_B\ T$ ($k_B\ T \approx 4.1\cdot10^{-21}$ J for $T = 300$ K). For example, using Eq. 5.1.20 with $\theta = 0$ and considering a water molecule ($m = 1.85$ D gas phase) with the distance to an ion $r = 0.235$ nm (the estimated distance between a Na^+ ion and a water molecule in vacuum), the interaction energy in vacuum has the following value:

$$A = \frac{-(1.602\ 10^{-19})\cdot(1.85)\cdot(3.336\ 10^{-30})}{4\pi(8.854\ 10^{-12})(0.235\ 10^{-9})^2} = -1.609\ 10^{-19}\ \mathrm{J} \tag{5.1.21}$$

This resulting value for A is about 40 times (in absolute value) higher than $k_B\,T$. However, at an ion–dipole distance of about 1.5 nm, $A \approx k_B\,T$ (in absolute value). For a molecule with $m = 1.5$ D interacting at the distance of 0.4 nm with an ion with $z = 1$, the free energy $A \approx 4.50\cdot10^{-20}$ J, which is equivalent to 27.1 kJ/mol. For the same interacting distance, this energy is higher than the sum of other interactions not involving ions (Eq. 5.1.47) and shows the importance of the presence of ions in systems involved in various separations. Multiplying with $\mathcal{N}$ the previous value for A, the resulting value is about 96 kJ mol^{-1} or about 23 kcal mol^{-1}. This high energy explains the orientation of dipoles of the solvent around the ions in solution. In the case of water, this process is known as hydration of the ions, and a hydration number can be associated with different ions. A solvation zone is also present around the ions in a solution.

The assumption made for obtaining Eq. 5.1.21 for the free energy A that the dipole–ion interaction is present in a fixed position is valid when the ions and the dipoles are in close proximity, but it becomes a strong simplification when the distances are larger. When A given by Eq. 5.1.20 (for $\theta = 0$) is of the order of k_BT, a Boltzmann angle-averaged expression for A should replace Eq. 5.1.20. This expression is obtained from *potential distribution theorem* (e.g., Ref. [5]), and in this case, the interaction energy between an ion and a dipole has the following expression:

$$A \approx -\frac{(ze)^2 \cdot m^2}{6k_b\, T\, (4\pi\varepsilon_0\varepsilon)^2\, r^4} \tag{5.1.22}$$

The values for A generated using Eq. 5.1.22 are smaller than those obtained using Eq. 5.1.20. At a distance $r = 1.5$ nm between the ion and dipole, using Eq. 5.1.22, the resulting value is about $A = -6.3\cdot10^{-22}$ J (almost 7 times smaller in absolute value than k_BT for T 300 K). This result indicates that outside of the first layer of solvating molecules that surround an ion, the molecules of a polar solvent are likely to rotate freely and not be further clustered around the ion.

One important observation regarding the expression of A given by Eq. 5.1.22 is its dependence on temperature. This indicates that the total energy $E_{i\text{-}d}$ for the ion–dipole interaction is not anymore equal with A. From the following formula:

$$E = A - T\left(\frac{\partial A}{\partial T}\right)_V \tag{5.1.23}$$

for the expression of total energy $E_{i\text{-}d}$, and noticing that $\left(\frac{\partial A}{\partial T}\right) = \frac{(ze)^2 \cdot m^2}{6k_b\, T^2\, (4\pi\varepsilon_0\varepsilon)^2\, r^4}$ the result for the total energy is given by the following expression:

$$E_{i-d} \approx -\frac{(ze)^2 \cdot m^2}{3k_b\, T\, (4\pi\varepsilon_0\varepsilon)^2\, r^4} \tag{5.1.24}$$

The part of energy that is not available as free energy of interaction is associated with the entropy in the system.

Dipole to dipole interactions

Two polar molecules interact through Coulomb type forces, similarly to the interaction of ions and dipoles. Assuming the dipoles in a fixed position (not free rotating), the same procedure as for the interaction between an ion and a dipole can be followed. The resulting energy for two dipoles is given by the following formula:

$$A = -\frac{m_1 m_2}{4\pi\varepsilon_0\varepsilon r^3}\,(2\cos\theta_1\cos\theta_2 - \sin\theta_1\sin\theta_2\cos\varphi) \tag{5.1.25}$$

where $m_1 = d_1q_1$, $m_2 = d_2q_2$, and the angles θ_1, θ_2, and φ are defined in Fig. 5.1.3.

The expression 5.1.25 of A for $\theta_1 = 0$, $\theta_2 = 0$ (φ irrelevant in this case) becomes the following:

$$A = -\frac{m_1 m_2}{2\pi\varepsilon_0\varepsilon r^3} \tag{5.1.26}$$

The evaluation of this energy, for example, for two adjacent water molecules (at a distance $r = 0.28$ nm) gives $A \approx -3.1\cdot10^{-20}$ J, indicating a quite strong interaction for the water molecules. The same calculation for $r = 0.55$ nm gives $A \approx -4.1\cdot10^{-21}$ J. The comparison of these results with $k_BT \approx 4.1\cdot10^{-21}$ J for $T = 300$ K shows

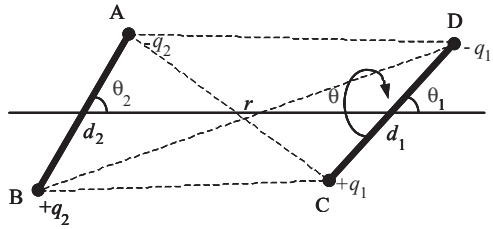


FIGURE 5.1.3 Schematic drawing of the interaction between two fixed dipoles m_1 and m_2.

that the dipole–dipole interaction becomes closer to the thermal energy of a harmonic oscillator as the distance between the interacting molecules increases. For this reason, it is expected that the dipole orientation fails to be optimal when the energy A calculated with formula 5.1.26 becomes closer to k_BT, and an angle-averaged expression for A should replace Eq. 5.1.26. The angle-averaged expression of energy A for the dipole–dipole interaction is the following:

$$A = -\frac{m_1^2 m_2^2}{3k_BT\,(4\pi\varepsilon_0\varepsilon)^2 r^6} \qquad (5.1.27)$$

For a distance $r = 0.28$ nm, $A \approx -2.0\cdot10^{-20}$ J, only slightly lower than the one generated with Eq. 5.1.26, but the energy calculated with Eq. 5.1.27 has a much faster decrease as the distance r increases. For $r = 0.55$ nm, when Eq. 5.1.26 indicates an energy equal to that of thermal energy of a harmonic oscillator k_BT, the value of energy calculated with formula 5.1.27 is $A \approx -3.4\cdot10^{-22}$ J. The angle-averaged energy between two dipoles is usually referred as *Keesom interaction*.

Similar to the case of ion–dipole interaction, the angle-averaged expression for the energy $E_{d\text{-}d}$ of two dipoles is temperature dependent. From expression 5.1.23 that relates E and A, it can be obtained that the total energy of the interaction is double that given by Eq. 5.1.27, which can be written as follows:

$$E_{d-d} = -\frac{2\,m_1^2 m_2^2}{3k_BT\,(4\pi\varepsilon_0\varepsilon)^2 r^6} \qquad (5.1.28)$$

The decrease (in absolute value) of the calculated E_{d-d} for two molecules as a function of temperature is shown, as an example, in Fig. 5.1.4. The calculations showing the molar energy (where $E_{d-d} = 2\,\mathcal{N}\,A$) were based on the value $m = 1.5$ D and on an intermolecular distance of 0.3 nm.

As seen from Fig. 5.1.4, the increase in temperature decreases (the absolute value of) the interaction energy.

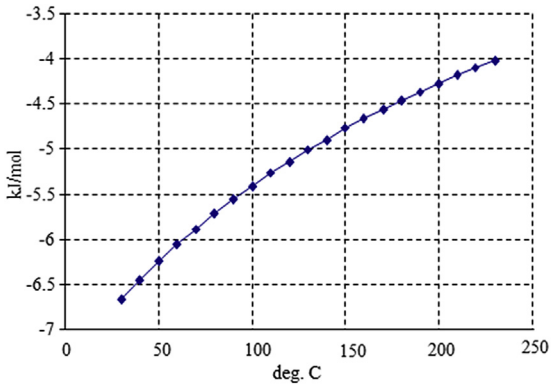


FIGURE 5.1.4 The temperature dependence of dipole-dipole interaction energy E_{d-d} for two molecules at 3 Å distance.

Polarizability of molecules

Under the influence of an electrical field $\vec{\mathbb{E}}$, molecules and also atoms show a displacement of the electron cloud relative to the center of positive charges (nucleus for the atoms), generating an induced dipole moment $\vec{m}_{ind}$. *Polarizability* (in electric field) α is defined by the ratio as follows:

$$\alpha = \frac{\vec{m}_{ind}}{\vec{\mathbb{E}}} \qquad (5.1.29)$$

The SI units of polarizability α are $C^2\ m^2\ J^{-1}$ ($C^2m^2J^{-1} = C\ m^2\ V^{-1}$), but more common values are expressed in units of $4\pi\varepsilon_0(Å)^3 = 4\pi\varepsilon_0 10^{-30}\ m^3 = 1.11\cdot10^{-40}\ C^2\ m^2\ J^{-1}$ (sometimes for polarizability expressed in $4\pi\varepsilon_0(Å)^3$ the symbol α' is used and the polarizability is indicated as polarizability volume). Tables of polarizability (usually given as polarizability volume), as well as procedures for its estimation using semiempirical formulas or quantum mechanical calculations [7], are reported in the literature. As defined by Eq. 5.1.29, polarizability is a scalar quantity indicating that the electric field produces polarization only in the direction of the field. However, polarizability can also be defined as a tensor when the electrical field

generates moments of dipole in different directions from that of field $\vec{\mathbb{E}}$. Besides the scalar and tensor values for the polarizability, the components $\alpha(XX)$, $\alpha(YY)$, and $\alpha(ZZ)$ of the tensor (for the components for the vector of the dipole moment field $\vec{m}$ and of the electric field $\vec{\mathbb{E}}$) are sometimes indicated. Polarizability is generated from the displacement of the electron cloud (*electronic polarizability*), and for the molecules that have a permanent dipole, also from *orientational polarizability* that is generated from the orientation of the molecule dipoles.

A simple evaluation of electronic polarizability α_0 can be obtained considering that an electron is displaced by the length d from the initial position, in the electrical field $\vec{\mathbb{E}}$, as indicated schematically in Fig. 5.1.5.

In this case, the induced dipole moment will be $m_{ind} = \alpha_0 \vec{\mathbb{E}} = d\ e$ (where d is the projection of the distance between charges $+e$ and $-e$ in the molecule and α_0 is electronic polarizability). The force between the two charges in the presence of a field is given by expression 5.1.4, which can be written as follows (where L is the radius of the molecule):

$$F = \frac{e^2}{4\pi\varepsilon_0 L^2}\sin\theta = \frac{e^2 d}{4\pi\varepsilon_0 L^3} = \frac{e\, m_{ind}}{4\pi\varepsilon_0 L^3} \quad (5.1.30)$$

Since the force is given by $F = e\,\mathbb{E}$ (written not using the vector notation for F or for $\mathbb{E}$) from Eq. 5.1.30, the result for the value for the induced dipole moment is $m_{ind} = 4\pi\varepsilon_0 L^3\ \mathbb{E}$. From this relation, the value for the electronic polarizability is obtained as follows:

$$\alpha_0 = 4\ \pi\varepsilon_0 L^3 \quad (5.1.31)$$

This formula can be used for the electronic polarizability not only for atoms, but also for molecules where L is the radius of the molecule. It is common to express the electronic polarizability in units $(4\pi\varepsilon_0)\cdot 10^{-30}\ \mathrm{m}^3$ (L being given in Å).

The expression for orientational polarizability α_{orient} for a molecule with the permanent dipole moment m is obtained from potential distribution theorem as Boltzmann angle-averaged expression in the following form:

$$\alpha_{orient} = m^2/3k_B T \quad (5.1.32)$$

The total polarizability is obtained as a sum of the two polarizability types from the following formula:

$$\alpha = 4\pi\varepsilon_0 L^3 + m^2/3k_B T \quad (5.1.33)$$

Eq. 5.1.33 is known as Debye–Langevin equation. Molecular polarizabilities can be obtained from molecular orbital calculations [7] or by semiempirical evaluations [8].

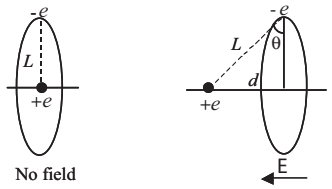


FIGURE 5.1.5 Schematic diagram of the displacement of an electron in an electric field.

Ion to molecule interactions

The influence of an ion placed at the distance r from a neutral molecule with α polarizability has as a result the induction of a dipole moment $m_{ind} = \alpha\ \mathbb{E}$ in the molecule. This induced dipole moment can be assumed as created by the displacement of a charge q at a distance d in the molecule. The resulting force (vector sign not shown) on the molecule is therefore given by the following formula:

$$F = q\frac{\mathrm{d}\mathbb{E}}{\mathrm{d}r} = \alpha\mathbb{E}\frac{\mathrm{d}\mathbb{E}}{\mathrm{d}r} \quad (5.1.34)$$

The free energy for the interaction of the ion and the molecule is therefore given by the following expression:

$$A = -\int Fdr = -0.5\,\alpha\,\mathbb{E}^2 \tag{5.1.35}$$

The intensity $\mathbb{E}$ of the field created by an ion with the charge ze is given using Eq. 5.1.6 by the following formula:

$$\mathbb{E} = \frac{ze}{4\pi\varepsilon_0\varepsilon r^2} \tag{5.1.36}$$

Introducing expression 5.1.36 for $\mathbb{E}$ in Eq. 5.1.35, the result is the following:

$$\begin{aligned} A &= -\frac{\alpha(ze)^2}{2(4\pi\varepsilon_0\varepsilon)^2 r^4} \\ &= -\frac{(ze)^2}{2(4\pi\varepsilon_0\varepsilon)^2 r^4}\left(\alpha_0 + \frac{m^2}{3k_bT}\right) \end{aligned} \tag{5.1.37}$$

The second term in Eq. 5.1.37 is identical with the value from Eq. 5.1.23, indicating the interaction between an ion and a permanent dipole.

Dipole to molecule interactions

The interaction of a dipole with a polarizable molecule can be evaluated using the same procedure as previously used for an ion to molecule interaction, considering that the induced dipole is generated by a charge q of the dipole. For a fixed dipole m oriented at an angle θ to the line joining it to a polarizable molecule, the intensity of the electrical field $\mathbb{E}$ generated is given by the following expression:

$$\mathbb{E} = \frac{m\left(1+3\cos^2\theta\right)^{1/2}}{4\pi\varepsilon_0\varepsilon r^3} \tag{5.1.38}$$

The free energy of interaction is given by Eq. 5.1.35, which will take the following form:

$$A = -\frac{m^2\alpha_0\left(1+3\cos^2\theta\right)}{2(4\pi\varepsilon_0\varepsilon)^2 r^6} \tag{5.1.39}$$

For the angle-averaged free energy, expression 5.1.39 becomes:

$$A = -\frac{m^2\alpha_0}{(4\pi\varepsilon_0\varepsilon)^2 r^6} \tag{5.1.40}$$

It can be noticed that replacing α_0 in Eq. 5.1.40 with α_{orient} given by Eq. 5.1.32, the resulting energy is Keesom energy given by Eq. 5.1.27.

For two molecules each possessing permanent dipole moments m_1 and m_2 and polarizabilities $\alpha_{0,1}$ and $\alpha_{0,2}$, the expression for the free energy of interaction becomes the following:

$$A = -\frac{m_1^2\alpha_{0,2} + m_2^2\alpha_{0,1}}{(4\pi\varepsilon_0\varepsilon)^2 r^6} \tag{5.1.41}$$

This energy is known as *Debye interaction energy*. Since the free energy in Eq. 5.1.41 does not depend on temperature, it can be concluded that the energy dipole–induced dipole $E_{d\text{-}id}$ of the system is given by the following expression:

$$E_{d-id} = -\frac{m_1^2\alpha_{0,2} + m_2^2\alpha_{0,1}}{(4\pi\varepsilon_0\varepsilon)^2 r^6} \tag{5.1.42}$$

Nonpolar molecule to molecule interactions

A considerably large number of molecules do not possess a permanent dipole moment (or have ionic character) or have a very small dipole moment. An example of a nonpolar molecule and the calculated point charges of its atoms is given in Fig. 5.1.6 for octane. The calculation of point charges in a molecule can be done by

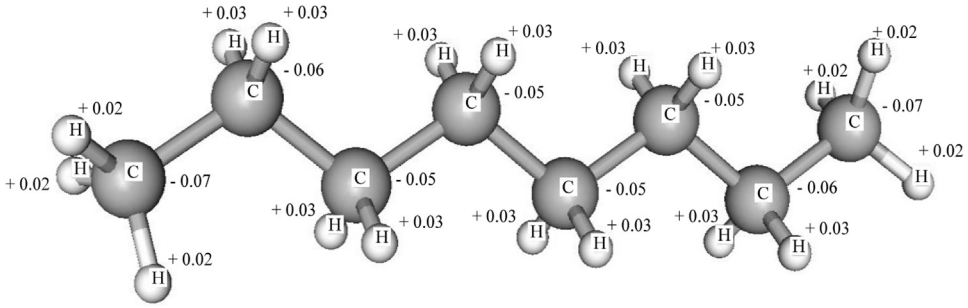


FIGURE 5.1.6 Calculated point charges in a molecule of octane (with MarvinSketch 5.4).

different techniques and may lead to different results [7]. The charges indicated in Fig. 5.1.6 were calculated using MarvinSketch 5.4 Plug-ins [9]. The same charges calculated, for example, with an empirical molecular orbital method (e.g., MOPAC-7 with AM1 parametrization [10]) can lead to charges as high as +0.088 for hydrogens and −0.2 for carbons.

Since the electrons forming the C−H bonds are not perfectly distributed between the two atoms, small partial point charges are present for each atom. However, the dipole moment of such molecule is zero. Since water is the most common polar solvent and the nonpolar molecules or molecules with a very small dipole moment are not water soluble (and their surface does not get wet with water), such nonpolar compounds are frequently referred to as *hydrophobic*.

The interaction between two molecules that do not possess a dipole moment can be approached by considering that one molecule has the polarizability α_0, and the other possess an instantaneous dipole moment m_{inst} created by the orbiting frequency of its electrons. In this case, Eq. 5.1.41 can be applied for evaluating the free energy of their interaction. From quantum mechanical evaluations, it can be shown that $m_{inst} \approx {}^3/_4\ \alpha_0\ I$ for atoms and small molecules with the polarizability α_0 and I ionization potential (The ionization potential is typically expressed in e.V, 1 e.V. $= 1.60218 \cdot 10^{-19}$ J). This indicates that the free energy of interaction between two identical small molecules is given by the expression ($\varepsilon = 1$):

$$A = -\frac{3\alpha_0^2 I}{4(4\pi\varepsilon_0)^2 r^6} \tag{5.1.43}$$

For two dissimilar small atoms or molecules, the formula of free energy interaction becomes the following:

$$A = -\frac{3\alpha_{0,1}\alpha_{0,2}}{2(4\pi\varepsilon_0)^2 r^6}\,\frac{I_1 I_2}{(I_1 + I_2)} \tag{5.1.44}$$

The energy of interactions between nonpolar but polarizable molecules is known as *London dispersion energy*. Expression 5.1.44 for the free energy of interaction of two molecules is independent on temperature, and therefore, the dispersion energy $E_d = A$, where A is given by Eq. 5.1.44. Also, formula 5.1.44 does not include a dielectric constant ε for the medium.

The application of London formulas to the interaction of larger molecules or to asymmetric molecules leads to badly underestimated results mainly because the dispersion forces do not act anymore between the centers of the molecules but between various centers of electronic polarization within each molecule. More elaborate calculations, including in the evaluation the sum of each local interaction, can be done for obtaining more accurate results. For this reason, the total dispersion energy E_d for a polyatomic molecule

composed from j polarizable groups interacting with a surrounding phase composed from k polarizable groups (connected or not in a molecule) is obtained as a sum of all possible interactions:

$$E_d = \sum_j \sum_k (E_d)_{j,k} \tag{5.1.45}$$

Hydrophobic character can be attributed not only to a whole molecule, but also to a moiety of a molecule such as in the case of a fatty acid that has a long hydrocarbon chain attached to a carboxyl group. The hydrocarbon chain can be referred to as a hydrophobic moiety and the carboxyl group as a polar one, although the whole molecule has a permanent dipole moment. An example of such molecule is octanoic acid. The calculated point charges for octanoic acid (using MarvinSketch 5.4 Plug-ins [9]) are shown in Fig. 5.1.7:

As shown on the octanoic acid molecule, relative large point charges are present on the carboxylic group, but very rapidly along the hydrocarbon chain the point charges become equal to those in octane molecule. The dipole moment for octanoic acid is $m = 2.05$ D (calculated using MOPAC 7). However, although polar, the interactions attributed to the long hydrophobic chain cannot be neglected. The same separation of interactions from different parts of a polyatomic molecule as described for the polarizable interactions can be extended to other interactions. For example, when considering a molecule with polar groups and with a hydrophobic moiety (as in the example of octanoic acid), the polar part of interaction and the dispersion energy caused by the polarizability of specific molecular moieties must be added together.

Unified view of interactions in the absence of ions

In the absence of ions, the total interaction of molecules in a medium with dielectric constant ε is given by the following expression:

$$E_T = E_{d-d} + E_{d-id} + E_d \tag{5.1.46}$$

$$E_T = \frac{1}{(4\pi\varepsilon_0)^2}\left(\frac{2m_1^2\, m_2^2}{3k_BT\varepsilon^2} + \frac{m_1^2\alpha_{0,2} + m_2^2\,\alpha_{0,1}}{\varepsilon^2} + \frac{3\alpha_{0,1}\alpha_{0,2}}{2}\frac{I_1 I_2}{I_1 + I_2}\right)\frac{1}{r^6} \tag{5.1.47}$$

The evaluation of E_T at 300 K for an idealized pair of molecules with $m = 1.5$ D, $\alpha_0 = 4.2$ ($\cdot 1.11 \cdot 10^{-40}$ $C^2m^2J^{-1}$), $\varepsilon = 1$, and $I = 10.9$ e.V ($1.60218 \cdot 10^{-19}$ J) for a distance varying between 3 Å and 7.7 Å generated the graph shown in Fig. 5.1.8 (energy expressed per mol after multiplication of Eq. 5.1.47 with $\mathcal{N}$).

As shown in Fig. 5.1.8, the dispersion energy E_d has the largest contribution to E_T (about 69%), followed by the dipole–dipole energy (E_{d-d} represents about 25% of E_T), and followed by dipole-induced dipole interaction E_{d-id},

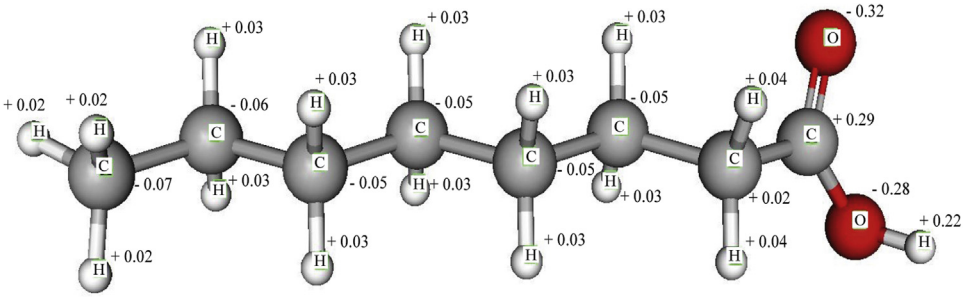


FIGURE 5.1.7 Calculated point charges in a molecule of octanoic acid (with MarvinSketch 5.4).

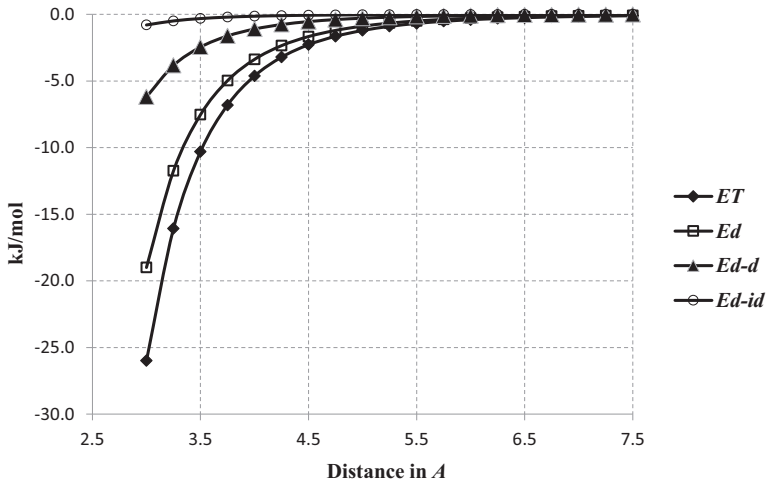


FIGURE 5.1.8 Theoretical calculation of E_{d-d}, E_{d-id}, E_d, and E_T for an idealized pair of molecules with $m = 1.5$ D, $\alpha_0 = 4.2$ ($\cdot 1.11 \cdot 10^{-40}$ $C^2m^2J^{-1}$), $\varepsilon = 1$, and $I = 10.9$ e.V, at 300 K.

which accounts for only about 6% of the total energy. As expected, the interaction energy decreases (in absolute value) as the distance between the molecules increases. For small molecules in gas phase, the above calculations were proven relatively close to experimental data obtained based on the deviation from perfect gas law (van der Waals coefficients). For this reason, the forces leading to the energy E_T given by Eq. 5.1.47 are frequently indicated as van der Waals forces. However, for condensed phase, the theory must be further reevaluated.

Lennard–Jones potential

Even assuming no free charges in a medium, based on Eq. 5.1.47, the result seems to indicate that the only interaction between molecules would be the attraction with an energy proportional with $1/r^6$. However, in reality, the attraction between molecules is maximum to a specific distance r, and at very small molecular distances, a strong repulsive force intervenes, not allowing the overlapping of the electronic clouds of the two molecules. One approach to explain why atoms cannot approach each other beyond a specific distance is to consider that the atoms are considered as hard, incompressible spheres. The radius of this sphere is known as *van der Waals radius* of the atom. A specific distance between atoms is also maintained between covalently bound atoms. The distance between two atoms in a molecule can be expressed as the sum of their covalent bond radii, equal with the *bond length*. Single bond covalent radii of atoms are about 0.08 nm shorter than their van der Waals radii, which can be explained by a slight deformation of the atom sphere when they are connected by a covalent bond. Based on the atomic van der Waals radii and the bond length, the van der Waals radii, volumes, and surface area of molecules can be calculated [11]. The volume of the molecule is not anymore a sphere, but in some instances molecular volumes are approximated with that of spheres. The molecular dimensions allow the calculation of a van der Waals *distance* between the centers of the interacting molecules (or atoms), and as an approximation, this distance can be obtained as the sum of van der Waals radii of the two "spherical" molecules. However, the true

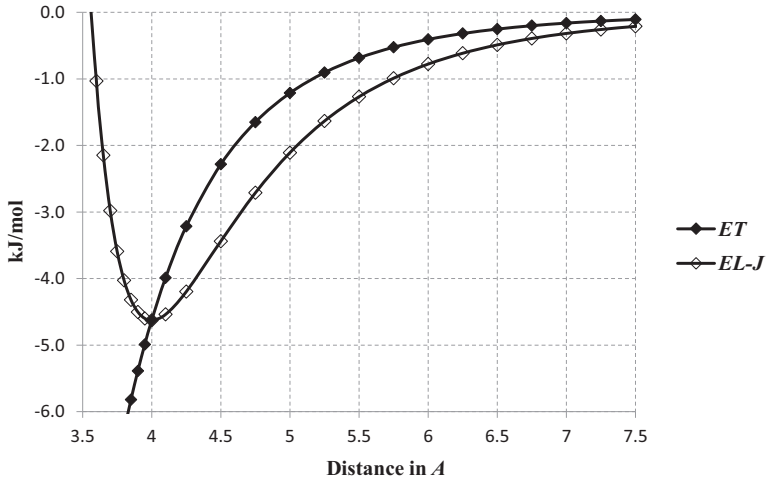


FIGURE 5.1.9 Variation of the interaction energy $E_{L\text{-}J}$ calculated with Eq. 5.1.49, and of E_T calculated with Eq. 5.1.47 between two molecules with $m = 1.5$ D, $\alpha_0 = 4.2$ ($\cdot 1.11 \cdot 10^{-40}$ $C^2m^2J^{-1}$), $\varepsilon = 1$, and $I = 10.9$ e.V, at 300 K, when $r_0 = 4$ Å.

shape of molecules can be very far from that of a sphere.

In order to account for the impossibility of two atoms or molecules to become closer to each other than their van der Waals radii, a repulsion potential must be included in the description of the total intermolecular interaction. One such potential is given by the semiempirical formula known as Lennard–Jones potential ($E_{L\text{-}J}$), which describes the interaction between molecules as a function of the distance r by the following expression:

$$E_{L-J}(r) = -\frac{A'}{r^6} + \frac{B'}{r^{12}} \tag{5.1.48}$$

(where A' and B' are compound specific parameters). An alternative to Eq. 5.1.48 is the following formula:

$$E_{L-J}(r) = -4E_T(r_0)\left[\left(\frac{r_0}{2^{1/6}r}\right)^{12} - \left(\frac{r_0}{2^{1/6}r}\right)^{6}\right] \tag{5.1.49}$$

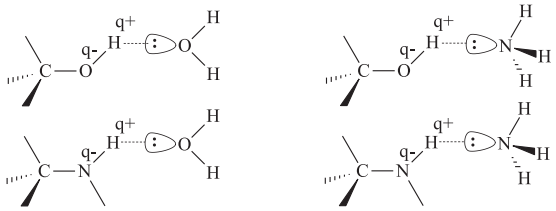
where E_T (having a negative value) is calculated using Eq. 5.1.47 for r_0 equal with van der Waals distance between the interacting particles (multiplication with $\mathcal{N}$ is necessary to obtain values per mol). The dependence of $E_{L\text{-}J}$ energy on the distance r between molecules is shown in Fig. 5.1.9, for $r_0 = 4$ Å, together with the values for E_T for various distances obtained with Eq. 5.1.47 (also shown in Fig. 5.1.8).

From Eq. 5.1.49 it can be seen that for $r = r_0$, the coefficients are $(1/2^{1/6})^{12} = 0.25$ and $(1/2^{1/6})^6 = 0.5$, such that $E_{L\text{-}J} = E_T$. The energy of van der Waals interaction as given by Eq. 5.1.49 depends on the distance r and also on the dipole moment m, polarizability α, and the ionization potential I, through E_T.

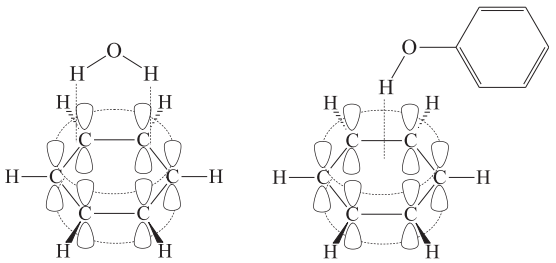
Hydrogen bond interactions

Another important type of molecular interaction is caused by the hydrogen bonds. The hydrogen bond appears to be partly electrostatic and partly covalent and involves an electron donor/electron acceptor interaction. In this bond, a hydrogen atom covalently bonded by atom A (A-H) is attracted by an atom containing a free electron pair :B. This leads to a strong polarization of the A-H bond and to electrostatic interactions between $H^{\delta+}$ and $:B^{\delta-}$. This type of interaction occurs, for example, between a molecular group, such as O–H or S–H, which carries a marked electric dipole moment and O

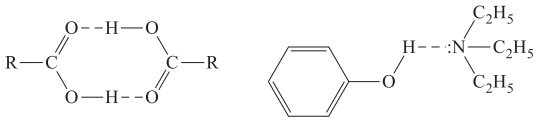
or N atoms from another molecule. This latter atom is characterized by the presence of at least one nonbonding orbital that can point toward the H-atom of the polar group of the molecule and is filled with a lone pair of electrons. The polar group that carries the H-atom is called the "donor," while the group containing O or N with a nonbonding orbital is called the "acceptor." This H-bond can be encountered in HPLC between different species from the mobile phase or between molecules interacting with groups in the stationary phase (residual silanol, amino, carboxyl, hydroxyl, etc.). Several hydrogen bond interactions are schematically depicted below:



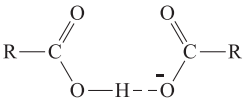
Generally, according to their energy of formation, H-bonds are classified into three main categories: weak, intermediate (or medium-strength), and strong H-bonds [12]. Weak H-bonds are characterized by low molar energy $\Delta E_{H\text{-}bond} < 20$ kJ/mol (the attraction energies have negative values and here the absolute values are given). They can be easily disrupted, and their formation is reversible. The average energy of a hydrogen bond from an OH group is 20–25 kJ/mol [3] and is 8–12 kJ/mol for NH_2 groups. Weak H-bonds occur when acceptors are not atoms with nonbonding orbitals, but a set of atoms with polarizable orbitals, such as π-orbitals extending, for instance, over aromatic rings. Such an H-bond, actually known as π hydrogen bond, occurs between a water molecule, as donor, and an aromatic ring, as an acceptor. This type of H-bond is also involved when the aromatic acceptor is replaced by other types of molecules that also exhibit π-orbitals, such as those containing double or triple carbon–carbon bonds. This may explain why the introduction of double, triple, or aromatic bonds in chemical structures leads to a decrease in the hydrophobic character of the molecule.



Intermediate H-bonds, $20 \text{ kJ/mol} < \Delta E_{H\text{-}bond} < 40$ kJ/mol, are usually encountered when carboxyl or phenolic groups are involved. The first example given below refers to the centrosymmetric dimers of carboxylic acids, which are found in their vapor form. Two examples of such bonds are shown below:

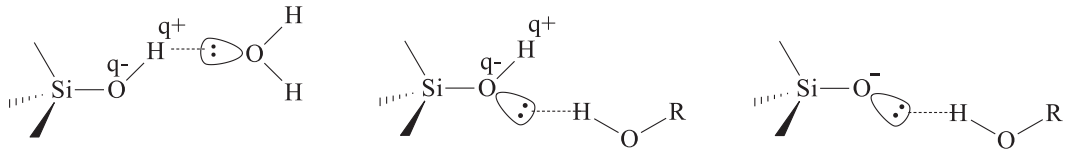


Strong H-bonds, $\Delta E_{H\text{-}bond} > 40$ kJ/mol, are not very common, and they occur when the acceptor is found in ionic form, as given in the following example:



The H-bonds are always present when water is present. Mobile phases in RP-LC typically have an aqueous component, and therefore, these types of forces are present together with polar–polar interactions, whether the target compounds are or are not dissociable. H-bonds

can interfere in RP-HPLC retention process through the presence of free silanol groups present on the surface of silica base for the bonded stationary phases. In such case, there are several possibilities for the formation of H-bonds between the OH groups from the stationary phase and the solute that participates to the retention process, as schematically indicated below:



This type of interaction may be blocked, for example, by the addition of triethylamine in the mobile phase, which may block the residual silanol groups and improves the peak symmetry in HPLC.

In certain systems, the energy of hydrogen bonds can be much higher (e.g., in HF), approaching 150 kJ/mol, which is comparable to that of a covalent bond (210–420 kJ/mol). A comparison of the energy of hydrogen bonds with other types of interactions can be seen in Table 5.1.1.

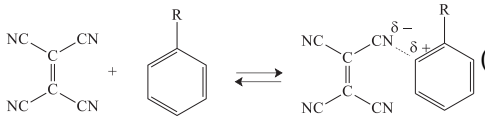
TABLE 5.1.1 Typical values for different interaction energies[a].

Interaction type	Energy (kJ/mol)	Energy (kcal/mol)
Dispersion	8–30	2–7
Dipole–induced dipole	4–8	1–2
Dipole–dipole	4–13	1–3
Hydrogen bonding	20–40	5–10
Ion–dipole	60–130	15–30
Ionic	200–800	50–200
Covalent	200–400	50–100

[a] *Note: The energies involved in the formation of the bonds are considered with negative values.*

Charge transfer or donor–acceptor interactions

One more type of interaction is the one that occurs between electron pair donor and electron pair acceptor compounds (EPD-EPA or charge transfer interaction). This type of interaction leads to complexes with a mesomeric structure between one formed by two noninteracting molecules (except for van der Waals forces) D and A, and one with two components with ionic interactions D^+A^-. The electron donor molecules (Lewis bases) and the electron acceptor molecules (Lewis acids) can be n, σ, or π donors or, respectively, acceptors. Their interacting energy can be as low as about 10 kJ/mol but can reach up to 180 kJ/mol. An example of this type of interaction is that between tetracyanoethylene and an alkylbenzene, the formula of the complex being written as a resonance between the structures:



(5.1.50)

In most cases when donor acceptor interactions take place, the ionic forms as shown in the previous reaction cannot be detected. Only very strong Lewis acids and strong Lewis bases have the tendency to form bonds mainly based on electrostatic interactions. For most organic molecules with a π electronic system, the molecular orbitals involved in the charge transfer are the π orbitals. These π orbitals are those with

the highest energy in the molecule. For this reason, the π donor π acceptor systems are frequently mentioned between compounds although they are weak Lewis acids and bases [13]. Only partial charges can be assumed in these cases as existent in the molecule. For example, NO_2 is an electron withdrawing group, while OCH_3 and Cl are electron donor groups. The π system of an aromatic ring containing one or more electron withdrawing groups will become a π-acceptor system and will be prone for donor–acceptor type interactions with a π-donor compound, although they are weak Lewis acids and bases, respectively. As an example, silver cations interact specifically with unsaturated compounds to form weak charge transfer complexes with olefinic double bonds. This type of interaction is known to be applied by introducing of Ag^+ in the mobile phase for the separation of cis-trans olefins for improving separation in reversed-phase HPLC [14].

Stacking and inclusion in supermolecular systems

Intermolecular interactions may lead to specific arrangements for certain molecules such as in the stacking of consecutive aromatic base pairs in DNA. The multiple types of intermolecular forces that lead to stacking are difficult to differentiate, but since the effect is encountered, for example, in molecules with flat aromatic rings systems, the interaction is attributed to π–π interactions. Ab-initio calculations indicate that the interaction of localized aromatic orbitals from different molecules has a minor contribution to stacking, and only common van der Waals forces of types previously described are responsible for this process. It is possible that since the molecules with extended π electron systems have flat surfaces, this improves the steric closeness for action of typical van der Waals forces and influences the molecular association. Nevertheless, π–π interactions even between molecules with small differences in the electron densities are indicated sometimes as contributing to the retention process in HPLC. When more significant differences are present between the electron densities of two molecules with π–electron systems, for example, when the stationary phase contains bonded dinitrophenyl groups and the solute is an aromatic compound with electron donor substituents, the π–π interactions are more important for explaining molecular stacking. The presence of π–π interactions for phases with phenyl or cyano groups may or may not play a role in the separation.

Inclusion is another type of supermolecular association, besides stacking. Specific molecules indicated as host, such as cyclodextrins (α, β, and γ) and cucurbituril, contain cavities with dimensions comparable to that of small molecules and are able to form inclusion complexes with these small molecules. An example of a complex between a β-cyclodextrin (7 glucose units) and guest molecule *trans*-1,4-bis[(4-pyridyl)ethenyl]benzene is schematically shown in Fig. 5.1.10. Numerous other inclusion complexes are obtained from cyclodextrins.

The shape of cyclodextrins is that of a truncated cone. The two diameters of the internal cavity for α-cyclodextrin (with 6 glucose units) are 0.45 nm (narrow)–0.57 nm (large), for β-cyclodextrin (with 7 glucose units) are 0.62–0.78 nm, and for γ-cyclodextrin (with 8 glucose units) are 0.79–0.95 nm (although the cyclodextrins are fairly rigid molecules, these values are averages since molecular shape is changing due to vibrations and rotations). The depth of the cavity is about 0.79 nm and the wall thickness is about 0.38 nm. The shape of the inclusion molecule is typically very close to that of the cavity. The interactions in inclusion complexes comprise polar interactions, hydrogen bonding, and possibly charge transfer,

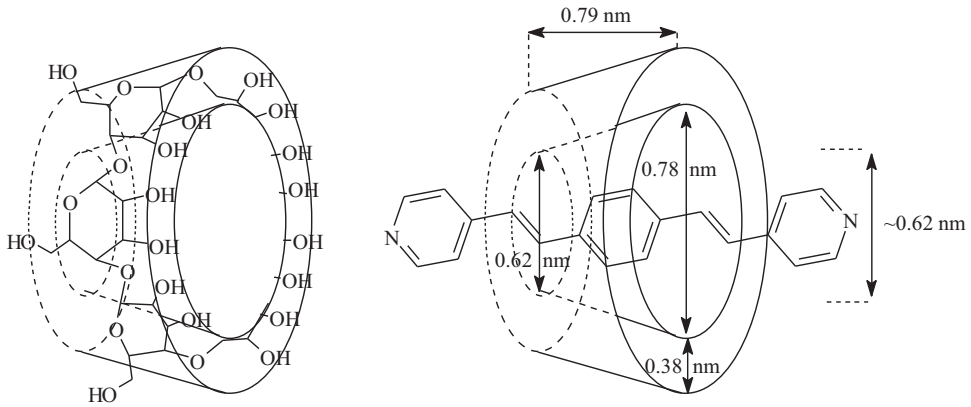


FIGURE 5.1.10 Schematic structure of the inclusion complex between a β-cyclodextrin and a guest molecule trans-1,4-bis [(4-pyridyl)ethenyl]benzene.

with the steric restriction of molecular movements (also based on the same types of interactions) leading to stable complexes. The reason for including these types of interactions separately from the typical ones, although these interactions are basically reduced to polar, hydrogen bonding, or charge transfer, is that only the special shape of the host molecule allows the formation of a stable complex. The interactions by themselves are rather weak to account for the high stability of the complex. Since the formation of the complex is highly dependent on the geometry of the guest molecule, differences can be seen in the formation of inclusion complexes between enantiomers.

Other types of bonds

In addition to the interactions previously indicated, some other specific types of intermolecular interactions are reported in the literature. One of these are the halogen bonds defined as the attractive interaction between an electrophilic region associated with a halogen atom in a molecule and a nucleophilic region in another (intramolecular halogen bonds are also possible) [15]. The halogen bond has similarities with the hydrogen bond. Various other types of intermolecular bonds are recognized in the literature. For example, coordinative bonds can be formed not only intramolecularly, but also intermolecular when a metal atom from one molecule interacts with another ligand molecule.

The effect of a solvent on molecular interactions

The effect of a solvent medium on molecular interactions was taken into consideration in the previous discussion solely by including the dielectric constant ε in the interaction formulas (ε is the dielectric constant of the medium relative to vacuum). However, in reality, the solvent effect is more complicated since the intrinsic dipole moment and polarizability of an isolated gas molecule may be different from that in a liquid (liquid state or solution), and because all the effects are taking place in a surrounding

with additional interactions besides the ones previously taken into consideration. Since dispersion forces (expressed as E_d energy) have typically the highest contribution to the molecular interactions, it is important to evaluate the true polarizability in a condensed medium. A solution to this problem is offered by replacing individual polarizability with that of a small sphere of radius d_X with a dielectric constant ε_X, in a field $\mathbb{E}$. The value of the induced dipole moment in this case is given by the following formula [16]:

$$m_{ind} = 4\pi\varepsilon_0\varepsilon\left(\frac{\varepsilon_X - \varepsilon}{\varepsilon_X + 2\varepsilon}\right)d_X^3\mathbb{E} \tag{5.1.51}$$

For a larger molecule that can be correctly approximated with a sphere, Eq. 5.1.49 leads to the following formula for the polarizability:

$$\alpha = \varepsilon_0\left(\frac{\varepsilon - 1}{\varepsilon + 2}\right)\frac{3M}{\rho\mathcal{N}} \tag{5.1.52}$$

where M is the molecular weight, ρ is the density, and ε is the dielectric constant of the bulk of the molecule. The formula does not give very good agreement for small highly polar molecules, but the agreement is good for larger polar molecules or weakly polar ones.

The polarizability can also be obtained using Clausius–Mossotti expression:

$$\alpha = \varepsilon_0\left(\frac{n^2 - 1}{n^2 + 2}\right)\frac{3M}{\rho\mathcal{N}} + \frac{m^2}{3k_BT} = \varepsilon R_m\frac{3}{\mathcal{N}} + \frac{m^2}{3k_BT} \tag{5.1.53}$$

where n is the refractive index of the bulk material in the visible range of frequencies, R_m is the molar refraction, and the other parameters are the same as previously defined (Lorenz–Lorentz equation, e.g., in Ref. [16]). Numerous other studies attempting to account for the influence of a solvent on the molecular interactions have been published [17–19]. One simple procedure for accounting for the solvent contributions is the use of Born model (e.g., Refs. [7,20]). In this model, a solvation energy E_{solv} is obtained based on the following expression:

$$E_{solv} = -\left(\sum_A\frac{q_A^2}{2r_A} + \sum_A\sum_{B>A}\frac{q_Aq_B}{r_{AB}}\right)\left(1 - \frac{1}{\varepsilon}\right) \tag{5.1.54}$$

where q_A and q_B are the partial point charges on the molecule, r_{AB} are the distances between atoms, r_A is an effective radius of each charged atom, and ε is the dielectric constant of the solvent [21]. The effective atomic radii represent a measure of the distance from the nucleus to the boundary of the surrounding cloud of electrons and are reported in the literature [22,23].

Successful results in the estimation of solvent effects were obtained considering interactions proportional with the solvent accessible area of the molecule. van der Waals surface area of the molecule (VSA) can be obtained from geometric considerations. The atoms are considered spheres of van der Waals radius, and the van der Waals area of the molecule is the sum of atomic areas minus the areas of interlocked adjacent atoms. However, the accessible area of a molecule is different from VSA since it may contain small gaps, pockets, and clefts which are sometimes too small to be penetrated even by a solvent molecule. To account for true interactions, the concept of solvent accessible surface area (ASA or SASA) was introduced [24,25]. This surface is obtained by hypothetically rolling a spherical probe of a diameter corresponding to the size of a solvent molecule on the original van der Waals surface (e.g., of a solvent molecule of radius 1.4 Å corresponding to water) [26]. The evaluation of interaction energy can be done using a solvation term proportional with the solvent accessible area of the molecule [27,28]. In this theory, the effective energy of interactions for a solute X having n atoms is divided in two contributions, one due to the solutes interaction,

E_X, and one due to the interactions with the solvent, E_{sol}, in a relation of the following form:

$$E(\boldsymbol{r}) = E_X(\boldsymbol{r}) + E_{sol}(\boldsymbol{r}) \tag{5.1.55}$$

where $\boldsymbol{r} = (r_1, r_2, r_3, \ldots, r_n)$ represents a vector indicating the position of each atom in the molecule X. The solvation component $E_{solv}(\boldsymbol{r})$ was found to be related to *SASA* by a relation of the following form:

$$E_{solv}(\boldsymbol{r}) = \sum_{i=1}^{n} \sigma_i A_i^{SASA} \tag{5.1.56}$$

where σ_i is an atomic solvation parameter and A_i^{SASA} is the solvent accessible area of the atom i. Selected values for σ_i for atoms in a molecule are given in Table 5.1.2 [19]. The values for A_i^{SASA} can be obtained using the following analytical expression:

$$A_i^{SASA} = S_i \prod_{j \neq i}^{n} \left[1 - \frac{p_i p_{i,j} b_{i,j}\left(r_{i,j}\right)}{S_i} \right] \tag{5.1.57}$$

In Eq. 5.1.57, the parameters are as follows: S_i is the *SASA* of an isolated atom of radius r_i and is given by the following formula:

$$S_i = 4\pi \left(r_i + r_{probe} \right)^2 \tag{5.1.58}$$

r_{probe} is the radius of the solvent probe. Some values for r_i are given in Table 5.1.2. The parameter $b_{ij}(r_{i,j})$ represents the *SASA* removed from S_i due to overlapping between atoms i and j at the distance $r_{i,j}$. The calculation of $b_{ij}(r_{i,j})$ can be done using the following expressions:

$$b_{i,j}\left(r_{i,j}\right) = 0 \text{ for } r_{i,j} > r_i + r_j + r_{probe} \tag{5.1.59a}$$

$$b_{i,j}\left(r_{i,j}\right) = \pi \left(r_i + r_{probe} \right) \left(r_i + r_j + 2r_{probe} - r_{i,j} \right) \times \left[1 + \left(r_j - r_i \right) r_{i,j}^{-1} \right] \quad \text{otherwise} \tag{5.1.59b}$$

For a solvent with $r_{probe} = 1.4$ Å, the parameter p_i is given in Table 5.1.2, and $p_{ij} = 0.8875$ if the atoms i and j are covalently bonded, and $p_{ij} = 0.3516$ otherwise.

TABLE 5.1.2 Several parameters utilized in the calculation of A^{SASA} for organic molecules [19].

Atom type	r_i	p_i	σ_i
Carbonyl carbon	1.72	1.554	0.012
Aliphatic carbon with 1 hydrogen	1.8	1.276	0.012
Aliphatic carbon with 2 hydrogens	1.9	1.045	0.012
Aliphatic carbon with 3 hydrogens	2	0.88	0.012
Aromatic carbon with 1 hydrogen	1.8	1.073	0.012
Amide nitrogen	1.55	1.028	20.06
Aromatic nitrogen with no hydrogens	1.55	1.028	20.06
Primary amine nitrogen	1.6	1.215	20.06
Nitrogen bound to 3 hydrogens	1.6	1.215	20.06
Guanidinium nitrogen	1.55	1.028	20.06
Proline nitrogen	1.55	1.028	20.06
Hydroxyl oxygen	1.52	1.08	20.06
Carbonyl oxygen	1.5	0.926	20.06
Carboxyl oxygen	1.7	0.922	20.06
Sulfur	1.8	1.121	0.012
Sulfur with 1 hydrogen	1.8	1.121	0.012
Polar hydrogen	1.1	1.128	0

The formulas for the calculation of *SASA* for organic molecules previously described are used in computer programs that provide values for this parameter for a variety of molecules (e.g., MarvinSketch 5.4.01 [9]). In addition to *SASA*, other similar parameters were introduced such as ASA_H, which is the solvent accessible surface area of all hydrophobic atoms (with formal partial charges in absolute value $q_i < 0.125$) and ASA_P, which is the solvent accessible area of all polar atoms (with formal partial charges in absolute value $q_i > 0.125$) [28]). The estimation of E_{solv} values were successfully used for the simulation of interactions of small proteins in

solution and also can be used to understand the interaction of small polar molecules with a solvent.

One additional complication related to molecules in solution is the formation of molecular associations or ionic associations with the solvent molecules. For example, ions in aqueous solutions are not free but solvated [21]. Within the solvent, the interactions between solvated molecules can be different from the one between free molecules, and attempts to calculate the interaction energies frequently lead to incorrect results.

Solvophobic effects

The solvophobic theory attempts to explain the interactions between polar solvents and nonpolar solutes. This theory is commonly invoked for explaining the lack of solubility of a nonpolar molecule in polar solvent and its higher solubility in a nonpolar solvent, although the interactions at the molecular level may indicate that the energy between a nonpolar molecule with a polar molecule (of a solvent) is larger (in absolute value) than between two nonpolar molecules (analyte and solvent). This difference would apparently indicate that polar solvents should be better solvents for nonpolar molecules. This is not the case, and the explanation is that in order to dissolve a nonpolar molecule in a polar solvent, polar↔polar interactions between the solvent molecules are replaced by nonpolar↔polar interactions. This process is schematically suggested in Fig. 5.1.11. In this figure, a pure solvent (H_2O) is initially shown with typical polar↔polar interactions taking place between the solvent molecules. In stage 2 of the process, a cavity is created in the solvent, and this would require energy to break a number of polar↔polar interactions. After the cavity is formed, a solute molecule is placed in it, leading to new interactions of the type nonpolar↔polar.

The dissolution process is controlled by the sum of free enthalpies (ΔG^0) necessary to create a cavity in the solvent (which is an endothermic process) and the enthalpies corresponding to the electrostatic and dispersive forces between the analyte molecule and the solvent (which is an exothermic process). If the total free enthalpy $\Delta G^0 < 0$ (exothermic process), then the analyte molecule will have affinity for the solvent, favoring solubility. If $\Delta G^0 > 0$, the process is not thermodynamically favored and the analyte molecules do not favor presence in the solvent. Since polar↔polar interactions are stronger than nonpolar↔polar ones, the solubility of nonpolar molecules is low in polar solvents. On the other hand, the cavity creation in a nonpolar solvent requires low energy (breaking nonpolar↔nonpolar interactions) and nonpolar molecules dissolve easily in nonpolar solvents in part due to the entropy increase during solubilization.

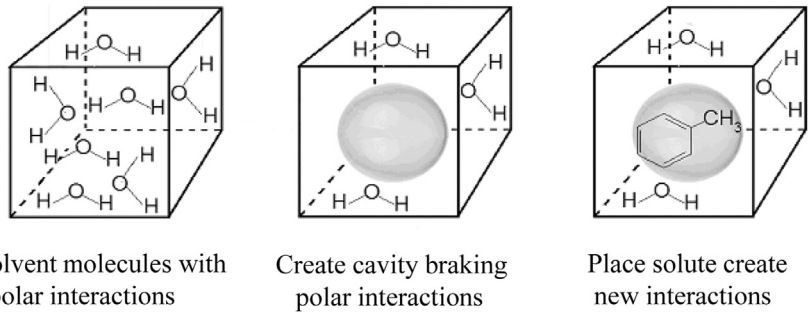


FIGURE 5.1.11 Schematic illustration of placing a hydrophobic molecule in a polar solvent.

The change in standard free enthalpy ΔG^0 is further discussed in terms of free energy the process taking place at a constant volume. The free energy $A^{sol}{}_{X,S}$ (symbol Δ and the index ^{0} for standard expressions are omitted for simplifying the notation) necessary for placing a molecular species X into a solution formed by molecules S can be expressed by the free energy required for the creation of the cavity in the solvent to accommodate the species X indicated as $A^{cav}{}_{X,S}$ plus the free energy of van der Waals interactions between the molecule j and the surrounding molecules S, indicated as $A^{vdw}{}_{X,S}$. For the description of free enthalpy G third term that accounts for the change in the free volume of the system (excluded volume expansion) should be added. The change in the free enthalpy for the dissolution process has the following expression:

$$G_X^{sol} = A_{X,S}^{cav} + A_{X,S}^{vdW} + RT\ln\left(RT/p_0V_S\right) \tag{5.1.60}$$

In expression 5.1.60 p_0 is the atmospheric pressure and V_S is the molar volume of the solvent equal to molecular mass (weight) divided by the density ρ. The expressions for both terms $A_{X,S}^{cav}$ and $A_{X,S}^{vdW}$ were reported in the literature [29–31]. The free energy (change) for the cavity formation in the solvent by the molecular species X can be expressed by the following formula [32]:

$$A_{X,S}^{cav} = \kappa_{X,S}^{e}\cdot\mathscr{A}_X\gamma'_S(1-W_S)\mathscr{N} \tag{5.1.61}$$

In Eq. 5.1.61, $\kappa_{X,S}^{e}$ is a special parameter (dependent on S) defined by formula 5.1.63, $\mathscr{A}_X$ is the surface of the cavity in the solvent necessary to accommodate the molecule X and can be taken as equal with van der Waals area $\mathscr{A}_X^{vdW}$ of molecule X when the whole molecule is hydrophobic, γ'_S is the surface tension of the solvent S, and W_S is a correction factor given by the following expression:

$$W_S = \left(1-\frac{\kappa_{X,S}^{s}}{\kappa_{X,S}^{e}}\right)\left(\frac{\mathrm{dln}\,\gamma'_S}{\mathrm{dln}\,T}+\frac{2}{3}C_{exp,X}T\right) \tag{5.1.62}$$

In expression 5.1.62, $C_{exp,X}$ is the coefficient of thermal expansion for the species X. The coefficient $\kappa_{X,S}^{e}$ expresses the ratio between the energy required for the formation of a suitable shaped cavity with a surface area $\mathscr{A}_X$ in the solvent S and the energy required to expand the planar surface of the solvent by the same area, which is approximately given by $\mathscr{A}_X\gamma'_S$. A similar coefficient must be developed for entropies. The coefficient $\kappa_{X,S}^{s}$ expresses the ratio between the entropy required for the formation of a suitably shaped cavity with a surface area $\mathscr{A}_X$ in the solvent and the entropy required to expand the planar surface of the solvent by the same area. The expression for $\kappa_{X,S}^{e}$ is the following:

$$\kappa_{X,S}^{e} = 1+\left(\kappa_S^{e}-1\right)\left(V_S/V_X\right)^{2/3} \tag{5.1.63}$$

where V_X is the molar volume for the species X and where κ_S^{e} corresponds to the pure solvent and is given by the following expression:

$$\kappa_S^{e} = \frac{\mathscr{N}^{1/3}\Delta\boldsymbol{H}_{vap,S}}{V_S^{2/3}\gamma'_S\left(1-\dfrac{\mathrm{dln}\,\gamma'_S}{\mathrm{dln}\,T}-\dfrac{2}{3}C_{exp,S}T\right)} \tag{5.1.64}$$

The values for $\Delta\boldsymbol{H}_{vap,S}$ (the heat or enthalpy of vaporization of the solvent) are available in the literature [33] or can be estimated [34]. Regarding entropies, a similar expression with 5.1.63 is valid for $\kappa_{X,S}^{s}$ and a similar one with 5.1.64 is valid for κ_S^{s} when $\Delta\boldsymbol{H}_{vap,S}$ is replaced by $\Delta S_{vap,S}$ of the pure solvent. Tables for (dimensionless) values of κ_S^{e} and for κ_S^{s} were reported in the literature [30] and are reproduced in Table 5.1.3. The resulting values for W_S following these calculations are relatively small.

By placing together formulas 5.1.61 and 5.1.63, and taking $W_s \approx 0$ based on its evaluation, the final expression of $A_{X,S}^{cav}$ is the following:

$$A_{X,S}^{cav} = \left[1+\left(\kappa_S^{e}-1\right)\left(V_S/V_X\right)^{2/3}\right]\mathscr{A}_X\gamma'_S\mathscr{N} \tag{5.1.65}$$

The expression for the interacting free energy $A_{X,S}^{cav}$ of the molecule X with the surrounding

TABLE 5.1.3 Parameters used for the calculation of solvophobic forces published in the literature for different solvents [30].

Solvent	$\kappa^e{}_S$	$\kappa^s{}_S$	D_S	$\gamma'{}_S$ dyn/cm	ε_S (at 25°C)	$\frac{d\ln \gamma/_S}{d\ln T}$	ω_S	$C_{exp,S}$ 10^3
Heptane	0.687	0.542	0.234	19.80	1.92	0.100	0.348	1.250
Isooctane	0.672	0.527	0.238	18.32	1.91	0.089	0.313	1.207
CCl_4	0.629	0.475	0.272	26.15	2.23	0.126	0.255	1.220
Cyclohexane	0.621	0.466	0.254	24.38	2.01	0.120	0.262	1.200
Benzene	0.629	0.469	0.293	28.20	2.27	0.139	0.215	1.220
Toluene	0.679	0.529	0.291	27.92	2.37	0.121	0.256	1.066
Aniline	0.972	1.127	0.335	42.79	6.85	0.105	0.256	0.850
n-Octanol	0.827	0.831	0.238	29.40	3.40	0.103	0.288	0.890
n-BuOH	1.089	0.931	0.241	24.20	17.10	0.108	0.252	0.940
EtOH	1.543	1.508	0.220	21.85	24.44	0.084	0.147	1.080
Nitromethane	0.808	0.580	0.231	36.47	38.20	0.168	0.192	1.192
MeOH	1.776	1.609	0.202	22.20	32.63	0.086	0.100	1.190
Water	1.277	1.235	0.205	72.00	77.46	0.157	0.023	0.257

solvent S when X is placed in the cavity is a relatively complicated problem, since this interaction must account for the interaction of the molecule X with the whole surrounding solvent. This energy consists of two different terms, one caused by electrostatic forces $A^{es}_{X,S}$ and the other caused by dispersion forces $A^{disp}_{X,S}$ such that:

$$A^{wdW}_{X,S} = A^{es}_{X,S} + A^{disp}_{X,S} \tag{5.1.66}$$

The expression for $A^{es}_{X,S}$ is obtained from the Onsager reaction field [35] and is given by the following formula:

$$A^{es}_{X,S} = -\frac{\mathcal{N}^2 m_X^2}{2V_X}\mathscr{D}_S\mathscr{P}_{X,S} \tag{5.1.67}$$

where:

$$\mathscr{D}_S = \frac{2(\varepsilon_S - 1)}{2\varepsilon_S + 1} \quad \text{and} \quad \mathscr{P}_{X,S} = \frac{V_X}{4\pi\varepsilon_0(V_X - \mathcal{N}\mathscr{D}_S\alpha_X)} \tag{5.1.68}$$

In Eq. 5.1.68, the common notations m_X for the dipole moment, α_X for polarizability of species X, and ε_S for the dielectric constant of the solvent were used.

The expression for $A^{disp}_{X,S}$ is obtained by using an effective pair potential that contains a correction from the gas phase potential of interaction of Lennard–Jones type [30,36]. Following a relatively elaborate calculation, the following formula is obtained:

$$A^{disp}_{X,S} = -\frac{27(1-x)}{8\pi}\left(Q\prime_{X,S} + Q\prime\prime_{X,S}\right)Y_{X,S}D_XD_S \tag{5.1.69}$$

In Eq. 5.1.68, x is a proportionality constant that relates the entropy of interaction to the energy of interaction and has a typical value of $x \approx 0.436$. The other parameters in Eq. 5.1.68 are as follows:

$$Y_{X,S} = 1.35\frac{I_XI_S}{I_X + I_S} \tag{5.1.70}$$

where I_X is the ionization potential of species X and I_S is the ionization potential of the molecule of solvent. The expression for D_X is given by the following formula:

$$D_X = \frac{n_X^2 - 1}{n_X^2 + 2} = \frac{4\pi}{3V_X}\mathcal{N}^2\alpha_X \tag{5.1.71}$$

where D_X is the so-called Clausius–Mossotti function for the molecular species X and where n_X is the refractive index for the compound X (Eq. 5.1.51) and α_X the (average) polarizability. A formula identical to 5.1.71 gives the value for D_S, where n_X and α_X are replaced, respectively, by n_S and α_S of the solvent.

The expression for $Q'_{X,S}$ (dimensionless) is the following:

$$Q'_{X,S} = \frac{V_X \mathfrak{B}_{X,S}}{\mathcal{N}} \tag{5.1.72}$$

$$\mathfrak{B}_{X,S} = \left[\frac{\overline{\sigma}^6}{(\overline{d} - \overline{l})^9}\left(\frac{t^2}{11} + \frac{t}{5} + \frac{1}{9}\right) - \frac{1}{(\overline{d} - \overline{l})^3}\left(\frac{t^2}{5} + \frac{t}{2} + \frac{1}{3}\right)\right] \tag{5.1.73}$$

where:

$$t = \frac{\overline{l}}{\overline{d} - \overline{l}} \quad \overline{d} = \frac{1}{2}(d_X + d_S) \quad \overline{l} = \frac{1}{2}(l_X + l_S)$$
$$\overline{\sigma} = \frac{1}{2}(\sigma_X + \sigma_S) \tag{5.1.74}$$

and d_X is the diameter of molecule X, d_S is the diameter of solvent molecule, l is Kihara parameter (e.g., Ref. [37]) and σ is London parameter (for solute X or solvent S). All these parameters can be estimated using the following formulas (shown for species X only, the formulas for the solvent molecules having the same expressions):

$$d_X = 1.74\left(\frac{3V_X}{4\mathcal{N}\pi}\right) \quad l_X = d_X\frac{0.24 + 7\omega_X}{3.24 + 7\omega_X}$$
$$\sigma_X = d_X\frac{2.66}{3.24 + 7\omega_X} \tag{5.1.75}$$

where ω_X is the acentric factor that can be calculated for nonpolar compounds based on the expression:

$$\omega_X = -\log p_{red} - 1 \tag{5.1.76}$$

where p_{red} is a reduced vapor pressure with $p_{red} = p/p_c$ (p_c is the critical pressure) at a reduced temperature $T/T_c = 0.7$. For polar compounds, the acentric factor is taken from a nonpolar model compound with similar geometry.

The value for $Q''_{X,S}$ is typically taken as $Q''_{X,S} \approx 0.1\ Q'_{X,S}$.

The resulting free energy of hydrophobic interactions leads to the following formula:

$$A_X^{sol} = A_{X,S}^{cav} + A_{X,S}^{es} + A_{X,S}^{disp} \tag{5.1.77}$$

In expression 5.1.77, $A_{X,S}^{cav}$ is given by formula 5.1.65, $A_{X,S}^{es}$ is given by formula 5.1.66, and $A_{X,S}^{disp}$ is given by formula 5.1.69. As previously indicated, an exothermic process takes place with negative values for the free energy A. The verification of the above formula was performed and reported in the literature [30–32,38,39]. Some values of the parameters used for the calculation of solvophobic free energy were published in the literature for different solvents [30] and are given in Table 5.1.3.

The evaluation of hydrophobic interactions by direct measurements is not very common. One of the few examples of direct measurements is that of two methane molecules. The van der Waals force between two methane molecules in vacuum is about $-2.5 \cdot 10^{-21}$ J. The same interaction in water is about $-14.0 \cdot 10^{-21}$ J. This effect is caused by the reduction of the surface exposed to water when the two hydrophobic molecules are together. Similar results were reported for other molecules, such as the interaction of two molecules of benzene in water with a free energy of -8.4 kJ mol^{-1} and that of cyclohexane with -11.3 kJ mol^{-1} when they form dimers [5].

The topic of solvophobic interactions is more complicated than summarized in this

section, and there is no simple theory to offer a complete picture of the subject. Polyatomic molecules, for example, can have one or more polar moieties and a hydrophobic part. These two different group characters in the same molecule are difficult to treat in a unified mathematical model. When attempting to apply the theory of interactions to separation problems as encountered in HPLC, the type of calculations presented in this section can be used only as directional information and no truly quantitative data can be generated due to the lack of information regarding the values of necessary parameters.

When the solvent is capable of forming hydrogen bonds, such in the case of water, the hydrogen bonds are disrupted in the solvent when a solute is dissolved. This component was not addressed completely in the previous theory, except through the value of γ'_S, the surface tension. The accommodation in solution of a molecule that cannot form hydrogen bonds (a hydrophobic molecule) does not necessarily break the hydrogen bonds, leading only to their reorientation. Not breaking the hydrogen bonds leads to a smaller enthalpic change than in the case of breaking the bonds. However, the molecules reorientation makes the entropic factor to be strongly affected during the dissolution of a nonpolar molecule in a polar solvent, at the same time as the enthalpic factor. For the dissolution of molecules in a polar solvent such as water, the free energy of transfer is roughly estimated as proportional with the surface area of the dissolved molecule. The number of reoriented molecules of the solvent is also influenced by the surface area of the hydrophobic molecule that is dissolved. The entropy change during the dissolution process is therefore expected to depend on the surface area of the dissolved molecule, as well.

Solvophobic interactions are key for the understanding of retention process in RP-HPLC. Also they explain numerous solubility effects. For example, the theory can explain why perfluorinated alkanes have much lower water solubility than the corresponding hydrocarbons, in spite of the fact that the polarity of C−F bond is much higher than that of C−H bond (and a stronger interaction of the solute with the water molecules is expected). The dispersion forces between water and fluorocarbons are not very different from those between water and hydrocarbons. This is caused by the fact that although the fluorine atoms are larger than the hydrogen atoms (van der Waals radius for H is 1.2 Å and for F is 1. Å), and are assumed with higher polarizability, the electronegativity of fluorine atoms reduces the polarizability of the electron system in the fluorinated compounds. The point of difference between hydrocarbons and fluorocarbons is that the C−F bond has a much larger dipole moment than does the C−H bond, leading to a stronger interaction with the dipolar water. Therefore, it would be expected that a fluorocarbon surface is more hydrophilic than that of the corresponding hydrocarbon. This effect is indeed noticed, for example, when bonded fluorinated hydrocarbons are used as stationary phases in RP-HPLC chromatography. These phases are still hydrophobic, but their hydrophobic character is lower than that of the corresponding hydrocarbon. The explanation for the poor fluorocarbon water solubility is that fluorocarbons have a larger molar volume (and molecular surface area) than the corresponding hydrocarbons. For example, a fluorocarbon with a surface area of 299.9 $Å^2$ (perfluorohexane) corresponds to a hydrocarbon (hexane) with surface area of 215.6 $Å^2$. The work done to form a cavity large enough to accommodate a fluorocarbon (A^{cav}) offsets the anticipated free-energy benefit from enhanced energetic interactions with water. An entropic effect due to movement restrictions imposed to the solvent molecules by the polar fluorocarbon may also contribute to the low fluorocarbon solubility in water [40].

Chaotropic and kosmotropic interactions

Besides solvent molecules that affect molecular interactions in solutions, these interactions can also be affected by the presence of other solutes. For example, hydrogen bonds are stronger in a nonpolar medium than in one with some polarity. The addition of certain solutes that increase the polarity of a solution may affect significantly other solutes interactions that form, for example, hydrogen bonds or aggregation through hydrophobic effects. These solutes are known as *chaotropes* (Section 4.5). Some inorganic ions can act as chaotropes, their disruptive character growing in the order: $H_2PO_4^- < HCOO^- < CH_3SO_4^- < CF_3COO^- < BF_4^- < ClO_4^- < PF_6^-$. In solutions of mixed solvents containing water, the chaotropes may also disrupt the solvation shell of other solutes, modifying their interactions [41].

The opposite effect of contributing to the stability and structure of water–water interactions is known as kosmotropic effect. This can be achieved by neutral molecules, such as carbohydrates, proline, tert-butanol, and by small or high charged ions, such as CO_3^{2-}, SO_4^{2-}, HPO_4^{2-}, Mg^{2+}, Li^+, Zn^{2+}, or Al^{3+}. Ionic kosmotropes are characterized by strong solvation energy leading to an increase of the overall cohesiveness of the solution, which is also reflected by the increase of the viscosity and density of the solution [42].

5.2 Forces between molecules and a charged surface

General comments

The evaluation of molecule and surface interactions may provide some insight regarding the adsorption process. Similarly to the case of intermolecular interactions, the evaluation of free energy in different processes takes into consideration both intramolecular forces and concepts from the continuum model. A simple theory for estimating the forces between the molecules and the surface is however difficult to develop except for a few models. For this reason, only estimations of such interactions are typically available in the literature (for a more detailed discussion on the subject, e.g., Refs. [13,43]).

Charge to charged-surface interactions

The charge on a surface is characterized by its charge density σ_s. This charge density generates an electric field $\vec{\mathbb{E}}$ perpendicular to the surface. The intensity of this field can be evaluated considering a circular strip of radius r and width dr on the charged surface (Fig. 5.2.1) positioned around point A, the perpendicular projection of a charge ze on the surface acting on a charge ze at the distance x from the surface.

The field generated by this narrow strip on the point ze is determined by the distance $(r^2 + x^2)^{1/2}$ and also by the angle θ (since the field is perpendicular to the surface). The total intensity of the field can be obtained by integrating over r (from 0 to ∞ for an infinite surface). The

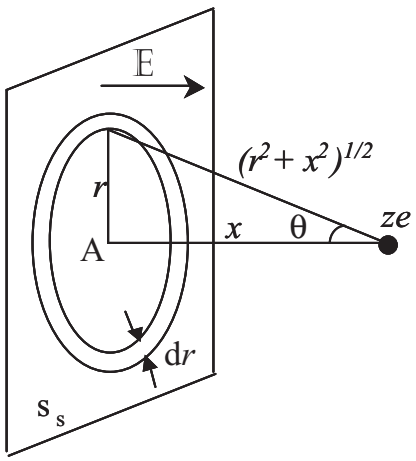


FIGURE 5.2.1 Schematic diagram of the interaction between a charged surface (charge density σ_s) and a charge ze at the fixed distance x.

expression for $\mathbb{E}$ (vector notation not used) will be the following:

$$\mathbb{E} = \sigma_s \int_0^\infty \frac{2\pi r \cos\theta\, dr}{4\pi\varepsilon_0\varepsilon(x^2+r^2)} = \frac{x\sigma_s}{2\varepsilon_0\varepsilon}\int_0^\infty \frac{rdr}{(x^2+r^2)^{3/2}} = \frac{\sigma_s}{2\varepsilon_0\varepsilon} \tag{5.2.1}$$

This relation indicates that the force between the charge ze and the surface is given by the following expression:

$$F = \frac{\sigma_s ze}{2\varepsilon_0\varepsilon} \tag{5.2.2}$$

Expression 5.2.2 indicates that the interaction between a charged surface and a particle with the charge ze is independent of the distance x between the particle and the charge. The energy to move a charge ze for the distance r in the field created by the surface will be given by formula $A = F\,r$, and this energy is given by the following formula:

$$A = \frac{\sigma_s zer}{2\varepsilon_0\varepsilon} \tag{5.2.3}$$

In Eq. 5.2.3, the sign of energy depends on the type of charges on the surface, a negative value indicating attraction and a positive one repulsion. The calculation of $E(r)$ for a charge e at the distance of 0.6 nm from a surface with the charge density of 0.03 C/m^2 indicates an energy of 98 kJ/mol showing that the interaction between a surface and a charge is of the same order of magnitude as the intermolecular interactions. In practice, since the medium always contains other charges with interfering fields, the true force between the charge and the surface decreases with the distance and it is much smaller.

Neutral molecule to charged-surface interactions

The interaction between a charged solid surface and a molecule j can be evaluated in a similar manner as it was done for the interaction between a charged surface and an ion with the charge ze. For this purpose, it is useful to consider first an interacting potential energy of the form (where C' is a constant):

$$E(r) = \frac{C'}{r^n} \tag{5.2.4}$$

An infinitesimal interaction should be further considered between a circular ring of cross-section $dx\,dy$ in the solid surface and the molecule X, as shown in Fig. 5.2.2. This ring volume is $2\pi ydxdy$. Using the notation ρ for the number density of molecules in the solid, the number of molecules in the ring will be $2\pi\rho ydxdy$.

The energy $E(r)$ of interaction between the molecule X at distance r and the surface will be given by the following expression [44]:

$$E(r) = -2\pi\rho C' \int_r^\infty dx \int_0^\infty \frac{ydy}{(x^2+y^2)^{n/2}} = \frac{\pi\rho C'}{(n-2)}\int_r^\infty \frac{dx}{x^{n-2}} = \frac{\pi\rho C'}{(n-2)(n-3)r^{n-3}} \tag{5.2.5}$$

Eq. 5.2.5 indicates that considering the Lennard–Jones potential given by Eq. 5.1.48,

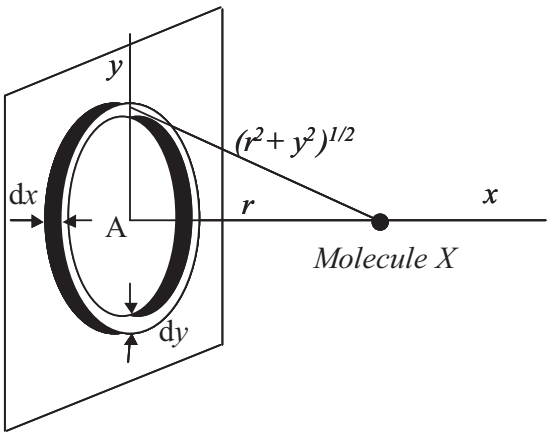


FIGURE 5.2.2 A circular ring of cross-section $dx\,dy$ taken in the solid surface interacting with molecule X.

the energy interaction between the molecules from a solid surface should be expressed by the following formula:

$$E(r) = -\frac{\pi\rho A'}{6r^3} + \frac{\pi\rho B'}{90r^9} \qquad (5.2.6)$$

Regarding Eq. 5.2.6, it should be noticed that it derives from a Lennard–Jones type potential that does not imply the existence of charges, but only of all other types of interactions. Using an expression of the type 5.1.49 for the estimation of parameters A' and B' in Lennard–Jones potential for molecules having optimum molecule to molecule interaction at $r_0 = 6.0$ Å for an idealized pair of molecules with $m = 1.5$ D, $\alpha_0 = 4.2$ (*$1.11 \cdot 10^{-40}$ $C^2m^2J^{-1}$), $\varepsilon = 1$, and $I = 10.9$ e.V, at 300 K the variation of Lennard–Jones molecule to molecule energy $E_{L\text{-}J}$ and that between surface to molecule energy E_{surf} (for $\rho = 1$) for a distance varying between 3 Å and 8 Å is shown in Fig. 5.2.3.

The results shown in Fig. 5.2.3 indicate that the interaction of a surface compared to that of a single molecule is basically of the same order of magnitude, with the surface acting more strongly than a single molecule, as expected. Also, it shows that the minimum of energy is closer to the surface as compared to the minimum for Lennard–Jones type potential. However, in reality, since the medium always contains other molecules with interfering interactions, the true force between the molecule and the surface decreases with the distance faster than indicated by Eq. 5.2.6. On the other hand, the interactions of ions in solution take place at larger distances. This was shown in Fig. 5.1.1 (even if the figure exaggerates to a certain extent the interaction range).

The interaction of the charges on a surface with individual molecules in a medium may explain some exclusion process seen in reversed-phase HPLC. The typical phases in RP-HPLC consist of a silica surface covered with the hydrophobic bonded phase, but also with a large number of silanol groups. The silanol groups have a slight acidic character, and the ionization process leads to the accumulation on the silica surface of small negative charges. These charges may act through Coulombic forces toward ionic species such as naphthalene sulfonic acids (that are almost completely ionized in solution). These types of molecules are excluded from the pores of the stationary phase. For this reason, for example, the retention

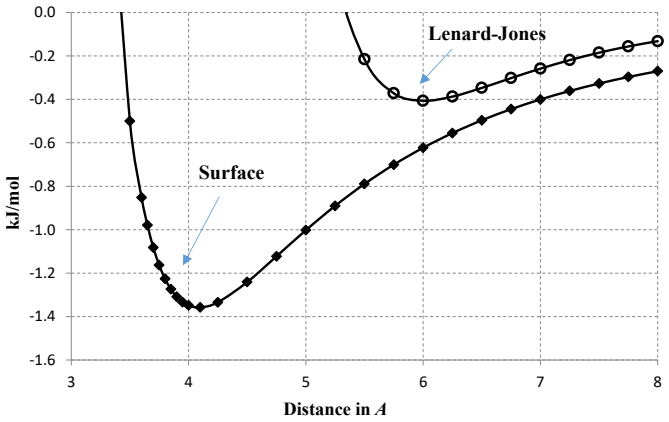


FIGURE 5.2.3 Variation of the interaction energy $E_{L\text{-}J}$ calculated with Eq. 5.1.49 and of E_{surf} calculated with Eq. 5.2.6 between two molecules with $m = 1.5$ D, $\alpha_0 = 4.2$ (*1.11 $10^{4\circ}C^2m^2J^{-1}$), $\varepsilon = 1$, and $I = 10.9$ e.V, at 300 K, when $r_0 = 6.0$ Å and $\rho = 1$.

times for naphthalene sulfonic acids are shorter than holdup time (dead time) t_0 for the column, as measured with small compounds that are not retained but also are not excluded from the stationary phase pores (such as uracil or thiourea) [45].

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CHAPTER

6

Characterization of analytes and matrices

6.1 Properties of solutes important for HPLC separation

Solutes classification based on their chemical structure

The chemical nature of the sample, mainly of its analytes but also of the matrix of the sample, is the main factor to consider in the selection of the HPLC type, and within a type of a specific HPLC method. For this reason, it is important to understand the characteristics of the analytes and of the matrix that influence the HPLC separation and detection. Depending on the number of atoms in a molecule, the solutes/analytes are initially divided into small molecules and macromolecules. The molecules are further classified based on their chemical structure. First, the organic and inorganic compounds are separately classified. Organic compounds are usually considered as those that contain in their molecule carbon and usually hydrogen. A small number of compounds that are considered organic do not contain hydrogen. Also, there are a few classes of compounds that contain carbon and are not considered organic (such as metal carbonates, carbides, and the oxides of carbon). Metal-organic compounds such as complexes of organic compounds with metals are usually considered organic. More complex compounds are also known. The classification in organic or inorganic compounds does not differentiate small molecules from large molecules (polymers). Many polymers such as polymeric carbohydrates, proteins, nucleic acids, and a wide variety of synthetic polymers are organic compounds. Inorganic polymers and polymers that contain inorganic and organic components such as silicones are also known. Samples in HPLC analysis may contain a variety of components with a variety of structures.

More detailed classifications for both organic and inorganic compounds are known. Classification of organic compounds includes various classes of molecules starting with simple compounds formed only from carbon and hydrogen such as saturated hydrocarbons (linear or branched), saturated cyclic hydrocarbons, unsaturated hydrocarbons with one or more double bonds, unsaturated hydrocarbons with triple bonds, aromatic hydrocarbons, etc. Combinations of all these structures are possible. On the hydrocarbon backbone, various functional groups can be attached. They can be classified based on the nature of atoms in the functional group or other criteria (such as monofunctional, bifunctional, etc.) This procedure will differentiate halogenated compounds, alcohols, enols, phenols, ethers, peroxy compounds, thiols, sulfides, amines, imines, a variety of other nitrogenous compounds (nitro, oximes, etc.), aldehydes, ketones, carboxylic acids, various derivatives of organic acids (such as esters,

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lactones, acyl chlorides, etc.), derivatives of carbonic acid (such as ureas, cyanates), sulfonic acids, etc. More than one functional group and more than one type of group can be attached on the hydrocarbon backbone. A special class of compounds is that of heterocycles (aromatic or not). Heterocycles can be classified based on the heteroatoms in the cycle such as oxygen (furans, pyrans), nitrogen (pyrrole, pyrazole, imidazole, triazole, pyridine, pyrazines, etc.), sulfur (thyophene), or different heteroatoms (oxazoles, thiazole, oxadiazoles, etc.). Organic molecules may have very complex structures and a simple classification is typically hard to make and at the same time less relevant. Specific classes of organic compounds, with similar properties, are also known, such as carbohydrates, amino acids, lipids, steroids, nucleotides, triterpenes, flavonoids, etc. In addition to all the classes of compounds containing C, H, O, N, or S, other types of organic compounds include those containing boron, silicon, arsenic, antimony, and metallic elements (organometallic compounds).

One particular aspect related to chemical structure is the isomerism. Isomers are molecules (or polyatomic ions) with the same number of atoms of each element but with distinct arrangements of atoms in space. The types of isomers can be classified as structural isomers in which bonds between the atoms differ, and stereoisomerism in which the bonds are the same but the relative positions of the atoms differ. HPLC is frequently utilized for isomer separation. A schematic description of some common isomerism is given in Fig. 6.1.1.

For macromolecules, the chemical structure also can be an important criterion of classification. Specific groups such as polymeric carbohydrates, lignins, tannins, Maillard browning polymers, proteins, nucleic acids, as well as various types of synthetic polymers can be differentiated based on their structure.

Inorganic compounds also cover a wide range of possibilities of combinations. These possibilities start with binary compounds such as oxides, sulfides, halides, to very complex structures. Acids, bases, and salts, coordination compounds, are among common inorganic materials. Inorganic polymers are also common in nature. The chemical nature of the analytes and of the matrix components is an important factor in the selection of the method of analysis. This is related to other properties determined by the chemical structure such as polarity, hydrophobicity, acid–base character, etc., that are further discussed.

Classification based on the role of the analyte in everyday life

Depending on the role/function in everyday life, analytes are frequently classified in various categories. No specific criteria are set for such classification, and only an enumeration of some of the most important categories is indicated here. It should also be mentioned that the classification based on the role in everyday life is frequently combined with that based on the analyte structure.

One common classification of sample is that as biological and nonbiological. The two groups are frequently further separated in multiple subgroups. Another classification is based on criteria related to environmental issues, and groups such as environmental pollutants, pesticides, herbicides, fungicides, etc., are considered. A large group of compounds classified based on their role in health issues is that of pharmaceuticals, metabolites, and biodegradation products. Numerous classes of pharmaceuticals are further differentiated such as antibiotics, anticancer, antiepileptic, steroids, analgesics, vitamins, etc. Metabolites and biodegradation products are also further classified based on their source, mechanism of production, etc. A classification based on the compound's role in toxicology or in forensic science is also utilized, with groups such as carcinogens, illicit drugs, poisons, other toxins, etc.

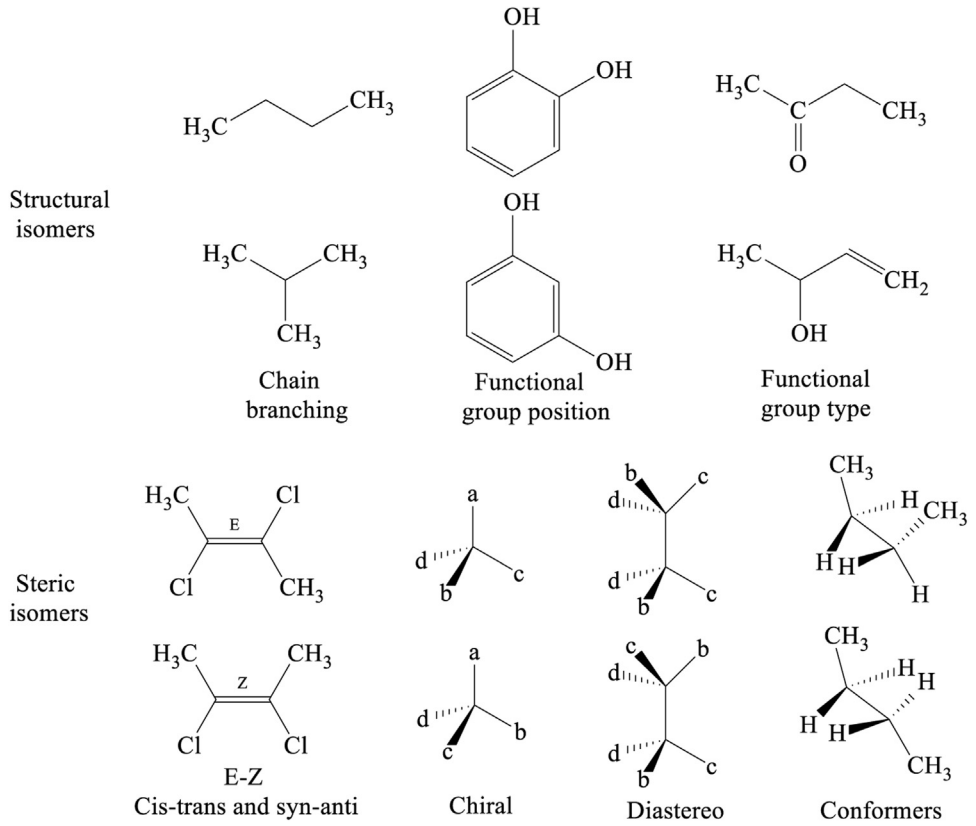

FIGURE 6.1.1 Different types of isomers with examples.

Food analysis and analysis of agricultural products plays an important role in everyday life, and as a result, classifications in groups related to these fields are also common. Analyte groups such as nutrients, flavors, toxins in food, main food components, etc., are frequently differentiated. Numerous other groups of compounds not listed previously are common. Among these can be mentioned flavors, polymer additives, solvents, surfactants, dyes, etc.

Although such classifications as those based on the role of the analyte in everyday life may appear not related to the HPLC separation technique, this classification is in fact extremely important. Most real-life samples are complex mixtures that must be separated and their components detected. The matrix which must be separated from the analytes depends very frequently on the group of the sample classified based on its everyday life criteria. For example, biological samples such as blood/plasma will contain proteins, while food products may contain large amounts of lipids or of carbohydrates. The selection of a separation frequently depends on the matrix that is described by the information regarding the class of sample. Also, the specificity of the separation and sensitivity of the analysis is frequently determined by the type of sample as classified based on its role in everyday life.

Comments on physico-chemical properties of analytes and matrices

Physico-chemical properties can be classified as molecular properties (e.g., molecular weight, dipole moment, polarizability, van der Waals volume, and surface area) and bulk properties (e.g., acidic or basic character in solution, octanol/water partition coefficient, solubility, etc.). The molecular properties and the bulk properties are related all depending on the chemical structure of the molecule, and various relations can be established between the molecular and the bulk properties. However, in some instances, these relations can be rather complicated and it is more convenient to use some of the bulk properties as independent parameters. Some of the physico-chemical properties of analytes and matrices of samples meaningful for HPLC separation are further discussed.

Molecular weight

An important classification of chemical compounds is based on molecular weight, and classes such as small molecules, oligomers, and polymers are classified based on the molecular weight. Molecular mass M of one molecule is its mass expressed in unified atomic mass units (1/12 of the mass of one atom of the isotope carbon-12, sometimes named dalton Da). Molecular weight (Mw) of a molecule is the ratio of the mass of the molecule to 1/12 of the mass of isotope carbon-12 (Mw is dimensionless) [1]. Mw and molecular mass M are numerically equal, but they are not the same parameter, although the terms are frequently used interchangeably. Molecular weight can be used to differentiate analytes as small molecules or as macromolecules. HPLC is used for the separation of both small and macromolecules. As a common definition, macromolecules are chemical compounds formed from at least 1000 atoms linked by covalent bonds. However, instead of number of atoms, an Mw higher than about 5000 is commonly used to indicate a macromolecule, and an Mw lower than about 2000 to indicate a small molecule. Between these two limits there is a gray area where, among others, the molecules known as oligomers are placed. The molecular weight is related to many molecular properties and plays a major role in the selection of HPLC type. Macromolecules are frequently separated using size exclusion chromatography (gel filtration and gel permeation). However, polymers are typically formed from a mixture of macromolecules with different degree of polymerization of one or more monomeric units. The average degree of polymerization (number of monomer units) and an average Mw is usually indicated for polymers.

Acidic or basic character of analytes

Many types of molecules contain functional groups likely to lose or gain protons under specific circumstances. The molecules capable of donating a proton are indicated as "Brønsted" acids, and those capable of accepting a proton are indicated as "Brønsted" bases. For most organic compounds, the dissociation with the formation or acceptance of protons is an equilibrium process (see Section 4.5). This dissociation is characterized by the acidity constant (or constants in case of polyprotic molecules) commonly expressed as $pK_a = -\log K_a$. Tables with acidity constants are readily available in the literature for common acids and bases. Similar to any equilibrium constant K_a is temperature dependent and its values are typically listed for 25°C. Various techniques are available for pK_a calculation [2]. Computer programs are also available for the calculation of pK_a (e.g., MarvinSketch [3]). For macromolecules with multiple groups ionizable in solution, the individual pK_a values for each functionality are not relevant parameters, the basic or acidic character of the molecule being a global property. The acidity or basicity constant for a compound

is a very important parameter in the decision of the type of chromatography that should be used for its separation. Also, the pH of the mobile phase is selected in many separations as dependent of the pK_a of the analytes, with the purpose of keeping the analyte in mainly one form or another.

van der Waals molecular volume and area

van der Waals molecular volume $\mathscr{V}^{vdW}$ and molecular area (surface) $\mathscr{A}^{vdW}$ are important parameters for the understanding of molecular interactions during a separation. This volume can be defined as the one occupied by a molecule that is impenetrable to other molecules at ordinary temperatures. In RP-HPLC in particular, van der Waals molecular area $\mathscr{A}^{vdW}$ plays an important role in the expression of the free energy for the partition equilibrium and directly to the value of retention factor for an analyte, as shown in Section 9.1 (see Eq. 9.1.16). The calculation of both van der Waals molecular volume and area starts with the concept of van der Waals radius of an atom. This is the radius of an imaginary sphere used to model the atoms describing its finite size. The radius is obtained based on results from gas kinetic collision cross-sections, gas critical volumes, crystal densities (extrapolated at 0 K), liquid state properties, X-ray diffraction data, etc. [4,5]. Several "mean" van der Waals radii (in Å) are indicated in Table 6.1.1.

TABLE 6.1.1 van der Waals radii r for several elements in Å.

H 1.20						
Li 1.82	Be 1.53	B 1.92	C 1.70	N 1.55	O 1.52	F 1.47
Na 2.27	Mg 1.73	Al 1.84	Si 2.10	P 1.80	S 1.80	Cl 1.75
K 2.75	Ca 2.31	Ga 1.87	Ge 2.11	As 1.85	Se 1.90	Br 1.85
Rb 3.03	Sr 2.49	In 1.93	Sn 2.17	Sb 2.06	Te 2.06	I 1.98

For most organic molecules, the atoms are placed at covalent bond distance, which is shorter than the sum of the van der Waals radii of the connected atoms. For this reason, the van der Waals molecular volume is smaller than the sum of the volume of each component atom. This is also true for the molecular surface. For a diatomic molecule with two atoms with r_1 and r_2 van der Waals radii, and the covalent bond distance l, the calculation of the molecular volume is obtained starting with the two interlocked spheres as shown in Fig. 6.1.2.

The volume of one sphere $\mathscr{V}_2^{vdW}$ (with radius r_2) can be calculated as $\mathscr{V}_2^{vdW} = 4/3\pi(r_2)^3$. The volume of a spherical segment $\Delta\mathscr{V}_1^{vdW} = \pi h_1^2(r_1 - h_1/3)$ must be added to $\mathscr{V}_2^{vdW}$ (atom with radius r_1) and the volume of another spherical segment $\Delta\mathscr{V}_{2-1}^{vdW} = \pi h_2^2(r_2 - h_2/3)$ must be subtracted from $\mathscr{V}_2^{vdW}$ to obtain the total volume. The values for h_1 and h_2 are obtained as $h_1 = r_1 + l - m$, $h_2 = r_2 - m$ and $m = \left(r_2^2 - r_1^2 + l^2\right)/(2l)$. The total van der Waals volume will be $\mathscr{V}^{vdw} = \mathscr{V}_2^{vdW} + \Delta\mathscr{V}_1^{vdW} - \Delta\mathscr{V}_{2-1}^{vdW}$. The volume of a molecule $v_X = V_X/\mathscr{N}$ is not the same

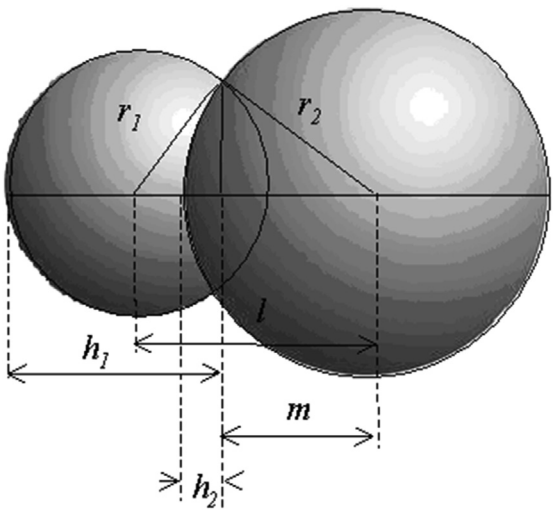

FIGURE 6.1.2 Description of the calculation of the volume of a diatomic molecule.

as van der Waals volume $\mathscr{V}^{vdw}$ since the volume of voids and the changes in the volume due to interactions must be included for the calculation of the value for v_X. van der Waals volume is usually measured in $Å^3$ or in nm^3.

van der Waals area of the molecule can be obtained as the sum of two spherical segments with $\mathscr{A}_1^{vdW} = 2\pi\, r_1 h_1$ and $\mathscr{A}_2^{vdW} = 2\pi r_2(2r_2 - h_2)$. Since direct calculations of van der Waals molecular volumes and areas are difficult for larger molecules, various approximations were developed for this purpose [6]. Computer programs are also available for the calculation of van der Waals volume and areas (e.g., MarvinSketch [3,7]). van der Waals surface area of a molecule is usually measured in $Å^2$ or in nm^2. Related to van der Waals volume is the molecular volume (McGowan calculated molecular volume [8]).

Molecular shape

Besides the numerical value of van der Waals molecular volume and area, the shape of molecules plays an important role in HPLC separations. The shape is characterized by numerous parameters such as area of projection on different plans, different parameters regarding accessibility to polar groups (polar surface area), solvent accessibility, etc., as well as a number of topological parameters (such as Balaban index, Harry index, eccentricity, etc. [9,10]). Various computer programs (e.g., Ref. [3]) are capable of calculating such parameters some with direct utility in predicting the results of separations. The high importance of molecular geometry is easily understood for the separations based on adsorption equilibrium. For example the spatial access to the polar phase and the orientation of different groups in the molecule which depend on molecule geometry determine the strength of interaction with the stationary phase. However, the steric component of a molecule is also important in RP-HPLC where the separation is mainly based on partition. As demonstrated by the utility of hydrophobic subtraction model for selectivity characterization in RP-HPLC, a steric factor must be included in the model to account for steric interactions. Some information regarding the importance of molecular shape for molecular interactions was given in Section 5.1.

For polymers, the molecular shape is a more complex problem, since they are formed from macromolecules in a range of Mw and shapes. The shape is characterized by various parameters such as hydrodynamic volume (the volume of a polymer coil when it is in solution), the degree to which the crystalline and amorphous regions are present, the tacticity (relative stereochemistry of adjacent chiral centers within the macromolecule), etc.

Molar volume

Molar volume V_{mol} (also noted only by V or V_X to indicate the molar volume of molecule X) is the volume occupied by 1 mole of a compound (or element) at a given temperature and pressure. The expression for V_{mol} is given by the following formula:

$$V_{mol} = \frac{Mw}{\rho} \text{ or with another notation } V_X = \frac{M_X}{\rho_X} \tag{6.1.1}$$

In Eq. 6.1.1, Mw is the molecular weight and ρ is the density of the compound (notations M_X and ρ_X are also used). Molar volume is related to the calculation of various other parameters used in separations. The volume of a molecule v_X is obtained from the molar volume based on the expression $v_X = V_X/\mathscr{N}$ ($\mathscr{N}$ is Avogadro number) and it is different from van der Waals volume $\mathscr{V}_X^{vdW}$ [11]. Molar volumes can also be defined for mixtures forming solutions. For a solution, an excess molar volume can

also be defined (in chemical thermodynamics, excess properties are properties of mixtures which quantify the nonideal behavior of real mixtures [12]).

Molecular polarity

Polarity is an important characteristic of molecules. The strict definition of polarity refers to the separation of the center of partial positive charges from that of partial negative charges in the molecule leading to an electric dipole. However, the term polarity is used with a wider meaning and besides the electric dipole of the molecule characterized by the dipole moment m, under the concept of polarity is included the polarizability characterized by parameter α, as well as the capability to form hydrogen bonds.

The definition of dipole moment m used for the description of molecular polarity is given by formula 5.1.12. The unit of dipole moment is the debye (D), and $1\ \mathrm{D} = 3.336 \cdot 10^{-30}$ C m. The interactions of the dipole moment with ions, other dipoles, and whole molecules were presented in Section 5.1, and the energy corresponding to such interactions is given in Table 5.1.1. The dipole moments of some compounds used as solvents are discussed in Section 7.2.

Polarizability α which describes the tendency of molecules to develop a dipole moment under the influence of an electric field, usually created by other molecules from the environment, is defined by Eq. 5.1.29. The common units of polarizability α are $4\pi\varepsilon_0(\text{Å})^3$, indicated as polarizability volume. The role of polarizability in the molecular interactions was also discussed in Section 5.1. Polarizability can be estimated based on different types of calculations (e.g., Refs. [13,14]). The polarizability of some compounds used as solvents is discussed in Section 7.2.

Hydrogen bond formation was also presented in Section 5.1, with some values of this type of interaction given in Table 5.1.1. Hydrogen bonding capability is frequently evaluated based on molecular composition, with practical observation such as for the interaction of molecules with solvents, the hydrogen bonds strength increasing in the order $CH_3CN < CH_3OH < H_2O$. Since hydrogen bonds have a higher energy than dipole–dipole or dipole–induce dipole interactions, even molecules with small m or α can have stronger polar interaction if they are capable of forming hydrogen bonds. The tendency of forming hydrogen bonds of some compounds used as solvents is discussed in Section 7.2.

Related to molecular polarity, one other parameter characterizing an analyte is the molar refraction given by the following expression:

$$R_{mol} = \left(\frac{n^2 - 1}{n^2 + 2}\right) V_{mol} \tag{6.1.2}$$

From molar refraction, polarizability is obtained based on Lorentz–Lorenz formula:

$$R_{mol} = \frac{4\pi}{3} \mathcal{N} \alpha \tag{6.1.3}$$

For the characterization of molecular polarity, besides the direct parameters m and α, various other associated parameters are utilized, such as octanol/water partition coefficient log K_{ow}, further presented in this section.

Partial charge distribution

The analysis of electronic population is a subject of considerable interest for the understanding of molecular properties (e.g., Refs. [14,15]). The partial charge distribution determines physico-chemical properties such as dipole moment m, ionization constants, reactivity, etc. Various approaches and procedures are reported in the literature for the calculation of partial charges [14,16,17]. Computer programs are also available for partial charges calculation (e.g., MarvinSketch [3] and other programs [18,19]). The values for partial charges (point charges)

are useful for the understanding of molecular polarity, although not used frequently in specific calculations related to separations. The potential application of this parameter could be in those LC separations where electrostatic interactions play an important role.

Isoelectric point

The isoelectric point (pI) is the pH value at which the molecule carries no electrical charge. The concept is particularly important for zwitterionic molecules such as amino acids, peptides, and proteins. For an amino acid, the isoelectric point is the average of pK_a values for the amine and the carboxyl group. In the case of amino acids with multiple groups ionizable in solution (e.g., lysine with two amino groups or aspartic acid with two acid groups), the isoelectric point is given by the average of the two pK_a of the acid and base that lose/gain a proton from the neutral form of the amino acid. This can be extended to the definition of pI of peptides and proteins. The pI value can be used to indicate the global basic or acidic character of a zwitterionic molecule, and compounds with p$I > 7$ can be considered basic, and those with p$I < 7$ can be considered acidic. The isoelectric for a simple amino acid, the pI, can be obtained using the average of pK_{a1} and pK_{a2} where pK_{a1} refers to the acid group and pK_{a2} to the amino group. The value can also be obtained experimentally from electrokinetic experiments or can be evaluated from partial charges on the molecule, obtained, for example, using semiempirical procedures (e.g., Ref. [13]). As an example, the variation of the charge on histidine molecule and the value of pI point (p$I = 7.69$) are shown in Fig. 6.1.3.

For complex molecules such as proteins, isoelectric point is useful in the description of acidic or basic character, where individual pK_a values are not relevant [20,21].

Octanol/water partition constant and its use for polarity estimation

A common characterization of polarity of molecules is based on a "bulk" property and not on molecular properties. This characterization is obtained for a compound X using the octanol/water partition constant $K_{ow}(X)$ (P is also a common notation for K_{ow} and for a compound X notations such as $K_{X,ow}$ can be used). Octanol/water partition constant is used for

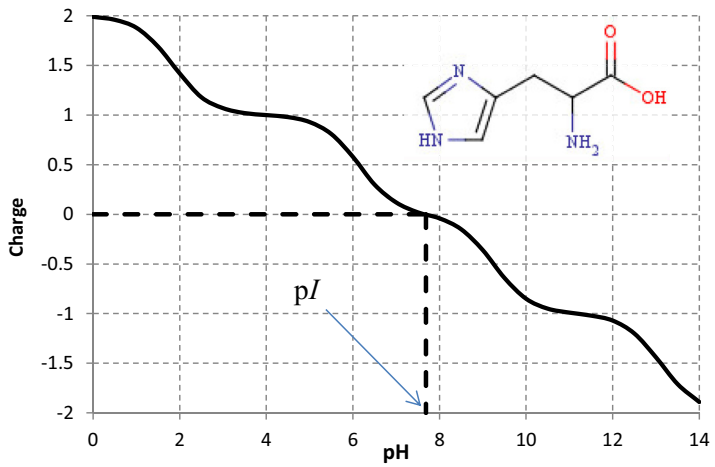

FIGURE 6.1.3 Isoelectric point for histidine.

the characterization of hydrophobicity of a compound X, which is the opposite of polar character. The expression for $K_{ow}(X)$ has been given by formula 4.1.22. The polar and/or hydrophobic character of a molecule is related to its K_{ow} value, with positive values for log K_{ow} indicating hydrophobic character, and with larger values for log K_{ow} showing more hydrophobicity. Molecules with low log K_{ow}, even if the values are positive, show the presence of some polar character, and negative values clearly indicate polar properties. However, there is not a direct relation between K_{ow} and the charge distribution in the molecule. Octanol/water coefficient is a "bulk" property referring to the whole material, while charge distributions (dipole moment, polarizability) are properties at molecular level. The application of the findings at the molecular level to the bulk level is not straightforward. Several properties of the continuum, such as dielectric constant of the bulk that affects interactions, should also be considered for interpreting the findings at the molecular level.

Octanol/water partition coefficient can also be used for the characterization of compounds that can be partially present in the form of ions or can exist in more than one form. These compounds are characterized by the distribution coefficient D_{ow} that was defined by Eq. 4.1.23. Octanol/water parameters (K_{ow} and D_{ow}) have a widespread utilization in separation science and also in other important fields of science, such as drug design and environmental studies. Values for K_{ow} are available for many compounds [22,23], can be calculated using computer programs (e.g., MarvinSketch 5.4.0.1, ChemAxon Ltd. [3], EPI Suite [23], ClogP [24], ACD/logPdb, KowWin [25], and SciLogP/Ultra), and can be obtained using additive fragment methodology [26–28]. Other methods for the estimation of K_{ow} are based on physicochemical molecular properties such as van der Waals molecular surface area [29], solvatochromic parameters [30], etc. The results generated by different methods of log K_{ow} typically generate similar results, and in good agreement with the experimental data. As an example, the dependence between calculated K_{ow} values using two different computer programs, MarvinSketch and EPI Suite, and K_{ow} experimental values is shown in Fig. 6.1.4. For allowing a uniform comparison between compounds, the K_{ow} values used in this book were the calculated ones based on MarvinSketch 5.4.0.1 program.

The distribution coefficient D_{ow} for species with groups ionizable in solution depends on pH as previously discussed in Section 4.5. For a simple acid with the formula HX, the distribution D_{HX} between an aqueous and an organic phase is given by Eq. 4.5.12. When the organic phase is octanol, this formula can be written as follows:

$$D_{ow}(HX) = \frac{C_{HX,o}}{C_{HX,w}\left(1 + K_a/\left[H^+\right]\right)} = K_{ow}(\mathrm{HX})\frac{1}{1 + 10^{pH-pK_a}} \tag{6.1.4}$$

Expression 6.1.4 indicates that at high pH, the value for $10^{-\mathrm{pH}}$ is extremely small and $D_{ow}(HX)$ is expected to be small. In acidic conditions, $K_a < 10^{-\mathrm{pH}}$ and the value for $D_{ow}(HX)$ becomes closer to that for $K_{ow}(HX)$. Similar evaluations can be performed for bases. As an example, the variation of log D_{ow} for histidine as a function of pH is given in Fig. 6.1.5. The figure also shows the variation of % content of different forms of histidine (ionized in solution) as a function of pH. As indicated in Section 4.5, histidine being an amphoteric compound has a low D_{ow} since even the at isoelectric point, p$I = 7.69$ (Form 3) the molecule has two opposed charges.

The values of log K_{ow} (and log D_{ow}) are very useful for the characterization of polar or hydrophobic character of small molecules. However, the same technique cannot be applied for quantitative characterization of polarity of polymers, where the log K_{ow} value loses its meaning. Proteins, for example, have the capability of folding,

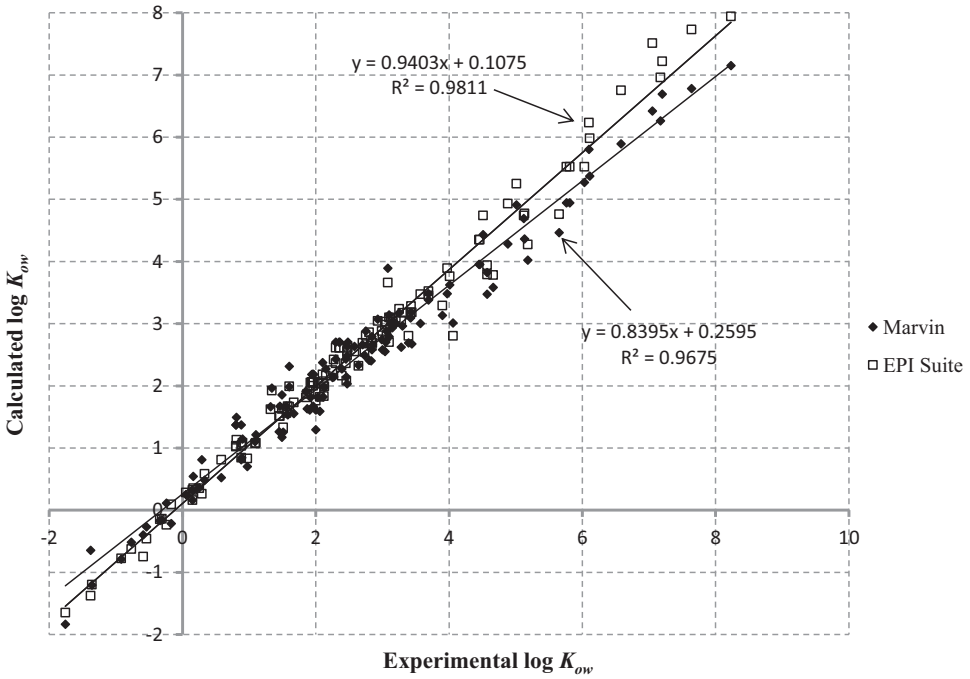

FIGURE 6.1.4 The dependence between the calculated K_{ow} (MarvinSketch and EPI Suite) and experimental K_{ow} values.

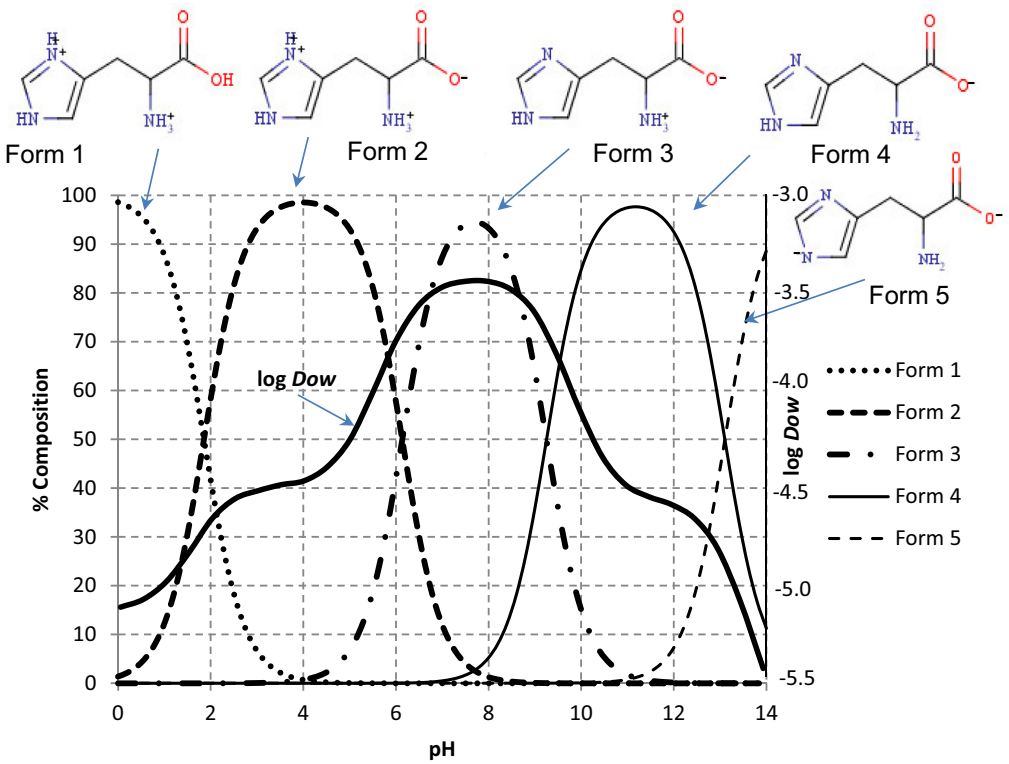

FIGURE 6.1.5 The variation of % content of different forms of histidine (ionized in solution) as a function of pH and the variation of log D_{ow}.

and the polar groups have the tendency to congregate in such a manner to maximize electrostatic interactions with the polar solvating medium. In a polar solvent like water or aqueous solutions of acids, the protein may change its tertiary and quaternary structure and expose polar side chains toward the solvent, the hydrophobic moieties being congregated toward a more hydrophobic core. The opposite effect may take place in the presence of organic solvents. This behavior would lead to a variable octanol/water partition.

For polymers, the rank of hydrophobicity can be based on the functional group identity and oligomer length [31]. For synthetic polymers, a convenient approach to estimate the hydrophobic character of their surface is to measure the contact angle with different solvents, such as water (wettability), ethyleneglycol, or diiodomethane. Contact angle values higher than 90° are assigned to hydrophobic polymers, while the values higher than 150° are specific to superhydrophobic polymers [32].

The extension of polarity characterization with octanol/water partition coefficient can be done to solvent mixtures and even to stationary phases, although such values do not have a true experimental meaning. For example, for a mixture of solvents, a hypothetical K_{ow}^{hyp} can be utilized for the description of the apparent hydrophobicity of the mixture. Such a value can be taken as being the weighted (by content) average of the K_{ow} values of the participating solvents (see Section 7.1). An experimental value for a solvent mixture cannot be obtained since the components of a solvent mixture (e.g., acetonitrile + water) would distribute independently between octanol and water and will not remain as an initial mixture. However, hypothetical values are still useful for hydrophobicity estimation.

For the extension of K_{ow} value to the characterization of a stationary phase, one possibility is to simulate the stationary phase with a model small molecule that contains the main characteristics of the phase (e.g., taking the bonded moiety and a small portion of a silica surface). The stationary phase can be characterized by a K_{ow}^{model} although the value of K_{ow}^{model} does not correspond to an experimental property. The use of K_{ow}^{model} for stationary phase characterization is further discussed in Section 8.2.

Thermodynamic parameters related to solubility of nonelectrolyte compounds

Solubility is a property related to separation, since the analyte must be soluble in the mobile phase for achieving retention and elution. In addition to that, the sample should be soluble in the solvent used for injection. The solubility is defined as the maximum amount of solute that dissolves in a fixed volume of a solvent at a given temperature and can be expressed as mol/L of solute, mole fraction x, and some other ways. This maximum amount of dissolved compound is attained when equilibrium is established between the solid substance and its dissolved form in a saturated solution. The equilibrium can be written as follows:

$$X_{\text{solid}} \leftrightarrow X_{\text{solution}} \tag{6.1.5}$$

The molar concentration in a solid $C(X)_{\text{solid}} = 1$; the constant governing the equilibrium Eq. 6.1.5 can be written in the following form:

$$K_{\text{solub}} = C(X)_{\text{solution}} \tag{6.1.6}$$

Expression 6.1.6 shows that the molar concentration of a saturated solution $C(X)_{\text{solution}}$ is constant (for a constant temperature). In a saturated solution in the presence of solid, by the addition of solvent, more solid dissolves (until the solution is not saturated anymore). The elimination of solvent leads to the formation of more solid.

The dissolution process of nonelectrolytes can be viewed hypothetically as formed from two

steps. For the dissolution of a solid species, the first step is melting (fusion) of the compound X to form a supercooled liquid, requiring the free enthalpy ΔG_{fus} to disrupt the molecular interaction from the solid lattice. The second step is the mixing of this liquid with the solvent S. This indicates that the free enthalpy of dissolution ΔG_{dis} can be written in the following form:

$$\Delta G_{dis} = \Delta G_{fus} + \Delta G_{mix} \tag{6.1.7}$$

From the expression $\Delta G = \Delta \boldsymbol{H} - T\Delta S$, and considering that at melting temperature $\Delta G_{fus}(T_{fus}) = 0$, the following is obtained: $\Delta \boldsymbol{H}_{fus} = T_{fus} \cdot \Delta S_{fus}$ (the value for ΔS_{fus} is typically taken using the approximation $\Delta S_{fus} \approx 13$ cal/mol deg). From the standard expression $\Delta G = \Delta \boldsymbol{H} - T\Delta S$ and using the value $\Delta S_{fus} = \Delta \boldsymbol{H}_{fus}/T_{fus}$, the expression for ΔG_{fus} at a given temperature T becomes the following:

$$\Delta G_{fus}(T) = \Delta \boldsymbol{H}_{fus}\left(1 - \frac{T}{T_{fus}}\right) \tag{6.1.8}$$

The formula for the free energy of mixing is given by the following expression:

$$\Delta G_{mix} = \Delta \boldsymbol{H}_{mix} - T\Delta S_{mix} \tag{6.1.9}$$

For regular solutions (e.g., Ref. [33]), the entropy of mixing is given by the following expression:

$$\Delta S_{mix} = -R\sum_{j} n_j \ln x_j \tag{6.1.10}$$

In Eq. 6.1.10, j is one component in the solution, n_j is the number of moles for j, and x_j is the mole fraction of component j $\left(x_j = n_j \Big/ \sum_{i}^{tot} n_i\right)$. Considering only two components (solute X and solvent S), estimating $x_S \approx 1$ (and $\ln x_S \approx 0$), and taking 1 mole of compound $n_X = 1$, the expression for ΔS_{mix} is the following:

$$\Delta S_{mix} = -R \ln x_X \tag{6.1.11}$$

At equilibrium between solid and solution, for constant pressure and temperature $\Delta G_{dis} = 0$. As a result, $\Delta G_{fus} + \Delta G_{mix} = 0$. Formulas for both ΔG_{fus} and ΔG_{mix} are previously indicated. The replacement of ΔG_{fus} with its expression given by formula 6.1.8 and of ΔG_{mix} with its Eq. 6.1.9 (where ΔS_{mix} is given by formula 6.1.11) leads to the following result:

$$\Delta \boldsymbol{H}_{fus}\left(1 - \frac{T}{T_{fus}}\right) + \Delta \boldsymbol{H}_{mix} + RT \ln x_X = 0 \tag{6.1.12}$$

From this expression, the following formula can be generated for the maximum amount of solute that dissolves in a fixed volume of solvent:

$$\ln x_X = -\frac{\Delta \boldsymbol{H}_{mix}}{RT} - \frac{\Delta \boldsymbol{H}_{fus}}{R}\left(\frac{1}{T} - \frac{1}{T_{fus}}\right) \tag{6.1.13}$$

For the evaluation of x_X, in Eq. 6.1.13, $\Delta \boldsymbol{H}_{mix}$ and $\Delta \boldsymbol{H}_{fus}$ must be estimated. The evaluation of $\Delta \boldsymbol{H}_{mix}$ can be done for nonpolar compounds based on the use of *Hildebrand solubility parameter*. This approximation considers first the dissolution in a solvent S of a vaporized molecule X. This dissolution can be viewed as the reverse of vaporization. The energy of vaporization can be separated into two terms, one accounting for the removal of a molecule from the liquid and the other for the formation of new interactions between X molecules remaining in solution. Assuming that a molecule in the liquid is surrounded by n other molecules and the energy for each interaction is E_{XX}, the energy term of removal will be nE_{XX}. The formation of new interactions $X-X$ in solution will have the same energy E_{XX}, but the number of such interactions will be $n/2$. The vaporization energy $\Delta E_{vap,X}$ is therefore given by the following expression:

$$\Delta E_{vap,X} = \mathcal{N}\frac{nE_{XX}}{2} \tag{6.1.14}$$

(where $\mathcal{N}$ is the Avogadro constant, $\mathcal{N} = 6.022 \cdot 10^{+23}\ \text{mol}^{-1}$). When the process of dissolution of a molecule X in the solvent S takes place, n/2 interactions $S-S$ will be broken, and n' interactions $X-S$ will be created (and $n' \approx n$). Therefore, indicating by E_{XS} the interaction energy between a molecule of solute and one of solvent, the dissolution energy is given by the following expression:

$$\Delta E_{sol,\,XS} = \mathcal{N}\left(\frac{nE_{SS}}{2} - nE_{XS}\right) \tag{6.1.15}$$

In the solution, an equal number of intermolecular interactions per unit volume can be assumed, such that $\mathcal{N} \cdot n = C_t \cdot V_X$, where V_X is the molar volume of species X ($V_X = Mw_X/\rho_X$ where ρ_X is the compound density) and C_t is a constant. With this assumption, Eq. 6.1.14 takes the following form:

$$\Delta E_{vap,\,X} = \frac{C_t V_X E_{XX}}{2} \tag{6.1.16}$$

Expression 6.1.16 can be written in the following form:

$$E_{XX} = \frac{2\Delta E_{vap,X}}{C_t V_X} \tag{6.1.17}$$

Expression 6.1.17 indicates that the molecular interaction E_{XX} in the liquid is proportional with the ratio of vaporization energy per unit molar volume. This ratio can be used to give a specific solubility parameter with the notation δ_X that has the following expression [34]:

$$\delta_X^2 = \frac{\Delta E_{vap,X}}{V_X} \tag{6.1.18}$$

Parameter δ_X is known as *Hildebrand solubility parameter* and the units for δ are $(\text{cal/cm}^3)^{1/2}$. The value of δ_X can be estimated using a number of procedures [34]. These include calculation from heats of vaporization $\Delta \boldsymbol{H}_{vap,X}$, which is related to $\Delta E_{vap,X}$ by the following formula:

$$\Delta E_{vap,X} \approx \Delta \boldsymbol{H}_{vap,X} - RT \tag{6.1.19}$$

where $\Delta \boldsymbol{H}_{vap,X}$ is either measured or estimated. Hildebrand solubility parameter δ_X can also be estimated from superficial tension γ' with the following empirical formula:

$$\delta_X \approx 4.1\left(\frac{\gamma'_X}{V_X^{1/3}}\right)^{0.43} \tag{6.1.20}$$

Values for Hildebrand solubility parameter are available in the literature [35].

This solubility parameter can be used as a measure of the intermolecular interactions per unit volume of a pure liquid based on the following relation:

$$E_{XX} = \frac{2\delta_X^2}{C_t} \tag{6.1.21}$$

For the solvent S, the parameter δ_S is defined similarly to δ_X and E_{SS} is expressed by a formula similar to Eq. 6.1.21 The energy for the interaction $X-S$ can be approximated as the geometric mean of E_{XX} and E_{SS} such that:

$$E_{XS} = \sqrt{E_{XX}E_{SS}} = \frac{2\delta_X\delta_S}{C_t} \tag{6.1.22}$$

The energy of mixing of a mole of X with a large quantity of pure S to form a dilute solution should be equal with the sum of $\Delta E_{vap,X}$ and $\Delta E_{sol,XS}$ given by expressions Eqs. 6.1.14 and 6.1.15. The use of the values for E_{XX}, E_{SS}, and E_{XS} as function of Hildebrand solubility parameters in this sum leads to the expression for the energy of mixing in the following form:

$$\Delta E_{mix,XS} = \mathcal{N}\left(\frac{n}{C_t}\right)\left(\delta_X^2 + \delta_S^2 - 2\delta_X\delta_S\right) \tag{6.1.23}$$

Assuming no volume variation during mixing at constant pressure, the energies $\Delta E_{mix,XS}$ can be taken as equal with the enthalpy (heat) of mixing $\Delta \boldsymbol{H}_{mix}$. In conclusion, Eq. 6.1.23 can be written (after including $\mathcal{N} \cdot n = C_t \cdot V_X$) for 1 mole of solute X in the following form:

$$\Delta \boldsymbol{H}_{mix} = V_X(\delta_X - \delta_S)^2 \tag{6.1.24}$$

For a solution where the concentration of X and S is comparable, Eq. 6.1.24 must be replaced by the following similar relation [36]:

$$\Delta \boldsymbol{H}_{mix} = (x_X V_X + x_S V_S)(\delta_X - \delta_S)^2 \phi_X \phi_S \tag{6.1.25}$$

where x_X and x_S are the mole fractions and ϕ_X and ϕ_S are the volume fractions of X and S, respectively (see Eq. 3.5.2). For x_X very small, Eq. 6.1.25 is reduced to Eq. 6.1.24, although the transformation is not straightforward due to the approximations that are involved. The use of Eq. 6.1.12 with the values for $\Delta \boldsymbol{H}_{mix}$ given by Eq. 6.1.24 allows an estimation of solubility of a nonelectrolyte.

Eq. 6.1.13 gives the formula for solubility (expressed as the maximum mole fraction of a solute in the solvent) for the formation of an ideal solution. The mole fraction x_X can be changed into molar concentration C_X using the following approximation:

$$x_X = \frac{n_X}{n_X + n_S} \approx \frac{n_X}{n_S} = \gamma_X C_X \frac{M_S}{1000 \rho_S} \tag{6.1.26}$$

where γ_X is the activity coefficient included to correct for the deviation from ideal solutions, M_S is the molecular mass (weight) of the solvent, and ρ_S is its density. Eq. 6.1.13 can now be written in the following form [37]:

$$\ln \frac{\gamma_X C_X M_S}{1000 \rho_s} = -\frac{\Delta \boldsymbol{H}_{mix}}{RT} - \frac{\Delta \boldsymbol{H}_{fus}}{R}\left(\frac{1}{T} - \frac{1}{T_{fus}}\right) \tag{6.1.27}$$

From Eqs. 6.1.13 and 6.1.24, and taking $\gamma_X \approx 1$, the solubility for nonelectrolytes can be estimated based on solubility parameters δ, and the expression for $\ln C_X$ is given by the following formula:

$$\ln C_X = -\frac{V_X}{RT}(\delta_X - \delta_S)^2 - \frac{\Delta \boldsymbol{H}_{fus}}{RT}\left(1 - \frac{T}{T_{fus}}\right) + \ln \frac{1000 \rho_S}{M_S} \tag{6.1.28}$$

Since at melting temperature $\Delta \boldsymbol{H}_{fus} \approx T_{fus} \cdot \Delta S_{fus}$, by taking $\Delta S_{fus} \approx 13$ cal/mol deg (the fusion entropy being relatively constant for many compounds), Eq. 6.1.28 can be estimated by the following formula:

$$\ln C_X = -\frac{V_X}{RT}(\delta_X - \delta_S)^2 + 6.54\left(1 - \frac{T_{fus,X}}{T}\right) + \ln \frac{1000 \rho_S}{M_S} \tag{6.1.29}$$

Eq. 6.1.29 is useful for the evaluation of a solid nonelectrolyte solubility into a solvent. The formula shows that a low value for the temperature of melting $T_{fus,X}$ is favorable to solubility (for nonpolar compounds), and that the increase in temperature T also favors solubility ($T > T_{fus}$).

Formulas 6.1.28 and 6.1.29 also indicate that when the difference between the solubility parameters ($\delta_X - \delta_S$) is larger the solubility is lower, which is in agreement with the experimental observation that similar compounds dissolve one in the other, while different ones are less likely to mix. The simple principle of "like-to-like" describes the conditions when the dissolution is favored. The presence of other compounds in the solution may influence the solubility through other interactions. Solubility of electrolytes is a separate subject (e.g., Ref. [38]).

Solubility parameter δ can be estimated using a number of procedures [34]. These include calculation from heats of vaporization $\Delta \boldsymbol{H}^{\text{vap}}$ with the following formula:

$$\delta \approx \left(\frac{\Delta \boldsymbol{H}^{\text{vap}} - RT}{V}\right)^{1/2} \tag{6.1.30}$$

where $\Delta \boldsymbol{H}^{\text{vap}}$ is either measured or estimated. Another approximate formula is based on the value of superficial tension γ' as follows:

$$\delta = k\left(\frac{\gamma'}{V^{1/3}}\right)^{0.43} \tag{6.1.31}$$

where $k \approx 4.1$. Estimations can also be done by other procedures. Molar volume can be calculated using the simple formula 6.1.1.

Solubility parameter δ as defined by Eq. 6.1.18 is related to the vaporization energy ΔE_i^{vap}. This energy is determined by different types of internal molecular interactions (see Section 5.1 for various intermolecular forces). As a result, it can be concluded that δ is also composed of contribution from different types of interactions and several partial δ values can be differentiated. These partial δ values are indicated as δ_d (for dispersion van der Waals forces), δ_p (for polar van der Waals forces), δ_a (for proton acceptor hydrogen bonding), δ_h (for proton donor hydrogen bonding), and known also as Hansen solubility parameters. Values for δ and partial δ describing different types of interaction were reported in the literature for various solvents and some of these values are listed in Appendix 6.1.1 (together with the compounds molar volume). Only a limited number of the listed solvents are actually used as components of a mobile phase.

An equation for calculating δ from the contributions of different types of interactions has been suggested and has the following expression [39]:

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_a^2 + \delta_h^2 \tag{6.1.32}$$

However, because partial δ are obtained from extrathermodynamic considerations, the value for δ obtained with formula 6.1.32 is not equal to δ obtained based on thermodynamic considerations, in some cases differing only slightly but in other cases showing significant differences [40]. In addition, δ_h is usually difficult to evaluate.

Solubility parameter δ can be used for the evaluation of solubility of a compound in a specific solvent and also for the evaluation of partition coefficients of a solute between two solvents [41]. Its main use is related to the estimation of solvent properties related to their "elution" capabilities in RP-HPLC.

Activity coefficient from enthalpy of mixing

For regular solutions, the chemical potential μ_X depends on the enthalpy of mixing by the following formula:

$$\mu_X = \mu_X^0 + \frac{1}{n_x}\Delta \boldsymbol{H}_{X,mix} + RT \ln x_X \tag{6.1.33}$$

At the same time since activity can be expressed as $a_X = \gamma_X \cdot x_X$ (see Eq. 4.1.5), the chemical potential is given by the following expressions:

$$\mu_X = \mu_X^0 + RT \ln a_X \tag{6.1.34}$$

and

$$\mu_X = \mu_X^0 + RT \ln x_X + RT \ln \gamma_X \tag{6.1.35}$$

The subtraction of expressions Eqs. 6.1.33 and 6.1.35 leads to the following formula:

$$\ln \gamma_X = \frac{1}{n_X RT}\Delta \boldsymbol{H}_{X,\,mix} \tag{6.1.36}$$

From Eq. 6.1.34 for the activity coefficient and Eq. 6.1.24 for the enthalpy of mixing of a mole of compound X with solvent S (where $n_X = 1$), the following expression is obtained:

$$\ln \gamma_X = \frac{V_X(\delta_X - \delta_S)^2}{RT} \tag{6.1.37}$$

(Eq. 6.1.25 should be used instead of Eq. 6.1.24 when the concentration of X and S is comparable). Tables of values for solubility parameter δ are available in the literature for a variety of compounds (see also Appendix 6.1.1).

Solubility in water from octanol/water partition constant

Water solubility is an important parameter since it is very common that mobile phases contain water. The evaluation of solubility of

different organic compounds in water based on thermodynamic consideration is typically much less precise compared to the estimation based on the values of parameter K_{ow}. Values for octanol/water partition constant K_{ow} are readily available. The estimation of solubility of organics in water from K_{ow} values is typically done using regression equations of the following form:

$$\log C_X = a \log K_{ow}(X) + b \qquad (6.1.38)$$

Expression 6.1.38 can also be written in the following form:

$$\log(1/C_X) = a' \log K_{ow}(X) - b' \qquad (6.1.39)$$

where C_X is the solubility in water and a, a', b. b' are empirical parameters. Some values for these parameters are given in Table 6.1.2.

Correlation between van der Waals molecular surface and octanol/water partition constant

The values for some bulk properties can be obtained based on molecular properties, and in certain cases, the relation is relatively simple. This is, for example, the case of the value of octanol/water partition constant that can be obtained from the values of van der Waals molecular surface [38]. Based on solvophobic type interactions described in Section 5.1, it is possible to show that between the value $\log K_{X,ow}$ and $\mathscr{A}_X^{vdW}$ the following relation can be established:

$$\log K_{X,ow} = a' \mathscr{A}_X^{vdW} + \sum_n c_n \qquad (6.1.40)$$

In Eq. 6.1.40, a' is a constant and $a' \approx 1.46 \cdot 10^{-2}$ (for $\mathscr{A}_X^{vdW}$ in Å^2) with K_{ow} calculated based on MarvinSketch (better fit is obtained for $a' \approx 1.57 \cdot 10^{-2}$ with K_{ow} calculated based on EPI Suite, and $a' \approx 1.63\ 10^{-2}$ with experimental K_{ow}). The value for c_n ($n = 1$ for a single substituent) depends on the nature of the substituent (polar or not) attached on the hydrophobic moiety of the compound X. Values for c_n for different organic functional groups (attached to an aliphatic hydrocarbon) are given in Table 6.1.3 [38].

TABLE 6.1.2 Regression equation for the estimation of water solubility based on log Kow [42].

Equation	Units for C_X	Used for chemical class
$\text{Log } C_X = -1.37 \log K_{ow} + 7.26$	μM/L	Aromatics, chlorinated hydrocarbons
$\text{Log } (1/C_X) = 1.113 \log K_{ow} - 0.926$	mol/L	Alcohols
$\text{Log } (1/C_X) = 1.229 \log K_{ow} - 0.720$	mol/L	Ketones
$\text{Log } (1/C_X) = 1.013 \log K_{ow} - 0.520$	mol/L	Esters
$\text{Log } (1/C_X) = 1.182 \log K_{ow} - 0.935$	mol/L	Ethers
$\text{Log } (1/C_X) = 1.221 \log K_{ow} - 0.832$	mol/L	Alkyl halides
$\text{Log } (1/C_X) = 1.294 \log K_{ow} - 1.043$	mol/L	Alkynes
$\text{Log } (1/C_X) = 1.294 \log K_{ow} - 0.248$	mol/L	Alkenes
$\text{Log } (1/C_X) = 0.966 \log K_{ow} - 0.339$	mol/L	Aromatics
$\text{Log } (1/C_X) = 1.214 \log K_{ow} - 0.850$	mol/L	Various
$\text{Log } (1/C_X) = 1.237 \log K_{ow} + 0.248$	mol/L	Alkanes

TABLE 6.1.3 Values for constant c in the calculation of K_{ow} (MarvinSketch values) from van der Waals surface area of the molecule[a] ($a' \approx 1.46 \cdot 10^{-2}$).

Group	c_n	Group	c_n
Aromatic ring[b]	0.055	Aliphatic secondary amine	−1.897
Alcohol	−1.444	Aliphatic tertiary amine	−2.200
Phenol	−0.470	Aromatic primary amine	−0.998
Aliphatic ether	−1.581	Aromatic secondary amine	−1.320
Aromatic/aliphatic ether	−0.825	Ketone	−1.512
Aliphatic acid	−1.375	Nitro aromatic	−0.612
Aromatic acid	−0.852	Chloro aromatic	0.357
Aliphatic primary amine	−1.650	Bromo aromatic	0.481
Aromatic ester	−1.100	Nitrile	−1.072

[a] *Note: each group is counted as many times as present.*
[b] *Note: Separated rings are counted individually; condensed rings are counted as one.*

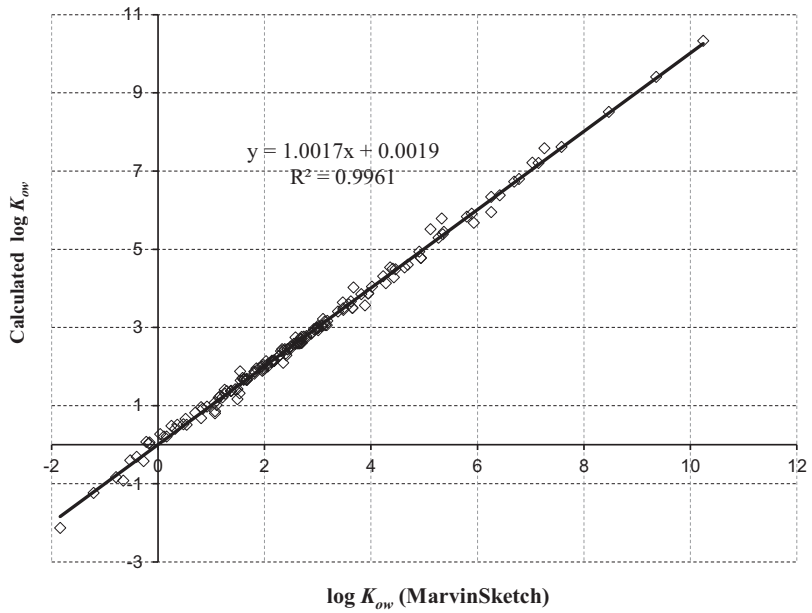

FIGURE 6.1.6 Correlation between log K_{ow} calculated and target values obtained using MarvinSketch. Calculation based on Eq. 6.1.40 with $a' = 1.46 \cdot 10^{-2}$ and the values for c_n indicated in Table 6.1.3.

The calculation of log K_{ow} based on Eq. 6.1.40 with the values for c_n indicated in Table 6.1.3 is shown in Fig. 6.1.6. The results are given for a set of 147 compounds, including mono, bi, tri, tetra, and pentafunctional ones, with identical or different functional groups with log K_{ow} target values obtained using MarvinSketch program (for which parameter a' was optimized).

Solvatochromic parameters for solute characterization

Solvatochromic parameters are parameters typically used for the characterization of solvents, but they can also be developed for solutes (see Section 7.1). Parameters α, β, and π^* for a number of compounds were listed in Appendix 7.1.4 related to solvent type properties, and more such parameters are available in the literature [43,44]. However, except for a limited number of analytes, the estimation of these parameters is difficult or impossible. This reduces the utility of formulas using such parameters to only directional information and comparison for analogous cases where the solvent is changed, while the analytes and the stationary phase remain the same. Accurate calculations, for example, of retention factor for a specific analyte, are not typically successful.

Other parameters for solute characterization

Several other parameters related to the separation in HPLC are used for the characterization of solutes. For example, in the development of certain models for the characterization of stationary phases, it was necessary to include special parameters for the characterization of solutes (analytes), in their interaction with the stationary phase. For example, in Section 9.7, it will be shown that the retention factor $k'(X)$ for a compound X is given by the following expression (see also Eq. 9.7.10):

$$\log k'(X) = \log k'_{EB} + \eta'(X)H_c + \sigma'(X)S^*_C + \beta'(X)A_c + \alpha'(X)B_c + \kappa'(X)C_c \tag{6.1.41}$$

In Eq. 6.1.41, $\log k'_{EB}$ is the retention factor for ethylbenzene, parameters H_c, S^*_c, A_c, B_c, and C_c describe the column characteristics, and the corresponding parameters $\eta'(X)$, $\sigma'(X)$, $\beta'(X)$, $\alpha'(X)$, and $\kappa'(X)$ describe solute properties: $\eta'(X)$-hydrophobicity, $\sigma'(X)$-steric interactions, $\beta'(X)$-hydrogen bonding acceptance, $\alpha'(X)$-hydrogen bonding donation, and $\kappa'(X)$-cation exchange or ion−ion interactions. Values of these parameters were reported in the literature for selected analytes [45−48]. However, these parameters were obtained for a specific mobile phase and were established with the specific purpose of column characterization. They were obtained from best fit regression lines, and are not readily available for solutes that were not in the "test" group.

Some other parameters characterizing the solute have importance in some HPLC applications. One such parameter is the diffusion coefficient D_X of the analyte. As shown in Section 3.1 (see, e.g., Eq. 3.1.34), the longitudinal diffusion in an HPLC column is proportional with D_X. Different theories and also empirical relations were developed for the estimation of diffusion coefficients. For example, Stokes theory empirically modified for better prediction gives the following formula for the diffusion coefficient for nonelectrolytes X in liquids B:

$$D_{X,B} = 7.4 \cdot 10^{-8} \frac{(\psi_B M_B)^{1/2} T}{\eta\, V_X^{0.6}} \tag{6.1.42}$$

where V_X is the molar volume of solute X (in cm^3 $mole^{-1}$), M_B is the molecular weight of solvent B, T is temperature in Kelvin degrees, η is the viscosity of the solution (in 10^{-4} g/cm s, or centipoise), and ψ_B is an "association" factor for the solvent (ψ_B is 1 for nonpolar solvents, 1.5 for ethanol, 1.9 for methanol, 2.6 for water).

Other formulas describing properties of the solutes involve parameters such as refractive index, diameter of the molecule, solvent accessible area SASA, Kihara parameter [49], rate of hydrolysis, etc. These parameters can be either found in the literature or estimated.

6.2 Physico-chemical properties related to detection

General comments

The main subject of present material being the separation in HPLC, the properties of the analytes important for detection are only summarily presented here. The detection in HPLC is based on certain physico-chemical properties that are different between the eluted molecules and the mobile phase such that the analytes can be detected and measured. For this reason, the choice of the type of detection in HPLC is determined by such particular physico-chemical properties. Among these properties are UV- or visible absorption, refractive index, fluorescence, molecular mass and fragmentation in a mass spectrometer, etc. Various instrumental procedures have been developed for measuring such properties, as described in Sections 2.1.

Although the physico-chemical property selected for detection does not affect directly the separation, the selection of the detector has indirect implications on the type of separation used in the HPLC method. For example, if a compound does not have good UV or visible absorption and the measurement is performed using a refractive index detector, the separation must be performed using a constant composition of mobile phase (isocratic separation). The lack of volatility is useful in choosing ELS detection when analytes do not have UV absorption. In case of using MS detection, also restrictions regarding the mobile phase composition are imposed, such that nonvolatile components must be absent from the mobile phase. The property selected for detection also has implications regarding the loading of the chromatographic column with sample. The sensitivity of detection is influenced by both the chemical nature of the analyte as well as by the type of detection selected for analysis. In some analyses, a larger amount of sample must be injected in the HPLC system in order to achieve the necessary sensitivity of the measurement. As indicated in Section 3.4, both the injection volume and the amount of sample have limitations in order to obtain a good separation. Together with the analytes, even after sample preparation, it is common that a certain amount of matrix is injected in the HPLC. The matrix must be separated by the same process as the analytes, and a larger matrix amount may affect adversely the separation. For sensitive detection, a more diluted sample can be injected in the HPLC system avoiding the adverse effect of excessive matrix on the separation [50].

Gas-phase acidity and basicity in MS process of ion formation

For MS detection, ions are formed in the ion source of the LC-MS or LC-MS/MS instrument. The ion formation is caused by protonation or deprotonation, adduct formation, or oxidation or reduction of the analyte molecules [51]. Ion formation when using ESI-MS or APCI-MS detection, in negative or positive modes, is controlled by the acidic/basic properties of analytes [52]. Since water is typically present in the effluent and H^+ ions are abundant for both ESI and APCI, the production of positive ions is the result of formation of molecular ions of the type MH^+ where M are the molecules of the analyte. In many methods using MS detection, a volatile acid such as HCOOH is also added in the mobile phase. In gas phase, the proton affinity is characterized by the gas-phase basicity (GPB) of the analyte. The formation of positive ions usually takes place by reactions of the following type:

$$M + H^+ \rightarrow [M - H]^+ \tag{6.2.1}$$

The ionization reaction Eq. 6.2.1 is characterized by the free enthalpy ΔG^0_{GPB} given by the following expression:

$$\Delta G^0_{GPB} = \Delta G^0\left([M-H]^+\right) - \Delta G^0(M) - \Delta G^0\left(H^+\right) \tag{6.2.2}$$

These reactions are always exergonic in gas phase. The proton affinities are conventionally quoted with the opposite sign from other thermodynamic properties (exothermic reactions having assigned a negative free enthalpy), and a positive value of ΔG^0_{GPB} indicates a release of energy by the system. The higher is the proton affinity, the stronger is the base and the weaker is the conjugate acid in the gas phase. Usually, ΔG^0_{GPB} for most organic compounds lies between 500 and 1000 kJ/mol.

Similar to the formation of positive ions, molecules of the type MH can be ionized to generate negative ions in a reaction as follows:

$$MH \rightarrow M^- + H^+ \tag{6.2.3}$$

Reaction Eq. 6.2.3 is related to the tendency of losing a proton, a property also known as gas-phase acidity (GPA), described by the variation of free enthalpy ΔG^0_{GPA}:

$$\Delta G^0_{GPA} = \Delta G^0(M^-) + \Delta G^0(H^+) - \Delta G^0(MH) \tag{6.2.4}$$

The values for ΔG^0_{GPA} for most organic compounds lie between 1300 and 1650 kJ/mol [53]. The larger values for ΔG^0_{GPA} than for ΔG^0_{GPB} are probably responsible for the lower sensitivity of negative ionization than of positive ionization in LC-MS. The GPB or GPA is energetically significantly different from the same ionization process in solutions, due to the energy contribution of hydration of ions in aqueous phase. A series of collision-induced dissociation experiments in ESI-MS showed that the GPB of the deprotonated analyte molecule M^- and the anion moiety play an important role in determining the stability of their adducts formed in gaseous phase [54].

The role of analyte polarity in MS detection

The difference in the polarity between the solvent and the analyte molecules favors the formation of positive ions (or negative ions when working in negative mode) from the analyte, and much less from the solvent. The ionization can be conducted to form either positive ions or to form negative ions from the analyte neutral molecules. The choice between positive or negative ionization mode depends on the nature of the compounds to be analyzed. Molecules with a basic character (e.g., amines, heterocycles containing nitrogen, etc.) have a tendency to form positive ions and are typically analyzed in positive mode. Other molecules such as acids and some oxygenated or halogenated compounds are typically analyzed in negative mode. Both ESI and APCI ionization techniques offer very reproducible generation of ions, can be used with a wide range of solvents as HPLC eluent, can work in a range of flow rates (e.g., 0.05−1.0 mL/min), and do not involve problems with capillary plugging. The ionization depends on the nature of mobile phase, and a change in the mobile phase composition may affect significantly the ionization yield (and therefore the detector response). To favor the process of positive ion formation, a volatile acid such as HCOOH is typically added in the mobile phase. The positive ions are attracted toward the curtain plate in the ion source, while the solvent molecules that are not charged are not attracted. Further desolvation and elimination of solvent molecules occur as the ions are directed toward the skimmer and further into the ion mass analyzer. For negative ion formation, salts such as $HCOONH_4$ or CH_3COONH_4 are usually added to favor the ionization of the analyte, although HCOOH can sometimes be used as additive even in negative mode ionization.

6.3 Properties of matrix related to HPLC separation and detection

General comments

The initial matrix of a sample is frequently modified by the sample preparation process that is commonly practiced before a core HPLC

analysis (e.g., Refs. [55,56]). However, even a "clean-up" sample may still contain a residual matrix that is injected in the HPLC system together with the analytes. To a considerable extent, the chemical nature of the matrix of an injected sample has a similar importance as the chemical nature of the analytes in an HPLC separation, and the same general properties as discussed in Section 6.1 for the analytes must be evaluated for the matrix.

The chemical nature of the matrix components (further discussion referring to the matrix injected in the HPLC system) can be similar to that of the analyte (e.g., both analytes and the matrix components are small molecules), but also can be very different (e.g., the analytes are small molecules, while the matrix is polymeric). The classification of the chemical nature of the matrix is similar to that of the analytes: (1) inorganic matrix, (2) polymeric organic matrix, (3) matrix consisting of organic molecules with no or very low polarity, (4) matrix of organic molecules with low to medium polarity, (5) highly polar compounds in the matrix, (6) ionic compounds in the matrix, and (7) compounds with amphiphilic properties. While the interest in a chemical analysis appears to be focused on analyte separation, a separation must also be performed between the matrix and analytes, although the matrix components do not need separation among themselves.

The type of chromatography that separates the matrix components from the analytes is frequently the same as the one used for analyte separation. When the analytes and the matrix have a similar nature, this is a straightforward task, although the separation must be good enough to avoid interferences. In some separations, the matrix components are different from the analytes and they are not well retained by the chromatographic column and are eluted at the beginning of the chromatogram, or the matrix may remain in the chromatographic column nut being eluted and require to be eliminated in a "column flushing" step.

Matrix effects on the separation in HPLC

In principle, the HPLC separation achieves the separation between the target compounds and the rest of the injected sample. There are, however, situations when this goal in not entirely achieved, and part of the injected sample matrix coelutes with the analytes. In some cases, where the detector allows good differentiation between the analyte and the components of the matrix, the separation (or complete separation) of the analyte from the matrix components is not even necessary. However, besides the effects on detection (further discussed), the components from the matrix may influence the equilibrium partition of analyte between the mobile and stationary phases. This effect can be significant when the amount of coeluting matrix components is high, leading to distorted and fronting or tailing peak for analyte. This effect is more pronounced when the sample load is close to the column capacity. One aspect that must be considered related to the separation of the matrix or matrix components from the analytes is related to the potential differences between the concentrations of the two. While the analytes may be in some cases present only in traces, the matrix may be present at much larger concentration. For the matrix at relatively low content in the sample, its presence may not affect separation. However, a larger concentration of the matrix must be taken into account related to the loading of the chromatographic column, and the distortion of the shape of chromatographic peaks (see Section 3.4 regarding the chromatographic sample loading).

The matrix can also influence some HPLC peak shapes similar to the influence of the sample solvent. The solvent of the sample, when it has weak eluting properties and is injected at larger volume (e.g., 30–50 μL), may leave all the analytes at the head of the chromatographic column before it is getting diluted with the mobile phase (see Section 7.6). This "focusing" of the analytes at the head of the column can

improve the peak shape. A sample solvent with strong eluting properties (and at larger injection volume) may produce the reverse effect. The analytes can be eluted from the column head until the sample solvent is getting diluted with the mobile phase. This can generate distorted peaks. The same effect can be seen with a matrix component in large proportion present in the sample, and for large injection volumes, when the matrix component may act as a solvent until it is diluted with the mobile phase. As an example, Fig. 6.3.1 shows the chromatogram of a standard sample containing 200 µg/mL lactic acid separated on a Synergy Hydro RP column with mobile phase 20 mM phosphate buffer at pH 2.9 and the same sample in the presence of 5 mg/mL propylene glycol (PG). Fig. 6.3.1 shows the change in the peak shape and of the retention time of the analyte (lactic acid) when PG in large amount is present in the sample, although PG does not influence the detection.

A common case where the matrix affects the retention process is ion chromatography (IC). IC applied to real environmental samples is limited by the matrix composition that influences the elution process of the ionic analytes as well as by the presence of high concentrations of other ions that are dominant in environmental samples [57].

Matrix effects on HPLC detection

The effect of matrix components coeluting or partly coeluting with analytes may induce modifications in the signal intensity measured by HPLC detectors. The amplitude of this effect (*ME*) can be calculated [58] from the ratio between the peak areas *A* measured for the same

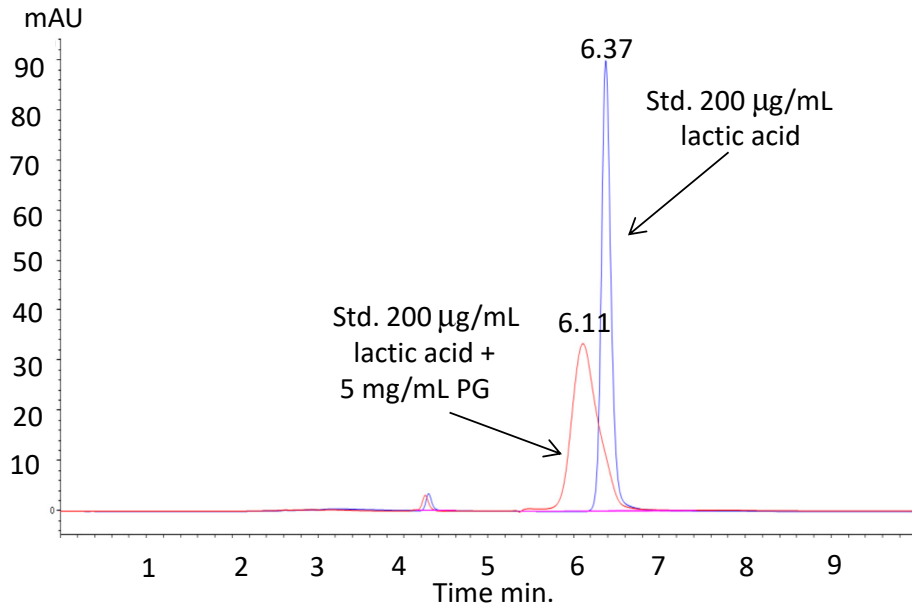

FIGURE 6.3.1 Chromatogram of a standard containing 200 µg/mL lactic acid on a Synergy Hydro RP column with mobile phase 20 mM phosphate buffer at pH 2.9 and the same sample in the presence of 5 mg/mL propylene glycol (PG) (UV detection at 215 nm).

amount of analyte injected together with the sample matrix $\left(A^{\text{analyte}}_{\text{with matrix}}\right)$ and in a pure standard solution $\left(A^{\text{analyte}}_{\text{as standard}}\right)$ of the analyte, following the expression:

$$ME = \frac{A^{\text{analyte}}_{\text{with matrix}}}{A^{\text{analyte}}_{\text{as standard}}} \times 100 \qquad (6.3.1)$$

A value of $ME = 100\%$ indicates the lack of matrix effect, $ME < 100\%$—ionization suppression in MS or fluorescence suppression, and $ME > 100\%$—ionization enhancement in MS or interference in UV, RI, etc. Experimental procedures to evaluate the matrix effect were also developed [59].

In practice, matrix effects depend on how good is the separation and may vary from sample to sample, and from analyte to analyte in the same injected sample. For MS detection, strong matrix effects may affect ionization suppression and *ME* can reach only a fraction of the original MS signal, while ionization enhancement can reach even twice the intensity of MS signal measured in solvent ($ME = 200\%$). Fluorescence detection in HPLC can also be affected by the unseparated matrix, usually decreased, although the matrix itself is not fluorescent [60,61]. In UV detection, the interference is caused by the lack of separation between the analyte and a component from the matrix that absorb at the same wavelength as the analyte and usually $ME > 100\%$. The use of RI detector always requires complete separation of the analyte from the matrix, since this type of detection being nonselective does not provide any possibility of differentiation between the analyte and a matrix component.

In cases of selective detection (e.g., a specific mass for MS detection is used or a specific wavelength for UV detection), it is possible that the HPLC separation does not require to isolate the matrix (or a matrix component) from the analyte, and an accurate measurement is still possible. However, very frequently although the matrix "is not seen" may still affect the detection. An exemplification of matrix influence on the analysis, when it is not well separated chromatographically, is that of four standards of deuterated tobacco-specific nitrosamines (TSNAs). The chromatogram of four TSNAs at concentrations of 1 ng/mL is given in Fig. 6.3.2. The analyzed compounds are D_4-nitrosonornicotine (NNN) (MRM transition 182.1 → 152.1), D_4-nitrosoanatabine (NAT) (MRM transition 194.1 → 164.1), D_4-nitrosoanabasine (NAB) (MRM transition 196.1 → 166.1), and D_4-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (MRM transition 212.1 → 126.1). The separation was performed on a Kinetex 1.7 μm EVO C18 column (100 × 2.1 mm). The elution was made using gradient with solution A aqueous 10 mM CH_3COONH_4 and solution B 0.1% CH_3COOH in acetonitrile [62].

The results from Fig. 6.3.2 demonstrate the high sensitivity of the LC-MS/MS technique in the absence of the interferences from the matrix. For the same sample containing 5% nicotine, in addition to the TSNAs at 1 ng/mL level, the chromatogram is shown in Fig. 6.3.3. Although the MS/MS conditions are not set for nicotine detection, and only the tail of nicotine peak overlaps with the TSNAs peaks, the presence of nicotine in the matrix of the sample significantly affects the detection as shown in Fig. 6.3.3 [50].

Matrix effects in LC-MS can be observed in case of many complex samples, such as for environmental or biological samples [63]. For biological samples, the interferences in LC-MS are mainly caused by the abundant content in phospholipids (e.g., glycerophosphocholines and lysophosphatidylcholines) that produce ion suppression by the competition with analyte molecules for space on the surface of droplets formed during the ESI process [64]. Atmospheric pressure chemical ionization (APCI) is less affected by suppression effects compared to

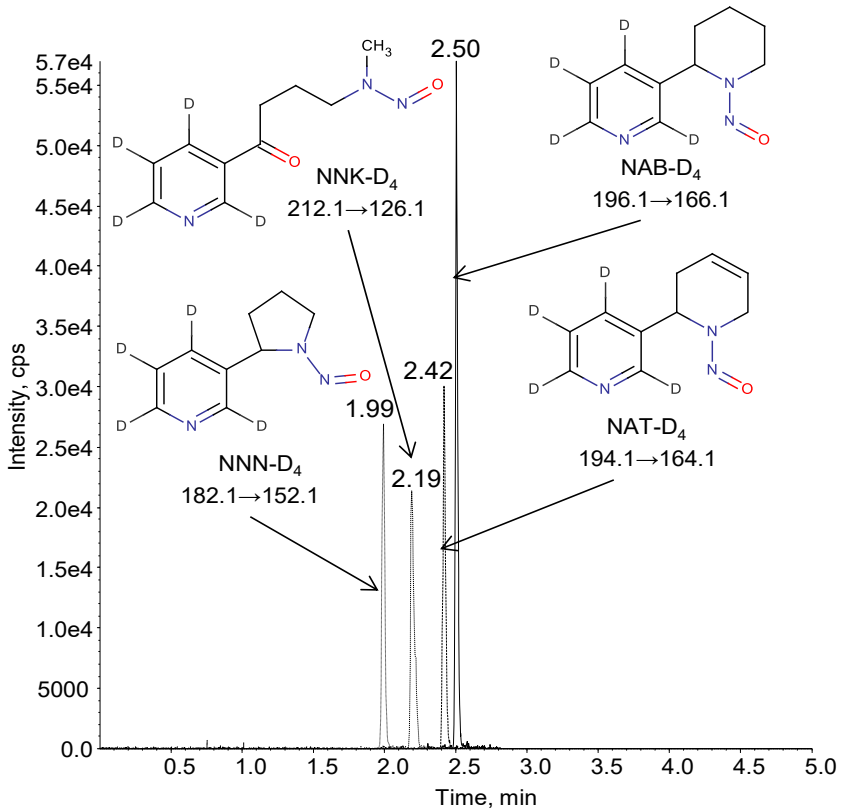

FIGURE 6.3.2 LC-M/MS chromatogram performed in MRM positive mode for a solution containing 1 ng/mL NNN-D_4, NNK-D_4, NAB-D_4, and NAT-D_4.

electrospray ionization (ESI), but when the sample matrix contains species with high basicity and surface activity, ion suppression effects will almost always be observable in LC-MS/MS analysis [65]. In order to eliminate or to reduce matrix effects for biological analyses in particular when using LC-MS techniques, some methods and adequate strategies can be applied [66]. Besides the optimization of HPLC procedure, one procedure to obtain accurate analytical results is to use for quantitation isotopically labeled standards that may be equally affected by the matrix as the analytes (see Section 18.2). In cases when the sensitivity of detection of the analytes is very high, sample dilution with solvent or with mobile phase used in LC separation can diminish the matrix effects [67]. However, the most effective approach for obtaining reliable results is the utilization of an efficient sample preparation method that removes large part of the matrix components that have the most significant effect on the detection of analyte. The strategies for this purpose include protein precipitation with different chemical agents, analyte isolation from sample matrix by liquid–liquid extraction, or by solid-phase extraction, and combination of different clean-up methodologies which are applied before the HPLC separation (e.g., Ref. [55]).

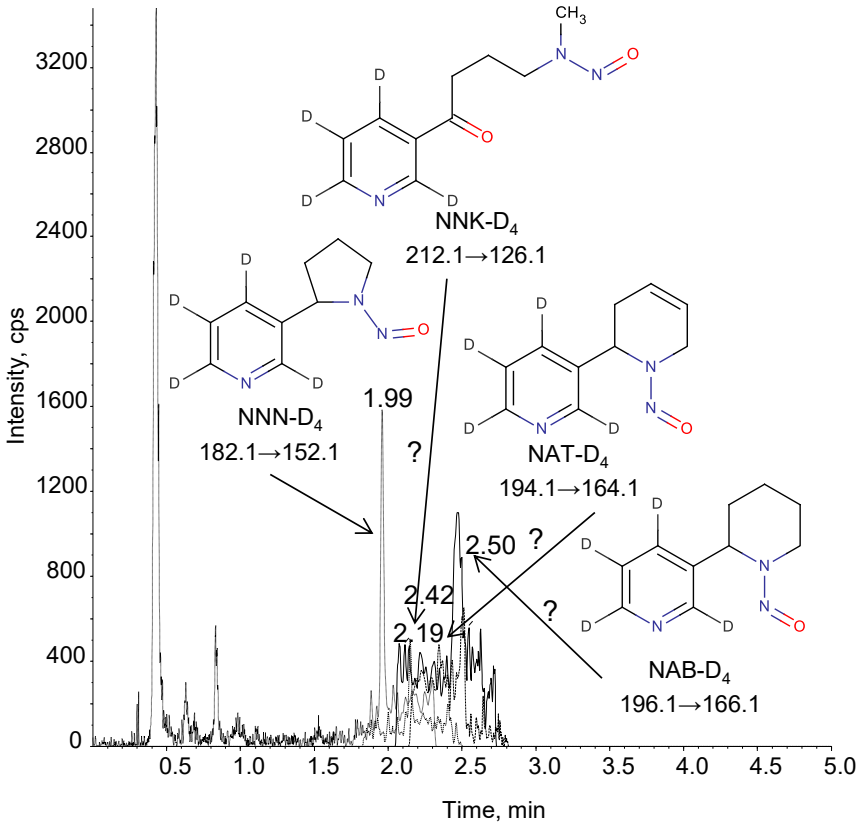

FIGURE 6.3.3 LC-M/MS chromatogram performed in MRM positive mode for a solution containing 5% nicotine and 1 ng/mL NNN-D_4, NNK-D_4, NAB-D_4, and NAT-D_4, showing ion suppression due to incomplete separation of a matrix component (nicotine).

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CHAPTER

7

Mobile phases and their properties

7.1 Characterization of liquids as eluents in HPLC

General comments

Various liquids are used in HPLC as mobile phases. Because these liquids are also dissolving the analytes, they are frequently indicated as solvents and not just as liquids. Some of the properties of these liquids (solvents) can be described by the same parameters as those used for the characterization of analytes, being just specific physico-chemical properties of a compound. Other properties are specific for the role of solvents as eluents in HPLC. In addition to the important role as eluents, liquids are necessary for dissolving the sample as it is injected in the HPLC system, as well as for other processes involved in sample preparation prior to HPLC analysis.

In case of solvent properties, one particular aspect is related to the properties of solvent mixtures. In most cases, the mobile phase in HPLC is made from a mixture of solvents that has a constant composition for isocratic HPLC separations or a variable one during the HPLC run in gradient separations. Some properties of solvent mixtures and the way they result from the properties of pure solvents that are mixed are further presented. Besides organic solvents, water without or with addition of buffers is frequently used in HPLC. The buffers role in HPLC is also discussed in this chapter.

One important characteristic of a solvent as mobile phase is its *elution strength* or simply *strength*. The strength of a solvent is a qualitative description of a property indicating how fast a solvent can elute a compound from a chromatographic column. This elution strength is related to how low is $k'(X)$ for a given compound X when eluted by the solvent from a given chromatographic column. Because the value of $k'(X)$ depends on the nature of X, the type of chromatographic column, and even on the conditions of the separation, its use for the characterization of solvent strength can be done only for comparing solvents and not as a parameter for characterization. Also, the concept of strength depends not only on the nature of the solvent but also on the type of chromatography (reversed phase-RP, HILIC, ion exchange, etc.). For example, a solvent that is a strong eluent in RP is a weak one in HILIC. Several parameters characterizing the solvent itself are used to conclude about its strength as an eluent, but these parameters must be corroborated with that of chromatographic type in which the solvent is utilized.

Among the parameters used for solvent characterization are Hildebrand solubility parameter δ [1], the octanol water partition coefficient K_{ow}

https://doi.org/10.1016/B978-0-323-91177-1.00002-8

(or log K_{ow}), the polarity parameter P' [2], solvatochromic parameters [3], as well as other solvent descriptors [4,5]. Additional properties of solvents such as surface tension γ', dipole moment m, polarizability α, and other general molecular properties are also important for HPLC separations. These properties are further discussed in this chapter in connection with solvents used as HPLC mobile phases. Detailed descriptions of various parameters characterizing the solvents are also available in the literature [2].

Besides the role as solvents, specific properties of liquids used as mobile phase are also important in HPLC. For example, solvent viscosity affects column backpressure and therefore separation conditions. As the mobile phase carries the separated analytes through the detector, other properties of the solvents used as mobile phase become of importance. These include the wavelength of light absorption (e.g., when UV-Vis detection is used), volatility (e.g., when ELSD is used), ionization capability (e.g., when MS detection is used), etc. The properties of solvents, related to the manner in which they affect detection, will be further discussed in this chapter. The properties of liquids as solvents are presented in this section.

Characterization of solvents with Hildebrand solubility parameter

Solubility of the solute in a liquid phase (solvent) plays a fundamental role in liquid chromatography [6]. The solubility based on thermodynamic concepts for analytes has been presented in Section 6.1, where Hildebrand solubility parameter δ for a compound X has been defined by Eq. 6.1.18, written as follows:

$$\delta_X = \left(\frac{\Delta E_{vap,X}}{V_X}\right)^{1/2} \tag{7.1.1}$$

Hildebrand solubility parameter also offers a criterion for describing the *elution strength* of a solvent. For describing the elution process based on the value of δ, the assumption should be made that the separation is based on a pure partition process. This assumption is valid for a reversed phase separation in which only hydrophobic interactions take place. However, since the theory of liquid–liquid partition is in many cases only an approximation model of the real chromatographic processes, the use of parameter δ for the characterization of mobile phase (δ_{mo}) and of the stationary phase (viewed as an immobilized liquid with δ_{st}) must be done with caution. A partition equilibrium of X between mobile and stationary phase is typically indicated as follows:

$$X_{mo} \rightleftarrows X_{st} \tag{7.1.2}$$

The equilibrium constant $K(X)$ for equilibrium 7.1.2 is given by Eq. 4.1.8, written for the partition of X between the two chromatographic phases as follows:

$$K(X) = \frac{\gamma_{X,mo}}{\gamma_{X,st}} \exp\left(-\frac{\mu^0_{X,st} - \mu^0_{X,mo}}{RT}\right) \tag{7.1.3}$$

Since the standard chemical potentials $\mu^0_{X,st}$ and $\mu^0_{X,mo}$ are independent of solution concentration, the values for these potentials must be selected. One choice for the standard state is that of the pure liquid X at the working temperature and pressure. In this case, $\mu^0_{X,st} = \mu^0_{X,mo} = \mu_X^{Liquid}$. The other choice is that of the pure solid $\mu^0_{X,st} = \mu^0_{X,mo} = \mu_X^{Solid}$. For both these choices, the result is $\Delta\mu^0_X = 0$. Another convention is to choose the standard state to be that of the pure liquid only for a solvent, with all the other components having a "fictitious" state with the properties that pure X would have, if its limiting low concentration properties in solution were to be retained in a pure substance. Selecting the choice of standard state $\mu^0_{X,st} = \mu^0_{X,mo}$, the expression of $K(X)$ is reduced to the following form:

$$K(X) = \frac{\gamma_{X,mo}}{\gamma_{X,st}} \tag{7.1.4}$$

The activity coefficients γ can be obtained based on Eq. 6.1.35 that expresses activity coefficient as a function of Hildebrand solubility parameter δ and molar volume V_X. From Eq. 7.1.4 and Eq. 6.1.35, the following formula can be obtained for $K(X)$:

$$K(X) = \exp\left\{V_X\left[(\delta_X - \delta_{mo})^2 - (\delta_X - \delta_{st})^2\right] / RT\right\} \quad (7.1.5)$$

Eq. 7.1.5 can be easily rearranged in the following form:

$$K(X) = \exp\{[V_X(\delta_{mo} - \delta_{st})(\delta_{st} + \delta_{mo} - 2\delta_X)] / RT\} \quad (7.1.6)$$

For the evaluation of the equilibrium constant (or of retention factor $k' = K\Psi$ when phase ratio Ψ is known), it is necessary to know the values of δ for the stationary phase, mobile phase, and analyte, which are seldom available. Also, even assuming that a pure liquid is adsorbed on a solid material and acts as stationary phase, the direct use of the values δ for this liquid in pure form may not correspond to the one of the liquids adsorbed on a solid surface. For these reasons, a true calculation is not usually feasible. Nevertheless, useful considerations can be made based on Eq. 7.1.6. With the assumption that the stationary phase is very nonpolar and the mobile phase (eluent) is polar, for RP-HPLC, it can be concluded that $\delta_{mo} > \delta_{st}$ (see the values in Appendix 6.1.1 which show that nonpolar compounds have lower δ than polar ones) and the term $\delta_{mo} - \delta_{st}$ is always positive. Also, in order to be retained on a hydrophobic column, the solute must be rather hydrophobic and its δ_X value should be lower than δ_{mo} and closer to δ_{st}. As a result, $(\delta_{st} + \delta_{mo} - 2\delta_X)$ is also positive. Eq. 7.1.6 shows that $K(X)$ for a solute is larger when the difference $(\delta_{mo} - \delta_{st})$ is larger, in other words, when the solvent used as eluent has a large δ_{mo} value. This can be easily exemplified by considering a hydrophobic compound retained on a C18 column, which is not eluted by water. As δ_{mo} of the eluent decreases, $K(X)$ decreases. When $2\delta_X > \delta_{st} + \delta_{mo}$, the equilibrium is rapidly displaced toward having the analyte in the mobile phase (eluent). This process can be visualized in Fig. 7.1.1 with a

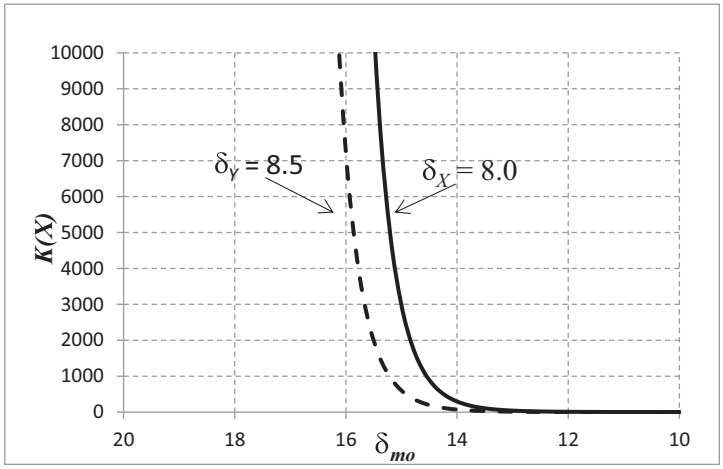


FIGURE 7.1.1 Exemplification of the variation of retention in an RP-HPLC separation as a function of δ_{mo}. (The value of equilibrium constant $K(X)$) as a function of the variation in δ_{mo} $(cal/cm^3)^{1/2}$ of the eluent for two analytes, one with $\delta_X = 8.0$ $(cal/cm^3)^{1/2}$ and the other with $\delta_Y = 8.5$ $(cal/cm^3)^{1/2}$, respectively, both with $V_X = 100$ cm^3/mol on a hypothetical stationary phase ($\delta_{st} = 6.0$ $(cal/cm^3)^{1/2}$).

hypothetical example of two analytes X and Y, one with $\delta_X = 8.0$ $(cal/cm^3)^{1/2}$, the other with $\delta_Y = 8.5$ $(cal/cm^3)^{1/2}$, and with $V_X = V_Y = 100\ cm^3/mol$. The analytes are supposed to be retained on a stationary phase with $\delta_{st} = 6.0$ $(cal/cm^3)^{1/2}$, and with the value of δ_{mo} of the solvent varying between 10 and 20 $(cal/cm^3)^{1/2}$. It can be seen that for $\delta_{mo} < 14$, neither compound is retained on the column (e.g., methanol will elute this compound). As δ_{mo} increases (e.g., for a water/methanol mixture), the value of K increases rapidly indicating retention on the stationary phase.

Eq. 7.1.6 can be related to the *strength* of an eluent for which the analyte is less retained in the column and therefore has a lower K. For RP-HPLC, the relation shows that an eluent with lower δ, which is typical for hydrophobic solvents, leads to lower $K(X)$ values for the analytes, indicating a "stronger" eluent. In a solvent with larger δ, the hydrophobic interactions in the liquid are expelling more efficiently the analyte from the mobile phase placing it in the stationary phase (which is a liquid with lower δ). However, the value of δ cannot be used directly for deciding if a compound is eluted or not from the chromatographic column. For example, acetonitrile has $\delta = 11.8$, while the stationary phase has a lower value for δ (e.g., a hydrocarbon such as isooctane has $\delta = 7$). Nevertheless, acetonitrile is capable of eluting many compounds from a stationary phase with a C8 (or C18) bonded phase.

In gradient RP-HPLC, the initial polarity of the mobile phase is typically high (e.g., water has $\delta = 21$ $(cal/cm^3)^{1/2}$), such that all analytes are retained on the column (large $K(X)$). As the content in organic phase increases, δ_{mo} decreases and the $K(X)$ values decrease. The compound slightly less hydrophobic ($\delta = 8.5$) is first eluted ($K(X)$ for $\delta = 8.5$ is low enough earlier than $K(X)$ for $\delta = 8.0$ as shown in Fig. 7.1.1). As δ_{mo} continues to decrease, the $K(X)$ for the compound with $\delta = 8.0$ also decreases sufficiently that the compound is eluted. The steepness of the decrease of $K(X)$ with the decrease of δ_{mo} can be related to the good separation capability of RP-HPLC in gradient elution.

In HILIC, the stationary phase is more polar than mobile phase and in Eq. 7.1.6 $\delta_{st} > \delta_{mo}$ (opposite than in RP-HPLC). In order to be retained on a polar column, the solute must be rather polar and its δ_X value should be higher than δ_{mo} and closer to δ_{st}. In conclusion, $(2\delta_X - \delta_{st} - \delta_{mo})$ is positive. Eq. 7.1.6 shows that $K(X)$ for a solute is larger when the difference $(\delta_{st} - \delta_{mo})$ is larger, in other words, when the solvent used as eluent has a small δ_{mo} value. This indicates that in HILIC the organic solvents are "weak" eluents, while polar solvents such as water are "strong" eluents. For this reason, parameter δ can be used for "strength" characterization, but this should be done depending on the type of separation.

Separation between two compounds X and Y can also be considered based on the differences in the values in their Hildebrand solubility parameters. The selectivity α is given by Eq. 3.2.2. The replacement in the formula for $\alpha(X,Y) = K(X)/K(Y)$, the expressions for $K(X)$ and $K(Y)$ given by Eq. 7.1.6, leads to the following expression:

$$\alpha(X,Y) = \exp\{(\delta_{st} - \delta_{mo}) \cdot [V_X(2\delta_X - \delta_{st} - \delta_{mo}) - V_Y(2\delta_Y - \delta_{st} - \delta_{mo})] / RT\} \quad (7.1.7)$$

Eq. 7.1.7 leads to the estimation of the value of α for a given separation (when the molar volumes V and parameters δ are known). The first factor in Eq. 7.1.7 shows that α increases as the difference between the δ parameters for the mobile phase and the stationary liquid phase increases ($\delta_{st} - \delta_{mo}$ is larger). The second factor shows that larger differences between V_X δ_X and V_Y δ_Y are larger and the value of α is also larger. These considerations are in accord with experimental observations that a stationary phase more different from the mobile phase

will lead to a higher selectivity between the species to be separated. As expected, the more different are the analytes (different V and different δ), the better is the separation. The formula also shows that at lower temperatures it can be expected to obtain a higher selectivity than at a higher temperature.

The interactions between the analyte, the mobile phase, and stationary phase, not being completely described by the solubility parameter δ, are important to consider in selecting a solvent and the values of the other solubility parameters: δ_d (dispersion), δ_p (polar), δ_a (proton acceptor), and δ_h (proton donor) for both analyte and eluent. For example, the comparison of acetonitrile (AcCN) and methanol (MeOH) using the values from Appendix 6.1.1 indicates that $\delta_{AcCN} = 11.8$ and $\delta_{MeOH} = 12.9$ that shows AcCN as being a stronger eluent in RP-HPLC. However, only δ_d (dispersion) and δ_p (polar) are larger for AcCN, while δ_a (proton acceptor) and δ_h (proton donor) are larger for MeOH. This indicates that differences between the two solvents can be expected to depend on the nature of the analytes to be separated. For example, the difference $\delta_X - \delta_{mo}$ is expected to be smaller for a compound containing OH groups, for $mo =$ MeOH as compared to $mo =$ AcCN. This indicates that for RP-HPLC although overall acetonitrile is a stronger eluent than methanol, for compounds capable of forming strong hydrogen bonds, methanol may also act a strong eluent. Unfortunately, quantitative estimations of eluent strengths cannot be made based on δ and partial δ values, and the inspecting of such values offers only guidance in the selection of a solvent for the mobile phase.

Characterization of a liquid based on its solubility parameter δ provides useful information regarding its solvent characteristics, but its utilization in HPLC is still rather limited. In addition, this approach does not account for the interactions between solute and solvent components. Also, mobile phases are seldom made using a single solvent, and the parameter δ is not defined for solvent mixtures. However, it can be approximated and a discussion on the characterization of solvent mixtures using Hildebrand solubility δ is given further in this section.

Miscibility of solvents and solubility one in another

One important factor regarding solvents selection is related to the miscibility between them (dissolution of one in another in any proportions) and to the range of solubility in case they are not miscible. This property must be considered in the preparation of mobile phases as a mixture of solvents, and also in the selection of the sample solvent that is injected in the mobile phase. For various solvents, the miscibility is typically based on experimental results. A chart showing various solvent's miscibility is given in Fig. 7.1.2.

An empirical procedure for evaluating solubility of a solvent in another is based on Hansen parameters [7]. Hansen parameters are based on the idea that like dissolves like. Each compound is characterized by three Hansen parameters: δ_d accounting for dispersion forces, δ_p accounting for dipolar interactions, and δ_h accounting for hydrogen bonding. These three parameters can be treated as coordinates for a point in a three-dimensional space also known as the Hansen space. The nearer the two molecules are in this three-dimensional space, the more likely they are to dissolve into each other. The Hildebrand parameters for nonpolar solvents are usually close in value to Hansen parameters, but Hansen parameters are also used for polar compounds.

Solubility of one solvent in another (when they are not miscible) is another useful parameter in HPLC. The general theory of solubility as developed for any nonionic compound and presented in Section 6.1 is also applicable to

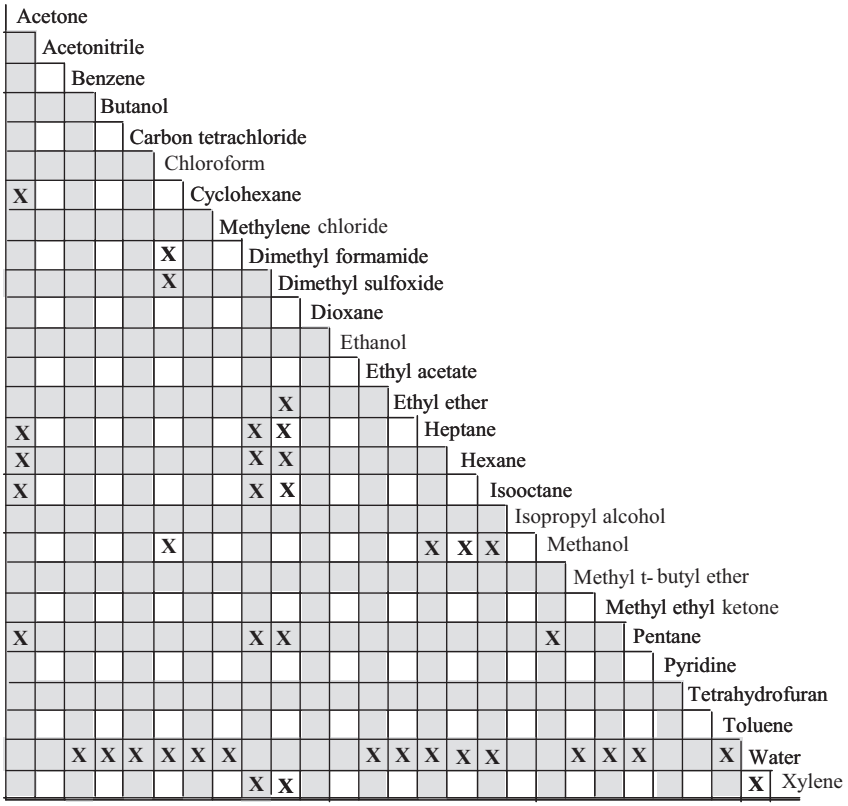


FIGURE 7.1.2 Miscibility chart of various solvents (X indicates lack of miscibility; the gray and white grid is not related to miscibility).

solvent solubility in another solvent. Some data regarding the solubility in water of several solvents are given in Appendix 7.1.1 (see Appendix to Chapter 7). Water is also soluble in a number of solvents, and the solubility of a solvent in water is not usually equal with the solubility of water in that solvent. As an example, octanol solubility in water is about 0.06% (at 20°C), while the solubility of water in octanol is about 4.89% (at 25°C). Other specific solubilities are found in the literature (e.g., Ref. [8]), as information on the web or estimated by certain programs (e.g., EPI Suite [9]). Solubility is also dependent on temperature, and according to Eq. 6.1.29 (that can be applied to any nonionic molecule), the increase in temperature favors the solubility.

Solvent characterization using octanol/water partition constant K_{ow}

Octanol/water partition coefficient K_{ow} is probably the most useful parameter for solvent characterization when the solvent is considered just a certain compound X, mainly because this parameter is readily available (see Section 6.1) [10] and can be calculated using computer programs if the experimental value is not known (e.g., MarvinSketch 5.4.0.1, ChemAxon Ltd. [11],

EPI Suite [9]). Also, log K_{ow} values can be obtained using additive fragment methodology [12,13] and by other procedures [14]. The use of octanol/water partition coefficient for the characterization of hydrophobicity (and polarity) of compounds has been already presented in Section 6.1. However, K_{ow} for various solvents does not correlate too well with other solvent characterizing parameters, as further shown.

For pure solvents, K_{ow} (log K_{ow}) can be used for the estimation of their hydrophobic character as it is done for any other compound. The values of log K_{ow} for a number of solvents are given in Appendix 7.1.2. These values were obtained using MarvinSketch computer package from ChemAxon Ltd [11]. A higher value for K_{ow} indicates a less polar compound and therefore the values for this parameter are growing in the opposite direction to δ. However, log K_{ow} and δ do not show a good correlation (for a set of 55 solvents, the correlation between log K_{ow} and δ has $R^2 = 0.4923$), as shown in Fig. 7.1.3.

For RP type separations, log K_{ow} of the solvent (log $K_{ow}(mo)$) can be used as a guidance regarding its "strength". For example, the strength of a solvent in RP-HPLC and the values for log $K_{ow}(mo)$ (see Appendix 7.1.2 for several common solvents) are in following the order: n-hexane log $K_{ow} = 3.13 >$ di-ethyl ether log $K_{ow} = 0.84 >$ tert-butanol log $K_{ow} = 0.54 >$ tetrahydrofuran log $K_{ow} = 0.53 >$ isopropanol log $K_{ow} = 0.25 >$ acetone log $K_{ow} = 0.11 >$ dioxane log $K_{ow} = -0.09 >$ ethanol log $K_{ow} = -0.16 >$ acetonitrile log $K_{ow} = -0.17$ (experimental -0.34) > methanol log $K_{ow} = -0.52$ (experimental -0.77) > dimethylformamide log $K_{ow} = -0.63 >$ water log $K_{ow} = -0.65$ (experimental -1.38). Although for RP-HPLC the elution "strength" of these solvents should follow the order of their log K_{ow} values, the changes in log $k'(X)$ (decrease for higher log $K_{ow}(mo)$) for different solvents are also dependent on the nature of X. For this reason, a higher or a lower log K_{ow} of the mobile phase influences differently different compounds.

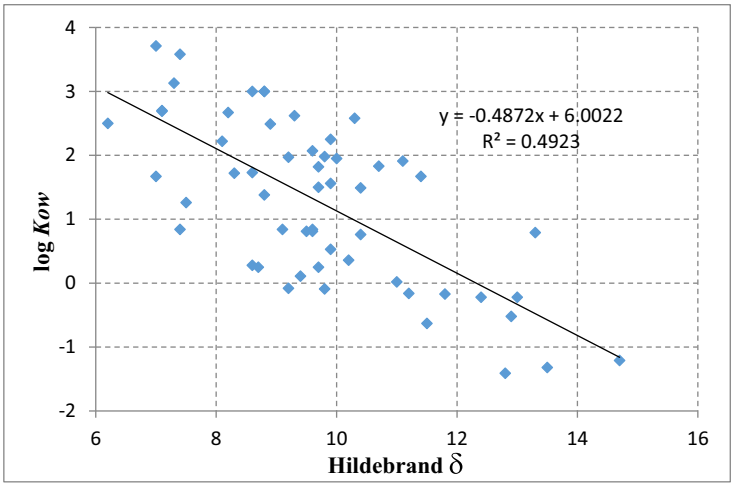


FIGURE 7.1.3 Correlation chart between log K_{ow} and Hildebrand solubility parameter δ.

Usually, larger k' values lead to better resolution, and this can be achieved by decreasing the strength of the mobile phase. However, the differences between solvents in influencing k', differently depending on the nature of the separated compounds, can be utilized for better separations, and this can be based on the use of weaker but in some cases even of stronger eluents.

One problem related to the use of log K_{ow} for the characterization of the hydrophobicity/polarity of the mobile phase in HPLC is that the mobile phase is typically made using a mixture of solvents (and sometimes also contains additives). However, a hypothetical K_{ow}^{hyp} can be utilized for the description of the apparent hydrophobicity of a solvent mixture, as further discussed in this section.

Solvent characterization based on liquid–gas partition

One possibility of characterizing a solvent is based on the measurement of "how good" the solvent is for dissolving a volatile solute. Such parameter is expected to be related to the dissolution energy for the compound X in the solvent S and to the energy of vaporization of compound X. For the characterization of solvent S, a set of trial solutes X can be selected followed by the measurement of the distribution constant $K_S(X)$ between the solvent and headspace. Experimentally, the distribution constants can be measured by adding a volume of test compound in a given volume of solvent S placed in a closed vial of a specific volume. A protocol for this procedure reported in the literature [15] used 5 μL mixture of ethanol, dioxane, and nitromethane in a chamber of 13.4 mL with 2 mL solvent S at 25°C. After equilibration, the concentration of each test compound X in the gas phase and in the liquid can be measured (e.g., by gas chromatography). The K_S value for each component in the mixture was calculated using the following formula:

$$K_S(X) = \frac{C_S(X)}{C_g(X)} \tag{7.1.8}$$

The selection of the three test compounds, ethanol, dioxane, and nitromethane was made on the assumption that they have different types of polar interactions with the molecules of tested solvent S. Dipole–dipole interactions are stronger with nitromethane, acidic–polar interactions are stronger with dioxane, and basic–polar interactions are stronger with ethanol.

The K_S values are further used in the calculation of a modified constant that is intended to eliminate the effect of the solvent molecular weight. The modification is done by the use of the solvent molar volume V_S (mL/mole) in the following expression:

$$K'_S(X) = K_S(X)V_S \tag{7.1.9}$$

The values K'_S are then used to calculate the coefficients K''_S, which are obtained with further correction of K'_S values with the intention to correct for nonpolar (dispersive) interactions. This is done with the following relation:

$$K''_S(X) = K'_S(X)/K'_\nu \tag{7.1.10}$$

where K'_ν is the estimated K'_S value, an n-alkane ($S = \nu$) whose molar volume is the same as that of the solute X. The values of K'_ν are calculated using the following expression:

$$\log K'_\nu = (V_X / 163)\log K_S(\text{octane}) \tag{7.1.11}$$

In Eq. 7.1.11, V_X is the molar volume of the solute (ethanol, dioxane, or nitromethane), K_S(octane) is the experimental distribution coefficient of n-octane in the evaluated solvent, and 163 is the molar volume of n-octane. The constants K''_S are further corrected to have zero value for n-hexane as a solvent. The resulting constants K''_S are used to measure the excess retention of a solute relative to an n-alkane of equivalent molar volume.

For any evaluated solvent S, an experimental polarity parameter (or chromatographic strength) P' is then defined by the following expression:

$$P'_S = \log K''_S(\text{ethanol}) + \log K''_S(\text{dioxane}) + \log K''_S(\text{nitromethane}) \quad (7.1.12)$$

Larger values for P'_S indicate a polar solvent (such as alcohol or water), and values close to zero show nonpolar solvents such as hexane, cyclohexane, etc. Solvent polarity can be used in selecting solvents in LC separations [16].

Parameter P'_S is not always sufficient for the characterization of solvent properties. The types of interactions that dominate solvent behavior can be quite different between solvents with the same P'. For example, a polar solvent and a solvent forming hydrogen bonds, although they may have identical P', may not act in the same manner toward different solutes. An additional separation parameter x_i was developed for solvent characterization, defined by the following formula:

$$x_i = \log\left[K''_S(i) / P'_S\right] \quad (7.1.13)$$

where i can be ethanol (x_e), dioxane (x_d), or nitromethane (x_n). Other solvents were also used for obtaining an x_i value, such as toluene (x_t) or methyl ethyl ketone (x_m) [2]. It can be assumed that the larger is x_i value for a specific compound, the higher is the similarity with the comparing solvent. However, the value of x_i also depends on P', and relatively large K''_S do not necessarily lead to large x_i values. For this reason, the values for x_i were used to group the solvents in nine main groups, the solvents in the same group having similar properties. These groups are (0) solvents with very low P' values (nonpolar), (1) aliphatic ethers, tetramethylguanidine and hexamethylphosphoric acid triamide, (2) aliphatic alcohols, (3) pyridine derivatives, tetrahydrofuran, amides, glycol ethers, and sulfoxides, (4) glycols, benzyl alcohol, acetic acid, and formamide, (5) methylene chloride and ethylene chloride, (6) tricresyl phosphate, aliphatic ketones and esters, and dioxane, (7) aromatic hydrocarbons, halo-substituted aromatic hydrocarbons, nitro compounds, and aromatic ethers, and (8) fluoroalkanols, *m*-cresol, water, and chloroform. Values for P' for some solvents classified in these groups are given in Appendix 7.1.2.

The parameters x_e for ethanol, x_d for dioxane, and x_n for nitromethane from Appendix 7.1.2 can be used to illustrate the separation of solvents in several groups, since in each group, the x_i values are close to each other. This can be illustrated in a triangular diagram. Since $x_e + x_d + x_n = 1$, their graphic representation can be done in a planar triangular diagram, and a representation in a tridimensional space is not necessary. The diagram is shown in Fig. 7.1.4. The compounds in each group are indicated by the group number. The x_e, x_d, and x_n values can be obtained from the graph.

As shown in the diagram given in Fig. 7.1.4, the solvents from the same group tend to cluster with x_e, x_d, and x_n values close to each other.

The polarity parameter P' is correlated, as expected, with Hildebrand solubility parameter δ. The correlation is positive and for a set of 34 compounds gives $R^2 = 0.7462$. This graph describing this correlation is shown in Fig. 7.1.5.

The relatively good correlation between δ and P' indicates that the two parameters provide basically similar information. The compounds with very low polarity P' such as carbon disulfide, cyclohexane, isooctane, and n-hexane that have $P' \approx 0$ do not correlate well with δ. The (negative) correlation between octanol/water partition coefficient (log K_{ow}) and polarity P' is not very good, as expected. The graph showing this correlation is given in Fig. 7.1.6 for 73 solvents.

Better guidance for selecting the appropriate solvent for elution or for dissolution of a

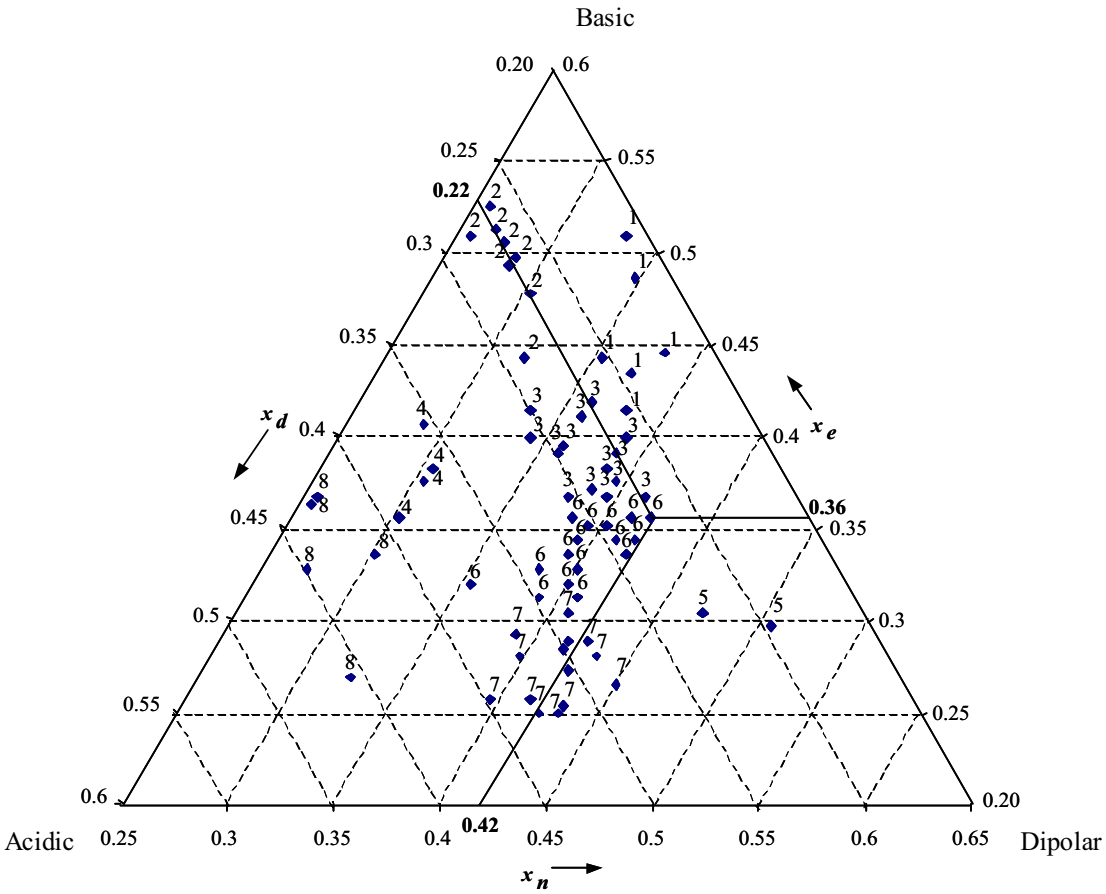


FIGURE 7.1.4 Triangular diagram showing groups (see numbers) of solvents clustered based on their x_e, x_d, and x_n values.

compound is obtained when using x_e, x_d, and x_n values for describing solvent similarities (see Appendix 7.1.2). The separation of solvent in classes based on these values indicates that specific types of interactions of analytes with solvent molecules, which are more prominent for a specific solvent than for another, are important in solvent characterization. These interactions include dispersion, dipole–dipole, hydrogen bonding, charge transfer, and ionic.

The utilization of polarity P' as well as of the parameters x_e, x_d, and x_n for solvent characterization is also basically only qualitative. It allows to place the solvent in a specific group of solvents and characterizes its polarity and type of polarity (basic–polar for ethanol, acidic–polar for dioxane, and dipole–dipole for nitromethane). Depending on the type of solvent desired, the selection should be made based on a higher or lower P', x_e, x_d, and x_n parameter. The strength of a solvent should be related to the type of stationary phase and the polarity of the analyte. For RP-HPLC, the solvents with similar properties as the analyte are typically stronger eluents as mobile phase than those that are dissimilar.

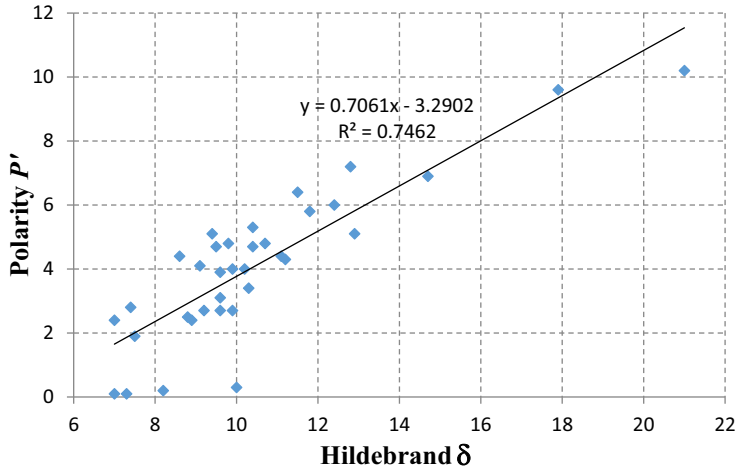


FIGURE 7.1.5 Correlation chart between polarity P' and Hildebrand solubility parameter δ.

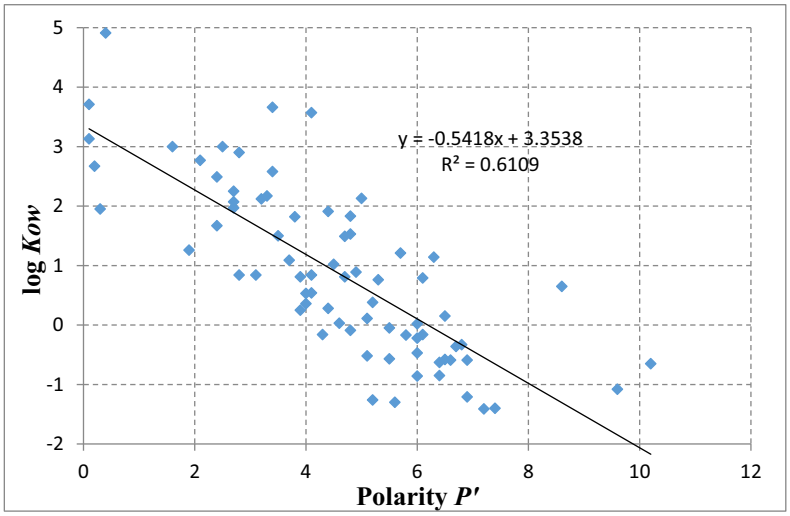


FIGURE 7.1.6 Correlation chart between octanol/water partition coefficient log K_{ow} and polarity P'.

Solvatochromic model and Kamlet–Taft parameters

Among other procedures for assessing the polarity of a solvent are the spectroscopic measurements. Such measurements can be based, for example, on the chemical shift in an NMR experiment or a change in the absorption spectrum in IR or UV-vis for a compound used as a "molecular probe." For example, solvents with a polar character can produce a bathochromic effect on the UV spectrum generated from a $\pi \rightarrow \pi^*$ transition in a compound. Other such effects under the influence of a solvent are known. The change in the position, intensity, or bandwidth that

occurs when a solute is transferred from the gas phase to a solvent is known as *solvatochromic* effect. One common scale to evaluate solvent polarity known as $E_T(30)$ scale is based on the variations in the maximum wavelength of absorption in visible of 2,6-diphenyl-4(2,4,6-triphenyl-N-pyridinium)phenolate (compound E_T-30). This test compound has a large solvatochromic effect, changing the absorption from 453 nm for water to 810 nm for diphenyl ether as a solvent (solutions are red in methanol and blue in acetonitrile). The polarity $E_T(30)$ is further calculated from the maximum of the wavelength absorbtion λ_{max} from the following expression [17]:

$$E_T(30) = \frac{28591}{\lambda_{max}(nm)} \tag{7.1.14}$$

From $E_T(30)$ values, a normalized parameter E_T^N can be obtained and is utilized for solvent characterization. This parameter is obtained from the following formula:

$$E_T^N = \frac{E_T(30) - 30.7}{32.4} \tag{7.1.15}$$

The normalized scale gives $E_T^N = 1$ for water and $E_T^N = 0$ for tetramethylsilane, which are the two extremes for $E_T(30)$ values. The $E_T(30)$ polarity has been reported for a considerable number of solvents [18,19]. The values for several common solvents are given in Appendix 7.1.3.

The effects involved in producing solvatochromic effects that are the base of $E_T(30)$ and E_T^N scales describe the polarity and polarizability but also the donor hydrogen bond formation ability of a solvent. Other compounds presenting solvatochromic effects accounting for other interactions are available. A set of parameters known as Kamlet–Taft solvatochromic parameters (π^*, α, and β) can be used for solvent characterization. A π^* scale was developed accounting for polarity and polarizability, which is not affected by hydrogen bonding or ion–dipole interactions based on the use of 4-ethyl-nitrobenzene as molecular probe. The $E_T(30)$ scale and the π^* scale are "single parameter" polarity scales and are based on the measurements of the property change of a single compound. Other such scales are known, some still based on the test of one compound (such as Nile Red), and others being "multiparameter" and based on the measurements on more than one compound as a probe. For example the solvent hydrogen-bond donor interactions can be described by an α scale developed on compounds such as several common dyes [20]. Hydrogen-bond acceptor interactions can be described by a β scale that was developed based on measurements on compounds such as 4-nitroaniline, N,N-diethyl-4-nitroaniline, 4-nitrophenol, and 4-nitroanisole [21]. Values for the parameters α, β, and π^* are available in the literature [22–24]. Some of these values for specific solvatochromic parameters α, β, and π^* are listed in Appendix 7.1.4.

As expected, solvatochromic parameter $E_T(30)$ is related to π^* and α, as well as to a polarizability correction parameter δ^* specific for different classes of solvents [24]. Solvatochromic parameter π^* has some correlation with the polarity P' obtained from liquid–gas partition, but $R^2 = 0.6475$. Also, π^* was found correlated with Hildebrand solubility parameter δ^2. However, the correlation remains acceptable only for compounds with low or medium polarity and is not applicable to polar compounds such as water or methanol. A dependence of $E_T(30)$ on other parameters has been verified to have the following form:

$$E_T(30) = 2.8591[10.60 + 5.12(\pi^* - 0.23\delta^*) + 5.78\alpha] \tag{7.1.16}$$

In Eq. 7.1.16, $\delta^* = 0$ for nonchlorinated aliphatic solvents, 0.5 for chlorinated aliphatic

compounds, 0.75 for water, and 1.0 for aromatic compounds. Several other correlations between solvatochromic parameters and the other solubility parameters were described [24]. For example, various relations were described between E_T^N and P', and also between π^*, P', and δ^* such as the following:

$$P' = 0.83 + 6.31(\pi^* - 0.30\,\delta^*) \qquad (7.1.17)$$

The values for α, β, and π^* were used in attempts to calculate retention factors in RP-HPLC, although the precision of the results was not very satisfactory [25]. Nevertheless, a number of studies were reported regarding the use of these parameters for solvent characterization [3,26].

Different from Hildebrand solubility parameter or octanol/water distribution constant, solvatochromic parameters can be measured directly for solvent mixtures [27]. These studies showed that the variations of E_T^N, α, β, and π^* are not linearly dependent with solvent composition ϕ for mixtures such as methanol/water or acetonitrile/water.

The values for α, β, and π^* were used in attempts to calculate retention factors in RP-HPLC, although the precision of the results was not satisfactory [3,25,28]. These parameters remain valuable as a guidance for selecting a solvent that displays stronger polarity and polarizability, stronger hydrogen-bond donor interactions or hydrogen-bond acceptor interactions.

Eluotropic strength

Besides solubility parameter δ, octanol/water log K_{ow}, and polarity P', some other parameters were developed for the characterization of the behavior of certain solvents, in particular related to HPLC applications. One such parameter is the eluotropic strength ε^0 [29]. This parameter has been developed in connection with the adsorption type equilibrium taking place in HPLC. Values for the eluotropic strength were available for stationary phases such as silica, alumina, fluorisil, and MgO. However, attempts were made to apply this parameter to reversed phase chromatography for which the partition type equilibrium is more applicable [30]. The descriptor "strength" of a solvent as a mobile phase component is frequently used in HPLC, but it does not refer to eluotropic strength ε^0.

Solvent characterization based on other parameters

Other methods for solvent characterization are reported in the literature (e.g., Ref. [31]). Considerable work has been invested in establishing relation between various properties of a solvent and of a solute for the calculation of a parameter *SP* describing the transfer of the solute from one solvent to another [5]. The desired descriptor *SP* can be, for example, log K_{ij} (where i and j are two different solvents such as octanol and water for K_{ow}) or log $k'(X)$. This descriptor can be defined by the following expression:

$$SP = c + eE + sS + aA + bB + vV \qquad (7.1.18)$$

In Eq. 7.1.18, the parameters c, e, s, a, b, and v characterize properties specific for the solvent(s) and E, S, A, B, and V for the solute. The value of c represents the value of the selected parameter for a reference compound. Each other pair is related to a specific type of interaction. For example, e and E are related to excess molar refraction which describes polarizability, s and S describe the dipolar effects, a describes hydrogen bond basicity of the solvent and A hydrogen bond acidity of the solute, b describes hydrogen bond acidity of the solvent and B hydrogen bond basicity of the solute, and v and V describe the molecular volume (McGowan calculated molecular volume [32]). Involving measurements

for different systems, lists of parameters were established. Parameters E, S, A, B, and V were established for numerous solutes based on results from LC separations, from liquid/liquid extraction, etc., using "training sets." To establish the values for the parameters c, e, s, a, b, and v, multiple linear regression has been utilized and the values for c, e, s, a, b, and v are reported for numerous solvent pairs [5]. As an example, for the water and an ideally dry solvent, the parameters c, e, s, a, b, and v are listed in Appendix 7.1.5. The values for various descriptors obtained with data from Appendix 7.1.5 refer to an ideal system where the solvent is dry and the water is pure. In reality, in extraction systems, the solvent is saturated with water and the water saturated with solvent. For solvents with low water solubility, the values from Appendix 7.1.5 correspond to a practical process, and for the other solvents, the parameters can be used only for solvent characterization (e.g., methanol and water are miscible and it is not possible to have a system dry methanol/water). Similar lists for other solvents and solutes are reported in the literature [5,33].

Solvent properties of liquid mixtures

In HPLC, it is very common that mixtures of liquids are used as mobile phases. Also, gradient separations with mobile phase composition changed during the chromatographic run are very common. It is therefore of considerable interest to estimate a specific parameter that characterizes solvent properties for a mixture of liquids. However, this is not always possible for parameters such as Hildebrand solubility parameter δ, which is based on an individual molecular property (vaporization energy) or K_{ow} that is specific for one compound.

For other parameters that can be truly defined for solvent mixtures, such as polarity or solvatochromic parameters, approximations can be directly obtained. For the polarity P' of a solvent, a rough first approximation of its value for a mixture of solvents is given by Eq. 7.1.19.

$$P'_{mix} = P'_1\phi_1 + P'_2\phi_2 + \ldots P'_n\phi_n \qquad (7.1.19)$$

where P'_1, P'_2, etc., are the polarities of individual solvents, and ϕ_1, ϕ_2, etc., are the volume fractions of each component of the mixture. However, Eq. 7.1.19 is only an approximation of polarity, and nonlinear variations of parameters with the composition are common for solvent mixtures.

For solvatochromic parameters α, β, π*, and E_T^N, the values for solvent mixtures can be obtained experimentally [27]. As an example, the variation of E_T^N for methanol/water and acetonitrile/water mixtures is shown in Fig. 7.1.7 [28]:

The variation of E_T^N with the solution composition for methanol/water mixture follows a quadratic dependence, while for acetonitrile/water mixture, the dependence is more complicated.

The variation of solvatochromic parameters π*, α, and β is again nonlinear for methanol/water mixtures, as shown in Fig. 7.1.8, and for acetonitrile/water, as shown in Fig. 7.1.9 [28].

The eluotropic strength ε^0 can also be defined for a mixture of two pure solvents A and B. This value can be obtained from the eluotropic strength of the pure solvents $\boldsymbol{\varepsilon}_A^0$ and $\boldsymbol{\varepsilon}_B^0$ [33].

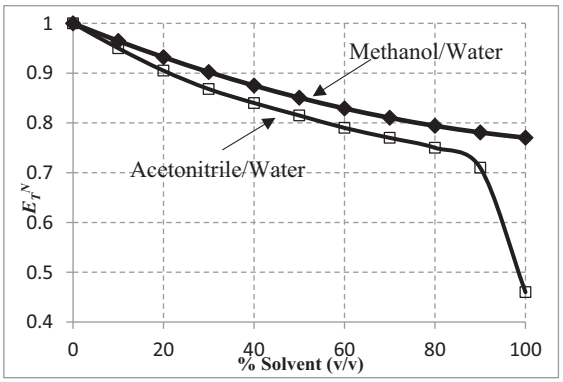


FIGURE 7.1.7 Variation of solvatochromic parameter E_T^N for methanol/water and acetonitrile/water mixtures.

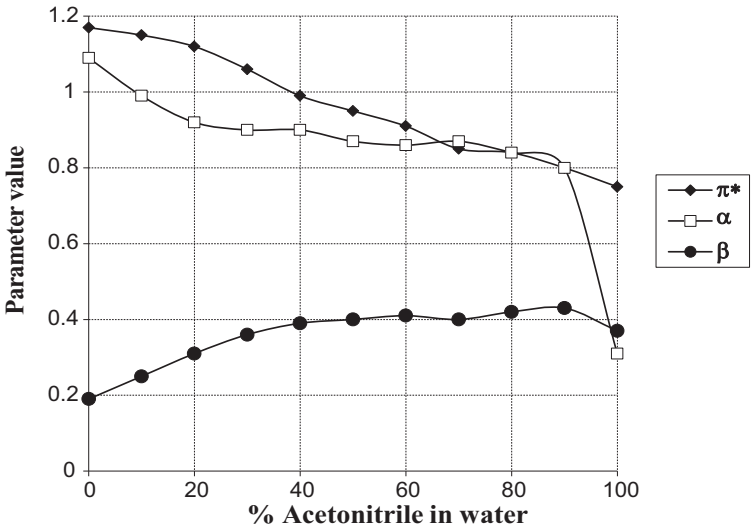


FIGURE 7.1.8 Variation of solvatochromic parameters π*, α, and β for acetonitrile/water.

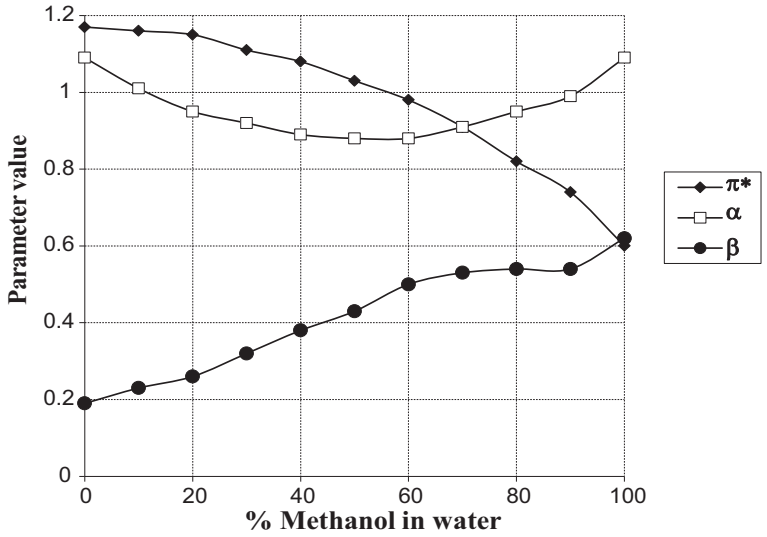


FIGURE 7.1.9 Variation of solvatochromic parameters π*, α, and β for methanol/water.

The main interest in the evaluation of specific parameters for the characterization of a solvent mixture is related to the prediction of the influence of composition changes on the analytes retention (typically characterized by log k'). A number of experimental results showed that a linear dependence exists between the mobile phase composition and log k' in RP-HPLC where

the equilibrium mechanism is mainly partition. This type of dependence is exemplified in Fig. 7.1.10, where the values for log k' are plotted versus the content in methanol in the mobile phase, for four compounds separated on a Purospher Star RP-18e column, 125 mm length, 4 mm inner diameter, and 5 μm particle size [34].

The trend lines for the data given in Fig. 7.1.10 show excellent linearity between log k' and volume fraction ϕ (%) values. For 4-chloro-4′-hydroxybenzophenone log $k' = 3.986 - 5.16\ 10^{-2}\ \phi$, $R^2 = 0.9991$, for 4-[(2-cyclopropyl-methoxy) ethyl]phenol log $k' = 3.051 - 4.21\ 10^{-2}\ \phi$, $R^2 = 0.9994$, for 4-(2-methoxyethyl)phenol log $k' = 1.886 - 3.12\ 10^{-2}\ \phi$, $R^2 = 0.9993$, and for phenol: log $k' = 1.484 - 2.52\ 10^{-2}\ \phi$, $R^2 = 0.9999$. The linearity between log k' and organic phase content (for RP-HPLC) is expressed by Eq. 3.5.3, written once more below [35,36]:

$$\log k'_{\phi}(X) = \log k'_{w}(X) - S(X)\phi \qquad (7.1.20)$$

where log k'_w (X) is the retention factor for the analyte X when separated with water as a mobile phase on a specific chromatographic column, and log k'_{ϕ} (X) is the retention factor for the same analyte in a mobile phase containing an organic modifier with the volume fraction ϕ. The values for k'_w (X) are not always measurable, and an extrapolation of the value can be obtained assuming relation Eq. 7.1.20 valid for $\phi \rightarrow 0$ starting with a certain mixture of water and a given solvent. Parameter S is assumed to be a constant specific for the given compound, given solvent, and given chromatographic column (a common approximation is $S \approx 0.25\ (M)^{1/2}$ where M is the molecular weight of the analyte). The calculated values of log k'_{ϕ} (X) depend on the stationary phase, the solvent A, and the nature of compound X. However, k'_w (X) is not obtained always the same from the extrapolation $\phi \rightarrow 0$ for different solvent/water mixtures. Although not necessarily very different, still different k'_w (X) can be obtained, for example, from a methanol/water mobile phase and from an acetonitrile/water mobile phase when $\phi \rightarrow 0$.

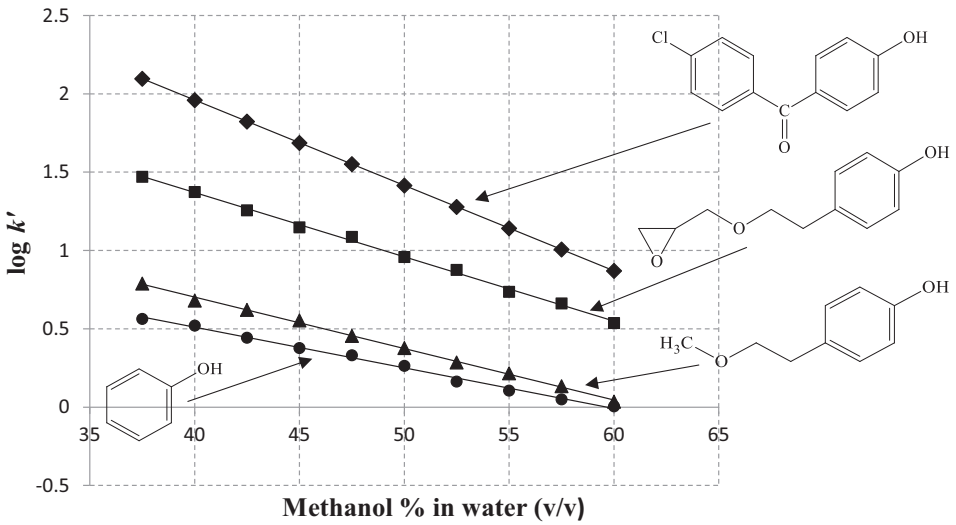


FIGURE 7.1.10 Variation of log k' with the composition of mobile phase methanol/water for four analyte, 4-chloro-4′-hydroxybenzophenone, 4-[(2-cyclopropylmethoxy)ethyl]phenol, 4-(2-methoxyethyl)phenol, and phenol (Purospher Star RP-18e, 125 mm length, 4 mm inner diameter, 5 μm particle size) [34].

For two volumetric fractions of the organic modifier ϕ_1 and ϕ_2, Eq. 7.1.20 leads to the following formula:

$$\log k'_{\phi_2}(X) = \log k'_{\phi_1}(X) - S(X)(\phi_2 - \phi_1) \tag{7.1.21}$$

Eq. 3.1.27 allows the calculation of $\log k'_{\phi_2}$ for the compound X in a solvent with the volume fraction ϕ_2 of organic component when the retention factor $\log k'_{\phi_1}$ is known for the mobile phase with the volume fraction ϕ_1. Variation of k' in gradient conditions is discussed in Section 7.7.

Graphs such as those shown in Fig. 7.1.10 can be used for the calculation of S and by extrapolation for the calculation of log k'_w (X). Several values for S for a water–methanol solvent mixture on different RP type columns are reported in the literature [37] and are given in Table 7.1.1.

The linear dependencies seen in Fig. 7.1.10 are not always followed, and different compounds and mobile phase systems may show large deviations from linearity [38]. For acetonitrile/water nonlinear variations of log k' as a function of ϕ are frequently obtained, which are better approximated with a quadratic equation [39]. Such nonlinear dependence is illustrated in Fig. 7.1.11 for the separation of chlorobenzene and 2,4-dinitrophenol on a Lichrospher 100 RP-10 column 250 × 4.0 mm with 5 µm particles with mobile phase acetonitrile/water at different compositions.

As shown in Fig. 7.1.11, the relation connecting the change in the log k' with the change in the composition of mobile phase is better expressed by the following formula:

$$\log k'_{\phi}(X) = \log k'_{w}(X) - S_1(X)\phi + S_2(X)\phi^2 \tag{7.1.22}$$

The coefficients $S_1(X)$ and $S_2(X)$ in Eq. 7.1.22 must be obtained experimentally similar to the case for the values for $S(X)$ for Eq. 7.1.20. Although the nonlinear dependences of log k' on solvent composition are not uncommon,

TABLE 7.1.1 Several values for S for different compounds X, reported in the literature for water/methanol mixtures [37] for RP-HPLC columns.

	Column				
Analyte	**Monomeric C18 (end-capped)**	**Monomeric C18 (not end-capped)**	**Polymeric C18 (end-capped)**	**Polymeric C18 (not end-capped)**	**Monomeric C8 (not end-capped)**
Phenol	2.21	2.97	2.52	2.35	3.13
Benzaldehyde	2.52	3.07	2.72	2.92	3.08
Acetophenone	2.82	3.63	3.04	3.08	3.39
Nitrobenzene	2.61	3.18	2.75	2.79	3.16
Methyl benzoate	3.17	3.82	3.46	3.44	3.78
Anisole	2.61	3.29	2.93	2.90	3.25
Fluorobenzene	2.70	3.27	3.07	2.90	3.28
Benzene	2.32	2.94	2.66	2.55	3.02
Toluene	2.9	3.52	3.23	3.13	3.56
Average	**2.65**	**3.29**	**2.94**	**2.90**	**3.29**

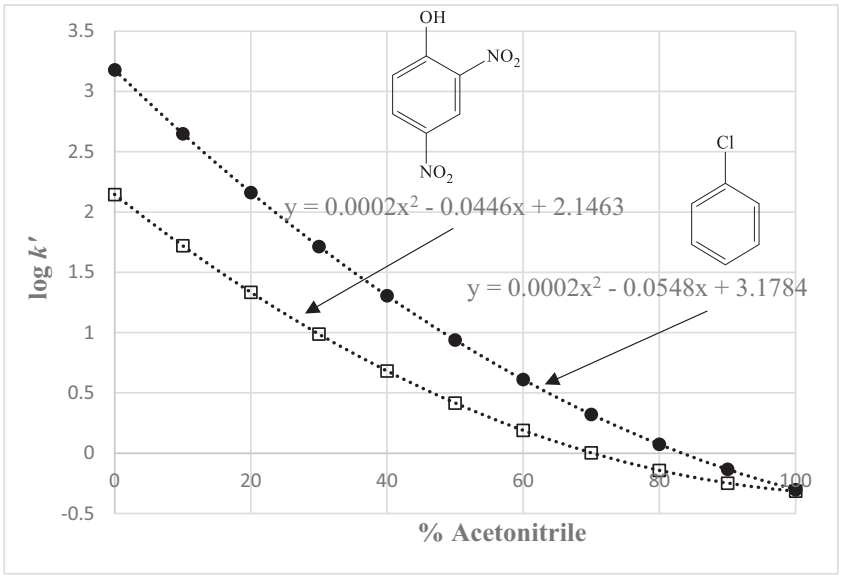


FIGURE 7.1.11 Variation of log k' with the composition of mobile phase acetonitrile/water for two analytes, chlorobenzene and 2,4-dinitrophenol.

linear relations are still frequently used to express the variation of the retention factor log k' as a function of organic content in the mobile phase ϕ. More complex expressions for log k'_ϕ of the form Eq. 7.1 20 and Eq. 7.1.22 are reported in the literature [40], and the lack of linearity is explained by different effects the most important being the contribution of adsorption type of equilibrium in addition to partition (see Sections 4.1 and 4.2).

For parameters defined based on a unique molecular property of a solvent, an approach to estimate their value for mixtures is to disregard the true meaning of parameters like δ or K_{ow} as to be unique to a compound. In this case, parameters δ or K_{ow} are assigned to a "hypothetical" liquid with identical property as the mixture.

The value for δ can be assigned to a "hypothetical" liquid with identical property as the mixture. The expression of δ for a mixture of solvents can be based on Eq. 7.1.20 that shows the variation of log k' for an RP-HPLC separation as a function of mobile phase composition ϕ. For a compound X, Eq. 7.1.20 for retention factor k' also holds for the equilibrium constant K_ϕ and can be written in the following form:

$$\log K_\phi(X) = \log K_w(X) - S(X)\phi \qquad (7.1.23)$$

Notations previously used include parameter $S(X)$ specific for a compound X, and the volumetric fraction of organic component ϕ. Equilibrium constant $K_w(X)$ corresponds to retention factor k'_w (X) in 100% water in the mobile phase. The use of Eq. 7.1.5 for K_ϕ (X) in Eq. 7.1.23 leads to the following relation for δ_{mo} in a solvent mixture versus δ_w in water:

$$(\delta_X - \delta_{mo})^2 = (\delta_X - \delta_w)^2 - S'(X)\phi \qquad (7.1.24)$$

where $S'(X)$ is given by the following formula:

$$S'(X) = \frac{2.303\ RT}{V_X} S(X) \qquad (7.1.25)$$

For relatively close values for δ_{mo} and δ_w, and assuming $2\delta_X\delta_{mo} \approx 2\delta_X\delta_w$, the following relation can be written:

$$\delta_{mo}^2 = \delta_w^2 - S'(X)\phi \tag{7.1.26}$$

From Eq. 7.1.26, the relation for Hildebrand parameters for two different mobile phases mo_1 and mo_2 with the volume fraction (for the same organic solvent) composition ϕ_1 and ϕ_2, respectively, can be obtained as follows:

$$\delta_{mo_1}^2 = \delta_{mo_2}^2 - S(X)(\phi_1 - \phi_2) \tag{7.1.27}$$

Regarding a hypothetical octanol/water parameter K_{ow}^{hyp} that can be utilized for the description of the apparent hydrophobicity of a solvent mixture X, such a value can be taken as being the weighed (by content) average of the K_{ow} values of the participating solvents. For example, for a liquid mixture containing a solvent X_1 with volume fraction $(1-\phi)$ plus another solvent X_2 at the volume fraction ϕ, and assuming a linear variation of $K_{ow}^{hyp}(X)$ for different volume fractions $(1-\phi)$ for X_1 and ϕ for X_2 the hypothetical $K_{ow}^{hyp}(X)$ can be defined by the following expression:

$$\log K_{ow}^{hyp}(X) = (1-\phi)\log K_{ow}(X_1) + \phi \log K_{ow}(X_2) \tag{7.1.28}$$

Based on Eq. 7.1.28 for a binary solvent, for example, water/acetonitrile, or water/methanol, the log $K_{ow}^{hyp}(X)$ can be estimated (water has log $K_{ow} = -1.38$, acetonitrile has log $K_{ow} = -0.34$, and methanol has log $K_{ow} = -0.77$).

7.2 Additional physical properties of liquids affecting separation

General comments

In addition to the elution power indicated as *strength*, a number of other physico-chemical properties of the liquids used as mobile phase play a role in the separation or indirectly in the values of parameters characterizing the separation. Among these properties are the following: (1) density ρ, (2) viscosity η, (3) diffusion coefficient D, (4) surface tension γ', (5) dielectric constant ε, (6) dipole moment m, (7) polarizability α, (8) solvent accessible surface area A^{SASA}, (9) hydrogen bonding capability, and (10) boiling point b.p. Some of these parameters were also presented in Chapter 6 related to the properties of the analytes, and considering the solvents just simply a chemical compound the same parameters can be used for their description.

Solvent density, viscosity, and diffusion coefficient

Density (volumetric mass density) of a substance is its mass q per volume V and it is given by the following formula:

$$\rho = \frac{q}{V} \tag{7.2.1}$$

In SI units, density is expressed as kg/m^3, but values 1000 times smaller expressed in g/mL are typically indicated. Density varies with temperature (density of water at 3.98°C is 1.000000 g/mL) and lists of density values are available in the literature for many compounds (e.g., Ref. [41]).

Viscosity of a fluid is a measure of its resistance to gradual deformation. Typically, the dynamic viscosity, which is usually of interest, is the measure of fluid resistance to shearing flow and it is measured in poise P (or centipoise cP). Viscosity of the mobile phase is an important parameter in HPLC separations, being related to the back pressure in the chromatographic column (see Eq. 3.1.76), as well as with the diffusion coefficient (see further Eq. 7.2.3). Also, experimental studies show that about twofold loss in theoretical plate number N of a column takes place for 2.5-fold increase in viscosity [42].

The ratio between dynamic viscosity and density of the fluid is known as kinematic viscosity.

Viscosity varies with temperature usually decreasing when temperature increases. Several models were developed for describing the variation of viscosity with the temperature. In Antoine equation the variation with temperature of viscosity is given by the following:

$$\eta(T) = a + \frac{b}{c - T} \qquad (7.2.2)$$

where T is temperature (in Kelvin deg) and η is viscosity (in cP). Parameters a, b, and c are empirical parameters for a specific liquid.

In Arrhenius model, the variation in the viscosity follows an equation similar to that for the rate constant:

$$\eta(T) = \eta_0 \exp(E / RT) \qquad (7.2.3)$$

In Eq. 7.2.3, η_0 and E are constants. As an example, the variation of viscosity of water (in cP) with inverse of the temperature (K) is given in Fig. 7.2.1. The fit for Eq. 7.2.3 is also shown in the figure.

Viscosity also varies for solvent mixtures depending on their composition. However, this variation is difficult to predict and depends on the interactions at the molecular level between the solvents. As an example, the variation of viscosity as a function of composition for water/methanol, water/tetrahydrofuran, and water/acetonitrile at 25°C is shown in Fig. 7.2.2.

The increase in viscosity of solvent mixture must be taken into consideration when working with gradient elution. The increase in viscosity during the gradient creates an increase in the column backpressure, and this may be a problem when working close to the upper limit of backpressure either acceptable for the pumps or for the column. As shown in Fig. 7.2.2, for water/methanol mixtures in particular, the viscosity becomes more than 1.5 times higher than that of water during a gradient reaching 40%–60% methanol. Data related to viscosity for a large number of solvents can be found in the literature [43]. The values of viscosity for several common solvents at 25°C are given in Appendix 7.2.1.

Another parameter with a role in HPLC separations is the diffusion coefficient. Different theories and also empirical relations were

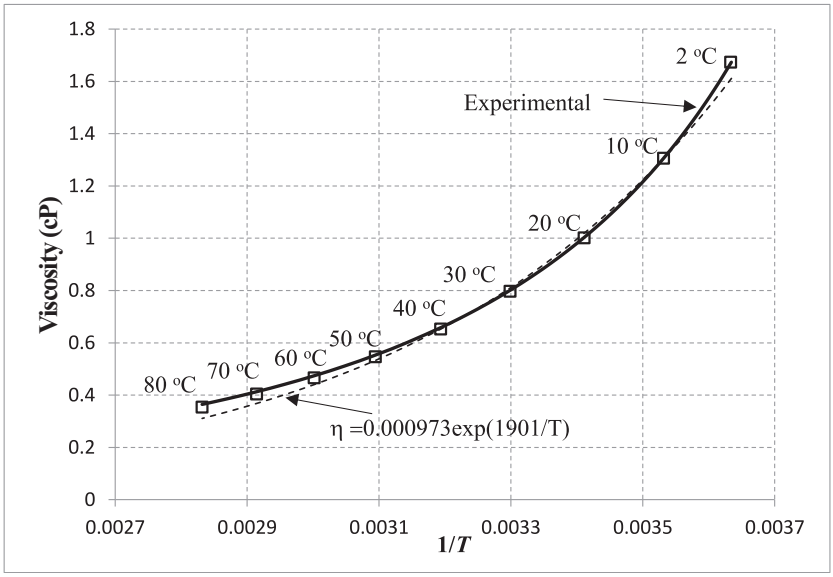


FIGURE 7.2.1 Variation of viscosity of water (in cP) with temperature.

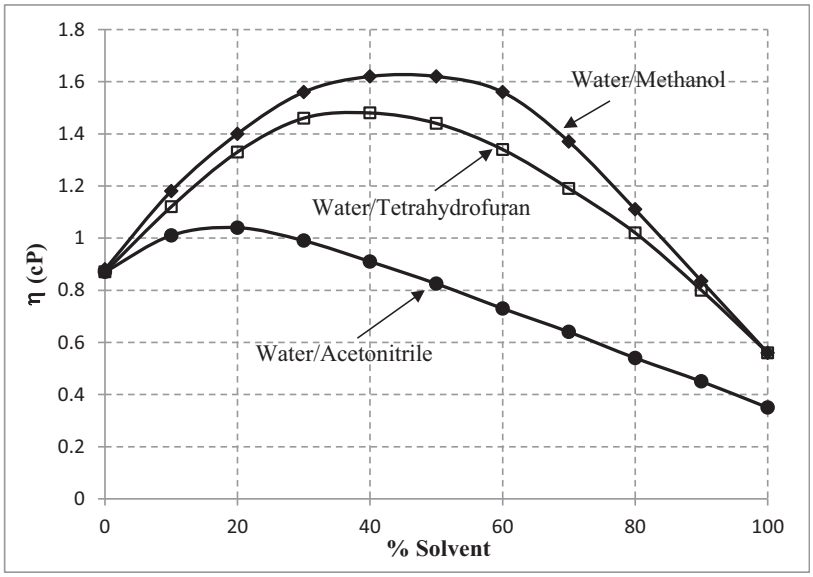


FIGURE 7.2.2 Variation of viscosity (in cP) for mixtures water/methanol, water/tetrahydrofuran, and water/acetonitrile as a function of composition, at 25°C.

developed for the estimation of diffusion coefficients. For example, Stokes theory empirically modified for better prediction gives the following formula for the diffusion coefficient for nonelectrolytes A in liquids B:

$$D_{A,B} = 7.4 \cdot 10^{-8} \frac{(\psi_B M_B)^{0.5} T}{\eta V_A^{0.6}} \qquad (7.2.4)$$

In Eq. 7.2.4, V_A is the molar volume of solute A in cm^3/mole (volume occupied by a mole of a substance and it is given by the expression: $V_A = M_A/\rho_A$), M_B is the molecular weight of solvent B, T is temperature in K, η is viscosity of the solution, and ψ_B is an association factor for the solvent (ψ_B is 1 for nonpolar solvents, 1,5 for ethanol, 1.9 for methanol, and 2.6 for water). The units for diffusion are cm^2/s and lists of diffusion coefficient are available in the literature (e.g., Ref. [44]). Some diffusion coefficients for a few solutes and liquids are given in Table 7.2.2.

TABLE 7.2.2 Some diffusion coefficients for liquids at 20°C.

Solute *A*	Solvent *B*	D_{AB} (cm^2/s)
CO_2	Water	$1.77\ 10^{-5}$
Ethanol	Water	$1.00\ 10^{-5}$
Phenol	Water	$0.84\ 10^{-5}$
Sucrose	Water	$0.45\ 10^{-5}$
NaCl	Water	$1.35\ 10^{-5}$
Phenol	Ethanol	$0.8\ 10^{-5}$
Phenol	Benzene	$1.54\ 10^{-5}$
Acetic acid	Benzene	$1.92\ 10^{-5}$

Surface tension

Surface tension is the tendency of liquid surfaces to shrink into the minimum surface area possible and it is measured in energy per unit surface (equivalent with force per unit length in

SI units dyne/cm). The importance of surface tension in HPLC separations is related to the energy in solvophobic interactions as indicated by Eq. 5.1.76 affecting the retention process in RP-HPLC. Because of the relatively high attraction of water molecules to each other through the hydrogen bonds, this solvent has a higher surface tension (72.8 mN/m at 20°C) than most other liquids.

Surface tension decreases linearly with the temperature. This dependence can be obtained from Eötvös rule, which can be written as follows:

$$\gamma'(T) = C_t V^{-2/3}(T_c - T) \tag{7.2.5}$$

where C_t is a constant, V is the molar volume, T_c is the critical temperature for the liquid, and T is the temperature of interest. From Eq. 7.2.5, the following expression of temperature dependence can be obtained:

$$\gamma'(T_2) = \gamma'(T_1) + C_t'(T_1 - T_2) \tag{7.2.6}$$

Eq. 7.2.6 (where C_t' is a temperature change coefficient) indicates that the increase in temperature ($T_2 > T_1$) leads to a decrease in surface tension. The values of γ' for several common solvents and the coefficient C_t' for the temperature dependence are given in Appendix 72.2.

The surface tension of solvent mixtures is difficult to predict. Several studies are reported in the literature providing results on γ' for different solvent mixtures [45,46]. As an example, the variation of surface tension with the organic phase concentration in water for several common solvents is shown in Fig. 7.2.3.

Changes in dynamic surface tension due to the presence of the analyte in the mobile phase found application in a detection technique indicated as DSTS [47].

Besides experimental values, the surface tension of liquid mixtures can also be estimated using various formulas based on thermodynamic principles [48,49]. Several empirical expressions were also suggested for the estimation of γ''.

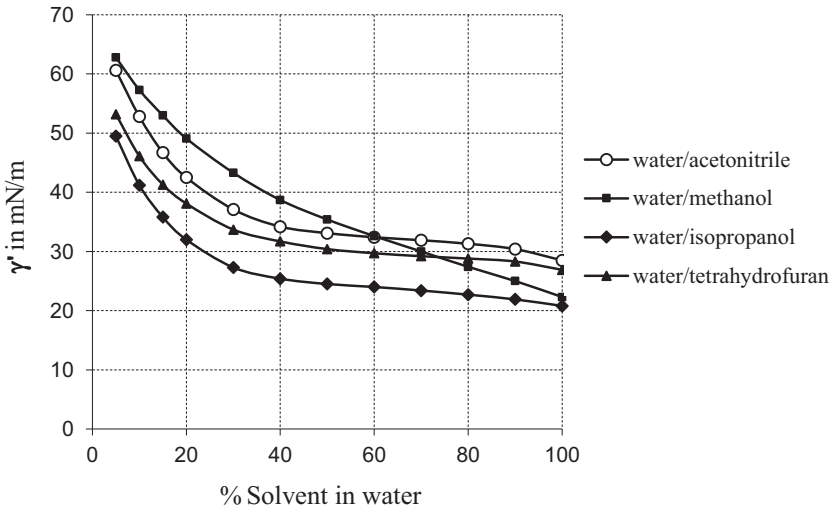


FIGURE 7.2.3 Variation of surface tension γ' (in mN/m = dyne/cm) with the organic solvent concentration in water for several common solvents used in HPLC.

For example, for water/acetonitrile mixtures, the estimation of γ' can be done using Eq. 7.2.7, and for the mixtures for water/methanol, the estimation can be done using Eq. 7.2.8.

$$\gamma' = 40.898 \exp\left(-C\%_{org} / 16.518\right) + 30.496 \tag{7.2.7}$$

$$\gamma' = 49.042 \exp\left(-C\%_{org} / 39.842\right) + 20.355 \tag{7.2.8}$$

In these formulas, $C\%_{org}$ is the % volume of organic component in the mobile phase (the results are in mN/m = dyne/cm).

Dielectric constant, dipole moment, and polarizability

Several solvents properties influence the interactions taking place in solutions and implicitly influence the elution properties of a mobile phase made with these solvents. Among these properties are the dielectric constant ε of the solvent, the dipole moment μ, and the polarizability α. Dielectric constant is the ratio of the permittivity of a substance to the permittivity of vacuum (permittivity is the measure of resistance that is encountered when forming an electric field in a medium). Dielectric constant is dimensionless (vacuum permittivity is $\varepsilon_0 = 8.854 \cdot 10^{-12}$ $CV^{-1}m^{-1}$). Dielectric constant of the solvent affects interactions in solution that involve ions and polar molecules, decreasing the intermolecular energy when the dielectric constant increases. Polar compounds are usually more soluble in solvents with higher dielectric constant. Also it affects molecular interactions (as indicated in Section 5.1), and in RP-HPLC, solvents with higher ε are usually weaker eluents. Dielectric constant also depends on temperature, typically decreasing as the temperature increases. Some values of the dielectric constant for several common solvents at 25°C are given in Appendix 7.2.3.

Dipole moment of a molecule has been discussed in Section 5.1, where it was also given its definition (see Eq. 5.1.12). Dipole moment of solvent molecules can be used for the characterization of a solvent polarity. The types of interactions for a number of HPLC types of separation including HILIC and to a lower extent RP are of polar type. For this reason, the dipole moment m is a parameter useful in solvent characterization. Dipole moment varies as a function of temperature, and for some compounds, it is different for the molecules in gas form and in liquid form. For molecular interactions used as a model in gas phase for the understanding of the separation process, the values of m in gas phase can be used. However, the values for the dipole moment in the liquid form seem more appropriate for solvent properties characterization. Values for the dipole moment (in debye, D) of several common liquids used in HPLC as mobile phase (or mobile phase additives) are listed in Appendix 7.2.3.

Polarizability was also defined in Section 5.1 (see Eq. 5.1.29). The dispersion energy between molecules has the largest contribution to the total interaction energy between nonionic molecules, being higher than dipole–dipole or dipole–induced dipole interactions. The intensity of dispersion interactions is dependent of molecular polarizability as shown by Eq. 5.1.44. Polarizability also affects the interaction of polar molecules, as shown by Eq. 5.1.42. As indicated in Section 5.1, the polarizability can be defined as a tensor when the electrical field generates moments of dipole in different directions from that of the field $\vec{\mathbb{E}}$. However, average molecular polarizability is the common value of interest [50]. The polarizabilities for several common solvents (expressed in $4\pi\varepsilon_0(\text{Å})^3$) are listed in Appendix 7.2.3. Experimentally, it has been proved that in binary water/acetonitrile

mixtures the salt polarizability depends on the molar fraction of acetonitrile by a nonlinear function. The two ions of the salts have different behaviors: the polarizability of cation is less affected by the organic content than the polarizability of anion [51].

Hydrogen bonding of solvent molecules

Hydrogen bonding was previously presented in Section 5.1 and the energy of this type of bonds was compared with other types of interactions in Table 5.1.1. The energy (enthalpy) of hydrogen bonds varies between 5 and 40 kJ/mol (see Section 5.1), being stronger than van der Waals interactions. It was shown that hydrogen bonding is based on electrostatic attraction between a hydrogen atom bound to a highly electronegative atom and another nearby electronegative atom. Hydrogen bonding is known, for example, to be formed to oxygen, nitrogen, or fluorine interacting with molecules that contain electronegative atoms (e.g., O, N, etc.). The molecules capable of forming hydrogen bonds contain donor, acceptor, or both types of groups for hydrogen bonding. Hydrogen bonding may be intermolecular but also intramolecular between different parts of the same molecule. For solvents that have small molecules, hydrogen bonding is typically intermolecular. The enthalpy of hydrogen bonding depends on the nature of the atoms to which the hydrogen in bound and also of the acceptor atom (e.g., O—H···N with about 29 kJ/mol, O—H···O with about 21 kJ/mol, N—H···N with about 13 kJ/mol, N—H···O with about 8 kJ/mol, etc.), but also on the specific molecules that interact, and of temperature. The enthalpy of hydrogen bonding can also be different for the same type of molecules in liquid state or gas state. Hydrogen bonding influences boiling point and viscosity of a solvent. The hydrogen bonds energy for liquid water (at 0°C) is estimated to about 23 kJ/mol, and it is lower in alcohols and even lower in amines. The enthalpy of hydrogen bonding in solvents plays an important role in their "elution strength," in specific types of chromatography. As already indicated, the water having strong intermolecular interactions is a weak eluent for RP-HPLC and a strong eluent for HILIC. For RP-HPLC, alcohols are stronger eluents than water, and they are weaker eluents in HILIC. This character is partly generated by the strength of the hydrogen bond enthalpy in each solvent [52—54].

Solvent boiling point

Solvent boiling point is not a frequent parameter of interest in HPLC separations. However, HPLC performed with the chromatographic column set at a higher temperature than ambient is not uncommon. The temperature set for the chromatographic process influences the separation and can be used to the advantage of a more convenient separation [55]. Among the advantages provided by elevated temperature are the increased speed of separation, the decrease of mobile phase viscosity, improved efficiency, changes in selectivity with the temperature [56,57], and the possibility to use only water as a mobile phase [58,59]. The boiling point of various solvents should be known in case the HPLC is performed at a higher than ambient temperature. Some boiling point values for several common solvents are given in Appendix 7.2.4.

7.3 Properties of liquids affecting HPLC detection

General comments

Besides the importance in the separation process, the mobile phase properties also play an important role related to the analyte detection in HPLC analysis. The mobile phase must differ

from eluted molecules by certain physico-chemical properties in order to be detected. These properties can be UV-absorption, fluorescence, molecular fragmentation in a mass spectrometer, refractive index (RI), or others that make the analytes detectable. For this reason, some physico-chemical properties of the mobile phase must be known, and the detection procedure must be selected such that the solvent (mobile phase) should not interfere with the detection. Several physico-chemical properties of the mobile phase relevant in the detection of analytes in HPLC are further discussed.

Refractive index

RI detection is commonly used for the analysis of compounds that do not have good absorption bands for UV light (see Section 1.3). For such compounds, other detection techniques can be used as well, such as MS or ELSD. However, RI is a convenient detection procedure since it offers good reproducibility and does not require expensive equipment. The sensitivity of the RI detection depends on the difference in the RI of the mobile phase and that of the analyte (besides other parameters related to the instrument construction). A list of values for the RI for a number of common solvents is given in Appendix 7.3.1. The RI depends on the wavelength of the incident beam, and the most accurate RI measurements are done with monochromatic light (usually 589 nm, the sodium D line), although white light is still commonly used for the measurements. The RI depends on temperature and typically decreases as temperature increases (for organic solvents, this decrease is about 0.0005 for 1°C and for water is about 0.0001). When RI is used as a detector in HPLC, only isocratic separations can be applied. Premixed solvents are used as mobile phase, as well. For RI detection, it is not recommended to generate the specific composition for the mobile phase by using two pumps and a mixing device. Small fluctuations in the RI of the mobile phase typically generate large oscillations in the RI detector response [60]. The RI of a binary solvent mixture is typically given by the following expression:

$$\frac{n_{mix}^2-1}{n_{mix}^2+2}=\phi\,\frac{n_1^2-1}{n_1^2+2}+(1-\phi)\frac{n_2^2-1}{n_2^2+2} \qquad (7.3.1)$$

In Eq. 7.3.1, n_1 and n_2 are the RIs of the individual solvents, n_{mix} is the RI of the mixture, and ϕ is the volume fractions of solvent 1.

UV cut-off

The measurement of the UV absorption of the analytes is probably the most frequent detection technique used in HPLC. The influence of the solvent on this type of measurement has two aspects. One is the requirement of the solvent to be "transparent" (to absorb very little light) in the region where the absorption of the analyte is measured. This "transparency" is characterized by the UV cut-off value, defined as the wavelength at which the absorbance A of the solvent versus air, in a 1 cm cell, is equal to unity (see Eq. 2.1.10). The UV absorption typically increases at wavelength approaching 200–210 nm, and the cut-off value indicates that at lower wavelength the absorption of light is too strong to allow the utilization of the solvent as mobile phase. The UV cut-off values for several common solvents are given in Appendix 7.3.1. A more accurate description of solvent absorption is obtained from the UV spectrum of the solvent. The low cut-off value of a solvent to be used as mobile phase is very important for the use of UV detection for compounds that do not absorb at higher wavelength values but have absorption in the range 205–215 nm such as many organic acids that have no other chromophores in the molecule except the COOH group.

A contribution to the cut-off value for a solvent may come not only from the solvent itself,

but also from certain impurities or additives that may be present in the solvent (such as BHT or phthalates). The potential UV absorption of the additives used as buffers should also be considered when choosing the mobile phase appropriate for detection. The addition of a common additive such as HCOOH practiced for enhancing ionization when the HPLC uses MS detection may influence, for example, the UV detection. When the same method is used with UV detection only or when both UV and MS detection are used in the method, the influence of HCOOH addition must be considered. The absorption UV-Vis spectrum in the wavelength range 190–420 nm of a solution 0.1% HCOOH in water for a 1 cm path length cell is shown in Fig. 7.3.1. The UV cut-off of several common additives and buffers is given in Appendix 7.3.2.

The second aspect regarding the solvent influence on the UV detection in HPLC is related to the possible modification of the absorption bands of the analyte. This effect is not seen for some analytes, but it is present for other analytes. For example, the solvents with a polar character may produce a bathochromic (higher wavelength) effect on the UV spectrum of the analytes with the absorption bands corresponding to a $\pi \rightarrow \pi^*$ transition. For such compounds, the maximum of the absorption band can increase with 1–20 nm when changing, for example, from hexane to ethanol as solvent. Compounds that form H-bonds with the solvent molecule will also exhibit a bathochromic effect. The increase of the accepting capacity in H-bond of the solvent will increase the maximum wavelength in the absorption spectrum of the analyte. On the other hand, the bands corresponding to $n \rightarrow \pi^*$ transitions may suffer a hypsochromic effect when the polarity of the solvent increases. The $n \rightarrow \pi^*$ transitions can also be influenced by pH changes due to the structural modification of the analyte. For example, a bathochromic shift can be seen for compounds with phenolic hydroxyls when the pH is modified from acid to basic values due to the modification in the compound dissociation status. For aromatic amines, the effect is hypsocromic when pH is modified from basic to acidic values (due to protonation). Besides the change of the maximum absorbance wavelength, the change in the value of the absorbance coefficient ε_λ is seen in certain solvents. Such changes may affect the selection of the optimum wavelength for detection.

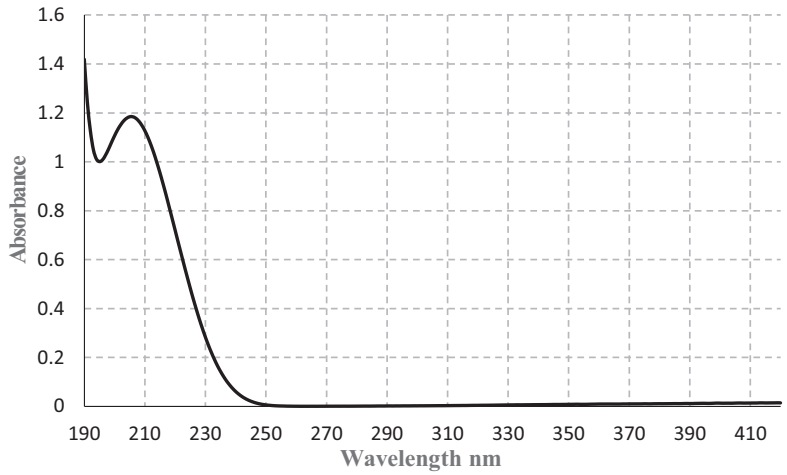


FIGURE 7.3.1 UV-Vis absorption spectrum for HCOOH (0.1% solution in water in 1 cm cell).

Fluorescence

The fluorescence is also influenced by the solvent composition. Both emission wavelength and fluorescence intensity for many compounds containing dissociable functional groups are dependent on pH. The fluorescence intensity can be influenced with as much as one order of magnitude and may shift the emission maximum when strong interactions with solvent occur. A shift of the emission band toward higher wavelengths is observed with the increase of the dielectric constant of the solvent. When the solvent absorbs radiation at the emission or absorption wavelengths, a significant decrease of detection sensitivity can be noticed. The presence of impurities in mobile phase, mainly the O_2, can produce a quenching of the fluorescence signal, mainly when the analyte concentration is close to the detection limit.

Solvent influence in MS detection

Solvent (mobile phase) selection for MS detection (electrospray ionization ESI and atmospheric pressure chemical ionization APCI) is very important since the ionization efficiency is strongly influenced by the solvent [61]. This is translated to different peak intensities at different solvent compositions, consequently the solvent composition affecting quantitation. Also, different analytes have different ionization characteristics, and compounds with basic character (even very weak basic character) are typically analyzed in positive mode, while compounds with acidic character (even very weak acidic character) are typically analyzed in negative mode (see Section 2.3). The formation of ions in ESI and in APCI depends on gas-phase basicity (GPB) or gas-phase acidity (GPA) of the analyte, but also of the mobile phase molecules. The competing of solvent in the process of ion formation is not desirable, but on the other hand, the formation of analyte ions by proton transfer from the ionized solvent can be an important process.

The MS sensitivity depends on various parameters such as the type of detection (ESI or APCI), the nature of analytes, the nature and proportion of the solvents used in the mobile phase, the additives from the mobile phase (including those influencing the pH), and the instrumental conditions applied to the MS. For a specific analyte, different combinations of these parameters may lead to different results, such that the optimization process of MS sensitivity cannot be obtained by a simple change of one parameter at a time. For this reason, the selection of the mobile phase with the purpose of obtaining good sensitivity in MS detection is a complex problem. A detailed discussion of the subject is beyond the purpose of the present book. For this reason, only general aspects of the problem are further discussed.

Several characteristics of the mobile phase influence ionization of the analytes. Among these are volatility, surface tension, viscosity, conductivity, ionic strength, dielectric constant, electrolyte concentration, pH, and the potential of gas-phase ion−molecule reactions. These properties must be considered in relation to the chemical and physical properties of the analyte, including pK_a, hydrophobicity, surface activity, ion solvation energy, and GPB or GPA, depending on the use of positive or negative ionization mode. Also the operational parameters of the instrument such as the flow rate and temperature of mobile phase are important. An arbitrary selection of the mobile-phase composition in LC-ESI/MS is not possible since only polar solvents and volatile additives can be used for obtaining good sensitivity. This requirement limits the use of additives present in the mobile phase to a few acids, bases, and salts. These additives may be necessary either for achieving the separation in the HPLC column or for enhancing ionization in the MS source or for both. Among the additives frequently used in LC-ESI/MS are

HCOOH, CH_3COOH, CF_3COOH, salts such as $HCOONH_4$, CH_3COONH_4, $(NH_4)_2CO_3$, or basic compounds such as ammonia or volatile amines. It is not possible to use in LC-MS additives/buffers containing H_3PO_4, H_2SO_4, or their salts with ions such as NH_4^+, Na^+, K^+, etc. Such additives produce small nonvolatile particles in the ion source, affecting the MS detection.

Regarding the solvents from the mobile phase, the following ones are compatible with both APCI and ESI interfaces: water, alcohols (as long as viscosity does not increase too much), acetonitrile, tetrahydrofuran, acetone, dimethylformamide, and to a lesser extent (basically only for APCI) dicholoromethane and chloroform. For positive ionization mode, the addition to the mobile phase of a volatile acid, usually HCOOH at 0.01%−0.2% levels, is common, while for negative ionization mode the addition of a volatile salt such as $HCOONH_4$, CH_3COONH_4, and $(NH_4)_2CO_3$ at 40−50 mM level is typically practiced. Addition of a low level of HCOOH can also be used for negative ionization mode. An organic solvent in mixture with water is common as a mobile phase. Generally, the use of only aprotic solvents is not suitable with the ESI interface. Pure water is also a poorer solvent for ESI than water mixed with organic solvents such as methanol and acetonitrile, even if the level of the organic component is as low as 5%. Due to pure water's relative high viscosity, the electrophoretic mobility of ions is lower, leading to inefficient charge separation and difficulties in producing a stable spray. Also, the evaporation of water from the charged droplet is slower than the evaporation of an organic solvent.

For many separations, it is possible to achieve relatively similar results either with the mobile phase water/acetonitrile or water/methanol (see Section 9.5). A replacement of acetonitrile with methanol or of methanol with acetonitrile may influence not only the separation, but also the ionization efficiency in MS detection. Acetonitrile is an aprotic solvent, while methanol is a protic solvent. By comparing, for example, the solvatochromic parameters, α, that describes the hydrogen-bond donor capability of the solvents, the values are $\alpha = 0.19$ for acetonitrile and $\alpha = 0.93$ for methanol (see Appendix 7.1.4) indicating significant difference. Regarding the set of parameter described by Eq. 7.1.18 [5], the parameters a related to basicity are $a = -1.566$ for acetonitrile and $a = 0.08$ for methanol, again showing significant difference (see Appendix 7.1.5). These differences may influence the formation of ions in the MS source.

Unlike ESI, which requires mobile phases based on polar or medium polar solvents, in APCI both polar and nonpolar solvents can be used. For this reason, APCI can be chosen as interface in NP-LC or in convergence chromatography (CC) with MS detection, while ESI is not recommended for this type of HPLC. The solvents commonly used in NP-LC/MS-APCI include n-hexane, 2-propanol, methanol, ethanol, iso-hexane, iso-octane, tetrahydrofuran, chloroform, ethoxynonafluorobutane, with additives such as formic acid, acetic acid, trifluoroacetic acid, or ammonia, diethylamine, triethylamine, and dimethylethylamine (depending on positive or negative ionization mode). The possible suppression effect of strongly acidic or basic additives depends on the analytes, but in general it must be avoided. In APCI, it is recommended, when possible, to replace acetonitrile with methanol in order to enhance detection sensitivity. This can be explained by the stronger basicity character of acetonitrile compared to that of methanol, which competes with target analytes for protonation. Additionally, acetonitrile tends to polymerize in APCI plasma, coating the corona needle with an insulating layer after several hours in operation. Dimethylformamide content in mobile phase is recommended to be lower than 10% when using API electrospray, and a high signal background can be noticed when using this solvent with the APCI interface. Chlorinated

hydrocarbons can enhance the ionization yield only for the APCI interface.

The formation of multiple molecular ions, especially due to the formation of sodium ion adducts, is commonly observed in electrospray mass spectrometry and may make it difficult to obtain good reproducibility and sensitive quantitation. In negative ionization mode, alkylamine additives could improve detection by suppression of multiple molecular ions through preferential formation of a predominant alkylamine adduct ion [62].

Solvent properties related to other detection techniques

Several detection techniques which depend on an evaporative process (evaporative light scattering detector (ELSD), charged aerosol detector (CAD), condensation nucleation light scattering detection (CNLSD), etc.) also require a totally volatile mobile phase [63]. In general, the easier it is to evaporate the mobile phases, the higher is the sensitivity for those detectors. However, the detection process in ELSD and mainly in cCAD is more complex than simple evaporation, and the sensitivity of the detection may depend on a number of additional factors [64].

7.4 Buffers and additives

General comments

Retention in HPLC of compounds that have acidic, basic, or amphoteric character is highly dependent on pH. This is mainly caused by the change in the compound structure due to the pH changes (see Section 6.1). It is common that within 2 pH units, the structure of a compound is changed by 98%, for example, from a completely neutral state into an ionized form. The change in the molecular structure of a molecule in solution as a result of solution pH has been previously discussed in Section 4.5, and the modification of log D_{ow} (log K_{ow}) as a result of pH modification was discussed in Section 6.1 (see, e.g., Fig. 6.1.4). The variation of log D_{ow} is associated with a different retention, for example, in RP-HPLC. The ionization status of a compound can be even more important regarding its retention in a polar (HILIC) or in an ion exchange separation than it is in RP-HPLC. The change in the retention of a compound at different pH values of the mobile phase can be an undesirable effect, and then effort will be made to maintain a constant pH during the separation. In many cases, however, the pH change of the mobile phase can be used to the advantage of achieving a specific separation. The pH of the mobile phase is achieved by adding buffers and/or additives.

For isocratic separations, the pH of the mobile phase can be adjusted before using it, when the mobile phase is prepared. However, some specific compositions of the mobile phase, even if maintained constant, can be generated by mixing two different solutions. From the starting pH of the two solutions, the resulting mixture has the desired pH value. The variation of the pH of a mobile phase during a gradient separation can be achieved by different procedures. For RP-HPLC, for example, one procedure is the modification of the ratio of two solutions, one being an aqueous (or partially aqueous) buffer solution A and the other an organic (or partially organic) solution B that does not contain buffers or additives. It is common in such cases to control only the pH of the aqueous phase (buffer A) and not the other one. The resulting pH of the mobile phase is frequently unknown and in gradient conditions may vary during the separation. To avoid such change, it is necessary to have the same pH for both solutions making the gradient. Another procedure to obtain a mobile phase with the desired pH consists of the modification of the ratio of two totally or partially aqueous solutions A and B, each one buffered at a different pH. These

solutions with different pH may have the same or different contents of the organic phase. Gradients with different pH and different organic contents can also be achieved by mixing more than two solutions. However, three or four solution gradients, although sometimes utilized, are not common.

Besides buffers, the pH of the mobile phase can be adjusted by simply adding a base or an acid. The compounds used in such additions can be classified as additives. Besides additives used for pH modification, other compounds can be added to the mobile phase and they do not affect directly the pH. However, they are necessary for other purposes either related to separation or to other aspects of the HPLC analysis. The addition of salts, for example, may modify the ionic strength of the mobile phase and through this procedure the separation. Addition of salts in the mobile phase in RP-HPLC, for example, may improve the peak shape in the chromatograms. However, the salt concentration (such as phosphates of Na^+ or K^+) must be done considering the limited solubility of these salts in organic solvents. In the case of MS detection, nonvolatile salts cannot be used in the mobile phase. In case of ion pair, hydrophobic interaction, or displacement chromatography, the additives are part of the separation process, and such additives are discussed in association with each specific technique. Some aspects related to additives are also discussed in this section.

Buffer pH

A solution of a weak acid *HA* and its conjugate base A^-, or a solution of a weak base *B* and its conjugated acid BH^+, has the capability to show resistance to pH changes upon the addition of a (small amount of a) strong acid or base. These types of solutions are known as buffers. The weak acids and bases involved in buffer preparation can be monoprotic or polyprotic and can be inorganic or organic. In water solutions, strong acids and strong bases are completely dissociated and the concentration of H^+ (or OH^-) is practically equal to the concentration of the acid (or base). Weak acids and weak bases are only partially dissociated, and for a weak acid, for example, the H^+ concentration is given by Eq. 4.5.7 that shows the pH varying much less with the acid concentration. Based on this concept, the addition of a small amount of a strong acid to a buffer solution will not change the pH in accordance to the change in strong acid concentration, but in accordance to a weak acid addition. This process can be followed quantitatively considering that for a mixture of a weak acid *HA* and its salt (e.g., as Na^+ salt) the mass balance for the solution requires that

$$C_{HA} + C_{NaA} = [HA] + [A^-] \tag{7.4.1}$$

In Eq. 7.4.1, C_{HA} and C_{NaA} are the analytical molar concentrations and $[HA]$ and $[A^-]$ are the molar concentrations in the solution upon dissociation and after equilibrium is established. On the other hand, the electrical neutrality (for a monoprotic acid) of the solution requires that

$$[Na^+] + [H^+] = [A^-] + [OH^-] \tag{7.4.2}$$

Since the salt is assumed to be completely dissociated, $[Na^+] = C_{\mathrm{NaA}}$ and Eq. 7.4.2 gives the following:

$$[A^-] = C_{NaA} + [H^+] - [OH^-] \tag{7.4.3}$$

From Eq. 7.4.1 and Eq. 7.4.3, it is easy to obtain the following expression for $[HA]$:

$$[HA] = C_{HA} - [H^+] + [OH^-] \tag{7.4.4}$$

Since the molar concentration of an acid and its conjugate base in practical applications is typically much larger than the difference $[H^+]$ - $[OH^-]$, it is common to use for Eq. 7.4.3 and Eq. 7.4.4 the simplifications:

$$[A^-] = C_{NaA}$$

and

$$[HA] = C_{HA} \tag{7.4.5}$$

With these simplifications, the expression for the dissociation constant of an acid can be written in the following form:

$$K_a = \frac{[H^+][A^-]}{[HA]} = [H^+]\frac{C_{NaA}}{C_{HA}} \tag{7.4.6}$$

Eq. 7.4.6 gives the pH value for a buffer made as a mixture of a weak acid and its salt and can be written in the following form:

$$pH = pK_a + \log\frac{C_{NaA}}{C_{HA}} \tag{7.4.7}$$

Eq. 7.4.7 is known as Henderson–Hasselbach equation. For a weak base in the presence of its salt, a relation similar to Eq. 7.4.7 can be written as follows:

$$pH = 14 - pK_b - \log\frac{C_{BHX}}{C_B} \tag{7.4.8}$$

Different from the pH of a strong acid that is completely dissociated and has $\mathrm{pH} = -\log C_{HA}$, or a strong base that has $\mathrm{pH} = 14 + \log C_B$, the buffer solution changes only a little upon the addition of a small amount of a strong acid or base. Also, Eq. 7.4.7 (and Eq. 7.4.8) indicates that the pH of the buffer does not depend on the concentration of the two buffer components (C_{NaA} and C_{HA}) but only on their ratio. Therefore, the buffer pH is not affected (within a certain range) by dilution. Formulas similar to Eq. 7.4.7 or Eq. 7.4.8 can be developed for polyprotic acids and bases.

Buffer capacity

The variation of the pH of a buffer solution when a strong acid or a strong base is added is described by *buffer capacity*. Several definitions of this property are known. Buffer capacity β can be defined as the number of moles of a strong acid or base that causes 1.00 L of buffer to change the pH by one unit. Another definition that describes buffer capacity even for small changes is given by the following formula:

$$\beta = \frac{\mathrm{d}n}{\mathrm{dpH}} \tag{7.4.9}$$

where n is the number of equivalents (moles for a monoprotic base or acid) of a strong base or acid added to the buffer in the infinitesimal amount $\mathrm{d}n$ to change the pH by d(pH). It can be assumed that the addition of a base (e.g., NaOH) leads to the increase of C_{NaA} and $\mathrm{d}n = \mathrm{d}C_{\mathrm{NaA}}$. (or the addition of an acid leads to the decrease of C_{NaA}). With the notation $C_{buf} = [HA] + [A^-]$ and the value for $[HA] = C_{HA}$ obtained from Eq. 7.4.6, the value for C_{buf} can be written as follows:

$$C_{buf} = \frac{[H^+][A^-]}{K_a} + [A^-] \tag{7.4.10}$$

Eq. 7.4.10 can be rearranged to give the concentration of $[A^-]$ as a function of C_{buf}, $[H^+]$, and K_a. From $\mathrm{d}n = \mathrm{d}C_{\mathrm{NaA}}$ where C_{NaA} is given by Eq. 7.4.3, and by replacing in C_{NaA} the value for $[A^-]$ from Eq. 7.4.10, it can be concluded that

$$\beta = \frac{\mathrm{d}C_{NaA}}{\mathrm{dpH}} = \frac{\mathrm{d}\left(\frac{K_w}{[H^+]} - [H^+] + \frac{C_{buf}K_a}{K_a + [H^+]}\right)}{\mathrm{d}[H^+]}\frac{\mathrm{d}[H^+]}{\mathrm{dpH}} \tag{7.4.11}$$

The calculation of the derivatives leads to the following formula:

$$\beta = 2.303\left(\frac{K_w}{[H^+]} + [H^+] + \frac{C_{buf}\,K_a[H^+]}{(K_a + [H^+])^2}\right) \tag{7.4.12}$$

Eq. 7.4.12 indicates that the buffer capacity β has a relatively complicated dependence on pH and acid dissociation constant K_a, and shows

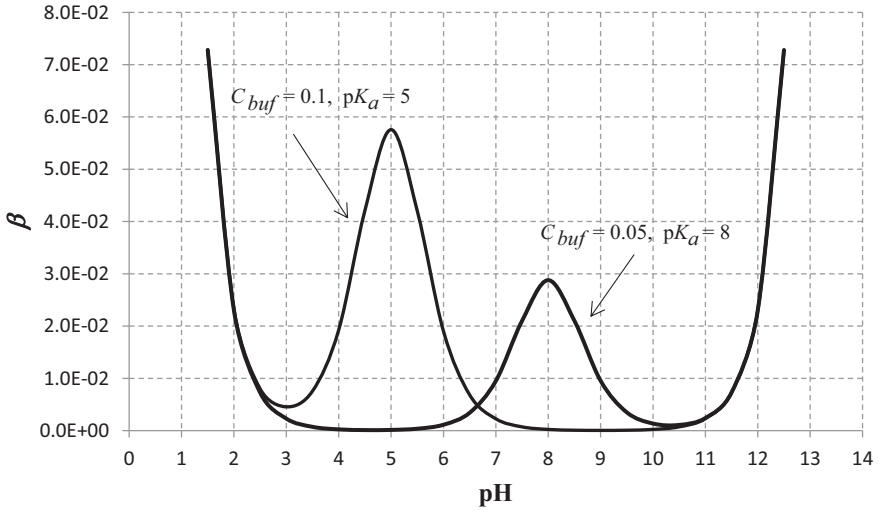


FIGURE 7.4.1 Variation of β with the solution pH and concentration.

that a higher buffer concentration C_{buf} is associated with a higher buffer capacity. Fig. 7.4.1 shows the variation of buffer capacity β with the pK_a and with the buffer concentration C_{buff}.

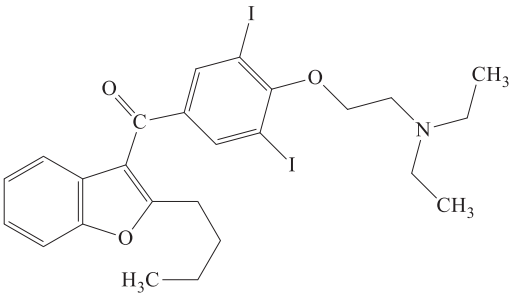
From Eq. 7.4.9 (and since $dn = dC_{NaA}$), the following formula can be obtained:

$$\mathrm{dpH} = \frac{dC_{NaA}}{\beta} \qquad (7.4.13)$$

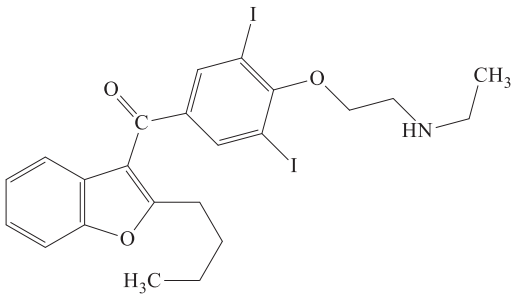
Eq. 7.4.13 indicates that a higher buffer capacity β is associated with a smaller variation in pH for an addition of a strong base or strong acid that affects the concentration of *NaA*. For concentrated solution of an acid or base when a small addition of acid or base is made, the pH does not change and this explains the large β values at the extreme pH values. For a middle pH range where the buffer is utilized, β has a local maximum centered at the pK_a value of the weak acid making the buffer, and the maximum is higher when the concentration of the buffer C_{buf} is higher.

Buffers are useful for several purposes such as (1) to maintain a specific media for the separation process, (2) to keep constant the protonation/deprotonation equilibria involving the analyte molecules, and (3) to maintain a constant pH value of the mobile phase even when injecting samples of different pH values. One example showing the effect of a buffer proportion in the mobile phase is given below for a separation of antiarrhythmic drugs amiodarone and desethylamiodarone (formulas given in Fig. 7.4.2).

The separation was done on a Purospher RP-18e (125 mm × 4 mm x 5 μm) in isocratic conditions and several chromatograms for different buffers proportion in the mobile phases are shown in Fig. 7.4.3. The mobile phases were selected at different proportions of an aqueous buffer with pH = 3 (obtained from 0.1% H_3PO_4 and trimethylamine) and acetonitrile. The flow rate for the separation was 1 mL/min, detection at 240 nm, and injection volume 1 μL of standards in methanol with 250 μg/mL of each compound. The acidic pH has the role of changing neutral molecules that have a high hydrophobic character (log $K_{ow} = 7.64$ for amiodarone and log $K_{ow} = 6.90$ desethylamiodarone) to molecules with lower hydrophobicity (log $D_{ow} = 4.14$ for amiodarone at pH = 3 and log $D_{ow} = 3.65$ for desethylamiodarone at pH = 3) and therefore with lower retention times on the RP-HPLC column.



amiodarone log K_{ow} = 7.64



desethylamiodarone log K_{ow} = 6.90

FIGURE 7.4.2 Formulas for amiodarone and desethylamiodarone.

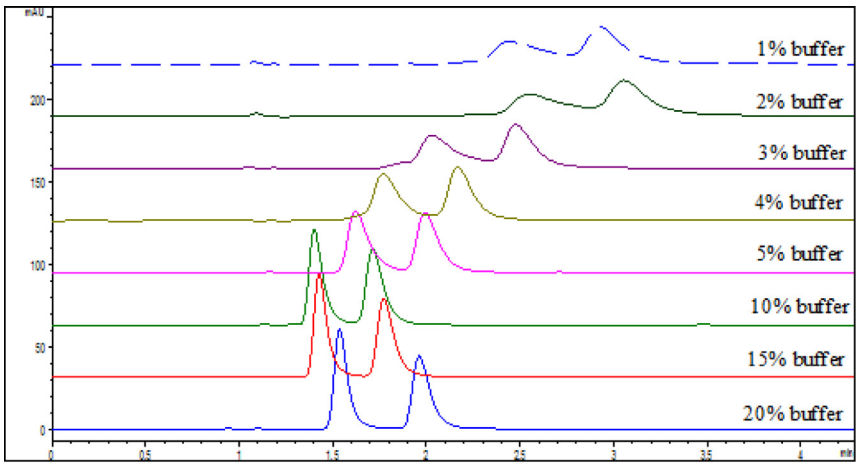


FIGURE 7.4.3 A set of chromatograms for the separation of amiodarone and desethylamiodarone at different buffer content in the mobile phase [65].

Initial decrease in buffer content (between 20% and 10% buffer content) and increase in acetonitrile leads to a decrease in the retention time, as expected for a "stronger" eluant. However, further decrease in buffer content at lower proportion (at 5% or less v/v) reduces the capacity of buffering and protonating the injected molecules, such that their retention time increases although the content of acetonitrile from the mobile phase increases.

Common buffers used in HPLC

The pH of a buffer solution is selected to maintain a desired pH value for the mobile phase. However, the stability of the stationary phase, which is frequently limited to the pH range 2–8, is also imposing the utilization of buffers in the range 2–8, although buffers in the range 1–12.9 are reported in the literature (e.g., HCl + glycine for pH as low as 1.04 and

NaOH + glycine for pH as high as 12.97) [66]. The pH range of utilization is related to the pK_a value for the weak acid or base used in the buffer. From Eq. 7.4.7, it can be seen that for equal concentrations of the weak acid and of its salt, the pH of the buffer solution is $pH = pK_a$. Also, the maximum buffer capacity occurs for $pH = pK_a$, and for this reason, the pK_a of the common weak acids and bases used for buffers preparation is of interest. Such values of pK_a are given in Appendix 7.4.1. The same appendix gives a list of some common buffers used in HPLC and their pH working range.

One common problem related to buffers is their stability in time. Some buffers are stable, but the stability of the pH of other buffer solutions is not always good. This is, for example, the case of buffers that contain NH_4OH as a base. Due to the volatility of ammonia, this can be eliminated in time from the solution, and the pH of such buffers may decrease (significantly). Ammonia can also be eliminated from a buffer solution during the solvent sparging (if this is performed). In such situations, the pH of the buffer is not the same at different times during its utilization, which can produce significant variations in the separation. The pH of buffers containing NH_4OH, for example, can vary as much as one pH unit in several days. Fresh buffers must be prepared at set time intervals in order to avoid this problem. Besides the variation in the pH, the stability of buffers in time may also be related to the growth of microorganisms. Some buffers may act as media for microorganism growth, and the same solution of a buffer has only a limited utilization time. This growth can be delayed by the addition of small amounts of NaN_3 and/or by refrigerating the buffer solutions during storage. The growth of microorganisms may slightly modify the buffer pH, but the main problem is related to the clogging of the filters (frits) present along the path of the mobile phase, even when the mobile phase was initially free of particles.

One important aspect when using buffers in HPLC is the buffer concentration. Buffer concentration in HPLC is usually made with the salt concentration of 50 mM or lower, and buffers of 10 mM concentration are common. However, addition of larger concentrations of acids or bases to achieve the desired pH is sometimes utilized [67]. The buffer concentration has an effect on the separation, on column stability, and also on the detector response [68]. In the description of HPLC methods that use buffers, in addition to the buffer pH, the concentration must be indicated.

Buffers in partially aqueous solvent mixtures

Mobile phases in several types of HPLC usually consist of an aqueous component and an organic phase component. When a specific pH is desired for the mobile phase, it is a common practice to make only the aqueous component with a desired pH, while the organic component is typically used without a buffer addition. Adding a buffer that sometimes contain of an inorganic salt to the organic component is not possible due to solubility problems. For additives such as an organic acid (e.g., HCOOH or CH_3COOH in mobile phases used with MS detection), it is common to have them added to both aqueous phase and organic phase, when no solubility problem is encountered. The change in buffer pH and concentration always must be considered when the mobile phase composition changes in a gradient separation.

When the buffer is added only to the aqueous component of the mobile phase, the final pH of the mobile phase is modified from the nominal value of the pH of the buffer, although a dilution of the buffer (C_{buf}) affects only the buffer capacity but not the pH (as indicated by Eq. 7.4.7). The cause of this change is that the buffer pH calculated for an aqueous solution is not the same as

the pH in a solution that is partially composed of water and an organic miscible solvent (such as methanol, ethanol, acetonitrile, etc.). For water, the dissociation constant (ionic product) K_w is given by $K_w = [H^+][OH^-] = 10^{-14}$ at 25°C (more correctly $K_w = a_H + a_{OH^-}$). Neutral is defined as the state at which $[H^+]$ equals $[OH^-]$, which occurs when $[H^+] = 10^{-7}$ equivalent with a pH of 7. For methanol, for example, the autoprotolysis constant is $K_{CH3OH} = [H^+][CH_3O^-] = 10^{-16.6}$. In methanol, neutral pH should be taken when $[H^+]$ equals $[CH_3O^-]$, which occurs when $[H^+] = 10^{-8.3}$ or a pH of 8.3. In conclusion, methanol–water mixtures have autoprotolysis constants $K_{H2O/CH3OH}$ between 10^{-14} (water) and $10^{-16.6}$ (methanol), and the neutral in these mixtures ranges from pH = 7 to pH = 8.3. In aqueous basic solutions, the anion is OH^-, and in basic solutions that contain high concentrations of methanol, the anion will be a mixture of OH^- and CH_3O^- [69].

A common procedure for determining the pH of aqueous/organic solutions is to directly measure it after mixing the aqueous buffer and the organic modifier. For a pH measured in a 100% aqueous solution with the electrode calibrated with aqueous standard buffers, the pH is accurate (and can be indicated as ${}^{w}_{w}$pH). For the pH measured in an organic/aqueous mixture with the electrode calibrated with aqueous buffers, a value indicated as ${}^{s}_{w}$pH is obtained (the upper index indicates the nature of the measured solution and the lower index for the calibration solution for the electrode). However, this is not a correct value. A correct value would be obtained only if the pH is measured with an electrode calibrated with buffers prepared in the same solvent as the one used for the mobile phase. In such case, a ${}^{s}_{s}$pH value would be obtained. This measurement requires the knowledge of the pH value of the reference buffers prepared at different partial aqueous compositions, which is not usually available [70]. A correction can be obtained for ${}^{s}_{w}$pH since between the values ${}^{s}_{s}$pH and ${}^{s}_{w}$pH there is a difference given by a term δ that is constant for each mobile phase composition. The following relation can be used for ${}^{s}_{s}$pH calculation:

$$ {}^{s}_{s}\mathrm{pH} = {}^{s}_{w}\mathrm{pH} - \delta \tag{7.4.14} $$

The parameter δ for methanol–water–based mobile phases can be estimated from the solvent composition (volumetric organic phase content ϕ expressed by the formula $\phi = \frac{V_{organic}}{V_{organic} + V_{water}}$) with the following empirical equation [71]:

$$ \delta = \frac{0.09\phi_{MeOH} - 0.11\phi^2_{MeOH}}{1 - 3.15\phi_{MeOH} + 3.51\phi^2_{MeOH} - 1.35\phi^3_{MeOH}} \tag{7.4.15} $$

The parameter δ for acetonitrile–water–based mobile phase is the following:

$$ \delta = \frac{-0.446\phi^2_{AcCN}}{1 - 1.316\phi_{AcCN} + 0.433\phi^2_{AcCN}} \tag{7.4.16} $$

The variation of δ for methanol and acetonitrile is shown in Fig. 7.4.4.

The organic content ϕ in a mobile phase also influences the dissociation of acids and bases. For example, methoxide ion is a more potent nucleophile than hydroxide, such that basic compounds in methanol–water mixtures can show different chemical behavior than in water alone, even when the hydrogen ion activity is the same. This fact is relevant to column stability and sample stability in basic methanol–water mixtures. When the content of the organic solvent in solution increases, the dielectric constant and the activity coefficients decrease. In the presence of the organic component from mobile phase, the acidic/basic properties of solutes are modified in different proportions, depending on solute

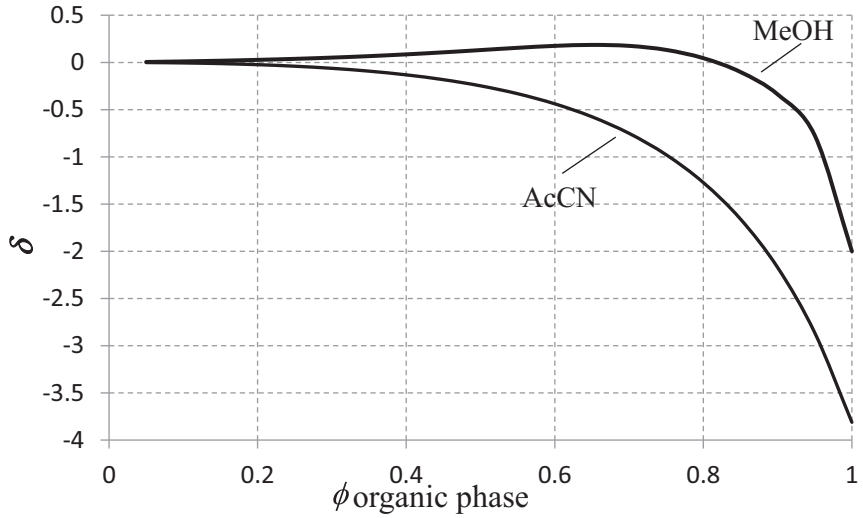


FIGURE 7.4.4 Variation of pH correction δ for methanol and acetonitrile at different volume fractions ϕ of the organic solvent.

structure and the nature of the organic modifier. A linear relationship between $pK_a(w)$ of a solute in pure water and $pK_a(s)$ of the same solute in a solvent s, which may contain water and another solvent, can be written as the following empirical relation:

$$pK_a(s) = \alpha_s\, pK_a(w) + \beta_s \tag{7.4.17}$$

In Eq. 7.4.17, the intercept β_s is related to the differences in basic character, dielectric constants, and specific solvation interactions (e.g., hydrogen bonding) of the solute and the solvent s and water, respectively. The slope α_s is related to the differences between specific solvation interactions, which depend on the solvent and solute. Thus, for an organic modifier, the values of α_s and β_s are calculated for sets of compounds according to the following equations dependent on volume fraction of the solvent (ϕ_s) in the mobile phase composition:

$$\alpha_s = \frac{1 + \alpha_1\phi_s + \alpha_2\phi_s^2}{1 + \alpha_3\phi_s + \alpha_4\phi_s^2} \tag{7.4.18}$$

and

$$\beta_s = \frac{1 + \beta_1\phi_s + \beta_2\phi_s^2}{1 + \beta_3\phi_s + \beta_4\phi_s^2} \tag{7.4.19}$$

where α_1, α_2, α_3, α_4, β_1, β_2, β_3, and β_4 are parameters obtained by numerical best fit techniques for all acids or bases from the same family. For example, the values of the best fit parameters for several classes of compounds and a mobile phase consisting of water–acetonitrile are given in Table 7.4.1 [72].

Besides dependences as given by Eq. 7.4.17, different other approximations were proposed to estimate the $pK_a(s)$ values in water/organic solvent mixtures. Simpler formulas such as linear or quadratic dependencies were suggested for this purpose, with expressions dependent on $pK_a(w)$ and volume fraction of the solvent (ϕ_s) as given below:

$$pK_a(s) = A' + B'\phi_s \tag{7.4.20}$$

$$pK_a(s) = A' + B'\phi_s + C'\phi_s^2 \tag{7.4.21}$$

TABLE 7.4.1 The fitting parameters for predicting the slope (α_s) and the intercept (β_s) of the correlation between $pK_a(w)$ and $pK_a(s)$, as given in Eq. 7.4.17 [72].

Compounds	α_1	α_2	α_3	α_4	β_1	β_2	β_3	β_4
Aliphatic carboxylic acids	9.97	−8.59	8.83	−8.72	−0.68	9.94	8.45	−8.59
Aromatic carboxylic acids	−2.42	3.14	−1.98	2.12	9.97	−9.12	5.96	−6.90
Phenols	10.05	−10.04	7.97	−8.37	−5.33	9.95	0.19	−0.70
Amines	−0.73	−0.27	−0.87	−0.12	−1.82	2.25	−1.75	0.90
Pyridines	−1.67	0.67	−1.66	0.67	−1.78	1.89	−0.58	−0.40

where $A' \approx pK_a(w)$ and where B' and C' are empirical parameters dependent on a specific compound for which pK_a(s) is needed [73]. Examples of the values of these parameters are given in Table 7.4.2 for methanol/water solutions, as a function of methanol content.

Other such estimations for $pK_a(s)$ based on $pK_a(w)$ and ϕ are reported in the literature [72,74].

Besides pH, the addition of an organic modifier also affects the buffer capacity β. Organic solvents have the effect of decreasing buffer capacity and also of shifting the pH where β has a maximum. For methanol, for example, this shift is toward higher pH values [69].

The influence of temperature on the pH of buffers

The pH of a buffer is also influenced by temperature. The first pH variation is related to the ionic product of water that depends on temperature and $pK_w = 13.99$ is only at 25°C. At 0°C $pK_w = 14.95$ and at 75°C $pK_w = 12.70$ (with a nonlinear variation). This variation indicates that the neutral pH = 7 is only at 25°C. The dissociation of weak acids and bases also depends on temperature, such that pK_a typically listed for 25°C is not the same at a different temperature. As a result, depending on the nature of

TABLE 7.4.2 Polynomial equations describing the dependences of $pK_a(s)$ of some buffers currently used in RP-LC as a function of the MeOH content in mobile phase.

pK_a	Equation in solvent/water	R^2
pK_1 (H_3PO_4)	$pK(s) = 2.127 + 2.16 \cdot 10^{-2}\phi_{MeOH} + 6.81 \cdot 10^{-5}\phi^2_{MeOH}$	0.9982
pK_2 ($H_2PO_4^-$)	$pK(s) = 7.202 + 1.27 \cdot 10^{-2}\phi_{MeOH} + 2.14 \cdot 10^{-4}\phi^2_{MeOH}$	0.9997
pK_1 (citric acid)	$pK(s) = 3.121 + 1.56 \cdot 10^{-2}\phi_{MeOH} + 6.47 \cdot 10^{-5}\phi^2_{MeOH}$	0.9992
pK_2 (citric acid)	$pK(s) = 4.756 + 1.62 \cdot 10^{-2}\phi_{MeOH} + 9.33 \cdot 10^{-5}\phi^2_{MeOH}$	0.9986
pK_3 (citric acid)	$pK(s) = 6.391 + 2.15 \cdot 10^{-2}\phi_{MeOH} + 7.65 \cdot 10^{-5}\phi^2_{MeOH}$	0.9996
pK (acetic acid)	$pK(s) = 4.757 + 1.26 \cdot 10^{-2}\phi_{MeOH} + 1.09 \cdot 10^{-4}\phi^2_{MeOH}$	0.9998
pK (NH_4^+)	$pK(s) = 9.238 - 5.87 \cdot 10^{-3}\phi_{MeOH} - 2.22 \cdot 10^{-5}\phi^2_{MeOH}$	0.9991

the buffer, the temperature change may influence the pH differently.

Another aspect is related to the measurement of pH using a glass electrode. As indicated by Eq. 2.1.15, the electrode potential used for pH measurement is temperature dependent. The result is a combination of effects and a detailed description of pH variation with temperature is not usually practical. However, it is common that the pH of buffer solution decreases as the temperature increases. For example, the variation of pH with temperature for a tris (tris(hydroxymethyl)aminomethane)/HCl buffer with a concentration of 50 mM in the range 5 to 35°C decreases with about 0.81 pH units. For other buffer solutions such that of a standard solution of 0.050 M of pure potassium hydrogen phthalate in water, the pH slightly increases in this range with about 0.05 units [75]. Additional complexity of pH variation with temperature appears when the mobile phase is partially organic. In such cases, only experimental measurements performed considering the influence of temperature and of organic composition on both the solution and the measuring electrode (typically glass electrode) must be considered [76]. This variation with temperature of buffer pH is not usually indicated in HPLC analytical procedures with the goal of measuring specific analytes. In most HPLC separations, the pH for the buffer solutions is indicated for room temperature (25°C). Since the pH varies with the temperature, this effect must be considered when performing a separation at a different temperature than the one where the buffer pH was measured, and any adverse effect of temperature increase must be avoided.

Solubility of buffers in partially organic mobile phases

The choice of a mobile phase composition (nature and concentrations) containing buffers must take into consideration the solubility of the buffer in the presence of the organic modifier. Binary mobile phases that are the most common in HPLC are frequently made having one aqueous or mainly aqueous component and an organic component.

It is common that the buffer is made only in the aqueous or mainly aqueous component of the mobile phase. For aqueous component, the buffer is made in water. For making the partially aqueous components, there are two alternatives: (a) the buffer is made in water and the organic component is added afterward and (b) the mainly aqueous component is made by mixing water with an organic solvent, and the buffer components are subsequently added. Since the pH of a partially aqueous solution is improperly measured with a common glass electrode, it is preferable to use alternative (a) with a precise pH measurement, although changes in this pH occur when the organic component is added. Nevertheless, both procedures are sometimes described for different HPLC methods. Further pH change may occur during a gradient separation, when more organic component is added to the mobile phase.

Since buffers are made using acids, bases, and salts, and these are sometimes inorganic compounds, their solubility in the organic or partially organic phase can be low. This solubility depends on the nature of the buffer, its concentration, and the nature and percentage of the organic modifier in mobile phase composition. For inorganic salts, the solubility depends mainly on the nature of the cation, and their solubility trend in partial organic solvents follows approximately the solubility in water: $NH_4^+ > K^+ > Na^+$. The alkylammonium cation has a higher solubility in organic solvents, such as methanol or acetonitrile, due to affinity of the alkyl chain toward these solvents. As an example, several solubility values for potassium phosphate type buffers in solvents that are commonly used in HPLC are given in Appendix 7.4.2 [77]. As a result of lower solubility of many

buffers in organic solvents, the buffer concentration should be selected at the lowest acceptable concentration (although this leads to a decrease in buffer capacity). Particular attention must be given to buffer solubility when used in gradient separations and the organic content of the mobile phase is increased during the chromatographic run (e.g., in RP-HPLC). A higher content of organic component is usually associated with a decrease in buffer solubility, and the buffer concentration must be adjusted appropriately to avoid any precipitation of salts in the chromatographic system.

Additives

A variety of compounds can be added to the mobile phase for modifying its properties. In some cases, the solubility of the additives is good in the mobile phase, but since some additives are inorganic salts, their solubility in aqueous/organic phases is limited and should be carefully monitored when the organic content increases in the mobile phase during a gradient separation. As an example, the solubility of K_2HPO_4 in water/organic solvent is indicated in Appendix 7.4.5.

The additives have various functions and they can be classified in several groups: (1) additives used for the modification of the pH of mobile phase, (2) additives for the modification of ion strength, (3) ion pairing compounds used in IPC, (4) surfactants, (5) chiral additives in the mobile phase for chiral separations on nonchiral columns, (6) other compounds that form adducts or complexes with the analytes to make them amenable for detection, (7) compounds added to maintain a specific form of stationary phase, (8) preservatives, (9) additives used for enhancing MS ionization, and (10) other additive types.

(1) Certain acids or bases can be added to the mobile phase for pH modification, without participating in a buffer system. Examples of such acids include H_3PO_4, H_2SO_4, HCOOH, CH_3COOH, CF_3COOH, C_2F_5COOH, ammonium hydroxide, pyrrolidine, pyridine, triethylamine, ethanolamine or quaternary ammonium hydroxides, etc. Such additions are sometimes necessary for keeping the mobile phase very acidic or very basic. The pK_a for several acids and the pK_b for several bases are given in Appendix 7.4.3. The pK_b for quaternary ammonium hydroxides is significantly lower than for the corresponding trialkylamines. For example, a 25% solution in water of tetramethylammonium hydroxide (TMAH) has a pH higher than 13. For the pH in water solutions of a weak acid, the pH is obtained using Eq. 4.5.7:

$$pH = -0.5 \cdot (\log C_{HX} - pK_a) \qquad (7.4.22)$$

As the concentration of additives is usually low, some attention must be paid to the pK_a or pK_b of certain additives in order to not generate a pH that may destroy the HPLC column. The use of additives in the presence of organic components in the mobile phase must be taken into consideration when using strong acids (such as CF_3COOH) or strong bases (e.g., TMAH) in the mobile phase.

(2) Ion strength may be important in some separations, and this can be modified by adding certain salts to the mobile phase. Among this type of additives are certain chaotropes that can disrupt the hydrogen bonding and solvation of ions (see Section 4.5). Addition of specific additives that modify the ion strength may influence the chromatographic peak shape. For example, in many RP-HPLC separations, a specific pH of the mobile phase is obtained using a buffer consisting of KH_2PO_4 and H_3PO_4. The same pH can be generated by using a specific amount of HCOOH, and the replacement is necessary for the MS detection when nonvolatile salts/acids cannot be used in the mobile phase. However, the peak shape in the separation is typically affected negatively by such a change.

Since the pH is not different in the two cases, the effect can be assumed as caused by the change in the ion strength of the mobile phase.

(3) In ion paring chromatography, an ion pairing agent (IPA or hetaeron) is added for producing ion pairs with the analyte (or to attach to the stationary phase) (see Section 10.2). A variety of ion pairing agents are reported in the literature (e.g., Ref. [78]).

(4) Surfactants can be added in the mobile phase, and a specific type of chromatography indicated as micellar liquid chromatography (see Section 10.4) uses a concentration of the surfactant in the mobile phase above the critical micellar concentration [79]. For this type of chromatography the solutes in the mobile phase are involved in three types of equilibria: a) between aqueous mobile phase and stationary phase, b) between mobile phase and micellar mobile pseudophase, c) between micellar pseudophase and stationary phase. Stationary phase may also suffer modifications in its character due to the adsorption of the surfactant on it. This type of chromatography may provide better separation in specific separations [80].

(5) Chiral additives can be used in the mobile phase with separation on nonchiral stationary phases (see Section 14.5). These additives are used to form diastereoisomeric complexes with the chiral analytes. Different such agents are reported in the literature [81].

(6) Formation of the ion pairs for IPC analysis and chiral additives is in fact part of a larger group of additives that form complexes or adducts with the analytes making them amenable for detection. Some of these additives are derivatization reagents added in the mobile phase that can contribute to formation of colored or fluorescent compounds (e.g., Ref. [82]). Other compounds form adducts that can be detected, for example, by LC-MS/MS [83].

(7) Some additives are used for maintaining a specific structure of the stationary phase such as in ion-mediated chromatography where the stationary phase is a cation exchange material in metal form; the mobile phase must contain a low level of that metal ion. Also, a low level of a strong acid can be used in the mobile phase, for example, for maintaining an ion exchange column in H^+ form.

(8) Preservatives are sometimes added to the mobile phase for avoiding the growth of microorganisms (e.g., in buffered solution of phosphates or acetates) or for avoiding the formation of epoxides (e.g., in mobile phases containing tetrahydrofuran or ethers). As a preservative against microorganisms' growth, NaN_3 dissolved in the mobile phase (5–10 mg/L) can be used.

(9) It is common to use special additives for enhancing MS ionization in LC-MS and LC-MS/MS techniques. The proton transfer process is strongly influenced by the presence of additives and as a result the detection sensitivity is significantly affected by them. The most common such additives typically added at concentrations around 0.1% are HCOOH, when the ionization is performed in positive mode, and $HCOONH_4$, when ionization is performed in negative mode. However, other additives such as CHF_2COOH, CF_3COOH, CCl_3COOH, and CH_3COOH, are used for positive ionization mode, and $HCOONH_4$, CH_3COONH_4, NH_4HCO_3, and even HCOOH are used in negative ionization mode. Some additives such as CF_3COOH are sometimes indicated as suppressing ionization (in positive mode), but this compound can be necessary in the mobile phase for decreasing the pH, and the result in affecting the ionization can be positive or negative, depending on the analyte.

(10) Among other additives in the mobile phase are certain organic modifiers that affect the

"strength" of the mobile phase (e.g., Ref. [84]). For example, small amounts of alcohols with a long aliphatic chain can be added in a mobile phase containing methanol and water, with the result of obtaining a "stronger" mobile phase for RP separations [85]. Such additives can be considered as solvents making the mobile phase, but their low level of addition classifies them more like additives. The use of deep eutectic solvents (DESs) as additives in the mobile phase is not a common practice [86], but may improve the chromatographic performances (peak symmetry and efficiency) [87] and fulfills the green requirements when applied to HPLC separations [88,89].

Influence of buffers and additives on column stability and properties

The properties of the stationary phases are critical regarding their resilience to extreme pH values of the mobile phase (see Chapter 8). However, the chemical stability of the silica-based stationary phase is also affected by the type and concentration of the used buffers. The effect of different buffers leading to the deterioration of silica backbone may be caused by the combined effects of pH and the capacity of complexation of the buffer. A study performed to evaluate column stability [90] used 6 L of eluent containing 50% CH_3OH and 50% buffer with pH = 10 (v/v) with a column packed with a C18 stationary phase. The effect on column chemical stability was measured as the amount of dissolved silica. A mobile phase based on phosphate buffer dissolved about 110 mg/column, a carbonate-based buffer dissolved about 40 mg/column, and for borate- and glycine-based buffers the dissolution was almost unobservable. This showed that the nature of the buffer composition can influence substantially the longevity of RP columns. Different other studies [91] have shown that column deterioration is best prevented by sodium as the buffering cation. In contrast, potassium is a more aggressive buffering cation compared to sodium and ammonium. These effects seem to be caused by an increase in the pH for the aqueous/organic solution upon the addition of the organic phase, which is different for buffers with different chemical nature, although in water their pH is the same [92]. The longevity of silica-based RP columns depends also on the concentration (ion strength) of the buffer. Even at pH = 7, it was proved that by passing 10 L of mobile phase containing buffer/ACN = 50/50 (v/v), the dissolution of silica support is about 175 mg/column for a buffer concentration of 10 mM/L, 220 mg/column for a concentration of 50 mmol/L, and 325 mg/column for 250 mM/L. Therefore, in order to prevent early column failure, low buffer concentrations are recommended [91].

In order to maintain the longevity of silica-based columns apart from the choice of the resistant columns (see Chapter 8), the selection of the nature and concentration of the buffer in the eluent is very important. Typically, borate and organic-based buffers such as glycine, in low concentration combined with the properly selected counterion, substantially prevent early column degradation of silica-based RP columns. On the other hand, the use of phosphate and carbonate as buffering anions leads to a faster dissolution of the silica support. Specific columns may display a different type of interaction depending on the mobile phase pH. This is, for example, the case of weak cation exchange columns and weak anion exchange columns. The ionic character of the stationary phase containing, for example, carboxyl groups can be basically eliminated by adjusting the pH of the mobile phase within two pH units of pK_a of the acidic groups. The column will act as a polar type column and can be used in HILIC type separations.

Suitability of buffers and additives for the detection in HPLC

In any HPLC method, the buffers and the additives from the mobile phase must be acceptable for the detection procedure used in the

method. Even if a separation may be done preferably with the mobile phase containing a specific buffer and/or additives, the mobile phase is rendered useless if the detection cannot be performed properly. Most types of detection can be affected by buffers, including the main detection techniques such as UV, fluorescence, and all techniques based on evaporative processes (MS, ELSD, and cCAD). Even in RI detection, a high concentration of a buffer may reduce the technique sensitivity.

The presence of a buffer in the mobile phase may produce a change in the UV transparency of the mobile phase, modifying the UV cut-off value and also may change the wavelength of absorption for a specific compound. Since many HPLC methods use UV detection, the UV cut-off for specific buffers and at a specific concentration is important. The cut-off UV values for several buffers are given in Appendix 7.4.4.

For fluorescence detection, solution pH is also very important. Both emission wavelength and fluorescence intensity for many compounds are dependent on pH. When different structures are possible for a compound, it is very common that the fluorescence of one species is different from that of the other. For this reason, the mobile phase pH must be carefully controlled in most methods using fluorescence detection. Also, fluorescence may be quenched by specific additives. Oxidants must be avoided from the mobile phase, and specific trace metals may influence fluorescence.

For all detection techniques that use an evaporative step, the use of nonvolatile buffers in the mobile phase is not acceptable even when used at low concentrations. Among these nonvolatile materials are acids such as H_3PO_4, H_2SO_4, H_4BO_4, the salts of these acids, nonvolatile salts of volatile acids such as HCOOK or CH_3COOK, and nonvolatile bases. MS detection, in particular, being a common detection technique and having excellent qualities regarding sensitivity and selectivity, requires volatile buffers. Chemical properties and concentration of the buffers, as well as pH, have a significant effect on analyte response in ESI. Some buffers acceptable or not for use with evaporative detectors are indicated in Appendix 7.4.4.

Although nonvolatile additives at typical concentrations used for buffers (e.g., 10 mM) are not useable in techniques that use an evaporative step, traces of nonvolatile additives (e.g., in the range of 10−50 μM) can be tolerated in techniques such as mass spectrometry with ESI or APCI ionization (e.g., Ref. [83]). Buffers at these concentrations would have an extremely low buffer capacity and therefore are not utilized.

7.5 General use of solvents as mobile phase

General comments

The use of specific solvents in different types of HPLC is further presented in Part 2 of this book. Only some general aspects related to solvent purity, the relation between different solvent properties and the solvent use as a mobile phase, and the degassing of the mobile phase in HPLC are further presented.

Solvent purity in HPLC

Solvents used in HPLC must be very pure unless a known impurity is present in the solvent and it does not affect in any way the HPLC analysis. Solvent impurities may affect the HPLC analysis in various ways. The impurity may generate (1) interaction with the analytes, (2) problems with the separation, (3) problems with the detection, and (4) deterioration of the HPLC equipment.

Solvent impurities may come from additives, solvent decomposition (e.g., in case of peroxide formation in ethers, or hydrolysis of esters), or can be present from the solvent synthesis. As an example, chlorinated compounds may hydrolyze to form HCl, which can produce some decomposition of the analytes. It has been demonstrated, for example, that the presence of

ppm levels of formaldehyde in methanol can cause significant degradation of certain pharmaceutical compounds and the HPLC grade solvents are not always suitable for HPLC analysis in drug product analysis [93]. The appearance of ghost peaks may be a result of unexpected reactions between acetonitrile and labile compounds catalyzed by the traces of alkaline impurities [94]. The presence of impurities may also affect the separation by producing changes in the retention time compared to a pure solvent. Detection is affected by solvent impurities, either by increasing the signal background, by quenching the fluorescence signal, or by ion suppression in MS detection [95]. Equipment deterioration is also possible mainly for long-term use, for example, from the HCl present in some old chlorinated solvents.

Besides the impurities dissolved in solvents, small insoluble particles may be present in some solvents. These impurities must be eliminated by filtration, which is typically performed using 0.45 μm pore filters. The filters must be made from materials perfectly inert to the solvent (e.g., PTFE). However, many prefiltered solvents for HPLC are commercially available.

Flow rate, temperature, and degassing of mobile phase

The flow rates for the mobile phase in analytical HPLC can be kept constant during the chromatographic run or they may be part of a gradient program where the flow rate is increased or decreased. The values for the flow rate are selected depending on several constraints: (1) the optimization of separation, (2) the type of chromatographic column, (3) the mobile phase properties, and (4) the detector constrains (e.g., with MS detectors where the sensitivity depends on the flow rate).

(1) In theory, the volumetric flow rate U in a chromatographic column should be selected close to the optimum for efficiency as indicated based on van Deemter equation (see Eq. 3.1.70). By construction, the commercial columns usually provide good efficiency in a range of flow rates that also accommodates other requirements such as acceptable run times (not longer than 15–25 min) and backpressures in the range of capabilities for the HPLC or UPLC system. Chromatographic columns of UPLC type (small particles 1.6–2.7 μm) are typically used with flow rates in the range of 0.1 mL/min to 0.6 mL/min. Lower or higher flow rates are sometimes utilized. Common columns utilized in HPLC (e.g., with particles of 3–10 μm) allow larger flow rates in the range of 0.5 mL/min to 2–3 mL/min. Flows lower than 0.1 mL/min in common columns are limited by too much deviation from optimum flow rate indicated by Eq. 3.1.70 and also by the precision of delivering a correct mobile phase composition when the flows are lower than 0.1 mL/min. For larger flow rates, limitations also come from deviation from optimum flow rate indicated by Eq. 3.1.70, but mainly from maximum acceptable backpressure of the HPLC system (see Section 8.2). Fast HPLC has become useful in laboratories when required high throughput outcome and generally this relies on monolithic columns [96].

(2) The mobile phase properties also must be considered when selecting a flow rate. For example, mobile phase viscosity is important regarding the maximum flow rate in the chromatographic system because of the limitations in backpressure (difference between the pressure at the column inlet and that at the outlet). The limits in backpressure and the relation with the flow rate in the column are also discussed in Section 8.2 related to the column properties.

The backpressure in a chromatographic column is described by Darcy equation given by Eq. 3.1.76. The replacement in this formula of

linear flow rate u with volumetric flow rate U generates the following formula:

$$\Delta p = \frac{4\eta U \phi_r L}{\varepsilon^* \pi d^2 d_p^2} \qquad (7.5.1)$$

Eq. 7.5.1 indicates that the backpressure is proportional with the mobile phase viscosity h. Viscosity of various solvents was presented in Section 7.2 and the variation of this parameter is indicated by Eq. 7.2.3. The modern UPLC instruments are capable of generating high pressures (up to 1200–1300 bar), and even for columns with small particles (that produce high Dp values) flow rates up to 1 mL/min can be generated. However, other factors may impose a limitation in this backpressure such as the possible collapse of stationary phase (see Section 8.1). In addition, pressures higher than 600–700 bar may require attention to the connectors standing such pressures (e.g., use of metal tubing and connectors and not PEEK tubing and metal connectors). Since mobile phase viscosity decreases with the increase in temperature, a procedure to decrease the backpressure is the use of heating of the chromatographic column (and of the mobile phase) at temperatures up to 65–70°C. Other properties of the mobile phase such as volatility are also important related to column heating and the detector limitations in particular when evaporative techniques are used in the detector.

(3) The detectors may impose limitations on flow rate, especially when evaporative techniques are used. Large flow rate cannot be accommodated by some detectors such as MS with ESI or APCI ionization sources, where the mobile phase must be transferred as dry aerosols. It is common for LC-MS systems, for example, to require flow rates in the range of 0.1–0.6 mL/min, although some systems can work with flow rates around 1 mL/min. Such detectors are well suited for being used with UPLC systems where the lower flow rates are also required by the high backpressure generated by the columns with small particles (see Eq. 8.2.9).

The influence of temperature on a separation has been discussed in Section 4.6, and the selection of a specific temperature is usually done for one of the following reasons: (1) modify separation (although the increase in temperature typically decreases the value of retention factor k'), (2) decrease the solvent viscosity and reduces backpressure, and (3) maintain specific analytes in solution (such as certain polymers soluble only at a higher temperature of the solvent). The limitations in the choice of a specific temperature for the mobile phase are determined by the mobile phase boiling point, mobile phase chemical stability, as well as by column stability at elevated temperatures. Common HPLC columns are designed to work in the range between 10 and 70°C, and the manufactures typically specify the temperatures limits. A mobile phase can be used at a desired temperature when it is stable and does not boil at the temperature and pressure of the chromatographic column.

Degassing of the mobile phase is common in HPLC and was presented in Section 2.1. Most modern HPLC instruments have a "degasser" unit in-line with the flow of mobile phase that assures the elimination of dissolved gases from the mobile phase, as discussed in Section 2.1. Optionally, degassing can be done off-line by sparging an inert gas like helium through the solvent bottles. Solubility of various gasses in solvents is reported in the literature (e.g., Refs. [97,98]). The degassing may eliminate unintentionally some volatile components from the solvents and the volatility of mobile phase components must be evaluated before performing off-line degassing.

The use of "green" solvents as mobile phase in HPLC

The frequent use of HPLC as an analytical technique opened the discussion about the amount of organic waste from this technique

and its impact on environment. The organic waste refers to the organic solvents as well as some toxic additives in the mobile phase (e.g., trifluoroacetic acid, trimethylamine) used in HPLC. Some possibilities of implementation of "green chemistry" rules to HPLC are reported in the literature [99]. The main problem to implement "green chemistry" is that the separation performances obtained with common solvents such as methanol or acetonitrile are rarely attained by other solvents that are classified as "green." A solution to reduce or replace the harmful organic solvents commonly used in HPLC is to use as mobile phase less harmful, or more environmentally friendly solvents such as ethanol pure [100,101] or mixed with propylene carbonate, ethyl lactate, ethyl acetate, or some ionic liquids [102]. The separation performances (elution order, retention time, peak efficiency, or symmetry) achieved with these organic components in the mobile phase can be only slightly modified as compared to the separation based on acetonitrile in the mobile phase for several HPLC types (RP, ion-pairing, and HILIC) [103]. Moreover, the replacement of acetonitrile with a mixture of ethanol and propylene carbonate has no effect on the analyte detectability by UV-vis or MS [104]. Another possibility is to use water as the sole component of the mobile phase [59] at normal or elevated temperatures [105], or mixture of water, ethanol, and liquid CO_2 [106]. Other solvents were however used as "green" alternative. For example, a neutral deep eutectic solvent, produced from choline chloride and ethylene glycol, was employed for melamine monitoring in milk matrix using micellar liquid chromatography [107].

Among other the strategies to reduce the consumption of organic solvents is, for example, to decrease the flow rate of the mobile phase, which is feasible when the internal diameter of the column is reduced, as shown in Section 8.2 (see Eq. 8.2.6). Another alternative is to use parameters such as temperature to modulate separation optimizing it from the "green" point of view [108].

7.6 Solvents for sample injection and for needle wash

General comments

The introduction of sample in the HPLC is typically performed by placing a precisely measured volume of a solution of the sample in the mobile phase generated by the pumps (see Section 2.2). The sample must be completely dissolved, and for this purpose, a single solvent or a mixture of miscible solvents can be used to contain the sample. The sample solution must be free of any particles and should have a high stability, not undergoing solvolysis/hydrolysis or high tendency to evaporate when placed in capped vials with septum that was already punctured if the sample was previously injected (see Section 2.2).

The role of sample solvent in the chromatographic process

Besides the role of dissolving and loading the sample, the liquid used for sample dissolution also may have contribution to the chromatographic process. The injection volume V_{inj} is directly related to the amount of sample delivered to the HPLC system and therefore to the detector response (see Section 2.2). Also, the peak width (characterized, for example, by peak width W_b) is affected by the injection volume [109]. The theory about peak broadening in HPLC is developed considering that no contribution to W_b is due to injection volume, which is assumed to be extremely small. However, the correct value for W_b should include the width of the injected sample besides the broadening due to various random processes taking place in the chromatographic separation (see Eq. 3.1.52). As a result, the larger injection volumes produce a widening of the chromatographic peaks. Larger injection volumes may be necessary to place a larger amount of sample in the chromatographic system in order to improve sensitivity.

The use of a sample solvent (diluent) with the same composition as the mobile phase (initial mobile phase composition in case of gradient separation) is a common practice in HPLC. With this choice, the mobile phase composition is not modified by the injection, and no additional effect regarding the chromatographic process take place. Volumes typically used in HPLC in the range from 1 μL up to 25 μL affect very little the value of W_b or that of the peak shape. For UPLC, where the peaks are narrower, the injection volumes should be kept relatively low in order to preserve efficiency, and injections smaller than 3–4 μL do not affect the peak width.

However, the use of mobile phase as sample diluent is not always possible due to the low solubility of analytes or due to sample preparation procedures that deliver the processed sample in a specific solvent. For example, when sample processing such as liquid–liquid extraction (LLE), solid-phase extraction (SPE), QuEChERS ("Quick, Easy, Cheap, Effective, Rugged, and Safe"), or stir bar sorptive extraction (SBSE) is used, the analytes are frequently present in a different solvent than mobile phase since this solvent is selected based on sample processing requirements and not HPLC preference.

When the sample solvent is different from the mobile phase, an initial requirement is that the sample solvent must be miscible with the mobile phase [110]. Also, it is important that the analytes are also soluble in the mobile phase at the concentration reached when placing them in it. Another important requirement is that the pH of the solution injected into the mobile phase is in the range of pH stability of the chromatographic column. Some analytes may be present in a solution with very low or very high pH. Such solutions may destroy the stationary phase at the head of the chromatographic column. The intensity of this effect is difficult to estimate since it depends on the volume of the injected sample, on the actual pH of the sample solution, on the buffer capacity of the mobile phase, and on the resilience of the stationary phase to the excessive pH. When the injection volume of the sample is small (2–3 μL) and the buffer capacity of the mobile phase is good, marginally excessive pH values of the injected sample may be acceptable. However, with repeated injections they may have a damaging effect in time on the chromatographic column and the use of a guard column or precolumn is highly recommended. When these three requirements are not fulfilled, the peak shape, the separation, and quantitation in HPLC can be significantly affected, and the life of the chromatographic column significantly shortened.

The differences between the sample solvent and mobile phase may affect peak retention time and peak shape, and this depends on the nature of the two liquids (chemical and physical properties), the injected volume, and to a smaller extent the flow rate of the mobile phase. Regarding the physical properties, the viscosity of the two solvents plays an important role, and sample solvents with high viscosity seem to affect the most the peak shape. The mutual solubility of mobile phase and sample solvent also plays a role in affecting the peak shape. Regarding the chemical nature of the solvents, there are three model possibilities regarding the differences between the nature of mobile phase and sample solvent: (1) the sample solvent is an eluent similar with the mobile phase, (2) the sample solvent is a "weaker" eluent, or (3) the sample solvent is a "stronger" eluent.

(1) When the sample solvent is a similar eluent with the mobile phase, only small differences regarding the elution process will occur, and these are typically not significant. In UPLC, larger volumes of the injected sample (e.g., 10 μL or larger) can produce a noticeable peak broadening because in UPLC the peaks are narrow (e.g., W_b of 2–3 s). In common HPLC,

volumes up to 20–25 μL usually do not produce a significant peak broadening, but larger injection volumes may increase W_b.

(2) For the sample solvent, a "weaker" eluent than the mobile phase, a concentration of the sample at the column head may take place. This effect depends on the injection volume and sample solvent nature. Typically, small injection volumes such as 5–10 μL (for HPLC) do not produce a noticeable concentration effect. However, for larger injection volumes (used, e.g., with diluted samples), and with a very "weak" sample solvent, this effect may reduce the peak broadening caused by the large injection (sample on-column focusing). For a short period of time, the liquid reaching the head of chromatographic column is the sample solvent and this is unable to elute the sample components that are retained as a very narrow zone at the head of the column. In such cases, the volume of the injection may not influence the column efficiency. This effect is known as adsorption compression [111]. This property can be used for injecting larger volumes of a diluted sample, when a larger amount of sample is necessary for increasing sensitivity. Loading capacity of the column (maximum amount of sample including analytes and matrix acceptable for a column) should not be exceeded when larger volumes are injected.

(3) The use of a "stronger" solvent for sample injection may generate significant problems regarding the peak shape. The sample solvent plug may act as a strong eluent before it is diluted enough by the flowing mobile phase and may produce a widening of the region where the sample is distributed at the head of the chromatographic column. This process generates wider chromatographic peaks and very frequently peak shape distortion sometimes generating double peaks or very poorly shaped peaks [112]. Peak distortion was noticed in particular when the strong eluting solvent has a very different viscosity than the mobile phase and the mixing of the two is slower. Very small injection volumes may help in avoiding peak distortion.

A sample solvent that is a strong eluent is frequently used because stronger eluents are usually good solvents for the sample. For example, in RP-HPLC, acetonitrile or methanol is commonly used as sample solvent. In case of some hydrophobic compounds analyzed by RP-HPLC that are not soluble or are poorly soluble in a partial water solvent, the use of "strong" eluents as sample solvent is necessary. In order to avoid visible peak broadening or peak distortion, such solvents must be injected only at low volume (e.g., 1–3 μL). Solvents with high viscosity that mix more slowly with the mobile phase require even smaller injection volumes.

One possibility to improve the peak shape when this is distorted because of a "strong" sample solvent is the dilution of the sample with mobile phase and injecting a proportionally larger volume [109]. This method is known as precolumn dilution, and a mixer chamber can be utilized, followed by larger volume injection in order to reach low detection limits [113].

One alternative to avoid the use of strong solvents for carrying the sample is to change the solvent and redissolve the sample in a "weaker" solvent. Also, for the reduction of the sample volume when a larger volume is necessary because the sample is too diluted and a specific sensitivity must be attained, the sample solution may need to be concentrated. Evaporation of the diluted sample to concentrate the target analytes can be applied at this point of sample preparation. However, this operation can be time-consuming and can affect precision and analyte recovery (e.g., some analyte evaporation may occur simultaneously with the solvent or the analyte may suffer decomposition when evaporating temperature is too high). In these cases, other alternative paths may be selected for

sample preparation, or higher sensitivity for the detector may be evaluated such that the injection volume does not need to be increased [114].

The problem of strong eluting solvents used for sample dissolution is common in RP-HPLC. In HILIC, the separations can also be influenced by the sample solvent [115]. However, in HILIC separations, organic solvents are weak eluents under HILIC conditions so that polar analytes are accumulated in a narrow zone at the head of the column (sample on-column focusing). This is an advantage for the analysis of polar drugs in biological samples, for instance, when the proteins are precipitated with acetonitrile or with methanol. The organic supernatant can then be directly injected into the HILIC column, avoiding the step of solvent evaporation and the residue reconstitution. Injection of large volume of aqueous sample solvent of high elution strength should be avoided in HILIC. The volume of polar solvents for the sample should be limited by the same rules as nonpolar ones in the case of RP-HPLC. The use of large volumes of polar solvents for the sample injection may lead to broad or split peaks. An organic content of more than 50% for the sample solvent is typically recommended in HILIC [116,117].

For other types of chromatographic separations, such as size exclusion (SEC), sample viscosity and injection volume play an even more important role in the separation than for RP or HILIC [118] In SEC, sample concentration can be high and polymeric content can significantly influence the viscosity of the sample. Also, larger volumes than in other types of chromatography are typically utilized in SEC. For this reason, some specific restrictions are necessary regarding injection in SEC. For example, the relative viscosity of the sample in SEC should not exceed double that of the mobile phase (for a dilute aqueous buffer, this corresponds to a concentration of protein of about 70 mg/mL). The volume load in SEC should not exceed 1%–5% of the total column volume, although larger volumes are sometimes injected. Only injections that do not exceed 2% of the column volume were usually proven to maintain good resolution [119].

Sample solvent may also be important for chiral separations. Some samples can be dissolved in only a specific solvent, and the solvent may not be adequate for being injected in the column. For example, proteins are usually soluble in water and in DMSO. Since water can disturb the chiral separation, DMSO must be used for protein dissolution when injected in a chiral column. However, the chiral stationary phase must be stable under repeated DMSO injection. Some chiral columns such as Chiralpak IA are recommended as fulfilling such requirement [120].

An interesting possibility to focus the analyte at the head of the chromatographic column consists of using as sample solvent a compound that is very strongly retained at the column head with the analytes less retained than the solvent itself [114,121]. During the elution process, the sample solvent is retained at the column head longer than the analytes. For achieving focusing by this procedure, the following conditions are necessary: (a) the sample solvent must be hydrophobic (such as i-octane, hexane, heptane, octane, benzene), (b) the mobile phase must have a high content of water in order to avoid the dissolution of the solvent, and (c) the analytes must have a lower hydrophobicity than the sample solvent [122,123]. An example of column focusing by this approach is given by injecting up to 500 μL of 50 μg 4-hydroxy-3-t-butyl anisole dissolved in i-octane on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm particle size) with the mobile phase containing 40% ACN and 60% water with 0.1% H_3PO_4, at 25°C. The flow rate for the separation was 1.5 mL/min and the detection was performed at 291 nm. After each chromatographic run the column was washed with 100% AcCN and conditioned at

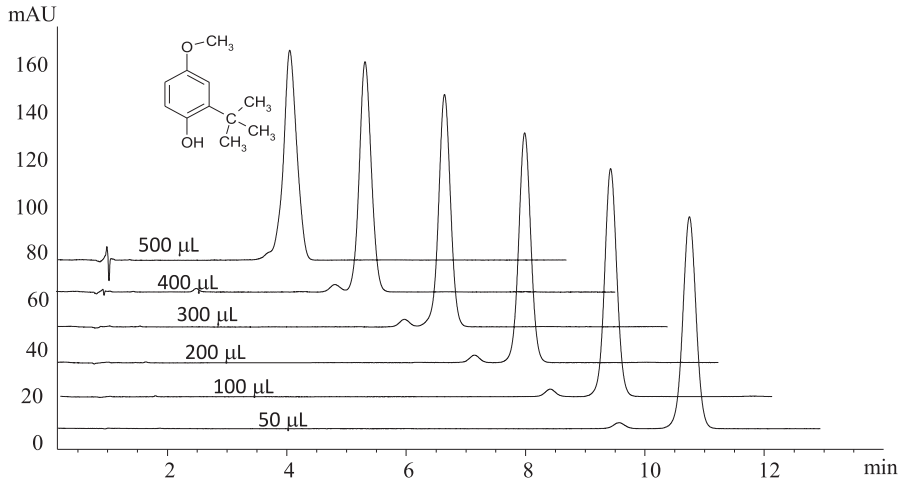


FIGURE 7.6.1 Separation of 50 µg 4-hydroxy-3-t-butyl anisole on a zorbax eclipse XDB-C18 column with large injections (50−500 µL) of the sample in i-octane in a partially aqueous mobile phase (traces offset by 10 mAU) [125].

the initial mobile phase composition [124]. The results are shown in Fig. 7.6.1 [125]. Larger injection volumes reduce the retention time of the analyte, probably because the portion of the analytical column covered by the sample solvent does not retain the analyte.

Effect of sample solvent on detection

Sample solvent may also have an effect on the response of specific detectors. For example, the signal of RI or of electrochemical detectors can be significantly influenced by the sample solvent. In some cases, the "elution" of sample solvent can generate a large signal (sometimes negative), which can adversely affect the signal measurement for the analytes, in particular when the sample solvent elutes close to the analyte. The problem of detector response to the sample solvent is addressed in various studies (e.g., Refs. [126,127]).

Solvents for the needle wash

In most HPLC (and UPLC) systems, the autosamplers have the capability to wash the needle utilized for taking the sample from the sample vial and placing it in the flow of the mobile phase. The needle wash is necessary for avoiding carryover from sample to sample. The presence of a carryover problem can be seen (e.g., as a ghost peak) in the chromatogram of a blank sample injected after a series of samples that may contain high concentrations of analytes [128]. Carry over depends on the type of analysis, HPLC system and the type of detection [129]. Most carry-over problems come from the injection part of the chromatographic system, and they can have several sources: (a) adsorption of components from sample to the needle, (b) needle seat design, (c) improper or worn-out sealing of the needle, (d) worn out of rotor/stator of injection valve, (e) tubing fittings contaminated with sample, and (f) contaminated parts of the column (fittings, frits, and connection with detector). The limit for carry over should be less than 0.05% for good quantification results. The needle is washed inside with the mobile phase. However, for external needle wash, an auxiliary solvent is used. This solvent selected for the external needle wash depends on the sample and mobile phase chemistries, making

sure that all solutions/buffers are miscible and soluble. The composition of the needle wash solvent should be the most solubilizing compatible solvent. It is common to select for the needle wash a solvent or a mixture of solvents similar to the mobile phase composition, but with higher content in the stronger eluent. It should be verified experimentally that in using the needle wash, no carryover of the samples take place.

7.7 Gradient elution

General comments

Gradient separations in HPLC are very frequently utilized. In common gradient HPLC, the composition of the mobile phase is changed during the chromatographic run (flow rate gradients are also possible). The composition change may be in the % of the organic components in water, in pH, in ionic strength, etc. [130]. More complicated gradients with changes in the nature and composition of the mobile phase are also applied for some separations but more common are the binary gradients in which only two components are modified during the chromatographic run. The major applications of gradients in HPLC are for RP, HIC, and also for HILIC [42]. In other separation techniques such as IC and NPC, the gradients are also used, but in size exclusion and chiral separations, the gradients are not usually applied. The change in the mobile phase composition in gradient HPLC can be done in a linear mode (see Eq. 2.1.1), nonlinear mode (see Eqs. 2.1.3 and 2.1.4), or even as step gradient (sudden change in mobile phase composition). Multiple gradient ramps are also possible during one chromatographic run. Examples of different common gradient shapes with the increase in organic component in the mobile phase as practiced in RP-HPLC are illustrated in Fig. 7.7.1.

In Section 2.2, it was indicated that in practice the change in the mobile phase composition takes place with a certain delay (dwell time t_D) caused by small dead volumes existent in the HPLC mobile phase path before the column itself, and that actual changes are less sharp than it is shown in Fig. 7.7.1 due to various mixing effects. Also, the HPLC pump system has some influence on the gradient actual shape. The gradient generated with HPLC system with low pressure mixing is less sharp compared to those obtained with high pressure mixing.

As shown in Fig. 7.7.1, it is common for a gradient to include at the beginning of the run a short time section where the mobile phase composition is kept constant (isocratic). This isocratic hold also includes the dwell time. This section of isocratic hold is chosen as having the "weakest" solvent in the chromatogram, and it is designed for the retention of the least retained compounds in the sample (for RP-HPLC the lowest organic content). The gradient slopes are designed to achieve the desired separation in the optimal (or close to optimal) retention times. Following the gradient slope, the gradient program typically includes a section with the "strongest" mobile phase, with the role of eluting all sample components from the

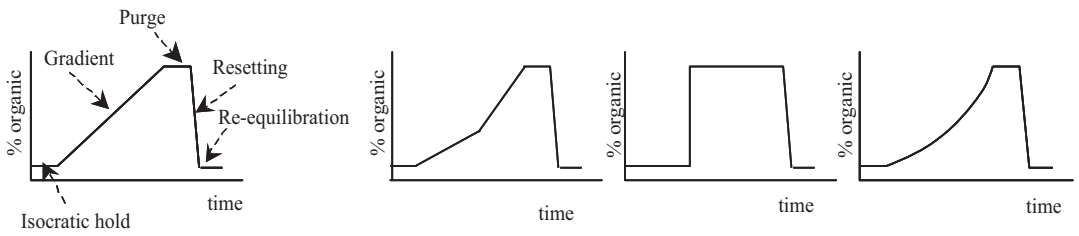


FIGURE 7.7.1 Examples of different common gradient shapes with the increase in organic component in the mobile phase.

stationary phase. After this section, the mobile phase composition is restored to the initial conditions to make the system ready for the next injection. A reequilibrating time is necessary for restoring the stationary phase to the initial conditions, which are not attained instantaneously. This reequilibration time depends on the column dimensions and also on the type of column and mobile phase. It is common to divide the volume necessary for column reequilibration into two portions, the system wash-out and the "true" column equilibration volumes. One empirical rule to establish these necessary volumes uses the following formula:

$$V_{equil} = 3\,V_{total} + 5\,V_{column} \quad \text{where } V_{column} = \frac{\pi\varepsilon^*}{4} d^2 L \text{ and } V_{total} \approx 650\ \mu L \text{ to } 3000\ \mu L \tag{7.7.1}$$

In Eq. 7.7.1, ε^* is the column porosity and $\varepsilon^* \approx 0.7$ (for typical 5 μm particles columns) as shown in Table 3.1.1 (When the column i.d. and the column length are expressed in mm and the volume is expressed in mL (cm^3), a factor of 10^{-3} must be included in the calculation). The dwell volume for common HPLC systems is between 500 μL and 3 mL. The equilibration time is obtained from V_{equil} based on the flow rate from the formula $t_{equil} = V_{equil}/U$. For example, for a column 2.1 × 150 mm and a small system volume, an equilibration time of minimum 4 min is necessary at 1 mL/min flow rate. However, Eq. 7.7.1 seems to give an overestimation of the necessary volume of solvent to reequilibrate a column. In many cases, only two column volumes of the initial eluent are sufficient for the column reequilibration. In case of ion pairing, the volume of initial eluent can be higher due to the slow equilibration process in this HPLC type [131].

In modern HPLC instruments, the gradients are obtained from the solvent supply system by computer-controlled pumping based on a gradient time table. One example of a gradient time table that was used for the separation by RP-HPLC of several hydroxybenzenes from cigarette smoke is given in Table 7.7.1. The separation was performed on a Beckman Ultrasphere ODS column 15 cm × 4.6 mm i.d., 5 μm particle size. Mobile phase was composed of solution A: water with 4% acetonitrile and with1% acetic acid and solution B: acetonitrile with 1% acetic acid.

The diagram of this gradient change is shown in Fig. 7.7.2. The detection for the analytes was done using an FLD and the resulting chromatogram is shown in Fig. 7.7.3 [132].

TABLE 7.7.1 Time table for the gradient separation of hydroxybenzenes from cigarette smoke.

Time min	Solvent B %	Solvent C %	Solvent D %	Flow rate mL/min	Max pressure Barr
0.0	0	0	0	1.4	400
0.5	0	0	0	1.4	400
10.5	31	0	0	1.4	400
15.5	100	0	0	1.4	400
20.0	100	0	0	1.4	400
20.2	0	0	0	1.4	400
22.0	0	0	0	1.4	400

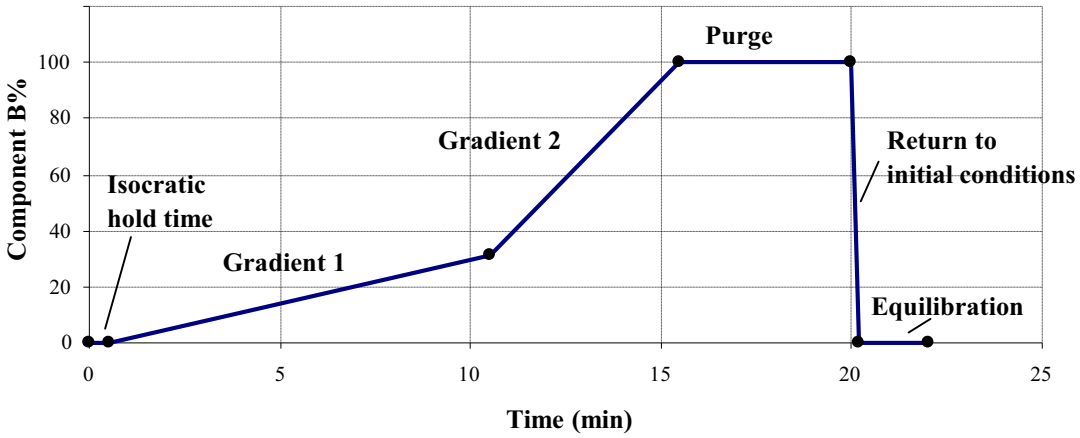


FIGURE 7.7.2 Gradient profile for a separation of hydroxybenzenes on an ultrasphere ODS column 15 cm × 4.6 mm i.d., 5 μm particle size.

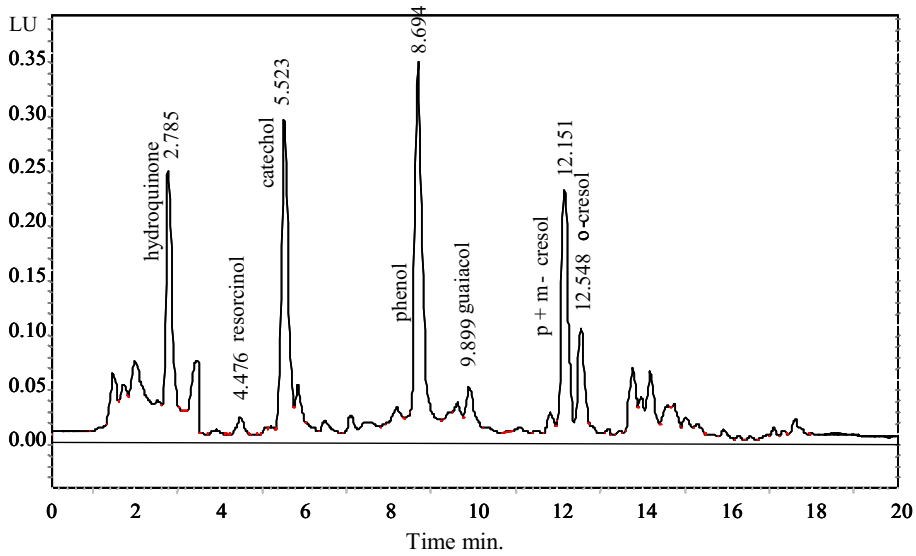


FIGURE 7.7.3 Chromatogram showing the separation of several hydroxybenzenes from cigarette smoke using gradient elution.

Gradient of solvent composition

The modification of solvent composition of the mobile phase is the most common type of gradient and this is typically done by mixing two solvents. In RP-HPLC, the starting composition has a low organic content, which is increased for along the chromatographic run. In multistep gradients, it is possible to encounter regions with a reversed gradient but this is uncommon. In HILIC, the starting composition has a high organic content. During the modification of organic phase composition,

it should be verified that the solvents are perfectly miscible, and also that their mixture is still a good solvent for the buffers and additives. In some applications, it is recommended that solution A contains a small proportion of solution B from the start of the gradient. For example, in a gradient made with two solvents such as water and methanol, it is recommended that solution A contains 5% methanol, while solution B is pure methanol. This assures an easier mixing of the gradient components.

As the organic content increases, the solubility of some additives decreases, in particular that of inorganic salts. Since some buffers and additives are frequently added in only one of the mobile phase components, it should be understood that the concentration of these additives and buffers is also changing when the solvent composition is changing. The precipitation of some additives at increased organic phase content in the mobile phase can destroy a chromatographic column and plug the HPLC system. Particular attention must be given to the concentration of buffers and additives in order to have them in solution during the whole gradient program.

Gradient of pH or of additive concentration

Another type of gradient is the one in which the pH of the mobile phase is changed. This can be achieved without a change in the content in the organic component, or changing both the pH and the organic component content. The variation of the pH of a mobile phase can be achieved by different procedures. For RP-HPLC, for example, one procedure is the modification of the ratio of two solutions, one being an aqueous (or partially aqueous) buffer solution A and the other an organic (or partially organic) solution B that does not contain buffers or additives. Another procedure consists of the modification of the ratio of two partially aqueous solutions A and B, each one buffered at a different pH. These solutions with different pH may have the same or different contents of the organic phase. Gradients with different pH and different organic contents can also be achieved by mixing more than two solutions [133]. However, three or four solution gradients, although sometimes utilized, are not common. The ionization status of a compound can be even more important regarding its retention in a polar (HILIC) or in an ion exchange separation than it is in RP-HPLC. When the mobile phase does not contain an organic component (e.g., in ion chromatography), the gradient is achieved by mixing two solutions with different pH (and possibly different inorganic additive contents).

The pH change of the mobile phase is usually performed intentionally, but in some applications, the change is the result of mixing one solvent that contains the buffer, and another solvent not having an adjusted pH. In order to avoid an undesired pH change, it should be assured that the buffer is present in both gradient components. Although the increase in organic constituent still modifies the final pH, the change is expected to be smaller.

The use of gradient for additives is similar to that for buffers. Two common additives in HPLC are HCOOH and $HCOONH_4$ which are frequently used when the detection is performed using MS for enhancing the analytes ionization. However, opposite to a desired gradient of these additives, it is usually necessary to keep their concentration constant when other components of the mobile phase such as the content in organic phase are changing. To achieve a constant concentration of these additives during the gradient of other components of the mobile phase, it is necessary to add them in equal concentration in the components of the gradient (e.g., aqueous phase and organic phase).

Salt gradient can be applied in ion exchange HPLC for the elution of proteins (e.g., monoclonal antibodies). For example, a gradient elution consisting in the increase of the Na^+

content in the mobile phase will shift the adsorption/desorption equilibrium of monoclonal antibody on the ion exchange sites of the stationary phase to the desorption, and thus this species is eluting from the column [134,135].

Gradient in flow rate

Besides composition change, a change in the flow rate of mobile phase may be practiced in some applications. The need for such a change may appear as a result related, for example, from the excessive increase in backpressure as the mobile phase composition changes. Some solvent mixtures have an increased viscosity as compared to the initial viscosity of the mobile phase. For avoiding excessive backpressure, a slower flow rate can be used for the region with higher viscosity of the mobile phase. Another reason for changing the flow rate during the chromatographic run may be related to the requirements of the detector. For example, the MS detectors when using ESI type ionization, must use a specific flow rate for assuring a maximum yield of ions (see Section 2.2). The same flow required for the detector optimum may not be necessary or convenient for the whole chromatogram. It is possible to adjust a specific flow rate only for the time window where the analytes of interest are eluting, while the rest of the separation is performed at a different flow rate.

Gradient in separation temperature

The adsorption of a molecule is usually an exothermic process ($\Delta H < 0$), while desorption is an endothermic process ($\Delta H > 0$). These effects are neglected in HPLC considering that the heat capacities of the mobile and stationary phases are larger than the two enthalpies, and the thermal conductivity of the mobile phase is sufficiently large to maintain a uniform temperature inside the column. As a result, the conditions during HPLC process can be assumed to be isothermal. However, there is experimental evidence that the temperature may vary inside HPLC columns, especially in dynamic HPLC (see Section 4.7). Temperature fluctuation can modify the viscosity of mobile phase and consequently the pressure drop according to Darcy's law. The solubility and diffusivity of analyte molecules can also change [136]. The effect of heating is the acceleration of the migration speed of analyte molecules [137]. The gradient in separation temperature can be used in some instances for modifying the separation [57]. In particular for small molecules, the change in the temperature has a small effect on the separation. This effect is more pronounced for large molecules and it was used for modulating the separation, for example, in interaction polymer chromatography (see Section 15.6).

Usefulness of gradient vs. isocratic elution

Gradient elution is basically used for three main purposes: (1) Reduction of the total run time of separations, (2) Modification of retention times in a chromatographic run which does not provide a good separation between specific compounds, and (3) Cleaning and/or regeneration of the chromatographic column. Other more uncommon utilizations of gradient can be mentioned, such as loading of the column with a specific reagent.

(1) In common HPLC separation, the values for the retention factors k'_X for different solutes should be in the range $2 \leq k'_X \leq 10$ (values between 1 and 2 are sometimes acceptable). These values are necessary for performing the separation at acceptable retention times ($t_R = t_0 + k'_{t0}$), with typical V_0 values in the range given in Table 2.1.1 from which $t_0 = V_0/U$ can be obtained (for $U = 1$ mL/min $t_0 = V_0$). It is not uncommon that in a sample are present

compounds that have "low affinity" for the stationary phase and some with "very high affinity." For RP-HPLC, for example, in solvents with a higher organic content, some compounds with small hydrophobic moieties are poorly retained and may have $k' < 1$. Their retention is very sensitive to small (unintentional) changes in the mobile phase composition, leading to changes in the retention times. Also, interferences from unretained materials from the sample matrix are possible when peaks of interest elute too early. For these cases, a "weak" mobile phase is initially necessary. However, such mobile phase may not be acceptable for the elution of more hydrophobic compounds with larger k'_X. With a polar mobile phase, these compounds are eluted late in the chromatogram and therefore have broad peaks. Although these peaks are well separated, the separation is unnecessarily large, the peak integration is less accurate, and the run time is too long. In these cases, a gradient elution is necessary to shorten the separation time. A reduction in the retention factor and therefore of the retention time can be achieved using gradients, when the content in the stronger solvent is increased in the mobile phase (larger ϕ), as indicated be Eq. 3.5.3 and written below:

$$\log k'(X) = \log k'_w(X) - S(X)\phi \qquad (7.7.2)$$

In this way, the early eluting peaks in a chromatogram are subject to the "weak" solvent and they have $k'_X > 2$. The peaks that would elute at long retention times in a "weak" solvent are subject to a "stronger" solvent such that they have $k'_X < 10$. The shortening of the retention time for late eluting peaks is illustrated in Fig. 7.7.4 for RP-HPLC.

One additional advantage of shortening the retention time by using gradient elution is related to the peak shape in the chromatogram. At longer retention times, the peaks are

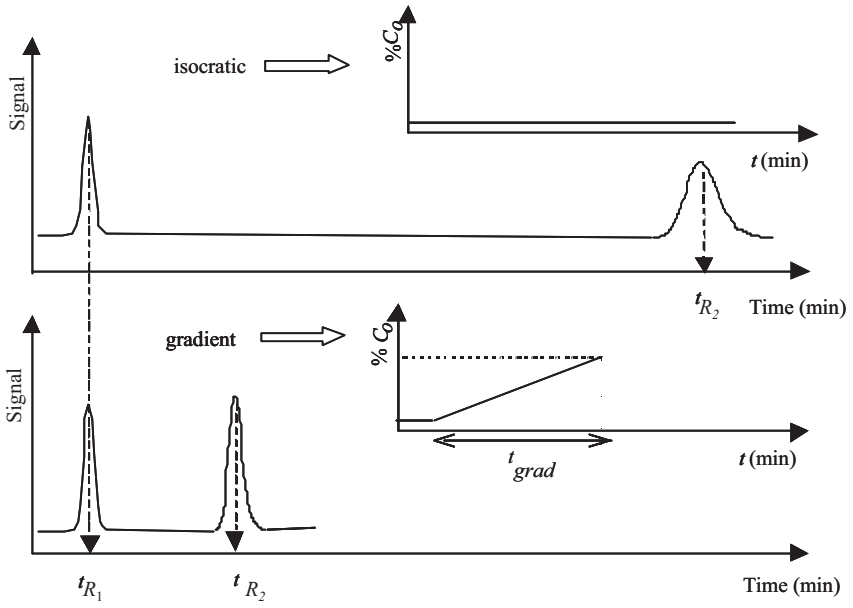


FIGURE 7.7.4 Difference in the retention of two hypothetic compounds in isocratic conditions and in gradient conditions, in an RP separation when the concentration of the organic solvent C_o is increased.

broadened, and shorter times help in obtaining sharper peaks. A detailed discussion on peak shape in chromatography is found in Chapter 3. Gradient elution also provides good repeatability of the retention times and also of the peak area, such that no adverse effects appear regarding quantitation with gradient separation, as compared to isocratic elution [39].

(2) The reduction of k'_X values for different analytes must be done such that the values for resolution remain $\alpha(X,Y) > 1.2$ for all the components of interest in the sample, and the value for resolution $R > 1$ (or better $R > 1.5$). However, R depends on k'_X (k^*) in addition to α and N, and smaller k'_X values may lead to poor separations. For this reason, when decreasing k'_X values by using a "stronger" mobile phase, this cannot be done without restrictions, and the gradient must maintain a good separation. The choice of a convenient gradient is relatively simple when an initial isocratic separation (or a gradient with small composition changes) provides a good separation. Starting with this separation, the slope of gradient is increased such that the change from a "weak eluting" mobile phase to a "strong eluting" mobile phase is done more rapidly. The new separation must remain acceptable regarding selectivity and resolution. The process can also start in reversed order, with a relatively poor separation in a rapid gradient and short retention times, which are changed to slower composition changes that would allow a better separation.

The expressions connecting k'_X to various parameters describing the mobile phase developed in Section 3.5 (e.g., Eqs. 3.5.20 and 3.5.21) also show that the variation in k'_X does not depend only on the mobile phase composition but also on the nature of the stationary phase and the nature of the analytes. When the composition of the mobile phase is changed (within a range) from "weak" to "strong" or when the pH and the ionic strength are changed, different components from the sample may be affected differently, and their retention times $t_{R,X}$ and $t_{R,Y}$ (and corresponding k^* for each analyte) may vary in different ways [138]. For this reason, it is possible that specific values for $\alpha(X,Y)$ increase, although both $k\times_X$ and $k\times_Y$ decrease. Different effects of increasing the mobile phase strength (% of organic component for RP-HPLC) are illustrated in Figs. 7.7.5a and 7.7.5b for two couples of hypothetic compounds (assuming linear variation of log k' with the % of organic solvent). For the first couple of compounds (A and B) shown in Fig. 7.7.5a, as the % of organic component in the mobile phase increases, log k'_A - log k'_B decreases, and the separation is worsened. For the second couple of compounds (C and D) shown in Fig. 7.7.5b, as the % of organic

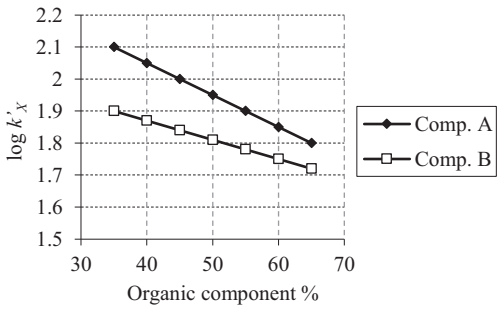


FIGURE 7.7.5A Variation of log k'_X as the content in organic phase increases and the separation worsens.

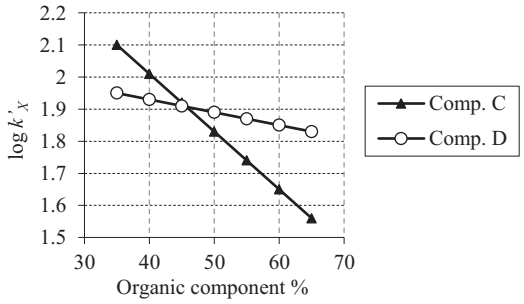


FIGURE 7.7.5B Variation of log k'_X as the content in organic phase increases and the separation first decreases and then improves.

component in the mobile phase increases, $\log k'_A$ - $\log k'_B$ has initially a decrease, but further on increases and the separation is improved. The effect shown in Fig. 7.7.5b is not uncommon, and improvements in the separation can sometimes be obtained by increasing the "strength" of the mobile phase, which lead at the same time to shorter retention times. Predictions of such changes are in general difficult to make, and the descriptions of analytical methods from the literature provide details on specific mobile phase compositions and gradients that assure a good separation [133]. This is the main cause for the need of "trial and error" strategy for optimizing some separations. The selection of a specific stationary phase in obtaining a desired separation is very important in such cases.

(3) In most gradients, a purge region is included for cleaning the column from any remaining solutes, not necessarily analytes. This region may have the highest concentration of the "strong" eluent. For example, in RP-HPLC, the purge region may consist of high concentration of organic phase or even of pure organic component. When pure organic component is not necessary for cleaning the column, a lower concentration should be used such that the reequilibration of the column is done faster.

The cleaning of the stationary phase from all injected sample components is part of the good care of the chromatographic column. However, this is not always convenient, and in some applications, it is recommended cleaning after a number of injections (analyzed samples) and not after each sample. In such cases, a separate gradient run (or isocratic run) with strong (possibly pure) solvents is applied, followed by a longer reequilibration of the stationary phase to initial condition.

For practical purposes, the reduction of the run time of a separation can be very useful. Contribution to achieving this goal, in RP, for example, can be obtained not only from using a mobile phase with a higher content of organic phase, but also from using sharper gradient changes. The slope of gradient, or its speed, can be optimized in order to assure best results such that the separation is not compromised and the analysis run time is shortened [139,140]. In practice, mainly when the gradients are very sharp, a gradient distortion can be noticed. This is manifested by large deviations from the sharpness of a required gradient and may be different from instrument to instrument. Low pressure mixing instruments tend to have more gradient distortions than high pressure mixing systems.

Besides the advantages, some problems may appear in gradient elution. One problem is related to the inability to use RI detection with gradients. Another problem may be the appearance of a drift in the baseline of the chromatogram. This drift depends on the selected solvents and on the detector, and it is not a common problem. For example, with MS detection the gradient elution typically works without problems, but in some cases, the background baseline changes and then the use of isocratic separation is preferable. Also, the gradient is not necessary when the analytes are well separated and the run time of the chromatogram is short enough for isocratic separation.

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CHAPTER

8

Analytical HPLC columns and their characteristics

8.1 Construction of an HPLC column

General comments

In HPLC, the separation media is usually contained in a chromatographic column. The chromatographic column is made from a tube (cylinder) filled with the stationary phase. A common stationary phase is made from a porous material in the form of particles on which an active phase is bonded, grafted, or coated [1]. Various physical dimensions of the tube containing the stationary phase are available, and the stationary phase can be of a number of types. The number of types of commercially available columns is very large (about 63,000) and various lists of vendors and brands of columns can be found on the web (e.g., Ref. [2]). For nano-, capillary-, and microflow UHPLC, either very narrow columns or special capillaries are used to contain the stationary phase [3,4]. Other separation devices are available (see, e.g., IonKey separation device [5]) such as cartridges and microfluidic chips that contain the stationary phase [6].

Progress is continuously being made in the construction of chromatographic columns, in particular regarding the stationary phase [7–13]. In addition to the chromatographic column, for its protection, it is common in HPLC to use a precolumn, or guard column, or cartridge installed between the injector and the chromatographic column. Several aspects of the construction of a chromatographic column (and of the guard column) are discussed in this section.

External body of the column

The external body of the column (empty column) is in the form of a tube made from stainless steel or a strong polymer (e.g., polyetheretherketone or PEEK). At the two ends of the column are special frits that keep the stationary phase from moving inside the body of the column, and also fittings that allow the connection of the column with the tubing connecting the column to the pumps and to the detector (see Section 2.2). The geometry and the design of the frits may influence peak efficiency [14].

The physical dimensions of common analytical chromatographic columns vary, and values for length (internal) L can be between 30 and 250 mm (common length 50, 100, 150, 250 mm), and internal diameters d for usual columns can be between 1 and 10 mm (e.g., 2.1, 3.0, or 4.6 mm). Other dimensions are possible, particularly when the column is designed for special tasks. The newer columns have the tendency of being shorter and narrower, as the solid particles that form the stationary phase are made smaller.

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The physical shape of the body of the chromatographic column may have some effect on the separation. At the column wall, the packing material has a different distribution compared to the middle of the column, and the migration rate of the sample band in the region of the wall is slightly different from the interior. The mobile phase flow at the walls is also different than that in the middle of the column. Such effects were studied in detail and reported in the literature [15–17].

Based on the internal diameter of the analytical column, they are sometimes classified in the literature as (1) standard (3.0–4.6 mm i.d.), (2) minibore (2.0–3.0 mm i.d.), (3) microbore (0.5–2.0 mm i.d.), (4) capillary (0.2–0.5 mm i.d.), and (5) nanoscale (0.05–0.2 mm i.d.). Miniaturized sample introduction techniques, pumps, and specialized detectors have been developed for microcolumn LC [18]. Columns on a chip were also made experimentally [19]. Larger columns are used for semipreparative and preparative purposes and capillary silica columns are used for nano LC. The empty volume of the column can be easily calculated as the volume of a cylinder $V = (\pi/4)\cdot d^2\cdot L$, and for analytical columns V ranges between 0.02 and 20 mL. The column is filled with the stationary phase, and a fraction of the empty column volume is filled with the mobile phase. This volume will give the dead (void) volume of the column, discussed in Section 3.1. Various dimensions of columns, empty column volumes, and typical void volumes are indicated in Table 3.1.1.

Commonly used column dimensions are the standard and the minibore. The columns with smaller diameter have the advantage of using less solvent since the flow rate U in narrower columns is usually lower in order to maintain the same linear flowrate u as in columns with larger diameter. For microbore columns, some problems with low loading capacity but also with a decrease efficiency are characteristic [20,21]. A study on optimization of column diameter showed that columns with $d = 1.5$ mm can be successfully used to achieve low solvent use and still have good column performance [22].

Regarding the dimensions of the guard column, they can have different dimensions depending on the manufacturer, some guard columns having the length of the active phase of a few mm working basically as filters, and other being as long as 2 cm with 4.6 mm diameter. The guard columns usually have a frit facing the injector that can be removable. The internal i.d. of guard columns are typically selected comparable to that of the analytical column and designed to minimize backpressure. Shorter guard column length is preferable, but it should be long enough to prevent strongly retained compounds that should not reach the main column.

Packing of particles in the chromatographic column

Most common stationary phases in HPLC consist of small particles. These particles are placed in the body of the column making the *particle packed columns*. Monolithic chromatographic columns are also manufactured, in which the stationary phase consist of a porous solid rod [23].

Some capillary and nanoscale columns have the stationary phase coated on the tubing wall (wall coated open tubular WCOT) [24]. Capillary and nanoscale columns are used mainly in electrochromatography [25]. However, a much larger number of commercial columns are made using particles that are packed in the body of the column. A compact and uniform packing of the chromatographic column with the particles is important since it affects the column plate number N. When using high pressures with a specific column, the stationary phase should not change its volume. If the volume of the stationary phase shrinks, a void volume can be formed at the head of the column, affecting significantly the column plate number. A denser bed of the stationary phase may also affect the backpressure under which the column must be operated, influencing the chromatographic conditions. Also, for certain

types of stationary phases, the separation itself can be drastically degraded if the stationary phase collapses under inappropriate elution conditions. The collapsing of the stationary phase has two damaging effects. One is the creation at the head of the column of a void space which produces a significant broadening of the chromatographic peaks. The other effect is the reduction of microchannels in the stationary phase with further increase in the backpressure. This type of problems can be encountered in particular for some polymeric stationary phases such as those used in size exclusion chromatography (SEC). Changes in the packing of the stationary phase, or its degradation, may occur in time, when the performance of the chromatographic column degrades. The reproducibility of the results of the analyses becomes a problem in such situations.

Different procedures of packing the columns with the stationary phase are used, depending on the stationary phase. Most commonly, the stationary phase is introduced in the column with mirror-finished wall as a slurry in a specially selected liquid (e.g., chloroform, cyclohaxanol, i-propanol) that allows the formation of a homogeneous suspension of the stationary phase and hinders the particle aggregation. Slurry packing involves the use of high pressure (usually 50% higher than the maximum pressure at which the column will be used) to push a dilute slurry of stationary phase through the column [26]. Based on the packing procedures, it is common for most columns to have a required direction for the mobile phase flow. Slurry packing capillary columns for UHPLC is more complicated and requires the use of high slurry concentration and sonication during packing that create homogeneous bed microstructures and yield highly efficient capillary columns [27]. In the case of capillary columns, several packing procedures are available, but slurry packing is also utilized [28].

Physical characteristics of the solid supports for the packed columns

A variety of stationary phases are used in HPLC. The material making the stationary phase in the form of particles or monoliths may act by itself as the active phase, but more frequently, the stationary phase is made from a porous rigid support on which the active phase is applied (chemically bonded, grafted, or physically coated). The most common solid support for stationary phase is made from porous hydrated silica, but other materials are also used as support such as hydrated zirconia, titania, ceramic hydroxyapatite, or organic polymers. The physical characteristics of the solid support are important assuring the contact between the mobile phase and the stationary phase. An in-depth study of the role of stationary phase support [29] shows that by the optimization of the solid support (e.g., modifications of internal particle structure) the column efficiency can be significantly improved. The main physical characteristics of the solid support in the form of particles include the following: (1) type of particles, (2) the dimension of particles, (3) shape of the particles, (4) the size distribution of the particles, (5) surface area of the particles, (6) pore size of the particles, (7) mechanical rigidity of the support, and (8) other solid support characteristics. For the monoliths, the particle type and dimensions are not applicable characteristics, but other ones such as surface area and pore size remain similar.

(1) There are three main types of particles: (1) porous (fully porous), (2) core-shell (superficially porous), and (3) pellicular (nonporous). Porous particles have a porous structure for the entire particle. Core-shell particles (also known as fused-core or superficially porous microspheres) have a solid nonporous core surrounded by a porous outer shell. For a solid core particle of 1.7−3 μm diameter, the porous shell may have 0.3−0.5 μm in depth. Pellicular particles are solid nonporous spheres covered with a thin layer of stationary phase. They typically offer only a small amount of active stationary phase and their use is limited to very small sample loads. In some cases, pellicular particles are used to load the guard columns. Both fully porous and core-shell particles are common. Fully porous particles

have the potential to offer a larger load capacity, depending however on other particles characteristics On the other hand, core-shell particles offer the possibility of achieving a higher theoretical plate number since the differences in the path length of two different molecules in the particle are potentially smaller. As a result, the broadening of the chromatographic peaks can be smaller for core-shell substrate. A simplified diagram of the three types of particles and the hypothetical paths of two molecules in a fully porous, core-shell, and a nonporous particle is shown in Fig. 8.1.1. The difference in the path of different molecules is shown smaller in core-shell particles compared to the fully porous ones, and the path lengths are shown equal for pellicular particles [30]. Core-shell particles are more and more common and are offered under different trade names by different suppliers (e.g., Cortecs from Waters, Kinetex from Phenomenex, TeicoSell and VancoShell that are chiral columns from Azip, etc.).

(2) The dimension of stationary phase particles d_p is an important parameter related to the column theoretical plate number and the backpressure of the column. Columns typically have particle with diameters of 5, 3, 2.1, 1.8, and 1.7 μm. In HPLC analytical columns, other values of particle dimensions are available but less common such as 10 μm particles. As shown in Section 8.2, the dimension of particles (diameter d_p) is a critical parameter influencing the number of theoretical plates N and all related parameters, as well as the column backpressure.

(3) The shape of particles can be irregular of spherical (ellipsoidal shapes were also evaluated [31]). Significant effort has been involved in generating particles as close as possible to spherical form [32–34]. For spherical particles, it is possible to have a much more homogeneous

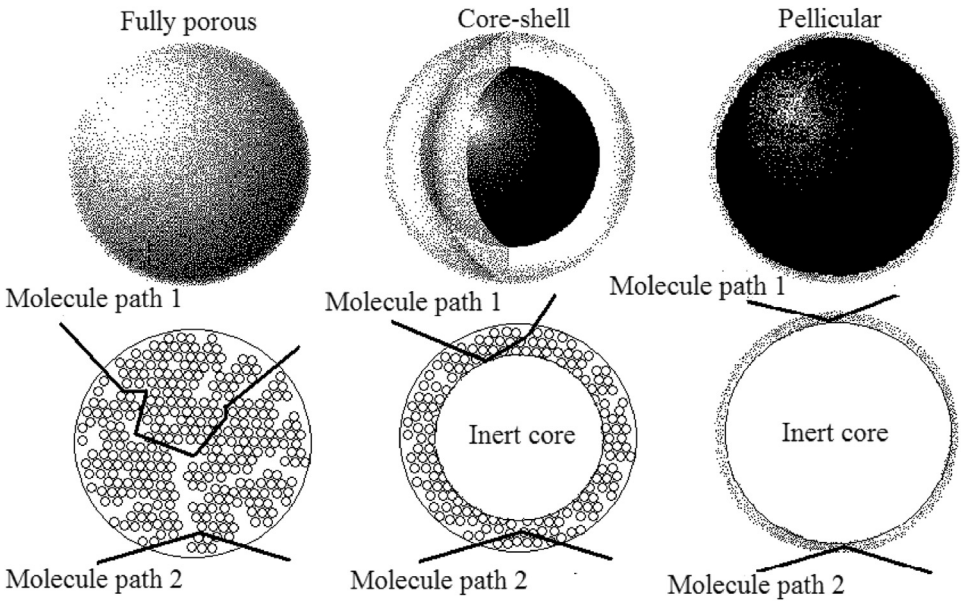


FIGURE 8.1.1 Schematic aspect of fully porous, core-shell, and pellicular (nonporous) particles. Hypothetical paths of two molecules in each particle type showing length differences are also illustrated.

stationary phase, and with less variation in the particle dimensions (less than 1%–2% difference in diameter). Several SEM pictures at different magnifications of the particles used in a C18 column with 5 μm diameter spherical particles are shown in Fig. 8.1.2.

(4) The uniformity of the dimensions of the particles is also an important physical characteristic for the solid particles that will lead to larger differences in the path within the solid particles of different molecules and consequently to peak broadening. The quality of

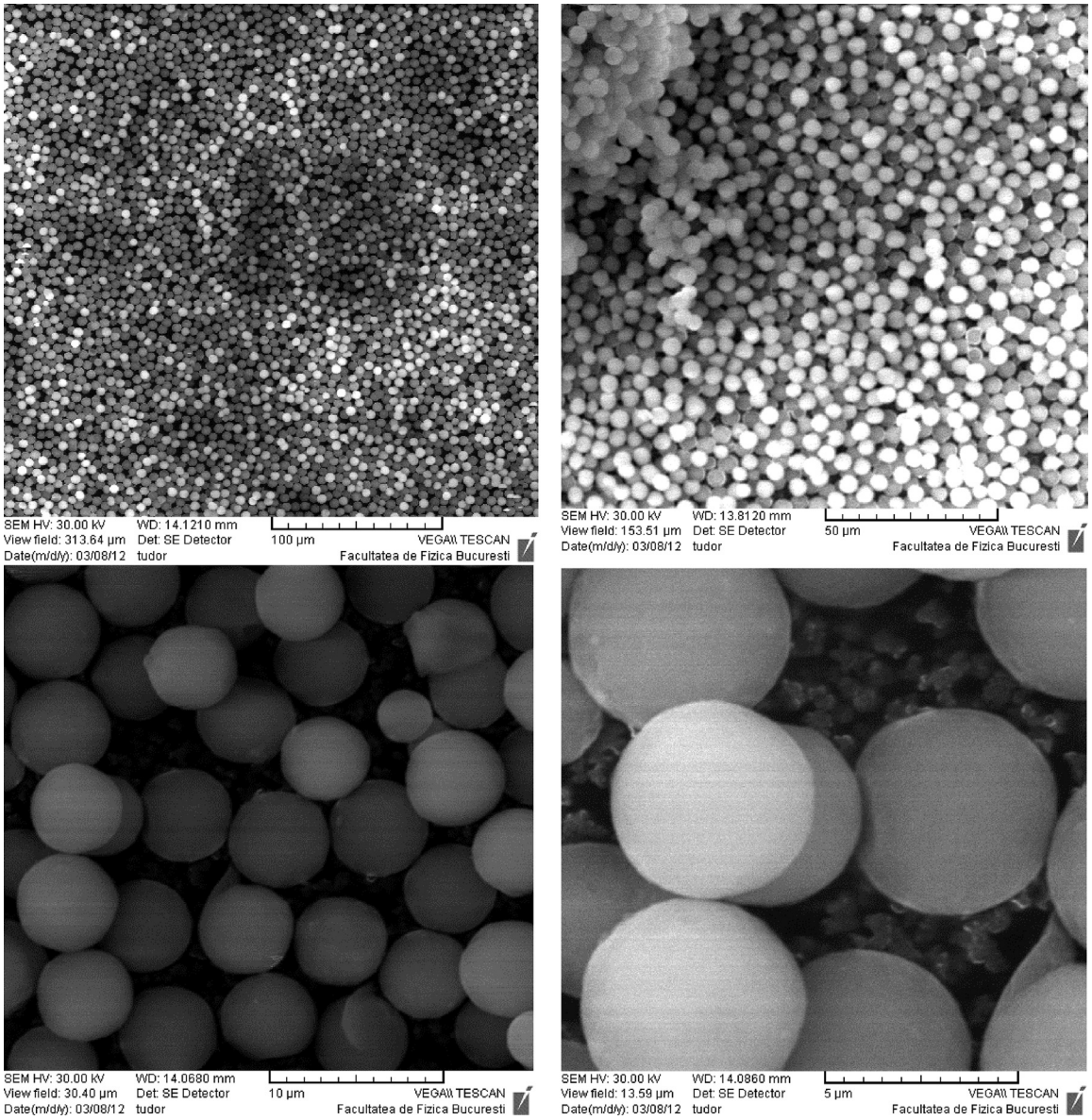


FIGURE 8.1.2 SEM pictures at different magnifications of 5 μm diameter particles used in a C18 column (the scale 100, 50, 10, and 5 μm is indicated on pictures).

the column regarding peak broadening is related to particle size homogeneity. Particle size distribution is typically described by the parameter d_{90}/d_{10}. The size of particles has ideally a Gaussian distribution. The value d_{10} indicates the particle size below which 10% of the material (by volume) is contained (same definition for d_{50} and d_{90}). The span of size distribution indicated as $(d_{90}-d_{10})/d_{50}$ indicates how far the 10% and 90% points are apart. Values for d_{90}/d_{10} lower than 1.2−1.3 indicates very good homogeneity. Values closer to $d_{90}/d_{10} = 1$ contribute to a higher theoretical plate number N (per column length) [35].

(5) Surface area of the stationary phase is a very important parameter regarding the column [36]. Since the active stationary phase is distributed on the surface of the solid support, a larger surface area is related to a larger amount of stationary phase for the same total volume of the space filled with mobile phase. For silica particles, for example, surface area varies between 100 m^2/g for low surface area particles, and 300 m^2/g for high surface area particles. The accessible surface area of RP stationary phase may reach 250 m^2/g when the mobile phase contains 5 or 10% methanol [37].

(6) The pore size (diameter) of the porous materials is also an important characteristic of the solid support. There are several classifications of pore size, but basically they are indicated as small (below 60 Å), medium (in the range 60−150 Å), and large (of about 300 Å or larger). The most common types used in HPLC are medium pore phases used for the separation of nonpolymeric molecules and large typically used for the separation of polymers (such as proteins). The pore size is important for being selected in agreement with the type of analyte to be separated on a column. For the separation of large molecules such as proteins, solid supports with large pores (300 Å or larger) are necessary in order to allow the intimate contact between the analyte and stationary phase. Pore size also plays an important role during silica derivatization through the differences in the reactivity of the −OH groups in small pores and in large pores, and also through steric effects. The pore volume is another solid support characteristic which is related to the pore size diameter [38].

In addition to the pore size, the porous support is also characterized by its tortuosity. Tortuosity τ is a parameter used to evaluate the shape of the channels (being tortuous) in the porous material and is related to the easiness of flow through stationary phase. A model for estimating tortuosity is the arc cord ratio where τ is taken as the ratio between the true length of a path and the distance between the ends of the path and can be determined using various experimental procedures [39]. Tortuosity is different between regular porous particles, core-shell particles, and monoliths [40].

(7) Structural rigidity of the solid support is an important parameter to be considered on a column. Inorganic supports such as hydrated silica or hydrated zirconia stand high pressures standing values between 9000 and 15,000 psi (600−1000 bar) without changes, depending also on the particle dimension. Specific high strength silica materials are used in the construction of some stationary phases (HSS technology). Other materials such as organic polymers are much more sensitive to higher pressure and specification of the columns must indicate the maximum acceptable pressure. Rigid organic polymeric particles typically stand pressure up to about 5000 psi, and soft gel columns must be used at even lower backpressures. Besides rigidity to high column backpressures, structural rigidity is also referred to the lack of volume variation when the solvent present in the mobile phase is changed. Inorganic supports for stationary phase keep a constant volume in different solvents, while organic polymeric phases swell and shrink

depending on the mobile phase solvents, these being a considerable disadvantage for the polymeric phases.

(8) Other aspects related to the solid support include the proper packing of the material in the body of the chromatographic column, the uniformity of network of channels, the absence of unintended fine particles, etc. The manufacture of highly efficient HPLC columns with precisely ordered morphology and uniform network of channels is utilizing 3D printers [41,42].

Chemical characteristics of the solid supports for the packed columns

The chromatographic columns are filled with the stationary phase consisting of particles or monoliths, and the particle being fully porous, core-shell, or pellicular. The stationary phase is usually made from a solid porous support on which the active phase is bonded, grafted, or coated. The solid support may be inert, but also may play a role in the separation. Regarding the guard columns, they should have preferably the same packing as the analytical column.

The chemical characteristics of the solid support include the following: (1) chemical nature of the support, (2) reactivity of the binding groups (e.g., −OH groups in case of silica or other hydrated oxides), (3) chemical resistance of the support to the mobile phase characteristics (e.g., pH), (4) chemical purity of the solid support, and (5) other chemical properties. These chemical aspects are further discussed in this section for each specific solid support.

(1) The most common material used as solid support for the particles in packed columns is porous hydrated silica ($SiO_2 \times H_2O$). Other hydrated oxides can be used as solid support for the stationary phase, such as hydrated zirconia ($ZrO_2 \times H_2O$), hydrated alumina ($Al_2O_3 \times H_2O$), etc., but their utilization is rather limited. Such materials offer a large surface covered with reactive groups (silanol or Si−OH in case of silica). The numerous reactive groups (Si−OH or Metal-OH) on the surface may act as active phase (bare silica can be used as a polar phase) but more frequently they are used for derivatization for covering of the solid support with the bonded stationary phase. This is done following chemical reactions that attach organic moieties on the solid surface. At the same time, these materials have a high rigidity and resilience to crashing and do not change their volume when placed in different solvents. Porous polymers are also used as support for stationary phases, but they may act as both support and active phase. Some other materials used as stationary phase act as both support and active phase, including metal organic frameworks [43−45].

(2) The reactive groups on the solid support play an important role in generating the active stationary phase. These groups are characterized by their nature and acidic/basic character, and related to that is their reactivity. Most inorganic supports have −OH groups as reactive sites. The density of the active groups versus that of backbone structure (e.g., −O−Si−O− groups) is an important chemical characteristic of the solid support (see Eq. 8.1.7).

Porous polymers are used frequently as stationary phases for SEC and some for ion exchange chromatography. For SEC, the polymers do not need specific functionalities, and the main characteristic of those phases is their tridimensional structure. For ion exchange purposes, ionic groups are covalently bonded in the body of the polymer. Some polymers are also used for stationary phase in reversed phase chromatography, but they are less resilient to high pressure than phases with inorganic supports and at higher pressure the phase may collapse.

(3) The solid support is affected by the acid or basic character of the mobile phase, as well as the potential, the nature of solvents making the mobile phase. For example, hydrated silica is resistant to acid and bases only in the range of pH between 2 and 8. Mobile phases with a lower or higher pH will lead to the damage of the chromatographic column. To improve the utilization range, several modifications of the silica were made, allowing the extension of this range. One such modification is the embedding of organic moieties within the structure of the solid support as achieved in ethylene-bridged structures (see reaction 8.1.6). Other procedures such as controlled surface charge procedure (CSH technology) are also used for this purpose [46]. The supports based on organic polymers are resistant to a wider range of pH, but they may have problems with incompatibility with specific solvents.

(4) The purity of the solid support is related mainly to the elimination from the hydrated SiO_2, or other support material of traces of metals such as iron or aluminum. The presence of transitional ions, for example in silica, may lead to various interactions with the analytes, through the *d* electrons of the metal that will superpose over the intended interactions (e.g., hydrophobic interactions with the active stationary phase). Even the presence of other metals in the silica structure may affect the types of interaction such as increase in ionic interactions with the analytes from the mobile phase. For example, the presence of Fe^{3+} and Al^{3+} in the silica matrix enhances the acidity of silanols that are in their close proximity, increasing the silanols ion-exchanging ability. These additional interactions lead to peak broadening and tailing.

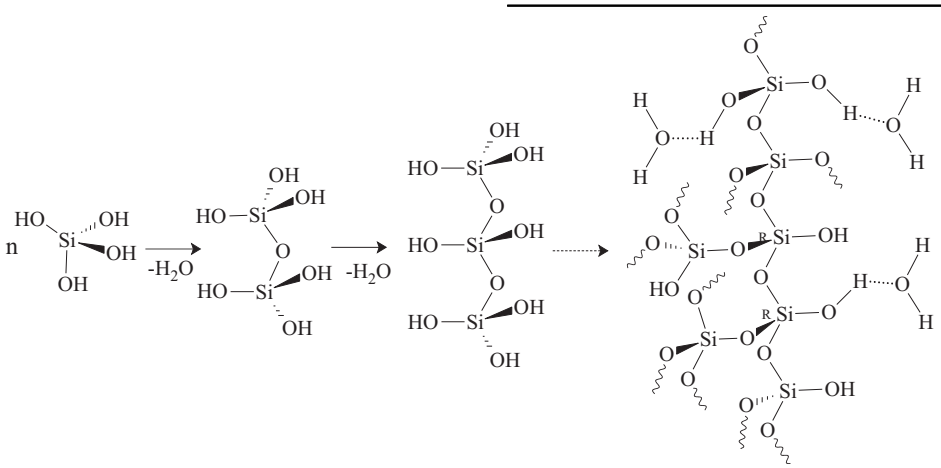
(5) Other chemical properties are related, for example, to other types of interactions with the analytes besides the one intended by the user. For example, for silica supports, many silanol groups remain on the silica support surface even after derivatization and *end-capping* (reaction of free silanol groups with blocking reagents). These silanol groups may be more or less acidic and may have interactions with the analytes. Also, in case of other hydrated oxides (such as hydrated ZrO_2), zirconium atoms from the solid backbone may interact with the analytes through its electrons from the d orbital.

Besides the reaction of the reactive groups with specific reagents for producing a bonded stationary phase, other procedures can be utilized to generate an active stationary phase. These procedures include coating or immobilizing a polymeric material on the solid support such as silica. The goal is to take advantage of the porosity of the material which has a large surface area, mechanical strength, and pores of appropriate dimensions, and at the same time to have a stationary phase of a different nature than the solid support for acting as a stationary phase.

Silica and ethylene-bridged silica as solid support for the stationary phase

Silica-based stationary phases are made using hydrated porous silica particles obtained from a chemical reaction that generates silicic acids (typically a controlled hydrolysis of an alkoxysilane or of a silicic acid salt). In extremely diluted solutions, several simple silicic acids were identified, such as metasilicic acid (H_2SiO_3), orthosilicic acid (H_4SiO_4), disilicic acid ($H_2Si_2O_5$), and pyrosilicic acid ($H_6Si_2O_7$). All these acids are very weak acids (e.g., orthosilicic acid has $pK_1 = 9.84$, $pK_2 = 13.2$). In more concentrated solutions, polysilicic acids with the general formula $[SiO_x(OH)_{4-2x}]_n$ are rapidly formed from the condensation reaction between the silanol groups that give rise to tridimensional

structures (precipitates in hydrated gel form) containing siloxane (Si—O—Si) groups. This is schematically shown in reaction 8.1.1.



(8.1.1)

The reaction of formation of silicic acid may start with acidic hydrolysis of salts such as Na_2SiO_3, Na_4SiO_4, K_4SiO_4, etc., or with the hydrolysis in the presence of an acid or a base (as catalysts) of several silicon compounds like SiH_4, $SiCl_4$, or of alkoxysilanes ($Si(OR)_4$ where $R = CH_3$, C_2H_5, C_3H_7, etc.). Tetraethyl orthosilicate $Si(OC_2H_5)_4$ is a common alkoxysilane used for the preparation of silica gels by hydrolysis. The following reactions schematically describe the formation of polysilicic acids from an alkoxysilane:

$$Si(OR)_4 + 4\ H_2O + H^+ \rightarrow Si(OH)_4 + 4\ ROH + H^+ \quad (8.1.2)$$

$$n\ Si(OH)_4 \rightarrow Si_n(O)_x(OH)_{4n-2x} + x\ H_2O \quad (8.1.3)$$

When the polysilicic acid is obtained by hydrolysis of alkoxysilanes, part of the alkoxy groups may remain attached to the silicon atom and further participate in the elimination reaction to form siloxane bonds. A partial hydrolysis of an alkoxysilane can be described schematically by the following reaction:

$$Si(OR)_4 + 3\ H_2O + H^+ \rightarrow Si(OR)(OH)_3 + 3\ ROH + H^+ \quad (8.1.4)$$

Polysilicic acids formed by hydrolysis are initially present in solution form (sol form) and a large number of water molecules are bound through hydrogen bonds or mechanically retained in the structure. The choice of the reactions and of the conditions used to obtain silica gels varies considerably depending on the intended use of a specific material [47,48]. As the molecules of polysilicic acid increase in size, the sol particles are growing and further changing in a system containing both a liquid phase and a solid phase with a morphology ranging from discrete particles to a continuous polymeric network filled with a large number of pores of different dimensions. This material generates a gel (hydrogel or alcogel depending on the groups still left unreacted) where most of the structures are cross-linked. The distinction between a material in sol form and that of a gel is not precise, and as the amount of water in the structure decreases and the polymeric network increases, the gel form becomes more obvious. The two processes taking place during the gel formation are the growth of sol particles and

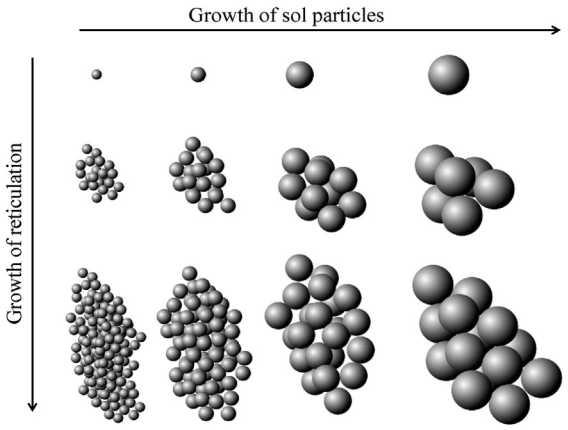


FIGURE 8.1.3 Schematic diagram of the two processes taking place during the gel formation: the growth of sol particles and the reticulation of the gel.

the reticulation of the gel. These processes are schematically pictured in Fig. 8.1.3.

Sol particles can grow isolated from each other, having forms close to small spheres. This capability allows the formation of particles with a controlled shape and size, amenable to be used for chromatography. The control of the size of sol particles and the elimination of water with the increase in the cross-linking of the polymeric structure can be achieved by selecting the concentration of reagents that form the polysilicic acid and also by controlling other parameters such as temperature, pH, gelling time, addition of electrolytes for flocculation, addition of detergents, specific solvents, etc. The process is very complex, and as a function of selected parameters, the growth of sol particles or of the reticulation can be favored, such that the properties of the final material may be different. The structure of the gel continues to change in a process known as aging. Aging affects the properties of the final material by several mechanisms known as polycondensation (further reactions of silanol groups to form siloxane bonds), syneresis or pore narrowing (the shrinkage of the gel network with the elimination of liquid from the pores), and coursing (the dissolution of small particles and the growth of larger ones).

Superficially porous particles are typically manufactured by adding solid silica cores during the sol formation. The hydrated silica is growing on the cores and further changing in a system containing a continuous polymeric network filled with a large number of pores of different dimensions. The pore sizes are mainly controlled by the size of the silica nanoparticles and the tortuous pore channel geometry is determined by how the nanoparticles randomly aggregate. A new process indicated as pseudomorphic transformation has been recently reported [49]. In this process, the superficially porous particles are made with a narrower particle size distribution, thinner porous layer, high surface area, and nontortuous pore channels oriented normal to the particle surface.

Following the gelling and aging process, the hydrogel (or alcogel) is typically washed and it is dried (converted into a xerogel) for obtaining a solid material. A silica gel, as first precipitated from water, may contain up to 300 mol of H_2O to 1 mol of SiO_2. The drying process consists of three stages. In the first stage, the gel loses water and its volume decreases with the volume of water that was evaporated. In this stage, the pore volume decreases and the stiffness of the dried gel increases. In stage two, the liquid from the pores of the gel is eliminated by migration to the surface of the material where it is evaporated. In stage three, the liquid escapes from the pores directly by evaporation. When dried below 150°C, surfaces containing a large number of silanol groups (≡Si−OH) are still present in the gel. When heated at 300 to 1000°C, the surface covered with silanol groups dehydroxylates to form siloxane (Si−O−Si) surfaces. The final properties of the dried material, such as density (porosity), hardness, active surface area, pore volume, and pore size distribution, depend on the initial hydrogel structure but also on the rate and temperature of drying.

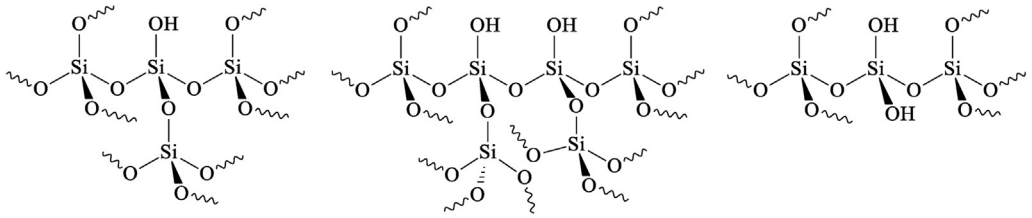


FIGURE 8.1.4 Schematic formulas for isolated, vicinal, and germinal silanols (〰is not 〰H).

The number of silanol groups (silanol density) on the material active surface is determined by the same parameters. The xerogel pores contain some water that is retained by strong adsorption forces. The silanol groups have weak acidic properties, which are slightly different from group to group depending on the fact that the groups are isolated, vicinal, or geminal. Schematic formulas for different types of silanols are shown in Fig. 8.1.4.

Silanol groups present at the surface of the silica particle play a major role in the use of silica gel as a solid support for the stationary phase in chromatography. Bare amorphous silica can be used in direct phase chromatography where a layer of water molecules adsorbed on the solid surface acts as a stationary phase. The silanol groups are active in the separation process, influencing this layer of adsorbed water. An adsorption equilibrium on the silanol groups is also postulated as a potential separation type. However, the main utilization of silica xerogels is related to the role of silanol groups as the place where other structural groups are introduced by derivatization in order to obtain modified silica for reversed-phase, chiral, hydrophilic, ionic exchange, and other type of stationary phases. The main property used for these derivatizations is the acidic character of −OH groups, which allows them to react with different derivatization reagents. Different silica gels have various surface characteristics regarding the density of silanol groups, and these can be derivatized to get attached functionalities to the surface. In general, a higher concentration of reactive hydroxyl groups is present in smaller pore silicas, typically with a pore diameter less than 50 Å, whereas less reactive hydroxyls predominate on larger pore silicas having pore diameters greater than 150 Å.

The pores considered for differentiating silicas are sometimes indicated as *mesopores*. Larger pores of 10−20 μm forming channels (through pores) with larger dimensions can also be present in silicas but they are in a small proportion. A common classification of silica gels is based on their average pore diameter and this differentiates Type A (fine pores), Type B (average pores), and Type C (large pores). The main characteristics of these silicas are given in Table 8.1.1.

TABLE 8.1.1 Typical classification of silica gels based on their pore size.

Property	Fine pores	Average pores (common in HPLC)	Large pore
Aspect	Transparent	Semitransparent	Milky
Average pore diameter nm	2.0−3.0	4.0−10.0	80−125
Pore volume mL/g	0.35−0.45	0.6−0.9	0.7−1.0
Surface area m^2/g	650−800	450−700	300−400

Silicas with characteristic values outside those indicated in Table 8.1.1 are also utilized in practice, and they can be still classified in the same types depending on the closeness of their characteristics to those indicated in the table. Type B (pore size) silicas are the most commonly used in HPLC, but Type A and Type C silicas are also used for specific purposes. The silicas for chromatographic use are available under various trade names (Davisil, Astrosil, Lichrospher, Kromasil, etc.).

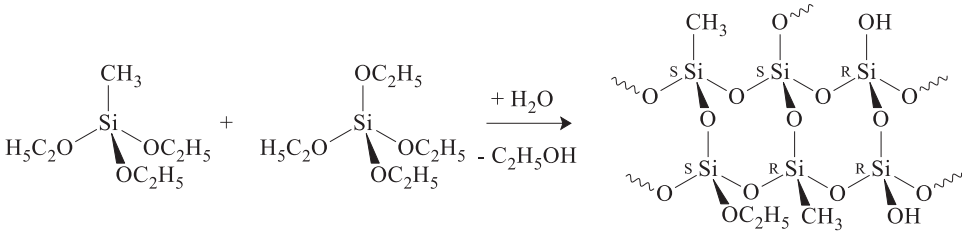
Other classifications of commercially available silica gels are also known (e.g., Davisil grades indicated in Table 8.1.2) that differentiate silicas based on both their pore size (22, 30, 60, and 150 Å) as well as on the size of particles.

Another important characteristic of silica solid supports is the purity. As previously indicated, traces of metallic ions such as Al^{3+}, Fe^{3+}, and Ni^{2+} are undesirable in the hydrated silica. For avoiding the impurity problem, very high purity silica has been produced, with a less acidic surface, and a more homogeneous distribution of silanol groups. This type of silica is indicated as *Type B*, while lower purity silica that has a higher metal content and more acidity is termed *Type A* silica.

TABLE 8.1.2 Classification of several high purity silicas using "Grade" description.

Grade (Davisil)	Pore size Å	Particle size mesh
633	60	200–425
635	60	60–100
636	60	35–60
643	150	200–425
646	150	35–60
12	22	28–200
62	150	60–200
923	30	100–200

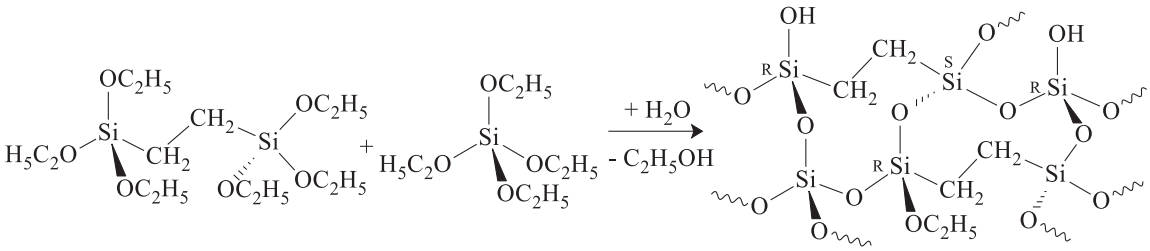
The pH range where typical silica is stable is between pH = 2 and 8. The pH values of the mobile phase outside this range are damaging common stationary phases with silica support. For this reason, a number of attempts were made to enlarge the range of pH stability. One such attempt is based on the use of hybrid organic–inorganic materials still based on a hydrated silica but also containing small organic groups that provide some protection to the access of mobile phase (excess of OH^- or H^+ ions) to the backbone of the stationary phase. The result is a silica base material that remains amenable to the derivatization necessary to make a bonded phase, but also having attached some organic fragments. One such material is obtained using the type of reaction 8.1.5:



(8.1.5)

Materials with even better stability than those having CH_3 groups in the silica were obtained using ethylene-bridged structures (indicated as BEH technology by Waters or TWIN technology or EVO by Phenomenex) [50] or propylene-bridged materials [51]. This type of silica-based support is prepared in two steps. In the first step, a polyethoxy-siloxane oil phase is prepared from hydrolytic condensation in acidic conditions of bis(triethoxysilyl)ethane and tetraethoxysilane

(TEOS) using a small amount of water. Highly spherical porous hybrid particles are then prepared by further condensation under alkaline conditions in an oil-in-water emulsion. The first step of these reactions can be schematically written as follows (reaction 8.1.6):



(8.1.6)

The resulting polyethoxysilane generates after further hydrolysis the desired material that can be derivatized to form the bonded phase. Once obtained, the silica-based organic/inorganic material is subjected to surface-ripening steps similar to those used for silica. Significant improvements at both low pH and high pH (some columns only up to pH = 8 such as for Acquity UPLC HSS T3 from Waters, but some up to pH = 12.0 such as Gemini NX-C18 from Phenomenex) were reported for stationary phases obtained with organic/inorganic phases. Hydrophobic columns (C18 or C8) obtained by the derivatization of silica with ethylene-bridged phases also have better behavior regarding wettability.

Combination of inorganic support with organic-bound groups capable of further polymerization can also be achieved by reacting the silica surface with reagents such as vinyltriethoxysilane or methacryloxypropyltriethoxysilane. In this case, a reactive group capable of further polymerization is attached to the silica surface, allowing a better shielding of the possible free silanol groups from the silica surface. Other procedures for covering the silica base material with an organic/inorganic layer are utilized for providing support materials with wide range of pH stability (e.g., in Kromasil Eternity columns that have an extended pH range up to 12).

Other modifications of the silica supports were reported, including a controlled surface charge procedure (CSH technology) [52]. This technology takes advantage that the silica surface is usually slightly negatively charged due to the dissociation of silanols. This charge can be neutralized by adding specific reagents such that the surface reactivity is decreased.

The silanol groups from the silica surface play an essential role in making the silica support amenable for binding the stationary phase. The amount of silanol groups on silica surface varies depending on silica preparation, but values in the range 7–8 μmol/m^2 are common. Related to the amount of silica, the amount of silanol groups δ_{OH} (in mmol/g) is in the range of 3–7 mmol OH groups per g of SiO_2. The number of OH groups per unit mass of silica depends linearly on surface area of the material following an expression of the following form [53–55]:

$$\alpha_{OH} = 602.214 \frac{\delta_{OH}}{S_{surf}} \qquad (8.1.7)$$

In Eq. 8.1.7, S_{surf} is the surface in m^2/g and α_{OH} indicated as *silanol density* is number of OH groups per unit surface area (nm^2). The value of α_{OH} was found to vary between 4.1 and 5.6 OH groups per nm^2 (with an average of 4.9 OH groups per nm^2) for the silica used as support in HPLC.

The binding of desired functionalities to the silica surface is usually performed using derivatization reactions and the derivatization can be done using a variety of reagents, depending on the nature of the desired type of stationary phase. After binding the desired moieties on the silica surface, a large number of unreacted −OH groups (silanol groups) still remain present on the silica surface. These groups may not be desirable, for example, when a totally hydrophobic stationary phase is desired. For this reason, several procedures were developed to react with the remaining active silanol groups, after the initial derivatization. The most common procedure to "block" the remaining silanol groups is the reaction indicated as end-capping. The end-capping is achieved by blocking as many as possible from the remaining −OH groups with small hydrophobic moieties such as $-Si(CH_3)_3$. The attachment of large fragments such as octadecyl to the silanol groups has a limited yield due to steric hindrances, while the attachment of small groups such as $-CH_3$ is more efficient.

Silica-based monolithic chromatographic columns

Monolithic stationary phases are made from a single piece (rod) of a solid porous material. Modified silica rods are probably the most common type of monolithic columns, but organic polymers are also used as monoliths. The monolithic rods are prepared by a polymerization or polycondensation process, either in situ in a column tube, such as in glass tubes or fused silica capillaries, or in a column mold. In situ preparation of monolithic columns has the advantage that no further encapsulation of the porous monolith in a tube resistant to the pressure of the solvent is needed. However, this approach is not compatible with monolithic silica columns having larger diameters (4.6 mm or more) due to the shrinkage which occurs during the sol-gel preparation process. When the monolithic silica rod is made in a mold, it has to be further clad with a suitable material such as PEEK (polyetheretherketone), to which the column end fittings can be attached for use in the HPLC process [56].

For the production of silica-based monoliths, the basic sol-gel process is similar to the preparation of solid supports for porous silica materials. The process is conducted as a sequential hydrolysis followed by a polycondensation of silane derivatives. Similar to the preparation of porous silica particles, either hydrogels or alcogels can be obtained. Used for this purpose are compounds such as TEOS or tetramethoxysilane (TMOS) in aqueous acid or basic medium with an appropriate porogenic solvent (e.g., polyethyleneglycol). The hydrolysis conditions and the choice of the porogenic solvent are essential for obtaining mesopores and macropores [57,58]. Monoliths having the surface covered with the desired functionalities can be directly obtained by the hydrolysis of the appropriate compounds such as trimethoxyoctylsilane or trimethoxyoctadecylsilane.

Monoliths have a porous structure characterized by mesopores (pores between 2 and 50 nm in diameter) and macropores (about 4000 to 20,000 nm in diameter), with silica skeleton of approximately 1−2 μm thick, and a void volume of almost 80% of the entire column volume. These pores provide monoliths with high permeability, a large number of channels (the macropores are typically interconnected, reason for which they are also indicated as through pores), and a high surface area (generated mainly by mesopores). The backbone of the monolithic column can easily be chemically derivatized for specific applications. Fig. 8.1.5 shows the SEM picture of the typical porous structure of a monolithic silica column.

Monolithic columns show an efficiency equivalent to 3−5 μm i.d. silica particles, but with a 30%−40% lower pressure drop. The flow through monolithic channels is closer to laminar, and thus they allow less eddy diffusion. The transport of an analyte through the monolithic bed is based mainly on perfusion, and in the monolithic media with a low proportion of mesopores, the analyte

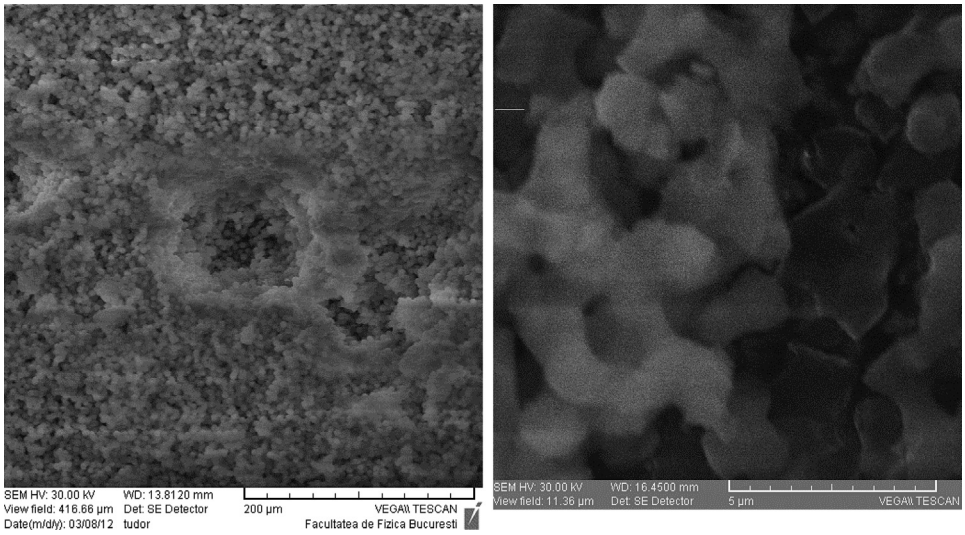


FIGURE 8.1.5 SEM picture of the typical porous structure in monolithic silica columns.

diffusion into and out of the pores does not significantly contribute to the band broadening. Due to the reduced diffusion in solute transfer through the monolithic bed, the peak efficiency does not decrease as sharply as in particle-packed columns when increasing linear flow rate of the mobile phase. This allows the application of higher flow rates and shorter analysis time, without a significant loss of plate numbers [59–61]. Monoliths having the surface covered with the desired functionalities can be obtained not only by derivatization of a silica monolith, but also by the hydrolysis of the appropriate compounds containing a desired group, such as trimethoxyoctylsilane or trimethoxyoctadecylsilane.

Core-shell particles in packed columns

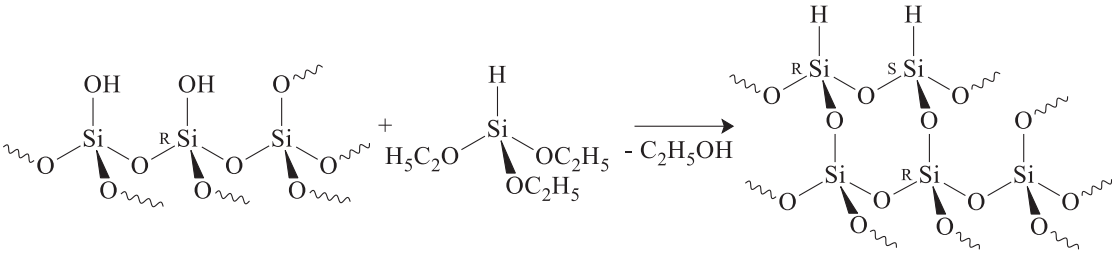
A number of procedures are used to obtain core-shell particles [62]. The core and the shell can be made of different materials although silica is the most commonly used for both the core and the shell, with the difference that the core is made of fused silica, while the shell is made from porous silica [63]. The core is usually a single sphere, but it can be an aggregation of several small spheres. The size of the core particle, the shell thickness, and the porosity in the shell are tuned in order to suit different types of chromatographic applications [64]. The particles can be synthesized by a two-step or by a multiple-step process. The core particles are synthesized first (e.g., as uniformly sized fused silica microspheres) and the shell is then added based on an electrostatic attraction procedure [65]. The thickness of the shell can be controlled by the number of deposition cycles that are applied to the core microspheres [64].

The porosity of the shell can vary, and medium size porosity (80–100 Å) utilized for the separation of nonpolymeric molecules [66], as well as larger size porosity (150–300 Å) used for separating larger molecules such as peptides and small proteins ($Mw < 15$ kDa) are available [67,68]. Larger superficially porous particles with a pore size of 400 Å or more allow large molecules ($Mw < 500$ kDa) unrestricted access to the phase and can be selected for protein separations [69,70].

Hydride-based silica

The presence of silanol groups on the surface of silica-based stationary phases even after

derivatization and end-capping prompted continuous effort to generate new stationary phases that do not have this problem. One suggested solution to the problem is the use of hydride silica as support for further derivatization [71]. Silica hydride support material is sometimes indicated as Type C silica. The basic chemical reaction for the synthesis process of hydride silica is the following (reaction 8.1.8):



(8.1.8)

Other procedures for the synthesis of silica hydride materials are known, such as reduction with $LiAlH_4$ of silica having chlorine bonded groups [71].

The presence of Si—H on the surface of the silica skeleton changes the polarity and the dimension of water layer adsorbed on it. It has been shown that the surface of hydride silica may form a very thin layer partially interrupted (average less than 0.5 water molecules), in contrast to bare silica that can adsorb 3—10 layers of water molecules [72]. Also, it has been shown that the Si—H group on the silica surface is stable in aqueous solutions in the range of pH from about 2 up to about 9, and it is not hydrolyzable as would be a small molecule of silane [73]. Unbonded silica hydride can be used as a normal phase (similar to a bare silica material), but further derivatization of the silica surface can be done using hydrosilylation reactions for the attachment of desired bonded phases. The silica hydride surface can be populated with hydride material up to 95% [74]. A number of studies were published in the literature regarding the preparation and the use of silica hydride stationary phases [75—77]. Silica surface covered with Si—H groups is sometimes indicated as *Type C* silica [78] and confusion with pore size Type C (large pores) should not be made. Hydride silica is also used for the monolithic columns [79]. Diamond hydride columns (for example, ANP Cogent Diamond Hydride column) are based on silica hydride support, by chemically bonding a small amount of carbon (up to 2%), which confers the surface a hydrophobic property.

Other inorganic support materials

Other inorganic materials used as solid support for stationary phases in HPLC are zirconia and titania (hydrated). Zirconium dioxide (ZrO_2), also known as zirconia, may exist in several crystallographic and amorphous forms and possesses both acidic and basic properties (amphoteric properties). Some physical properties (mechanical stability, high surface area, control of average pore diameter, control of particle diameter, swelling capability) as well as chemical properties (chemical stability over a large pH range 1—14) recommend this compound as substrate for stationary phases in HPLC. ZrO_2 can be prepared by precipitation from zirconium salts, zirconyl salts ($ZrOX_2$), or zirconium

alkoxides. The method of precipitation, pH, temperature, and the other parameters discussed at the formation of silica xerogels also influence the formation of zirconia xerogels that may consist of small (2–25 μm) spherical and rigid particles. Sol-gel–derived zirconia xerogels prepared via the hydrolysis of zirconium n-propoxide in methanol, ethanol, or 2-propanol are one of the most common approaches to prepare this support [80]. Other procedures to prepare zirconia supports can use zirconium oxide that is further treated with a base for hydration and reprecipitated in the presence of a surfactant and in specific physical conditions (temperature, stirring) that allow control of the particle properties. It is also possible to embed hydrated zirconium oxide in colloidal form in a mass of an organic polymer that is further destroyed by combustion.

The surface of zirconia xerogels is formed from Zr atoms bound to O atoms and also to OH groups, similar to the case of amorphous silica surfaces. The average concentration of Zr atoms on zirconia surface is about 12 μmol/m^2. The surface is covered with hydroxyl groups in a concentration of about 20–25 μmol/m^2 and is the site for further reactions when the derivatization is desired. The specific surface area of zirconia supports is dependent on the thermal treatment in the drying process, and this area decreases sharply when the drying is performed at temperatures higher than 300°C. Above this temperature, two processes are responsible for the decrease of area surface: microcrystallite growth and intercrystallite sintering. Temperature treatment also influences the porous structure of zirconia. When subjected to heat treatment of 150–300°C, a transitionally microporous material is obtained, at temperatures of 300–600°C, a transitionally macroporous material is obtained, and above 700°C, macroporous adsorbents with pores of 20–500 Å are obtained. These materials obtained at higher temperatures do not have enough OH groups necessary for derivatization and surface modification. Generally, the pore volume of various zirconia-based materials is much lower than that of silica materials, which makes silica to remain the preferred solid support used in manufacturing stationary phases for HPLC.

For the use of HPLC support, zirconia stationary phases pose a number of challenges that are different from those of silica. The OH groups bound to zirconium atom are much less acidic as compared to the silanol groups, although with the capability to act as a Brønsted acid. The lower acidity of zirconia makes this phase resilient to a wider pH range, between pH = 1 and pH = 10. Zirconia surface may also act as a Brønsted base, and also as a Lewis acid due to the d electrons of zirconium atoms that can interact with various ligands. The idealized surface of zirconia surface is suggested in Fig. 8.1.6:

Besides the behavior determined by the substituents attached to the zirconia surface, for example, that of a reverse phase when the surface is covered with hydrophobic substituents (e.g., C18), zirconia also exhibits polar and ionic interactions. For this reason, deactivation of zirconia surface, in particular the elimination of Lewis acid sites using a chelating agent, is applied to the newly developed zirconia stationary phases. The pore structure of zirconia is also somewhat different from that of silica, and leads

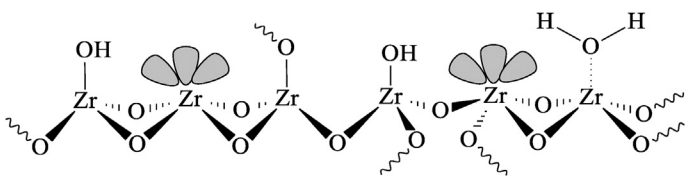


FIGURE 8.1.6 Idealized structure of hydrated zirconia surface.

in general to lower chromatographic performance compared to silica. Phases coated on zirconia with polymers are also known.

Similar to hydrated zirconium dioxide, hydrated titanium dioxide ($TiO_2 \times H_2O$) or titania can be used as solid support for stationary phases in HPLC [81]. Titania has hydroxyl groups on its surface that are only slightly acidic so that this can be used to separate basic molecules under normal-phase conditions [82]. Titania microspheres can be synthesized by hydrothermal methods and used as HPLC stationary phase [83] or can be used as a support for attaching hydrophobic moieties to the hydroxyl groups [84]. The preparation and use of titania monolithic columns was also reported [85].

There are also attempts to use other materials as solid support, which would have higher stability to high pH and to heating. Among these materials are Al_2O_3 (alumina) and also ThO_2 and CeO_2, although ThO_2 and CeO_2 have been studied, but columns made with them are not commercially available. The surface of alumina and its corresponding chemistry is more complex than silica. The basic spinel structure of Al_2O_3 often possesses defects that result in various arrangements of aluminum ions. Therefore, the hydration of its surface as well as the number of hydroxyl groups per unit area is determined by the specific three-dimensional structure of the oxide [86]. The advantage of Al_2O_3-based stationary phases is their stability up to pH = 12. Al_2O_3 is mostly used as support for phases containing Al_2O_3 covered on the surface with a polymeric layer (e.g., polystyrene–divinylbenzene copolymer). These columns are more stable in acidic medium, but they pose problems during the analysis of acidic compounds due to the mixed types of interactions they offer, one with the polymer and the other with the alumina surface. Alumina support was also evaluated for reaction with triethoxysilane (catalyzed by HCl) to obtain a silica hydride covered alumina.

Among other inorganic compounds that are used in chromatography are specific materials that have ion exchange properties. These materials include several groups of natural silicates such as zeolites. Zeolites are hydrated aluminosilicates with the general formula $M_xO \cdot Al_2O_3 \cdot nSiO_2 \cdot mH_2O$, where M is Na, K, Ca, etc. These compounds act as selective cation exchangers, the exchange process being associated with a molecular sieving mechanism. Several hydrated oxides also act as cation exchangers. Among these are hydrated oxides of Si, Al, Zr, Fe, Sn, etc. These hydrated oxides have both adsorbing and ion exchange properties. Other inorganic compounds, such as polymeric hydrated zirconium phosphate, hydroxyapatite, etc., also have ion exchange properties. Ceramic hydroxyapatite has been used in particular for protein separations [87,88].

Porous graphitic carbon and other carbon-based materials

Porous graphitic carbon is obtained by decomposing organic matrices typically using silica as a template, but also using some other procedures [89]. For example, the silica used as template is impregnated with a mixture of phenol and formaldehyde and then heated to 80–160°C to initiate the polycondensation. The characteristics of the silica gel as a template material determine the size and porosity of the particles that will be obtained. The polymer is then pyrolyzed under inert atmosphere (N_2) at 1000°C. Thus, highly porous amorphous carbon is produced. This carbon corresponds to what is normally called carbon black. After this step, the silica template is dissolved with a hot aqueous NaOH solution. The graphitization is realized through a thermal treatment at high temperature (about 2300°C) under inert atmosphere (Ar). This operation eliminates certain surface attached functional groups, produces a structural rearrangement of C atoms, and removes the micropores. After cooling down to 1000°C,

TABLE 8.1.3 Some properties of graphitic carbon.

Property	Characteristic
Particle shape	Spherical 3, 5, or 7 μm diameter
Porosity type	Porous 70%–75%
Specific surface area	Higher than 100 m^2/g
Pore volume	~0.7 m^3/g
Average pore diameter	~25 nm
Carbon load	100%
Mechanical strength	>400 bar

the replacement of argon by hydrogen can induce reactions between hydrogen and free radicals or functional groups still present at the carbon surface. By deactivating, the PGC surface becomes more uniform. The result is porous graphitic carbon, which has been commercialized (trade name of Hypercarb) [90]. Some physical properties of PGC are given in Table 8.1.3 [91].

An alternative to porous graphitic carbon are the phases made of carbon–clad zirconia or titania. This type of material can be produced either by high temperature graphitization of organic polymers covering the zirconia or titania support or by chemical vapor deposition of carbon on zirconia. However, in practice, the surface of this type of stationary phase is not perfectly homogeneous, and a significant proportion of the surface of zirconia still remains uncovered by carbon, thus creating highly acidic residual zirconia groups [92]. Other carbon-based materials such as carbon nanotubes, fullerenes, and nanodiamonds were also evaluated as stationary phase materials for HPLC [93].

Organic polymers used as support for stationary phases

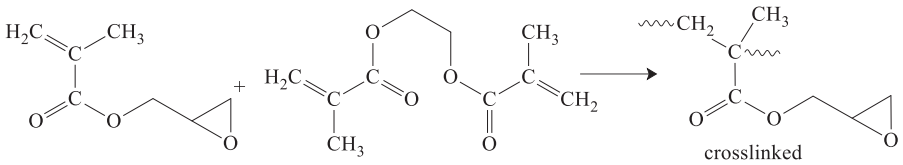
Porous polymers are used frequently as stationary phases for SEC and some for ion exchange chromatography. Some polymers were also used for reversed phase chromatography. For SEC, the polymers do not need specific functionalities, and the main characteristic of those phases is their tridimensional structure. Perfusion particles usually contain very large pores (e.g., 600–1000 nm diameter) connected to a network of smaller pores (30–100 nm diameter). The flow of mobile phase through a column filled with perfusion particles takes place through the particles and participates to a diffusion process. Polymers with controlled pore dimensions are frequently used as stationary phase for SEC. Also, the size of the polymer particles is important to control (similar to the case of silica particles). This can be achieved using monosized polymer seed particles [58].

One of the most common polymeric supports used as stationary phase is polystyrene cross-linked with divinylbenzene (PS-DVB) [94]. This material is obtained by suspension polymerization using a two-phase organic/aqueous system. The cross-linking polymerization is performed in the presence of inert diluents that are miscible with the starting monomers but must not dissolve in the aqueous phase. Submicrometer particles (microbeads) form as the styrene-divinylbenzene polymerizes and precipitates out of solution. The formed microbeads fuse together to form macroporous particles. Initially, a network of microporosity may be present in the microbeads, and polymerization conditions must be controlled to minimize this type of porosity because it results in a soft polymer that has a poor mechanical strength and high propensity of swelling. After the cross-linked PS-DVB porous particles are formed, any residual reactants, diluents, and surfactants must be removed by thorough washing.

Other polymers such as methacrylates and polyvinyl alcohol were also used. The organic polymers were used for both the preparation of particles, but also for the preparation of monolithic columns. Also, the stationary phases that are organic polymers and contain specific functionalities can be obtained either by derivatization or directly by the polymerization of a

monomer having already attached the desired functionality. For RP-HPLC, columns having C18, NH_2, or CN groups are commercially available, and those having COOH, SO_3H, NH_2, and NR_3^+ are commercially available for ion exchange chromatography.

A particular direction where effort was made to develop polymeric stationary phases is that of polymeric monoliths [95–98]. Monoliths were obtained from a variety of monomers, using or not a cross-linker. Initial monoliths were simply obtained from styrene and divinylbenzene that were copolymerized in a porogenic solvent and in the presence of a radical initiator. A new convenient procedure starts with glycidyl methacrylate, which is polymerized by UV, thermal, or γ-radiation initiation, in the presence of a cross-linker such as ethylene glycol dimethacrylate, and using a porogenic solvent and an initiator (e.g., 2,2′-azobis-isobutyronitrile) [99]. Solvents typically used as porogens are mixtures of cyclohexanol and dodecanol, a higher content of dodecanol leading to monoliths with larger pores [100]. The reactions taking place during polymerization are of the type shown below (reaction 8.1.9):



(8.1.9)

A number of methacrylate polymers evaluated for generating monoliths are listed in Table 8.1.4 [101].

Some of the polymers obtained from the monomers listed in Table 8.1.4 contain glycidyl groups. These groups are used for attaching ligands (such as C18 chains) and generate columns similar regarding the ligand with those having a silica base. The control of porosity of these polymers is achieved using specific porogenic solvents in specific proportions to the monomers. These porogens can be either good or bad solvents for the polymer. The initial reaction takes place in homogenous solution. If the porogen is a good solvent for the polymer, the phase separation takes place late and the pores are smaller. Otherwise, the polymer precipitates early, generating larger pores.

Several other polymers and copolymers based on the methacrylate polymers or copolymers are reported in the literature as used as monolith stationary phases in HPLC [101,102]. Among these are copolymers of glycidyl methacrylate with styrene, other alkyl methacrylates, N-vinyl-pyrrolidone, etc. Besides glycidyl methacrylate, other methacrylate polymers containing hydroxy instead of epoxy groups were used as starting material for monoliths synthesis.

Cross-linking and porogenic solvents in which the polymerization takes place have an essential role in creating an appropriate porous structure of the polymer. The changes in the ratio of monomeric precursors and of the porogenic diluents (low-density/high-density materials), the rate of initiation (initiator concentration, temperature, light intensity), as well as copolymerization dynamics of the monomer and cross-linker lead to fundamentally different materials, which pose problems with batch reproducibility.

A problem regarding organic polymer monoliths is related to the mass transfer properties of the material and not to the derivatization or the increase in the surface area of the material. The nanoporosity of solvated polymeric material is dependent on the solute/solvent–polymer interaction irrespective of the cross-linking density, and the polymers show swelling propensity in

TABLE 8.1.4 Methacrylate polymers evaluated for producing monolith columns for HPLC [101].

Monomer	Cross-linking compound
Glycidyl methacrylate	Ethylene glycol dimethacrylate
Glycidyl methacrylate with styrene	Ethylene glycol dimethacrylate
Glycidyl methacrylate with butyl methacrylate	Ethylene glycol dimethacrylate
Glycidyl methacrylate with octyl methacrylate	Ethylene glycol dimethacrylate
Glycidyl methacrylate with dodecyl methacrylate	Ethylene glycol dimethacrylate
Glycidyl methacrylate with N-vinyl-pyrrolidone	Ethylene glycol dimethacrylate
Glycidyl methacrylate with 2-hydroxyethylmethacrylate	Ethylene glycol dimethacrylate
Glycidyl methacrylate	Trimethylolpropane trimethacrylate with triethylene glycol dimethacrylate
Glycidyl methacrylate	Trimethylolpropane trimethacrylate
Glycidyl methacrylate with glycidyl methacrylate-peptide conjugate	Ethylene glycol dimethacrylate
Glycidyl methacrylate with [2-(methacryloyloxy)ethyl]-trimethylammonium	Ethylene glycol dimethacrylate
Methacrylic acid	Ethylene glycol dimethacrylate or trimethylolpropanetrimethacrylate
2-Hydroxyethyl methacrylate	Ethylene glycol dimethacrylate
Hydroxypropyl methacrylate	Ethylene glycol dimethacrylate
Ethyl methacrylate	Ethylene glycol dimethacrylate
Ethyl methacrylate	Trimethylolpropane triacrylate
Ethyl methacrylate with N,N-dipyrid-2-ylmethacrylate	Trimethylolpropane triacrylate
Butyl methacrylate with 2-acrylamido-2-methyl-1-propanesulfonic acid	Ethylene glycol dimethacrylate
Butyl methacrylate	Glycerol dimethacrylate or 1,4-butanedioldimethacrylate
Butyl methacrylate with 2-hydroxyethyl methacrylate	1,4-Butanediol dimethacrylate
Butyl methacrylate	Ethylene glycol dimethacrylate
Butyl methacrylate	Diethylene glycol dimethacrylate or triethylene glycol dimethacrylate
Hexyl methacrylate	Ethylene glycol dimethacrylate
Octyl methacrylate	Ethylene glycol dimethacrylate
n-Lauryl methacrylate	Ethylene glycol dimethacrylate
Stearyl methacrylate	Ethylene glycol dimethacrylate
n-Octadecyl methacrylate	Ethylene glycol dimethacrylate

(*Continued*)

TABLE 8.1.4 Methacrylate polymers evaluated for producing monolith columns for HPLC [101].—cont'd

Monomer	Cross-linking compound
Polyethylene glycol methyl ether acrylate	Ethylene glycol dimethacrylate or polyethylene glycol dimethacrylate
Glycerol dimethacrylate	Glycerol dimethacrylate
Trimethylolpropane trimethacrylate	Trimethylolpropane trimethacrylate
N,N-Dimethyl-N-methacryloxyethyl-N-(3-sulfopropyl) ammonium betaine	Ethylene glycol dimethacrylate
2-Diethylaminoethyl methacrylate	Ethylene glycol dimethacrylate
Sulfoethyl methacrylate	Poly(ethylene glycol) diacrylate

the hydro-organic solvents used in HPLC. Such porosity can be described as gel porosity. The nature of the cross-linking polymerization reactions, and the formation of porous scaffolds in general, inherently leads to a significant amount of gel porosity, resulting in mass transfer resistance of small molecules. This depends on the retention and size of analytes, as well as the linear chromatographic flow velocity. The fluid transport properties in the heterogeneous macropore space (flow dispersion) and the transport of small molecules in the swollen and porous polymer matrix are factors that are different from those in silica-based monolith stationary phases that have a large proportion of meso pores (through pore size is about 10−20 μm and meso pore size is around 20 nm) [102].

One special type of stationary phases in HPLC are made from capillary channeled polymers [103]. These stationary phases consist of fibers that are made from polymers such as polyamides, polyesters, polyesteramides, polyolefins, polyetherketones, polyacrylates, polysulfones, polycarbonates, or polyurethanes. The fibers are of 4DG (deep grooves) type with a cross section of 35 to 50 mm having 8 - 9 deep surface channels of 5-20 mm width that extend for the entire length of the fiber. Bundles of 1000-3000 of such fibers are placed linearly in the body of a column. Columns of this type lead to lower backpressure and can display good efficiency.

Derivatization of silica solid support

The active part of a stationary phase consists of specific organic groups bonded on silica support that interact with the analytes from the mobile phase. Such materials can be obtained by a variety of procedures. One set of procedures consists of the derivatization of silica surface and is presented in this subsection. Another set of procedures is based on direct synthesis of a silica type material containing the desired active groups. The widespread use of silica for obtaining stationary phases is based on its reactivity, large surface area, mechanical strength, and the possibility to generate porous silica particles of uniform desired dimensions. The most common type of silica used for preparing stationary phases is of Type B (related to purity). Regarding the pore size, silicas with medium pores (Type B related to pore size) are the most commonly used. Silicas with narrow pores (Type A related to pore size) and with large pores (Type C) are also used sometimes as support for the modern stationary phases, for special purposes. The derivatization of silica is based on reactions of its silanol groups with specific reagents that contain the desired molecular fragment to be attached to the surface. This fragment can be the final functionality or an intermediate one toward the desired bonded phase on the surface. Some schematic examples of fragment attachments to a silica surface are shown in Fig. 8.1.7.

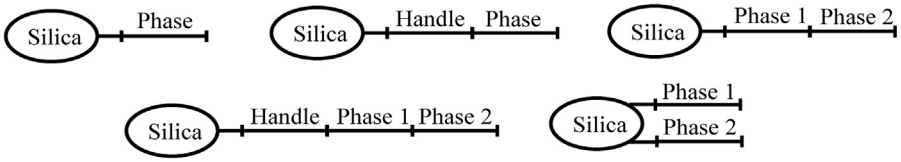


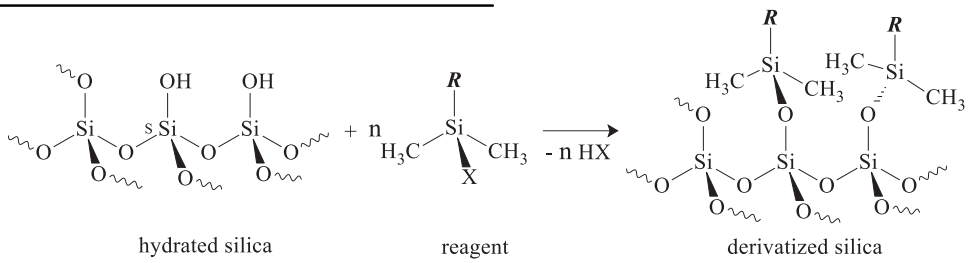
FIGURE 8.1.7 Schematic examples of fragment attachments to the silica surface.

The bonded phase obtained by derivatization of silica can have a hydrophobic character, a polar character, an ionic character, or a mixed type character (e.g., polar and nonpolar or nonpolar and ionic.) Different types of reagents and reactions can be used to derivatize the silanol groups from the silica surface, and some of these are further described in this section. The derivatization process can be performed on silica already shaped in particles of the desired dimensions and also can be performed on monoliths or on open tubular capillary or nanoscale columns.

A number of aspects related to the derivatization of silica support are important for the quality and properties of the generated stationary phase: (1) the type of bonded phase, (2) the type of reagent used for derivatization, (3) the vertical or horizontal type of derivatization, (4) the procedure to generate more complex stationary phases, (5) the extent of derivatization, (6) end-capping, and (7) use of special derivatization reactions.

(1) The type of bonded phase refers to the actual moiety that is bonded to the silica backbone. Various types of bonded phases are further discussed in more detail in the chapters dedicated to specific types of HPLC (RP-HPLC, HILIC, ion exchange, etc.). Groups such as octyl (C8) and octadecyl (C18) are very common for making hydrophobic phases, although other groups with hydrophobic character such as phenyl may be attached to the silica backbone. For partially polar phases but still with some hydrophobic character, groups such as cyanopropyl or hydrophobic chains with polar embedded groups (e.g., −O−) can be synthesized. Groups with a polar character or ionic character can also be bonded to the silica backbone typically using a short hydrophobic "handle" consisting, for example, of two or three aliphatic carbon chains. Also reactive groups can also be connected to the silica backbone through a short hydrophobic handle, making a material that can be further derivatized in another step. Among the groups utilized for second connection to the derivatized silica are the following: glycidyl, amino ($-NH_2$), azide ($-N_3$), and isothiocyanate (−NCS).

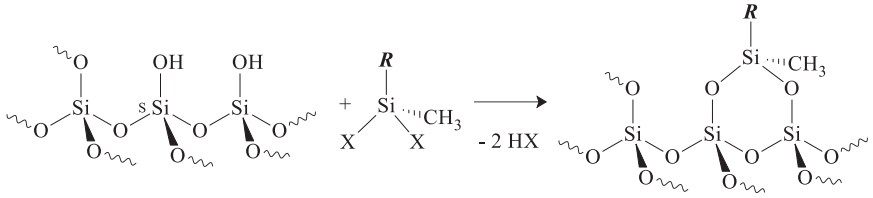
(2) Numerous types of reagents were used for hydrated silica derivatization. One group of reagents commonly used for derivatization consists of monofunctional silanization reagents (e.g., a chlorodimethylalkylsilane). This type of reagent allows the attachment of a silyl ligand ***R*** (such as dimethyloctadecylsilyl) to the silica backbone. The reaction is schematically described as follows (reaction 8.1.10):



(8.1.10)

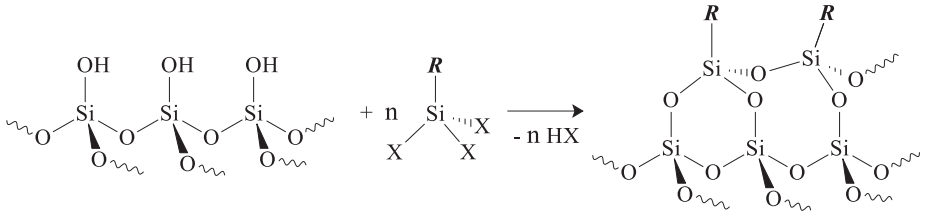
The reactive substituent X can be Cl, but also OCH_3, OC_2H_5, or other groups such as NH_2. The substituent ***R*** is the one determining the type of bonded phase (C8, C18, amino, cyano, and many others). The derivatization of silica surface with a monofunctional silanization reagent is typically indicated as *monomeric functionalization*, and this kind of derivatization leads to *brush* phases. Monomeric functionalization attaches a monomolecular layer of silyl ligands that form the active stationary phase on the silica surface. When the derivatization reagent is a chlorosilane, the presence in the reaction medium of organic bases (pyridine or triethylamine) is necessary to remove HCl formed as by-product. When alkoxydimethyl- ***R*** -silane is used as silanization reagent, toluene is a proper solvent for the reaction. In this case, the alcohol formed as reaction by-product should be removed from the surface, because it can subsequently react with silanol groups leading to alkoxy derivatized silica surface, which is unstable toward aqueous mobile phases. Monomeric functionalization (brush phases) leaves a considerable number of unreacted silanol groups on the solid support.

Another possibility of derivatization, which has the capability to involve in the reaction two or even three silanols from the silica surface, is the use of *di- or tri-functional reagents*. The hindrance in the reactions of Si—OH with reagents having larger ***R*** groups may be more frequent for silanols that are close to each other, such as vicinal ones. In monomeric functionalization, a large number of such silanols remain underivatized. The reagents having two or three reactive functional groups such as ethoxy or chloro have a higher capability to react with vicinal silanols, as shown schematically in reaction 8.1.11 where the reagent is difunctional.



(8.1.11)

For trifunctional reagents, the possibility to have all three reactive substituents connected to the silica surface is also possible [104]. A model for such derivatization is shown in reaction 8.1.12 where the reagent is trifunctional [105]:



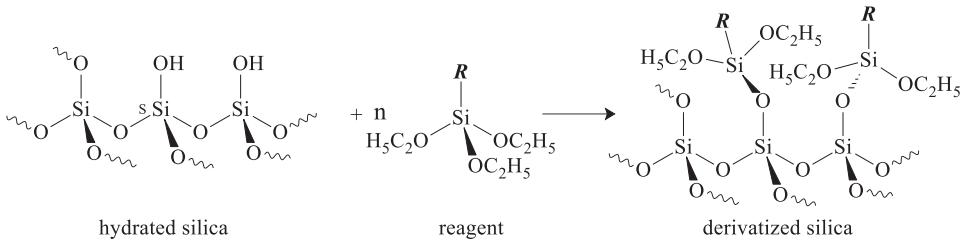
(8.1.12)

Both difunctional and trifunctional reagents may react at only one point with the silica surface, and the attached silyl ligands still have

reactive groups such that the treated surface retains additional capability for further reactions. A schematic description of a derivatization that leads to a surface containing reactive groups is shown below for a trifunctional triethoxysilane (reaction 8.1.13):

In reaction 8.1.13, it is shown that the resulting derivatized silica still contains the reactive groups $-OC_2H_5$.

A number of common reagents used for the derivatization of silica surfaces are shown in Table 8.1.5. These reagents may be used for attaching on the silica surface either



(8.1.13)

TABLE 8.1.5 Examples of reagents used for the derivatization of silica surface.

Reagent	Formula	Uses
Trimethylchlorosilane	$ClSi(CH_3)_3$	End-capping
Propyltrichlorosilane	$Cl_3SiCH_2CH_2CH_3$	Bonded phase
Octyltrichlorosilane	$Cl_3SiCH_2(CH_2)_6CH_3$	Bonded phase
Octadecyltrichlorosilane	$Cl_3SiCH_2(CH_2)_{16}CH_3$	Bonded phase
Phenyltriethoxysilane	$(CH_3CH_2O)_3SiC_6H_5$	Bonded phase
γ-Glycidoxypropyltrimethoxysilane	$(CH_3O)_3Si-(CH_3)_3-O-CH_2-HC(O)CH_2$	Bonded phase
γ-Aminopropyltriethoxysilane	$(CH_3CH_2O)_3SiCH_2CH_2CH_2NH_2$	Bonded phase
γ-Aminopropylmethyldiethoxysilane	$(CH_3CH_2O)_2CH_3SiCH_2CH_2CH_2NH_2$	Bonded phase
γ-Aminopropyldimethylethoxysilane	$(CH_3CH_2O)\cdot(CH_3)_2SiCH_2CH_2CH_2NH_2$	Bonded phase
Cyanopropyltriethoxysilane	$(CH_3CH_2O)_3SiCH_2CH_2CH_2CN$	Bonded phase
Nitropropyltriethoxysilane	$(CH_3CH_2O)_3SiCH_2CH_2CH_2NO_2$	Bonded phase
Dihydroxypropyltriethoxysilane	$(CH_3CH_2O)_3SiCH_2CH(OH)CH_2(OH)$	Bonded phase
3-Mercaptopropyltriethoxysilane	$(CH_3CH_2O)_3SiCH_2CH_2CH_2SH$	Bonded phase
3-Azidopropyltrimethoxysilane	$(CH_3O)_3SiCH_2CH_2CH_2N_3$	Bonded phase
Hexamethyldisilazane	$(CH_3)_3SiNHSi(CH_3)_3$	End-capping

hydrophilic or hydrophobic functionalities. Also, the same reagents used for the derivatization of silica gel particles can be used for the derivatization of silica monoliths. A much wider range of compounds than indicated in Table 8.1.5 can be attached to the silica surface by direct reaction with the silanol groups. Some of the attached groups may act as the active bonded phase on the silica surface. Among these groups are the alkyl, cyanopropyl, phenyl, etc. Other groups can be used as active bonded phase, but also as intermediates for further derivatizations. Among such groups are glycidyl, amino, and azido.

(3) The remaining reactive groups on the derivatized silica with di- or trifunctional reagents can be used for generating one layer of bonded phase or more layers (up to five), depending on derivatization conditions. The two types of derivatization are indicated as *horizontal polymerization* [106] and *vertical polymerization* [107]. Horizontal derivatization is sometimes indicated as generating an *oligomeric* phase, while vertical polymerization generates a *bulk* phase (a thicker layer of active phase bonded on silica surface).

In horizontal polymerization, the reactive groups of di- or trifunctional reagent are intended to react completely with the silica surface. For this purpose, the reaction medium is kept anhydrous, and because long substituents ***R*** on the reagent will lead to steric hindrance, a mixture of ***R*** groups including a desired one (e.g., octyl) and a short one (e.g., propyl) are used for derivatization. Also, higher temperatures may be used for increasing the yield of derivatization. The result is schematically shown in Fig. 8.1.8.

If water is present in the derivatization medium, a different type of derivatization takes place, indicated as *vertical polymerization*. Vertical polymerization generates a *bulk* phase and takes place with a three-step sequence. In the first step, the di- or trifunctional reagent reacts with silica to link trialkylethoxysilyl or dialkylchlorosilyl groups as shown in reaction 8.1.13. In the second step, the product is hydrolyzed in order to substitute the chlorine atom or the ethoxy groups with Si—OH groups as shown in the reaction (for ethoxy groups) 8.1.14:

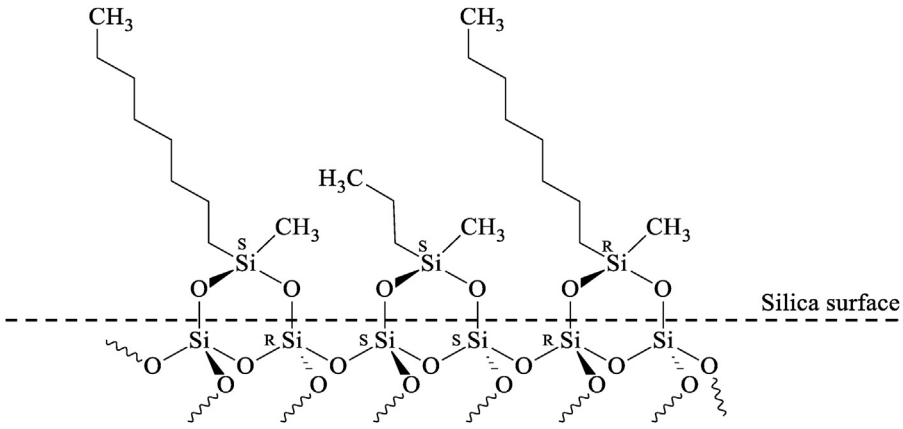
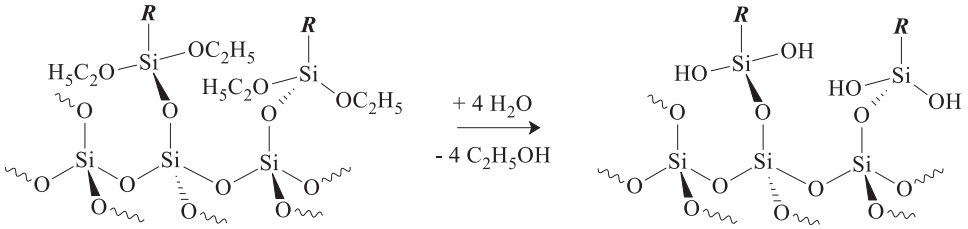
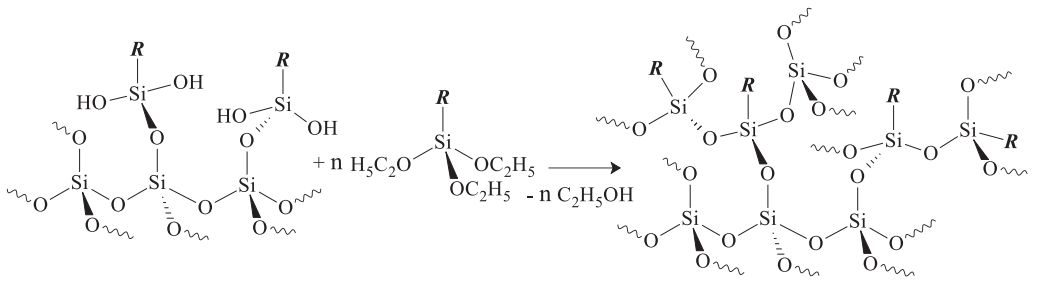


FIGURE 8.1.8 Schematic illustration of a horizontal polymerization showing octyl and propyl groups on silica surface.



(8.1.14)

The third step is subjecting the hydrolyzed surface to another derivatization reaction with difunctional or trifunctional silane as shown in the reaction 8.1.15:



(8.1.15)

an ordered packed bonded phase on silica surface. Schematic illustration of the structure of a vertical polymerization process is shown in Fig. 8.1.9.

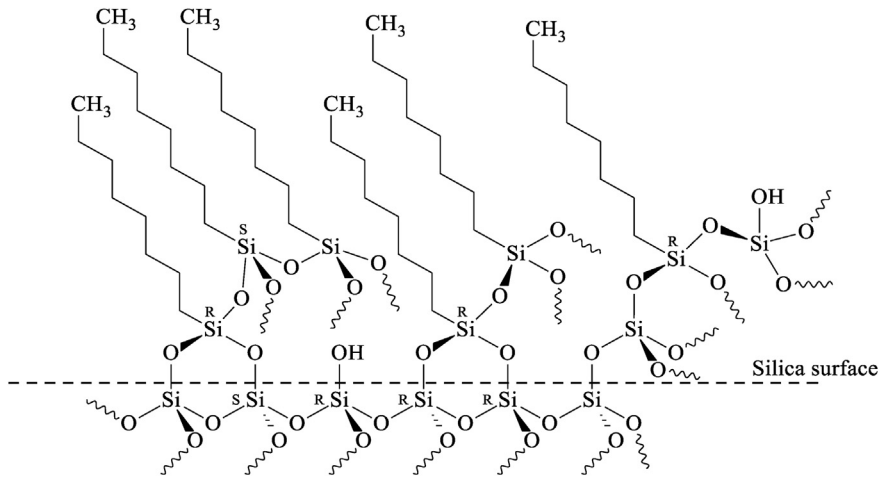


FIGURE 8.1.9 Schematic illustration of a vertical polymerization showing the octyl groups on silica.

This sequence is repeated several times (8–10 times). The result is a thick layer of the bonded phase on the silica surface. The alkyl chains form

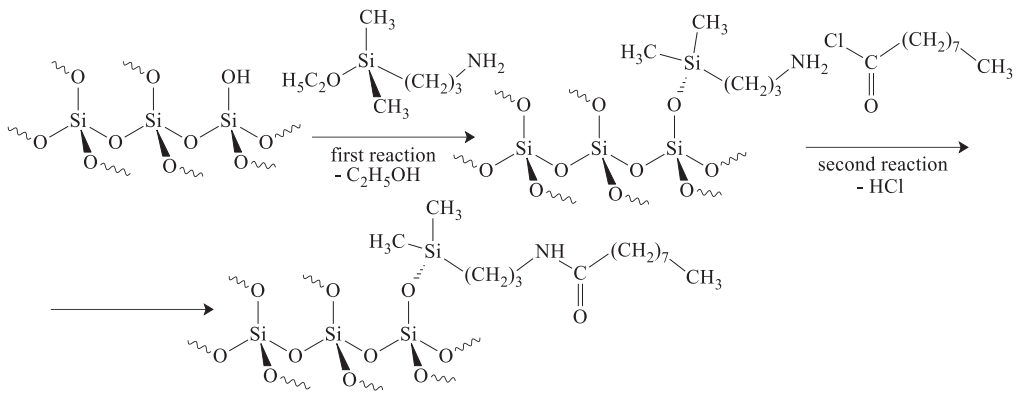
(4) More complex stationary phases, having more than one functionality, are very useful in certain separations. A common procedure to

prepare more complex desired stationary phases is to use a double step reaction of derivatization and further react a molecular fragment already attached to the silica surface with another reagent [108]. The initially attached functionality is either itself transformed into the desired functionality or can be used as a reactive "anchor" to attach a desired fragment that will form the stationary phase. By this procedure, phases indicated as polar embedded can be obtained. Such phases offer more complex interaction with the analytes. For example, the hydrophobic phases that have an amide embedded group can be obtained by an initial derivatization of hydrated silica with a silane containing a propylamino group, followed by a secondary derivatization with an acid chloride. This is schematically indicated in reaction 8.1.16.

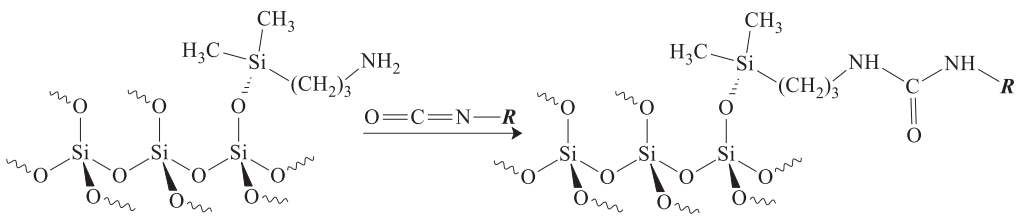
Other reactions with a silane reagent containing a short hydrocarbon "handle" such as propyl connected to a reactive group followed by a second reaction were used to produce a variety of bonded phases. For example, the preparation of octadecyl phases with polar embedded groups was obtained by a similar reaction [109].

The amino silica can also react with other compounds having the desired moiety acting as a stationary phase connected with a reactive group. For example, the reaction can take place with an isocyanate of a long carbon chain carboxylic acid (e.g., with $\boldsymbol{R}$ = C8, C18, etc.) as exemplified in reaction 8.1.17 [110].

Besides the amino groups attached to silica through a propyl handle, other reactive groups such as NH_2, CHO, CN, SH, etc., can be

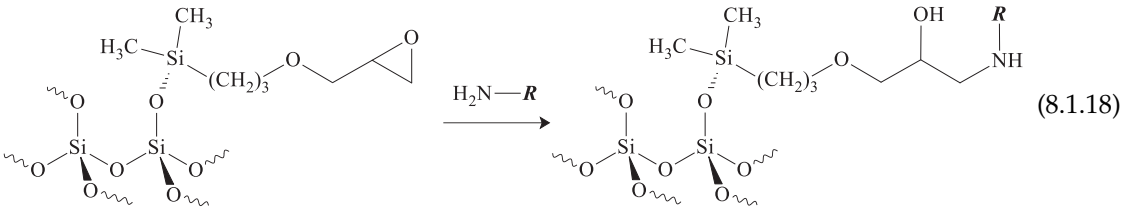


(8.1.16)



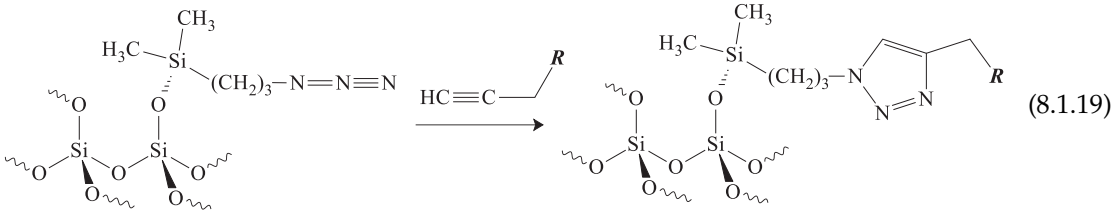
(8.1.17)

used in two step derivatization [111]. Also, the two-step derivatization can start with a glycidyl group on silica surface. An example of such reaction is given below (reaction 8.1.18):



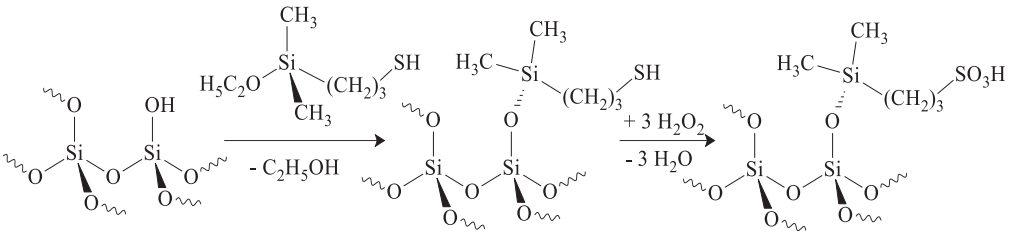
(8.1.18)

Silica derivatized with azido groups can participate in Huisgen cycloadditions (Click chemistry) as shown below (reaction 8.1.19):



(8.1.19)

Not only hydrophobic phases with embedded polar groups were obtained by this procedure, but also phases having macrocycles attached to silica or groups with special chiral properties. The two-step approach for the synthesis of polar embedded phases is common [109,112,113]. However, besides double reactions, polar embedded phases can be obtained by single step derivatization with appropriate reagents (e.g., Ascentis Express PR-Amide columns from Millipore-Sigma). Such reagents are silanes with a polar embedded functionality in a hydrophobic chain. For example, a phase containing an ether group in a hydrocarbon chain can be obtained by reaction of hydrated silica with triethoxy(propoxyoctyl)silane. Similarly, groups such as amide or carbamate can be embedded in a hydrophobic phase. A variety of other groups can be embedded in the structure of a stationary phase, such as anthracenyl [114], etc. Although the use of derivatized silica having a reactive group able to continue a coupling for obtaining more complicated stationary phases is common, other procedures of synthesis are also known. One example is the changing one attached group into another group as shown below for the case of obtaining a strong cation exchange stationary phase (reaction 8.1.20):



(8.1.20)

The use of fragments already attached to the phase and having a reactive group able to continue a coupling for obtaining more complicated stationary phases is much more widespread than the functionality change.

(5) The extent of derivatization is an important parameter for column selection. Silica offers a complex surface containing macropores, mezopores, and also micropores. Pore surface area accessibility is key to effective derivatization of silica. Several parameters are utilized to describe the derivatization extent. One such parameter applied for hydrophobic phases (e.g., C8 or C18) is the carbon load (*C*%) that can be determined from elemental analysis of the phase. Values of carbon load can vary between about 5% up to 25%. The carbon load is related to the number of micromoles of silyl ligands attached to the OH groups, which also characterize the extent of derivatization and is obtained with the following formula:

$$\delta_{\text{ligand}} = \frac{10^6 C\%}{(C_{\text{sil}} - C\%) M w_{\text{sil}} S_{\text{surf}}} \qquad (8.1.21)$$

In Eq. 8.1.21, δ_{ligand} is the amount in μmol of silyl ligands per m^2, *C*% is the carbon load, C_{sil} is the mole % of carbon in the silyl ligand, Mw_{sil} is the molecular weight of the silyl ligand, and S_{surf} is the surface area of the silica [115]. The value of δ_{ligand} for a ligand such as dimethyloctadecylsilyl is $\delta_{\text{ligand}} \approx 5.6$ μmol/m^2 (for $C\% = 20\%$, $C_{\text{sil}} = 77.4\%$, $Mw_{\text{sil}} = 310$, and $S_{\text{surf}} = 200$ m^2/g). The amount of silanol groups of 7–8 μmol OH/m^2 is not very different from that of the ligand amount. However, the value of 5.6 μmol/m^2 corresponds to a high *C*% load typical for stationary phases obtained for vertical polymerization. Because in vertical polymerization up to five alkylsilane ligands can connect to each other and not to the silica backbone, it results that a considerable number of silanol groups remain underivatized although the carbon load is high. Different stationary phase manufacturers indicate the distribution of bonded groups on the silica surface with descriptors such as XDB (extra dense bonding). Some other manufactures claim that very uniform bonding of the active phase is important for the column pH resilience (e.g., for InterSustain columns from GL Sciences).

(6) Since in both monomeric functionalized silica as well as in horizontal and vertical derivatized surfaces with di- and trifunctional reagents a significant number of free silanol groups remains on the silica backbone, attempts were made to reduce this number. Silanols affect column properties by generating polar interactions with the analytes. The free silanol groups can participate in the retention, generating tailing for compounds having besides hydrophobic moieties some polar groups (for example, amino compounds). These silanol groups may be intended to exist in particular types of columns, but frequently, a further derivatization is practiced to reduce the silanols number. This is performed by a separate procedure of blocking residual silanols by a subsequent derivatization with small groups such as trimethylsilyl (TMS) that have less steric hindrance during derivatization. This is performed using as reagent trimethylchlorosilane or hexamethyldisilazane. Repeated end-capping operations are not uncommon. Besides trimethylchlorosilane or hexamethyldisilazane, difunctional derivatization reagents with small ***R*** groups are also used for end-capping. Such reactions are typically performed at elevated temperatures to achieve a derivatization as complete as possible [116–118]. By end-capping, the carbon load *C*% of packing material does not significantly change, and thus its hydrophobic character for the reversed phase phases is kept almost constant. The density of end-capping groups

provides a specific character of the stationary phase different densities reducing to different degrees the remaining polar interactions possible with the specific column. End-capping with small polar groups is also possible for generating a "polar end-capped" phase, which offers additional interactions with the analytes [119]. End-capping can be performed on HPLC particles but also on monolithic columns [120]. End-capping with small polar groups is also practiced. End-capping is typical for hydrophobic columns, but HILIC columns can also be end-capped with small polar groups.

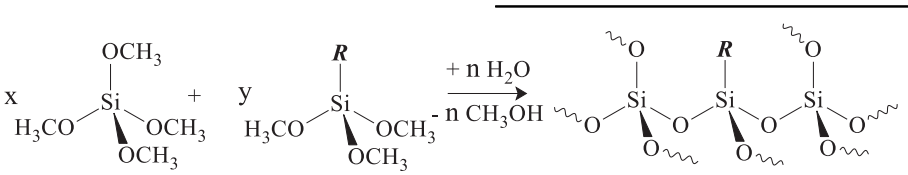
(7) Besides the reaction of hydrated silica with silanization reagents such as chlorodimethylalkyl silane, or alcoxydimethylalkyl silane, other reactions were used for binding the desired moiety to the silica surface. For example, one such method is based on a chlorination/organometalation two-step reaction sequence. In the first step, the Si—OH on the silica surface is converted into Si—Cl via a reaction with thionyl chloride. This step must be done under extremely dry conditions (usually done in a closed vessel purged with a dry gas-like nitrogen) because the presence of water traces leads to the reversal of the reaction, with hydroxyl groups replacing back the chloride bonded to the silica and regeneration of silanols. Then the substitution chain ***R*** can be attached to the surface using a Grignard's reagent or an organolithium compound. The resulting structures having ***R*** directly bonded to the siloxane matrix are more stable than structures based on substitution of H from silanol, which could be an advantage for the phases bonded by this procedure. However, due to the specific reaction condition from the first step and the formation of salts as by-products, the materials obtained using silica chlorination are less known as commercial packing materials for HPLC applications.

In addition to simple reagents used to derivatize the silica surface, more complicated compounds can be made reactive and used for reaction with the silanol groups. For example, cholesterol can react with allyl bromide to form a 3-allyl ether. In the presence of a catalyst (H_2PtCl_6), the double bond of the allyl ether reacts with the silanol groups attaching the cholesterol fragment to the silica surface (with a C_3 spacer) [121]. Special derivatization reactions are sometimes necessary for generating stationary phases with particular applications. Among these derivatizations are those designed for producing columns for chiral separations, or those used in affinity and immunoaffinity chromatography. Some of these columns have a porous silica support, and others have an organic polymeric support.

Direct synthesis of silica materials with an active bonded phase surface

Derivatized silica surfaces also can be obtained directly by the hydrolysis of the appropriate reagents, without having bare active silica prepared as first step, followed by further derivatization. For example, the hydrolysis of tetramethyl orthosilicate (tetramethoxysilane or TMOS) in the presence of octyltrimethoxysilane in an acidic medium generates a silica gel that contains octyl groups attached to the silica structure. The same procedure can be applied to obtain silica having C18 chains attached on its surface. Another example of direct reaction for obtaining a functionalized silica surface is the preparation of propylsulfonic acid functionalized mesoporous silica. This material can be prepared in a unique step starting with tetraethyl orthosilicate, 3-mercaptopropyltrimethoxysilane, and H_2O_2, in strong acidic conditions [122].

Direct hydrolysis of a mixture of silane derivatives (cohydrolysis) may generate the desired functional groups on the silica surface, but can also make the process more difficult to control



(8.1.22)

in particular in relation to the size and shape of stationary phase particles [123]. For this reason, cohydrolysis has been used more frequently for the preparation of monolith type materials where the control of particle size is not a critical step. The procedure leads to so-called organic–inorganic monoliths. One example of cohydrolysis reaction is given in reaction 8.1.22.

In reaction 8.1.22, the radical ***R*** can be octyl and octadecyl, but also other radicals. The procedure is also utilized for the preparation of monoliths with mixed mode functionalities. For example, a mixture of TMOS, aminopropyl-trimethoxysilane, and octyltrimethoxysilane can be hydrolyzed in acidic medium to generate a monolith with the active surface covered with both octyl and aminopropyl groups. In a similar manner, a mixed mode phase having octyl and cyano groups can be prepared, starting with TMOS, cyanopropyl-trimethoxysilane, and octyltrimethoxysilane [124]. Both structures are schematically shown in Fig. 8.1.10.

Derivatization of silica hydride supports

Silica hydride as a support material for HPLC has the advantage of reduced presence of silanol groups. Besides bare silica hydride, functionalized silica hydride is also used as a stationary phase. The attachment by covalent bonds of different functionalities of the silica hydride support is typically performed using a hydrosilation reaction. This reaction can be schematically written as follows (reaction 8.1.23):

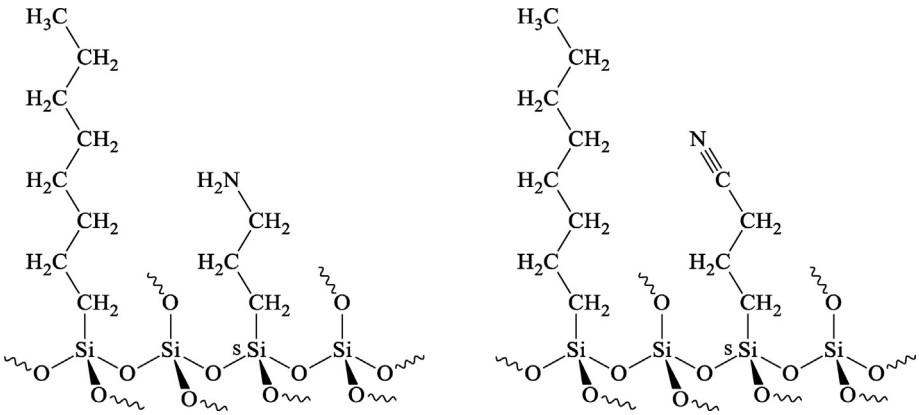
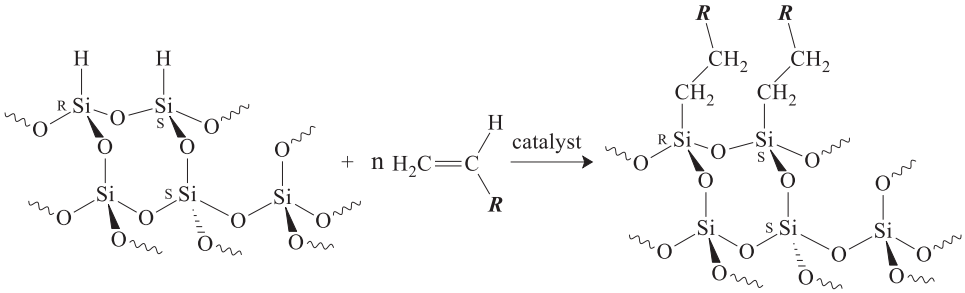
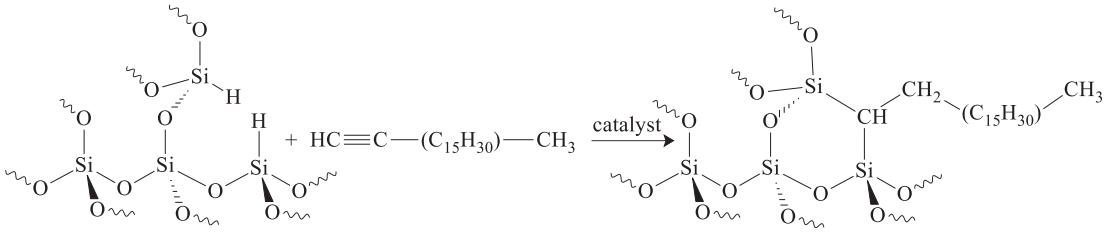


FIGURE 8.1.10 Schematic structure of a mixed mode phase with octyl and amino groups and a phase with octyl and cyano groups.



(8.1.23)

In reaction 8.1.23, the radical ***R*** can be selected to form a hydrophilic, hydrophobic, an ion exchange, or a chiral phase. The reaction takes place in the presence of a catalyst that can be $H_2[PtCl_6]$ or a free radical initiator [125]. Double attachment of the bonded groups can be obtained when using alkynes in the hydrosilation as shown in the reaction 8.1.24 [126]:



(8.1.24)

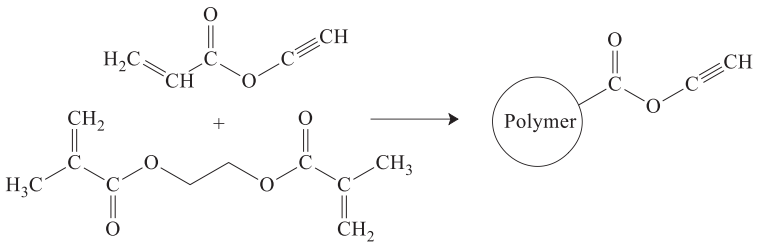
By the same procedure can be attached other functional groups, such as cyano, amino, fluorinated hydrocarbons, etc. [127–130]. Silica hydride phases can also be subject of end-capping.

The functionalities bound to silica hydride are not limited to simple groups. For example, a very stable structure based on Si–C bonds can be obtained from the reaction of hydride silica with vinylbenzo-18-crown-6-ether, in presence of $H_2[PtCl_6]$. This type of phase can be used in ion exclusion separations. Also, silica hydride can be used as solid support undergoing further reaction with an unsaturated hydrocarbon (containing either a double or a triple bond) in the presence of a catalyst. The resulting bonded phases do not have unreacted silanols that can produce undesirable interactions with the analytes.

Derivatization of presynthesized organic polymers

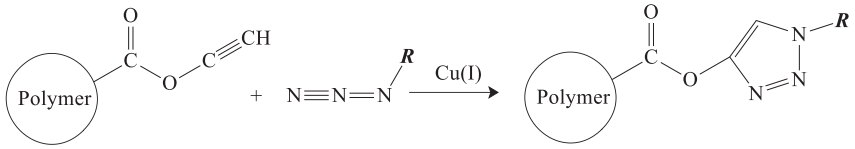
Various procedures were applied for the derivatization of a preformed polymer in order to obtain the desired functionalities on the surface of material forming the stationary phase. Specific derivatizations were performed on vinyl type polymers that have attached on the polymeric backbone—specific reactive groups, such as glycidyl or alkyne. Such reactions were

applied for the synthesis of particle type phases as well as monoliths. One example of synthesis starts with on the polymerization of ethylene dimethacrylate with propargyl acrylate as shown below (reaction 8.1.25):



(8.1.25)

The polymer with alkyne groups is further derivatized using a copper (I)-catalyzed (3 + 2) azide alkyne cycloaddition that is shown schematically below (reaction 8.1.26):



(8.1.26)

The radical ***R*** in reaction 8.1.26 can be, for example, a long hydrocarbon chain such as C18 [131].

Other examples include the synthesis of cation exchange resins, or anion exchange resins can be obtained by such derivatizations. The polymeric backbone of organic ion exchangers is usually obtained either by polycondensation or by polymerization. A common polycondensation reaction used to obtain polymeric resins is that between phenol and formaldehyde. Both acid and basic catalysts can be used in this reaction. In the presence of basic conditions, the reaction takes place more completely and usually leads to a tridimensional thermorigid polymer. The inert resin can be modified by direct sulfonation into a strong cation exchanger. The polycondensation in the presence of acid catalysts may leave a significant number of free $-CH_2OH$ groups. These can be further derivatized with $SOCl_2$, and the $-CH_2OH$ groups are changed into $-CH_2Cl$ groups. Upon treatment with $N(CH_3)_3$, the resin can be changed into a strong anion exchange material. Various other organic polymer–based stationary phase were synthesized. However, silica-based stationary phases have several advantages and are much more commonly used in HPLC [132].

Synthesis of organic polymers with active groups

Direct synthesis of polymers with active groups present in their molecular structure is a convenient procedure for obtaining polymer-based stationary phases. For this purpose, the polymerization is done starting with specific monomers that have the desired group. Sulfonated styrene can be used, for example, in a copolymerization reaction with divinylbenzene (usually in the presence of benzoyl peroxide) to form a polymer with sulfonic groups [133]. Copolymerization between acrylic acid or methacrylic acid and divinylbenzene generates a resin with carboxyl groups. This resin can be used as a weak cation exchange resin.

Synthesis of organic polymeric monoliths with active functionalities

A number of polymeric monoliths that can be further derivatized with desired functional groups were synthesized and utilized as stationary phases in HPLC [99]. A convenient procedure to perform such derivatizations starts with a polymer having glycidyl groups. Such polymer can be further derivatized using various reactions. Two such reactions, a hydrolysis and an amination are shown in Fig. 8.1.11:

Other reagents besides those indicated in Fig. 8.1.11 were used to react with the glycidyl groups bonded to a polymeric backbone such as diethylamine, other substituted amines, sulfuric acid, chloroacetic acid, thiobenzoic acid, etc. Biofunctionalization of the epoxy or hydroxy bearing monoliths was reported in the literature [134]. Reactions involving intermediate derivatization using a diamine and glutaraldehyde, carbonyldiimidazole, disuccinimidyl carbonate, etc., were used to bind ligands on the polymeric monolith.

Coated or immobilized (grafted) polymeric stationary phases on silica

Besides the derivatization techniques frequently utilized for binding a phase with desired chemical properties on the silica surface, other techniques are also used for the same purpose. The goal is to take advantage of silica, which has excellent properties for a stationary phase including a large surface area, mechanical strength, and pores of appropriate dimensions. These properties are difficult to achieve with other supports including inorganic or organic polymers. One simple procedure to cover silica with the desired active stationary phase, and also to cover some of the silanol groups not wanted in the separation, is a simple coating. For example, derivatized cellulosic materials can be coated on silica by simple adsorption using a proper solvent for the organic material (e.g., acetone, dichloromethane, or tetrahydrofuran [THF]) [135]. The coating must not alter the porous structure of silica, but at the same time may not be sufficient for assuring stability of the coated silica material since some spots may remain unprotected (uncovered). For this reason, some chemical reactions that assure good bonding of the coat can be performed, using, for example, the immobilization of a presynthesized polymer. A presynthesized polymer (polybutadiene, polysiloxane), still having some reactivity by interrupting the polymerization process before it is complete, can be immobilized on silica using gamma radiation, microwaves, or thermal treatment, possibly in the presence of a radical initiator [136]. The main disadvantage of this procedure for obtaining immobilized polymers on silica particles is the inhomogeneity of the polymeric covering such that some parts of the silica still remain uncovered, and the overall stability of the stationary phase is not significantly improved compared to standard bonded phases. Covalent bonding on the silica surface can also be achieved by using a derivatization procedure with a reactive polymer. In this case,

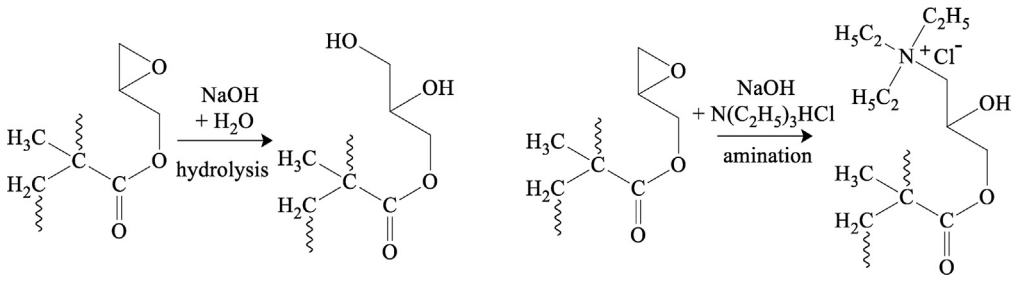


FIGURE 8.1.11 Various coupling reactions for polymers having glycidyl groups.

the silica is first derivatized, for example, with γ-aminopropyltriethoxysilane. This reaction is followed by derivatization with a polymer containing specific reactive groups [137,138]. This procedure indicated as grafting is used for example for binding chiral carbohydrates on a silica surface.

An alternative procedure that appears to be successful in obtaining stable phases with polymers immobilized on silica particles is to generate a layer of zirconia or titania on the silica before bonding the polymer [139,140]. The method of attaching zirconium or titanium oxide on silica starts with dry silica gel, which is treated with $ZrCl_4$ or $TiCl_4$ followed by exposure to ammonia. An alternative procedure consists of the treatment of silica gel with zirconium or titanium tetrabutoxide followed by a hydrolysis step of the adsorbed layer of tetrabutoxide on silica surface. This type of support is known as metalized silica. The polymers bonded on metalized silica include poly(methyloctylsiloxane), poly(methyltetradecylsiloxane), poly(methyldecyl-(2%−5%)-diphenylsiloxane), poly(dimethylsiloxane), etc. The bonding procedure involves the deposition on the metalized silica of the polymer dissolved in a solvent, followed by particle drying and exposure to gamma radiation, microwave radiation, or thermal treatment. Such phases show better resistance to mobile phases with high pH and good peak symmetry. Other procedures for obtaining immobilized polymers on silica are known. One such procedure uses, for example, vapor deposition of a silicone polymer of the base silica gel particles. This procedure leads to a quite homogenous monolayer of polymer. The polymer can be further derivatized to attach the desired bonded phase.

Metal-organic frameworks used as stationary phase for HPLC

Metal-organic frameworks (MOFs) are a type of coordination polymers formed from the reaction of a metal cation with a multifunctional organic molecule that acts as a linker. In special reacting conditions (usually long heating time and pressure), the resulting compound self-assembles in tridimensional structures with high porosity and uniform pore size. Example of metal ions used for MOF preparation are Cr^{3+} (used in MIL-101 [141]) or Zr^{4+} (used in UiO-66) in reaction with benzene-1,4-dicarboxylic acid (terephthalic acid). Other multifunctional organic molecules can be used for MOF synthesis, such as 4,4′-biphenyldicarboxylic acid. Also, the acid ligand may have additional functionalities (−OH, $-NH_2$, etc.). For example, 3-hydroxy-terephthalic acid, 2-amino-terephthaalic acid, 2,5-diaminoterephthalic acid, etc., can be used in the synthesis of MOFs. MOFs found utilization in various types of separations (SPE, GC, electrophoresis) and also in HPLC [43,45,142]. HPLC columns directly packed with MOFs are still a developing technique since the irregularity in the MOFs particles is a factor contributing to the low column efficiency. Core-shell particles with MOF coating were experimented for HPLC use [143,144].

8.2 Column properties affecting separation

General comments

The properties of a chromatographic column are determining the column performance regarding the separation. The manner these properties are connected to the column performance is basically discussed in this section. The list of properties and the column performance further presented are summarized in the scheme from Fig. 8.2.1.

Because of the importance of the chromatographic column in HPLC analysis, further information on columns and stationary phases is also presented in Part 2 of this book. Present section offers only a summary regarding stationary

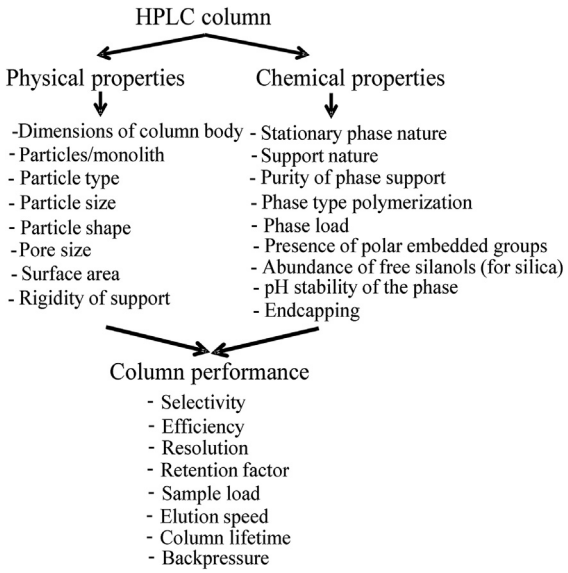


FIGURE 8.2.1 Aspects of column construction and their impact on column performance.

phase and column classification based on their physical and chemical properties. A standard classification of stationary phases has been proposed by US Pharmacopeia (USP) [145]. This classification uses codes indicated as L1, L2, L3, etc., and is shown in Appendix 8.2.1. The appendix also includes several examples of columns for each category [146]. The USP classification offers a view on complexity of columns used in HPLC. However, even this classification is not always appropriate for all column classifications. For example, core-shell type columns such as Kinetex XB-C18, or Poroshell 120 SB-C18, or certain zirconia-based columns such as ZirChrom-EZ are not easily classifiable using the criteria shown in Appendix 8.2.1.

Dimensions of the column body affecting separation

The dimensions of the chromatographic column (length and internal diameter) are usually selected based on the intended use, and they tend to be larger for small preparative scale (not industrial), and smaller for analytical purposes. Related to their dimensions, the columns are placed in several groups as indicated in Table 8.2.1.

A discussion about preparative and semipreparative columns is beyond the purpose of this book. Micro and nano LC-capillary columns are utilized for analytical purposes, but specific instrumentation is necessary for using them, such as syringe pumps and dedicated small volume cell detectors. Specific issues are related to such columns, and they are not very common in routine analytical HPLC practice [147]. For these reasons, the main interest in HPLC practice is in the conventional, narrowbore, and microbore analytical columns that will be further discussed.

TABLE 8.2.1 Classification of HPLC columns based on their dimensions.

Type	Inner diameter (mm)	Length (mm)	Typical flow rate (mL/min)	Sample loading
Small preparative scale	>25	300, larger	>20	>25 mg
Semipreparative	10	250, larger	5–10	10–20 mg
Analytical conventional	3, 4.6	50, 100, 150, 250, 300	0.5–2	50–200 μg
Analytical narrowbore	2, 2.1	50, 100, 150, 250	0.2–0.6	20–100 μg
Analytical microbore	1, 1.7, 1.8	50, 100	0.05–0.3	<5 μg
Micro LC-capillary	<0.5	50,100	1–10 μL/min	1 μg
Nano LC-capillary	<0.1	50	<1 μL/min	<0.1 μg

Among the chromatographic parameters affected by the column dimensions are (1) retention time t_R, (2) separation run time, (3) column efficiency (number of theoretical plates N), (4) column resolution R, (5) maximum sample load in the column, (6) column backpressure, and (7) column stability in time.

(1) The retention time t_R is affected by column dimensions. The dead time t_0 for a chromatographic column is given by Eq. 3.1.5 that shows that t_0 is directly proportional with the column length L. The linear flow rate u in the chromatographic column is related to the volumetric flow rate U by Eq. 3.1.3 where d is the column inner diameter and ε^* is a constant depending on column porosity, e.g., $\varepsilon^* \approx 0.7$. From Eq. 3.1.3, the following expression is immediately obtained:

$$u = \frac{4U}{\varepsilon^* \pi d^2} \tag{8.2.1}$$

Since U is the value set by the operator of the HPLC system, the following expression gives the dependence of t_0 on L, d, and the set volumetric flow rate:

$$t_0 = \frac{\varepsilon^* \pi d^2 L}{4U} \tag{8.2.2}$$

Expression 8.2.2 indicates that the column dead time is directly proportional with the square of the column diameter and the column length. The retention time $t_R(X)$ for a compound X is related to t_0 by Eq. 3.1.17 that can be written in the following form:

$$t_R(X) = \frac{\varepsilon^* \pi d^2 L}{4U}[1 + K(X)\Psi] \tag{8.2.3}$$

Expression 8.2.3 shows how the retention time t_R of a compound is changing when different column dimensions (d and L) are selected for a certain flow rate U, and the same type of stationary phase and mobile phase (ε^* that depends on physical properties of the stationary phase, $K(X)$ that depends on the analyte and the chemical nature of the stationary and mobile phase, and Ψ that depends mainly on the stationary phase structure). When no change is made to an HPLC separation except the column dimensions, Eq. 8.2.3 shows that the retention time of any analyte is directly proportional with the length and with the square of the internal diameter of the column. Since peak broadening depends on u and the change in column dimensions leads to a change in u when U is kept the same, the results of changing the column dimensions influence more than the retention time t_R. However, Eq. 8.2.3 provides a useful guidance regarding the expected t_R values when the column dimensions are changed.

In theory, the retention factor k' is not affected by the physical dimensions of the column because both t_0 and t_R are changing simultaneously (see Eq. 3.1.19) with column dimensions. However, the change in the column dimensions may affect (although not very much) the value of Ψ, by small changes in the void spaces inside the column and slight increase in the volume V_0 of the mobile phase. Overall, the separations can be slightly better with a column with a larger diameter because of a lower linear flow u for the same U and a value closer to the optimum for the theoretical plate number of the column (see Eq. 3.1.70). However, on columns with larger diameter, longer run times are generated and the peaks are broadening. Assuming broadening exclusively caused by diffusion (which is not the real case) based on expressions Eqs. 3.1.43, 3.1.46, and 3.1.50, the peak broadening (in time) W_b is given by the following formula:

$$W_b = 4\sigma_t = 4\frac{\sqrt{2Dt_R}}{L}t_R \tag{8.2.4}$$

Expression 8.2.4 shows that the peaks are broadening for longer retention times. Based on Eq. 3.1.56 for a fixed length of the column L, the value of N decreases when t_R are longer and as a result the resolution of the wider columns is lower. Also, broad peak integration may be performed with less accuracy, and method sensitivity may be affected (see Fig. 3.4.1) with smaller peak height for broader peaks at the same sample amount. For this reason, if other requirements are satisfied, a narrower column may offer advantages.

(2) The separation run time which is a time slightly longer than the retention time of the last peak in the chromatogram is directly related to Eq. 8.2.3. The expression shows that for achieving shorter separation run times, shorter and narrower columns are recommended.

(3) Column efficiency is also related to physical dimensions of a column. Expression 3.1.55 shows that the theoretical plate number N is directly proportional with the column length L. Therefore, longer columns have a larger number of theoretical plates. The height equivalent to a theoretical plate H is not directly affected by the column length. However, since the linear flow rate u changes with the column diameter, the optimum volumetric flow rate U_{opt} for which the minimum H value is obtained depends on d. This value $u_{opt} = \sqrt{B/C}$ (where B and C are parameters in van Deemter equation 3.1.67) will become for the volumetric flow rate U the following (see also Eq. 3.1.70 where explicit dependence on D and d_p is given):

$$U_{opt} = \frac{\varepsilon^* \pi d^2}{4}\sqrt{B/C} \tag{8.2.5}$$

Expression 8.2.5 shows that changes in d will affect the optimum flow rate for obtaining a minimum H. For maintaining the same linear flow rate u in two columns, when the column diameter d_1 is changed to d_2, the following expression for the volumetric flow rates can be obtained from Eq. 8.2.1:

$$U_2 = \frac{d_2^2}{d_1^2}U_1 \tag{8.2.6}$$

For narrower columns (smaller d_2 than d_1), a lower volumetric flow rate U_2 is necessary in order to achieve the same linear flow rate u in the two columns. Since the height of theoretical plate H is dependent on u (see van Deemter equation 3.1.67), the efficiency of the separation can be affected when changing columns with different diameters, even if the other parameters are kept identical but the flow rate is not adjusted properly.

(4) The resolution of the chromatographic column R is also affected by column dimensions. Expression 3.2.8 shows that the resolution (as measured for a compound X relative to previous peak) depends on a number of factors (nature of column stationary phase, nature of compounds to be separated, etc.) and also on the square root of the number of column theoretical plates N. Since N is directly proportional with the column length L, for the same compounds to be separated and identical columns except for their length, the variation of R can be described by the following formula:

$$R = Const \cdot L^{1/2} \tag{8.2.7}$$

Expression 8.2.7 indicates that longer columns offer better resolution, but only proportional to the square root of their length.

(5) The sample load of the column refers to two different parameters: the maximum injection volume of the sample and the

maximum amount of sample to be injected. Formulas for the maximum volume of sample were developed (see Eq. 3.4.17). These formulas show that larger columns allow larger injection volumes. Both column diameter and length influence the maximum injection volume, but the use of columns with larger diameter has more impact on volume load. Expression 3.4.18 indicates the following limit for the injected volume in the column:

$$V_{inj} < 0.035 \cdot d^2 d_p^{1/2} L^{1/2} (1 + k') \tag{8.2.8}$$

As a result, V_{inj} depends on the square of column diameter d and only on the square root of L. Columns with larger diameter can accommodate larger sample volumes. Too large amount of sample injected in the chromatographic column may affect the peak shape usually generating fronting (either steep front of the peak and shortened retention time, or peak less steep than the rear relative to the baseline due to nonideal equilibria in the chromatographic process).

The amount of sample injected is also limited by the amount of stationary phase but also by the nature of stationary phase through its capacity to retain the solute. In analytical HPLC, the sample load is preferred to be low, but the load depends on the detector sensitivity, which limits the minimum amount of analyte that can be injected. Approximate formulas (see Eq. 3.4.19) were developed for the estimation of maximum column load depending on column dimensions but also on other columns and analyte characteristics. The problem of the need of a larger sample load occurs when the sample has a larger amount of matrix ("dirty" matrix) that must be separated from very low levels of analyte. In such cases, a larger sample must be injected in the column to achieve an acceptable detection of the analyte, but at the same time a large amount of matrix must be accommodated by the column. For this reason, columns with more stationary phase (with larger diameter and length) are selected for sample that remains with a large amount of matrix even after a cleanup sample preparation process.

(6) The column backpressure is also affected by the column dimensions. The acceptable value for the backpressure of a column when working in typical conditions is a very important parameter. This parameter is limited by the maximum possible pressure generated by the HPLC pumps, and also by the structure of the stationary phase that may start collapsing above a specific pressure. The value of a column backpressure is determined by the particle size, the flow rate, the nature of the mobile phase, temperature, the column dimension, etc. A formula that governs backpressure in a packed bad column is given by Eq. 3.1.76 for linear flow rates and Eq. 7.5.1 for volumetric flows (Darcy equation):

$$\Delta p = \frac{4\eta U \phi_r L}{\varepsilon^* \pi d^2 d_p^2} \tag{8.2.9}$$

Expression 8.2.9 indicates the proportional increase in the backpressure of the column with the column length L. The backpressure also increases proportional to the decrease in the square of column diameter d. Also backpressure depends on the mobile phase viscosity η, and since the viscosity of liquids typically decreases as the temperature increases, an increased temperature of the mobile phase may be used to lower the backpressure of the column. The value of the column flow resistance factor ϕ_r depends on the structure of stationary phase. Instead of column resistance factor ϕ_r, a related value $\mathscr{K}^0 = \varepsilon^*/\phi_r$ indicated as flow permeability can

be used. The value of $\mathscr{K}^0$ is given by the following expression:

$$\mathscr{K}^0 = \frac{4\eta UL}{\pi \Delta p\, d^2 d_p^2} \qquad (8.2.10)$$

For practical purposes, instead of the specific flow permeability $\mathscr{K}^0$, a column permeability $\mathscr{K}^*$ related to $\mathscr{K}^0$ $\left(\mathscr{K}^* = \mathscr{K}^0 d_p^2 \Big/ \eta\right)$, but that can be directly measured, is defined by the following formula:

$$\mathscr{K}^* = \frac{uL}{\Delta p} = \frac{L^2}{t_0 \Delta p} \qquad (8.2.11)$$

The value for $\mathscr{K}^*$ can be measured for a given column based on Δp, t_0, and L and provides information on the column characteristics and allows the evaluation of $\mathscr{K}^0$ when the solvent viscosity η and particle dimensions d_p are known.

Monolithic columns compared to traditional columns have lower backpressure for similar flow conditions. Also, since the theoretical plate number N is larger for core-shell stationary phases as compared to fully porous particles, core-shell particle columns show lower backpressure for the same N as compared to columns with fully porous particles. For example, for a traditional C18 column with 1.7 μm particles, 50 mm long, 2.1 mm i.d., a typical working backpressure in CH_3CN/H_2O 50/50 is around 400 bar at 0.6 mL/min flow rate ($N \approx 270{,}000$ per m). A core-shell C18 column with 2.6 μm particles that has a similar N and identical length and i.d. develops pressures below 300 bar. However, for columns with particles of the same dimensions, the backpressure is about the same regardless the type of particles used for stationary phase. The backpressure of the column may be overcome by the capability of the pumps of the HPLC instrument, in particular in the case of new UPLC systems. However, concern about phase collapse must be taken into consideration, in particular for stationary phases used in size exclusion and phases with an organic polymeric support in general.

(7) The column stability in time can be estimated by the number of injections n_{inj} that can be made on a column, with unaltered results for the chromatography. This number of injections depends on numerous factors besides the column dimension. The type of column, the nature of mobile phase, the type of sample, the nature of the matrix, the degree of clean-up performed on the sample before injection, the injection volume, the sample concentration, etc., are factors influencing n_{inj}. In order to protect the columns from undesirable materials that may reach the stationary phase, filters and precolumns are commonly utilized in the sample path. The efficiency of the precolumn and the frequency with which this precolumn is changed also influence the number of injections n_{inj}. The only practical observation related to column dimensions is that the columns with larger amounts of stationary phase are in general more resilient to various factors that affect the column stability in time.

Physical properties of stationary phase affecting separation

Chromatographic columns can be classified based on physical properties of their stationary phase in specific types. The selection of a chromatographic column from one of these types is very important for the success of an HPLC analysis since physical properties of the stationary phase are close related to column performance. Table 8.2.2 summarizes a classification of columns based on stationary phase physical properties.

TABLE 8.2.2 Classification of HPLC columns based on stationary phase physical properties.

Stationary phase physical type	Property
Phases consisting of packed particles	Stationary phase made from small particles
Monolithic phases	Stationary phase made of a porous solid rod
Porous particle	Phases made with porous particles frequently based on silica
Core-shell particles	Solid nonporous core surrounded by a porous outer shell
Pellicular particles	Solid spheres covered with a thin layer of stationary phase
Standard diameter particles	3–5 μm diameter
Small particles	1.7–2.1 μm diameter
Uniform particles	Uniform spherical particles d_{90}/d_{10} close to 1 (<1.7–1.8)
Irregular particles	Irregular shape
Small/medium pore size particles	60–100 Å pore size
Large pore size particles	~300 Å pore size
Low/medium surface area particles	150–200 m^2/g surface area
Large surface area particles	~300 m^2/g surface area
Rigid phases particles	Rigid particles (stand 600–1000 bar pressure)
Soft phases particles	Particles collapse above 300–400 bar (or even less)

From the classification described in Table 8.2.2, some stationary phase types are very common in HPLC columns. Other types, such as pellicular particles are not so frequently utilized. Also, the monolithic phases, although with a considerable number of applications, are much less common than phases made from particles packed in the chromatographic column. For this reason, the main discussion will be related to packed chromatographic columns. Also, different types of chromatography depend differently on physical properties of the stationary phase. For reversed phase (RP), HILIC, and ion exchange, the physical properties of stationary phase are important, but the chemical nature of the phase is the main component assuring the separation. For SEC, the separation is based solely on the physical nature of stationary phase, and therefore its role is even more important than in other chromatography types.

Some of the aspects connecting physical properties of the stationary phase with column performance were already indicated in Section 8.1 where the construction of an HPLC column is presented. It was shown that physical properties of the stationary phase are important parameters related to most chromatographic characteristics of a chromatographic separation. Among the chromatographic parameters affected by the stationary phase physical properties are (1) retention time t_R, (2) retention factor k', (3) separation run time, (4) column efficiency (value for N), (5) column resolution R, (6) maximum sample load in the column, (7) column backpressure, (8) results reproducibility, (9) column stability in time, and (10) utilization for the separation of small or large molecules.

(1) The retention time is affected by several physical characteristics of stationary phase.

Expression 8.2.3 shows that t_R depends on phase ratio Ψ (defined by Eq. 3.1.18) and larger values for phase ratio are related to larger retention time. Phase ratio can be different between porous and core-shell columns, by having a different amount of stationary phase in the column. Also, larger surface area of the stationary phase may lead to a larger value for Ψ. Columns with smaller pores have larger surface area of stationary phase, while larger pores lead to smaller surface area of the stationary and therefore to lower Ψ. For columns with fully porous particles and those with the stationary phase with a larger surface area 200–300 m^2/g, longer retention times for the analytes are typically obtained. Since core-shell particles also have a surface area around 150 m^2/g, the differences in the retention times from columns with fully porous particles are not necessarily very large. A direct comparison of columns regarding retention time capability is difficult to make since these characteristics depend on many other parameters, in particular the dependence on equilibrium constant $K(X)$. An effect on retention time may also result from a combination column type plus flow rate in the column. Monolithic columns allow a reduction in elution time up to nine times due to the capability to use higher flow rates without having problems with the column backpressure. Due to a rapid mass transfer of the solutes between the bonded phase and the mobile phase, the decrease in the number of theoretical plates at higher flow rates, as predicted by van Deemter equation (see Fig. 8.2.2), is not as intense for monolithic columns. The use of larger flow rates may be limited in MS detection where flows around 0.3–0.6 mL/min are usually recommended.

(2) The value of retention factor k' is strongly influenced by the physical properties of stationary phase, in particular through the value of phase ratio Ψ. The phase ratio Ψ for an RP-HPLC column can be evaluated using different procedures. In one of these procedures [148], the following expression is utilized for the estimation of the volume of stationary phase:

$$V_{st} = \frac{\%CM_{sil}W_p}{1201.1\rho n_C} \qquad (8.2.12)$$

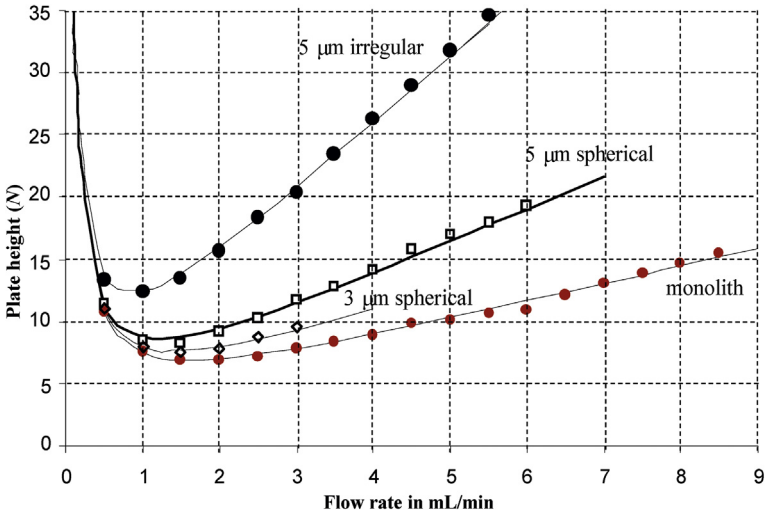


FIGURE 8.2.2 Variation in theoretical plate height H for different types of stationary phases (irregular particles 5 µm, spherical particles 5 µm, spherical particles 3 µm, and monolithic) exemplified for a C18 type stationary phase.

In Eq. 8.2.12, %C is the carbon load (g carbon per 100 g silica), M_{sil} is the molecular weight of the silane used for preparation of the stationary phase, W_p is the weight (g) of the packing, ρ is the density of the bonded alkyl groups (g/cm^3), and n_C is the number of carbon atoms per silane molecule. The use of this formula is straightforward when specific parameters are known for the utilized column. The carbon load is frequently reported by the column manufacturer, the density of the bonded alkyl groups is around 0.86 g/cm^3 [149], the weight of stationary phase can be estimated from column dimensions, and the number of carbons per silane molecule is typically known (except for some proprietary columns). Only the molecular weight of the silane used for preparation of the stationary phase requires some specific information. Eq. 8.2.12 shows that the value of Ψ depends on the phase load. Stationary phase is distributed on the surface of the solid support and a larger surface area leads to a larger value for the volume V_{st} of stationary phase. The pore size of the silica support is related to the surface area and therefore to the retention factor of the stationary phase. Larger pores are associated with a smaller surface area of the solid support and consequently to smaller retention factor. Other procedures based on solvophobic theory and octanol/water partition constant [150,151] or octane/mobile phase partition constant [152] showed that in fact the value of phase ratio also depends on the nature of mobile phase that is in contact with the stationary phase, and also on temperature [153,154]. However, Eq. 8.2.12 provides a guide useful for the estimation of Ψ.

(3) The run times are longer when the retention times are longer, and such longer times are typical for columns with larger dimensions (L and d as indicated by Eq. 8.2.3), but also for columns with porous particles and having larger surface area for the solid support of stationary phase translated in larger Ψ.

(4) Column efficiency (as expressed by theoretical plate height H or number of theoretical plates N) is a parameter highly dependent on physical characteristics of stationary phase, but also on some external factors such as how well the column is packed (with no void spaces). Expression 3.1.68 shows the dependence of H on particle size d_p of packed chromatographic column (one linear term and one quadratic). However, the formula does not take into account the shape of particles that also influences significantly the value of H (and N). An approximation of N based on d_p is given by Eq. 3.1.77 that indicates only that N is inversely proportional with d_p (and directly proportional with the column length L).

For core-shell particles, van Deemter equation 3.1.68 is applicable, but this expression does not give a direct account for the dependence of H on total core-shell particles diameter d_p [155,156]. For typical packed columns, the efficiency per meter N for the column can be between 20,000 and 150,000, and for core-shell columns, it can be as high as 300,000. Some other stationary phase physical properties are influencing the column efficiency, and differences are between packed and monolithic columns. The variation of theoretical plate height H (as function of the flow rate) for a monolith column versus several packed columns is shown in Fig. 8.2.2. The same figure shows the variation of H with the value for the flow rate for a C18 packed column having irregular particles, spherical particles of 5 μm diameter, or spherical particles of 3 μm diameter. The effect of particle shape and size on column efficiency indicates that irregular particles typically have a lower value for N (larger H), while spherical particles have higher values for N. As expected, smaller diameters

for spherical particles lead to higher N values (lower H).
For many columns, the number of theoretical plates N (for specified a test compound) is provided by the manufacturer. An estimation of N for common porous particles columns can be obtained using the following expression:

$$N \approx \frac{1000\,L}{Ct \cdot d_p} \tag{8.2.13}$$

In Eq. 8.2.13, Ct. is taken as 2, 2.5, or even 3, and for this reason, Eq. 8.2.13 offers only a very approximate value for N.

(5) Column resolution is an important parameter depending significantly on physical properties of stationary phase. Form Eq. 3.2.11 of the resolution R, it can be seen that both Ψ and N are present in the formula. Since these two parameters are influenced by the surface area of the solid support of stationary phase, as well as by particle size and shape, the result is that R depends in more than one way on physical properties of stationary phase. In Eq. 3.2.11, another parameter is the constant K for the analytes in the equilibrium between the mobile phase and the stationary phase. This constant depends mainly on chemical composition of the stationary phase.

(6) Maximum sample load in the column, regarding both the volume and the amount of sample, depends on column dimensions (L and d) and also on k' and N (see expressions Eqs. 3.4.17 and 3.4.18). These two parameters depend on stationary phase physical characteristics. In both expressions Eqs. 3.4.17 and 3.4.18, the larger values for N lead to lower acceptable injected volume and mass of the analyte. This result is caused by the fact that the peak shape of columns with high efficiency is narrower and more easily affected by large injections. However, peak shape is affected by a number of additional factors, and sample loads larger than recommended are frequently used when improvement in sensitivity are necessary.

(7) Column backpressure is affected by physical characteristics of stationary phase, as indicated by Eq. 8.2.9. Columns with larger particles (larger d_p) develop lower backpressure. Other aspects of column construction (through the value of ϕ_r) affect backpressure. However, development of UPLC systems with pumps that have the capability to produce higher pressures allows the use of columns with smaller particles for obtaining good column efficiency, in spite of incurring higher backpressures. In order to stand higher backpressures, the stationary phase of the column needs to have appropriate rigidity. If the stationary phase starts collapsing, an undesired space can be formed at the inlet side of the column, which can produce significant peak broadening. For softer stationary phases, or columns made from organic polymers, high pressures must be avoided.

(8) The short-term reproducibility of the results in HPLC may be affected by problems related to instrumentation (e.g., pumps and injector) and by the stability of the column. Protection of the column from excessive backpressure (e.g., by using too high flow rates), from precipitation of solids (e.g., of salts during gradient that changes the mobile phase to a higher organic content), from dewetting (use of high water content in the mobile phase in case of some hydrophobic columns), and from the use of mobile phase with pH outside column stability, will ensure short-term stability.

(9) The stability for longer periods of time of chromatographic column should consider two aspects of stability: (a) reproducibility of the column retention properties from run to run and (b) stability in time of the column when properly protected. Some of these properties are related to

the chemistry of stationary phase and some to the procedure of column packing. For resilience to high pressures, the stationary phase rigidity is very important to avoid the formation in time of a dead volume at the front end of the column. This dead volume can be caused by using high pressures, and once formed lead to significant peak broadening. For use in recommended pressure limits, and protection from "dirty matrix" that would not elute from the front end of the column, it is difficult to relate time stability to a specific stationary phase physical characteristic. Columns with larger particles seem to be more resilient in time than those with small particles. However, since columns with smaller particles have higher values for N, and typically generate narrower peaks, it is possible that the change of column quality is more obvious for such columns, but in fact the peak broadening takes place equally regardless the particle size. Column packing procedure is important for not allowing the formation in time of a void volume at the front of the column that reduces the column efficiency (as expressed by N).

Stability in time of the column is also related to avoiding extreme conditions of use for the column such as very high pressure, pH values for the mobile phase not exceeding the limits of recommended range, avoiding solvents not recommended to be used as mobile phase with the specific column, and avoiding precipitation of buffers in the column.

(10) For SEC, the pore and channels size in the stationary phase play the main role in the separation. However, even for other types of chromatography, such as RP-HPLC, the pore size is an important parameter regarding the utilization of the column for the separation of small or large molecules. For allowing the interaction of the analyte molecule with the stationary phase from inside the pores of the solid support, the molecule must be able to penetrate the pores. For this reason, depending on the application, stationary phases with medium pores (of about 100 Å) are typically used for the separation of small and medium molecules, and stationary phases with large pores (of about 300 Å) are used for the separation of large molecules, such as proteins.

Chemical characteristics of stationary phase affecting separation

The chemical properties of the stationary phase represent an essential factor assuring the chromatographic separation. Based on this nature (and that of the mobile phase), the separation is classified as reverse phase, polar phase, ion exchange, size exclusion, etc., and it is decided on which type of molecules the phase is used for the separation. In this section, only an overall view on the role of chemical properties of stationary phase is presented and the main focus is dedicated to bonded phases utilized in RP-HPLC and in HILIC. Details about stationary phase chemical properties are further discussed in more details in this book. This detailed discussion will also include the stationary phases used in ion exchange and size exclusion. The main chemical characteristic of the stationary phase is the chemical nature of the active phase which is critical for the utilization of the HPLC column. The nature of the active phase determines the value of $K(X)$ in the interaction with the analyte X, and as a result the values of retention factor k' and of t_R (see Eqs. 3.1.20 and 3.1.22), the value of selectivity α (see Eq. 3.2.2), and the resolution R (see Eq. 3.2.11). A summary classification of columns based on chemical nature of their stationary phase is given in Table 8.2.3.

In addition to the chemical nature of the phase, other chemical characteristics of the stationary phase are important. These characteristics include (1) chemical nature of the solid support (silica, zirconia, polymeric), (2) purity

TABLE 8.2.3 List of several types of stationary phases.

Types of stationary phase	Phase nature (examples)	Nature of support	End-capping
Hydrophobic, bonded	C4, C8, C18, other n-alkyl (e.g., C30), mixed alkyl, phenyl, diphenyl, mixed alkyl-aryl, cyclohexyl, fullerene, carbon (graphitized).	Silica	Yes (hydrophobic)
Hydrophobic, bonded	C8, C18, phenyl	Silica ethylene bridged	Yes (hydrophobic)
Hydrophobic	Polystyrene-divinylbenzene (PS-DVB), PS-DVB-methacrylate, DVB/methacrylate, polymethacrylate.	Polymeric	Not needed
Hydrophobic with polar group embedded, bonded	C18-carbamate, C8-carbamate, C8-amide, C14-amide, C18-amide, C16-sulfonamide, phenyl ether	Silica	Yes (hydrophobic)
Hydrophobic, polar end-capped, bonded	C18 polar end-capped	Silica	Yes (polar)
Hydrophobic with polar group embedded, polar end-capped, bonded	Ether-linked phenyl	Silica	Yes (polar)
Hydrophobic, bonded	C8, C18	Silica	No
Hydrophobic fluorinated, bonded	Pentafluorophenyl, perfluoroalkyl	Silica	
Hydrophobic, bonded	C8, C18	Zirconia	
Weakly polar, bonded	Cyano-propyl, pentafluorophenyl	Silica	Yes
Normal phase	Bare silica	Silica	No
HILIC, bonded	Amino, diol, zwitterionic, amide, urea, polyhydroxyl, diol with ether embedded	Silica	Yes/No
HILIC, bonded	Diol, amide	Silica ethylene bridged	No
HILIC	Polyamine	Polymeric	Not needed
Cation exchange, bonded	Sulfonic, carboxyl, phosphate	Silica	No
Cation exchange	Sulfonic, carboxyl	Polymeric	Not needed
Anion exchange, bonded	Ammonium quaternary $-NR_3X$ ($R = CH_3$, C_2H_5, $X = Cl$, OH), amino	Silica	No
Anion exchange	Ammonium quaternary $-NR_3X$ ($R = CH_3$, C_2H_5, $X = Cl$, OH), amino	Polymeric	Not needed
Anion exchange latex agglomerated	Ammonium quaternary $-NR_3X$ ($R = CH_3$, C_2H_5, $X = Cl$, OH), amino	Polymeric	Not needed
Ion-moderated	Sulfonic, carboxyl, Na^+, K^+, Ca^{2+} Pb^{2+}	Polymeric	Not needed

(*Continued*)

TABLE 8.2.3 List of several types of stationary phases.—cont'd

Types of stationary phase	Phase nature (examples)	Nature of support	End-capping
Ligand exchange		Various	
Gel filtration	Silica gel	Silica	
Gel filtration	Polystyrene/divinylbenzene, vinyl alcohol copolymers, polyamide, poly(hydroxymethacrylate)	Polymeric	
Gel permeation	Silica gel	Silica	
Gel permeation	Polystyrene/divinylbenzene, vinyl alcohol copolymers, polyamide, poly(hydroxymethacrylate)	Polymeric	
Displacement		Special	Special
Bioaffinity, affinity		Various	
Chiral, bonded	Chiral substituents such as (S)-valine, other bonded chiral selectors on silica.	Silica	
Chiral	Dextrans, cellulose, proteins,	Polymeric	
Other	Hydride	Silica	
Mixed mode	Various		

of phase support, (3) polymerization type of the phase (see Section 8.1), (4) load of bonded phase, (5) presence of polar embedded groups, (6) abundance of free silanol groups (for silica support), (7) pH stability of the phase, and (8) end-capping.

(1) The chemical nature of the solid support is related to a variety of properties of the chromatographic column, and it was in part discussed in Section 8.1. Silica columns are by far the most common due to the properties/performances of this material that make it adequate for support of the active phase in HPLC. Further discussions of the importance of the chemical nature of solid support are given in Part 2 of this book.

(2) For silica, the types of purity were discussed in Section 8.1. The presence of metals in the silica is a source of additional interactions between the stationary phase and the analytes in the mobile phase. These interactions are not intended and modify the properties of the stationary phase in an undesirable way. For stationary phases with a different support than silica, such as zirconia, such additional interactions besides those intended are even more difficult to avoid and contributed to the lower success of these types of columns.

(3) Horizontal and vertical polymerization lead to differences in the stationary phase characteristics even if the basic active groups (e.g., C8 or C18) are the same. Horizontal polymerization offers a lower phase load and consequently a lower Ψ value for the phase. However, the number of free silanol groups is reduced compared to vertical polymerization because the surface coverage with the bonded phase is more homogeneous. In vertical polymerization, a larger bonding layer is attached to the solid support, leading to a larger volume of stationary phase (V_{st}), but with a less homogeneous coverage of the silica backbone.

(4) The phase load on the solid support important in determining the phase ratio Ψ. The phase load depends on the procedure used to produce the stationary phase, and as previously indicated, horizontal polymerization leads to a lower phase load compared to vertical polymerization. However, even for vertical polymerization that is more common, the phase load may be very different from one column type to another. The phase load by itself, for example, for C8 or C18 columns is described by the carbon load %.

(5) The presence of embedded polar groups in a hydrophobic chain forming the active part of the stationary phase is used to provide (controlled) additional type of interactions in the separation process and modify the characteristics of the stationary phase to offer a different selectivity toward the analytes. A variety of such phases were evaluated in the literature and further discussion of this subject is given in Chapter 9.

(6) The initial abundance of silanol groups is a key factor for stationary phase synthesis but even after derivatization and bonding of the active phase (e.g., C18, C8, etc.) a significant number of silanol groups remain underivatized. The abundance of free silanol groups remaining on the columns is determined by the chemical structure of the initial silica support, the derivatization process leading to a specific density of the active groups of the phase, as well as on end-capping (see Section 8.1). Silanol groups offer polar type interactions and the stationary phase in some columns type is left intentionally present as active phase. Further discussion on the role of silanol groups in a chromatographic column will be given in Part 2 of this book.

(7) The pH stability of the stationary phase is an important parameter to consider during column utilization. The range of pH stability is specified by the column manufacturer, and as indicated in Section 8.1, it is determined by the construction of the column. Very acidic or very basic character of the mobile phase may lead to deterioration of the stationary phase, for example, by dissolving the silica at a higher pH values. Factors such as the nature of the solid support (silica, ethyl-bridged silica, organic polymeric), functional groups bonded on the solid support, end-capping type and density, and use of other procedure to enlarge the range of pH stability (such as CSH) are modifying the column stability to the pH of the mobile phase. Injection of samples with a pH outside the range of column recommended stability is possible within some limits. The buffer capacity of the mobile phase can bring the pH of the injected sample in the desired pH range. However, larger injection volume when the sample travels toward the column as an undiluted plug must be avoided when the sample injected solution has a pH outside column pH stability. Some examples of stability of different columns at the pH of the mobile phase are given in Table 8.2.4 [157].

The results from Table 8.2.4 show that not only the pH of the mobile phase influences the column stability but also the chemical nature of the buffer used to control the pH. For example, the acetate buffer and the phosphate buffer for pH = 7 produce a different result regarding column stability. Stability tests also indicated that the metal oxide layer (zirconia or titania) attached to the silica support increases the HPLC column lifetimes by making the stationary phase less susceptible to dissolution at higher pH (>8) of mobile phase [158].

(8) The end-capping is another chemical property important for column characteristics. The types of interactions between the stationary phase and the analytes in the mobile phase are controlled by end-capping. For hydrophobic phases where the separation is based mainly on hydrophobic interactions, the extent and type of end-capping determines the extent to which polar interactions (plus hydrogen bonding and ionic) also affect the separation. In this way, the end-capping has a direct effect on the value of

TABLE 8.2.4 Stability of several columns to mobile phase pH [157].

Column	Manufacturer	pH 2	pH 3	pH 6	pH 7	pH 7	pH 8	pH 9	pH 10	pH 11
Medium		TFA	Phosph.	Phosph.	Acetat.	Phosph.	Bicarb.	ammon./ bicarb	Ammon.	Tri-phosph.
XBridge C18	Waters	1	?	?	?	1	1	1	1	2
XTerra MS C18	Waters	1	?	3	1	3	1	1	?	?
XBridge phenyl	Waters	1	?	?	1	2	3	3	3	?
Luna C18	Phenomenex	1	?	1	2	2	2	2	?	?
Zorbax Bonus RP C18	Agilent	1	1	2	2	3	3	3	?	?
Sunfire C18	Waters	1	1	3	3	3	?	?	?	?
Zorbax SB C8	Agilent	1	?	?	?	1	2	?	?	?

Acetat., acetate buffer; *Ammon.*, ammonia; *Bicarb.*, bicarbonate buffer; *Phosph.*, phosphate buffer; *TFA*, trifluoroacetic acid; *Triphosph.*, sodium triphosphate. *1*, stable to more than 500 injections; *2*, stable to more than 200 but less than 500 injections; *3*, stable only to less than 200 injections; *?*, no data available.

$K(X)$ for a chromatographic column. End-capping also is important regarding the resilience of the stationary phase to the pH of the mobile phase, as well as the response of the stationary phase to phase collapse (see Chapter 9). Silanol activity on a stationary phase is usually an undesired property, and beside the effort to reduce it, various attempts were made to characterize it [159]. A silanol activity index can be obtained by determining the retention time of N,N-diethyltoluamide (DETA) relative to that of anthracene, with 100% acetonitrile as eluent. The retention of DETA is sensitive toward silanol activity, while anthracene retention behavior is assumed to be solely determined by hydrophobic interactions. The effect of end-capping on the use of HPLC columns is further discussed in Part 2 of this book.

As previously presented, among the chromatographic parameters affected by the chemistry of stationary phase are (1) selectivity α, (2) retention factor k', (3) retention time t_R, (4) separation run time, (5) column efficiency (number of theoretical plates N), (6) column resolution R, (7) maximum sample load in the column, (8) column backpressure, (9) column stability in time, and (10) low bleed of the column.

(1) Selectivity α is one of the main properties of the chromatographic column and an essential parameter for compound separation. Expression 3.2.2 shows that selectivity is determined by the ratio of constants governing the equilibrium of the analytes between the mobile and stationary phase. This equilibrium depends on the chemical nature of the analytes, of the stationary phase, and of the mobile phase. For this reason, the selection of a specific chemical nature for the stationary phase is a crucial component in achieving a desired separation. The choice of the chemical nature of the stationary phase is strictly correlated to the type of chromatography that should be used for a specific HPLC method. The classification of HPLC types such as reversed phase, normal phase, HILIC, ion exchange, etc., can be considered as based on stationary phase nature. Since the support for the stationary phase can also participate with interactions toward the analytes, the nature of this support must also be taken into consideration when selecting a specific column. Details regarding the selection of specific

columns are further presented in this book for different chromatography types.

(2) Retention factor k' (for a compound X) is another important parameter determined by the stationary phase nature. This parameter is affected through both $K(X)$ and Ψ for the specific stationary phase as shown by Eq. 3.1.20. While $K(X)$ strongly depends on both stationary and mobile phase, Ψ is mainly dependent on the stationary phase (although the mobile phase also plays a role in the value of Ψ, see, e.g., Ref. [150]). Higher k' improves resolution R and this would suggest that higher k' values are desirable. However, large k' is related to long t_R (see Eqs. 3.1.21 and 3.1.22) and consequently to long chromatographic runs (which are not highly desirable). Therefore, the selection of the chromatographic stationary phase should be done by compromising one aspect or the other of the separation.

(3) Retention time is strongly affected by the chemistry of the stationary phase, as previously described related to retention factor k'.

(4) Chromatographic run time is determined by the longest t_R in the chromatogram, and therefore strongly depends on the chemical nature of the stationary phase.

(5) The column efficiency (expressed by N) is affected by the chemical composition of the stationary phase, but the dependence is more complex. The rate of transfer of solute into and out of the stationary phase depends on the depth of a bonded stationary phase (see Eq. 3.1.64), but this is not the only source of peak broadening in HPLC [160,161]. Existence of multiple mechanisms of retention that may act differently on molecules also plays an important role on peak broadening (e.g., by producing tailing).

(6) Chemical properties of stationary phase are a key factor in determining the column resolution R. Eqs. 3.2.8 and 3.2.10 can be used to understand the dependence of R on the chemistry of stationary phase. Selectivity α, retention factor k', and the number of theoretical plates N are all affected by the chemical composition of stationary phase. As a result, the selection of a stationary phase/type of column for a specific separation must consider the way all these parameters (α, k', and N) are affected by the stationary phase chemical composition (nature of ligand, polymerization type, load of bonded phase, other effects such as end-capping).

(7) The maximum sample load in the column (volume of injection and mass of the sample) also depends on the stationary phase chemical nature. In Eqs. 3.4.18 and 3.4.19, retention factor k' and the number of theoretical plates N appear explicitly. However, the maximum sample load is affected by the separation mechanism on the column.

(8) Column backpressure is affected by the chemistry of the stationary phase through the column flow resistance factor ϕ_r (see Eq. 8.2.9). General rules regarding this dependence are more difficult to establish [162].

(9) Column stability is another factor significantly affected by the stationary phase chemical composition. The stability includes (a) reproducibility of the column retention properties from run to run, (b) resilience of the column to pH values of the mobile phase close to recommended limits, (c) stability of the column to different solvents, and (d) resilience of the column to heating (within a limited temperature range) [163]. For properly protected columns, the time stability depends on many characteristics, and it is not always related to a specific nature of the stationary phase. Regarding the resilience of the column to extreme conditions of use such as pH outside the range 2–7, the column chemical properties are very important. Also, the stability of the column to various solvents, including 100% water, depends on column chemical composition. This

chemical composition includes the chemical structure of the solid support, the bonded phase (or other column active phases), the end-capping, as well as other treatments of the stationary phase. For example, the structure of the solid support, such as silica, ethylene bridge silica, polymeric substrate, hydrated zirconia, etc., offer different pH stabilities, with silica the least resilient and polymeric support the most resilient. End-capping and other procedures for silica surface treatment such as controlled surface charge procedures (CSH technology) are also determining the resilience of the column to a wider pH range for the column [74]. The nature of bonded phase is also important for the type of solvent that can be used. Some hydrophobic columns, for example, cannot be used with 100% water in the mobile phase, since a dewetting/phase collapse may occur.

(10) The development of new detectors with high sensitivity such as MS and MS/MS detectors requires that columns do not generate any bleeding materials, even at very low levels [164]. Better derivatization procedures assure that columns do not generate any extraneous compounds in the eluting mobile phase.

Octanol/water distribution constant used to describe polarity of the active groups in the stationary phase

Octanol/water partition coefficient K_{ow} has been proven a useful procedure to describe the polarity of small molecules, and in HPLC separations, log K_{ow} correlates well with log k′ for RP-HPLC separations [165]. To a certain extent, the use of log K_{ow} can be extended to the characterization of the type of a stationary phase, although the polarity of a stationary phase is a concept much more complicated to define in comparison to polarity of an analyte or even of a mobile phase. It is common for a stationary phase, typically made with a specific bonded material, to not offer a uniform surface. For example, in the case of derivatized silica, the surface contains the groups from the bonded moieties, the groups from the end-capping process, the underivatized groups from the solid support (silanols), and also the groups of the solid support. For this reason, the phase characterization related to polarity of a specific stationary phase cannot be described by a parameter like log K_{ow}. The parameter log K_{ow} can be used only to understand the contribution to polarity of the moieties from the active phase of stationary phase.

Polarity of stationary phases is best described using a set of parameters obtained from the column behavior toward a set of "test" compounds and in a standard mobile phase. For example, the hydrophobic character of an RP column is well described by the retention factor for ethylbenzene (log k'_{EB}). The subject of stationary phases and columns characterization for specific types of HPLC is presented in more details in Part 2 of this book.

In an attempt to have a simpler procedure for characterizing the polarity of a stationary phase, one possibility is to use a simulation of the phase with a model small molecule [166]. Stationary phases can be seen as having a repetitive structure of a monomeric unit that forms the bonded material on silica surface. For example, a monomeric unit for a bonded phase on silica can be considered to be a short siloxane chain on which are bonded some silanol groups, a "functional" moiety (such as a C18 chain), and optionally some TMS groups that simulate end-capping. Such a monomeric unit can be small enough that its polarity can be estimated using the concept of a model octanol/water partition coefficient.

One advantage toward the qualitative estimation of a K_{ow}^{model} value for a stationary phase is offered by the existence of a simple procedure for the calculation of the values for log K_{ow} based on fragment additivity methodology [167]. In this procedure, the values of log K_{ow} for simple

molecules can be obtained (as an approximation) using the following formula:

$$\log K_{ow} = \sum nf_n \qquad (8.2.14)$$

In Eq. 8.2.14, f_n is a parameter associated with each molecular fragment (e.g., $-CH_2-$, $-O-$, $-NH-$, $-CONH-$, or larger fragments) and n is the number of occurrences of the fragments (in fact, formula for the calculation of log K_{ow} is more complex including a sum for the inclusion of different types of bonds) (e.g., Ref. [168]). Also, the value for f_n depends on which atom or cycle the fragment is connected to. In practice, Eq. 8.2.14 includes particular corrections to the contributions of various functional groups depending on the structural configuration (e.g., Ref. [169]).

Based on the repetitive structure of a stationary phase, Eq. 8.2.14 can be used to model a molecule for which the log K_{ow} value can be easily estimated. The value of log K_{ow} for the model molecule can be used to obtain some information about the importance of certain groups in the structure of a stationary phase. For a bonded phase on silica, the number of types of functionalities is rather limited, and this provides a simple procedure to evaluate the polarity of certain desired models. For this purpose, an initial simple model can be selected. Such an initial model for a silica with an alkyl (propyl) group as bonded phase can be the one shown in Fig. 8.2.3. The $-CH_3$ groups attached to the silicon atoms do not correspond to reality; however, their low polarity is mimicking better the polymeric (not hydrated) silica skeleton.

The selected small molecule shown in Fig. 8.2.3 to model a propyl bonded phase has log $K_{ow} = -0.26$. This small molecule can be modified in various ways, to generate units that can be assumed to represent the model unit structure of different types of stationary phases. The values for log K_{ow} of any model can be immediately obtained using the fragment additivity Eq. 8.2.14. Values for various fragments f_n that can be added to modify the monomer structure are given in Table 8.2.5.

The values from Table 8.2.5 for different fragments can be used showing, for example, the importance of blocking an OH with a TMS group (the replacement of a silanol H with TMS increases log K_{ow}^{model} value with 2.18). Also, comparison regarding the change in polarity by the attachment of specific groups to a hydrophobic chain can be based on such values. As indicated in Table 8.2.5, one additional free silanol group can reduce the log K_{ow}^{model} value of more than two CH_2 groups added to the bonded phase. This observation indicates the importance of free silanols on the silica support for the bonded phase. A higher density of the bonded phase would increase the value of log K_{ow}^{model} considerably. Values from Table 8.2.5 also show how imbedded groups decrease hydrophobicity of the aliphatic chain. The addition of polar groups at the end of an alkyl chain also shows that the polarity increases in the order

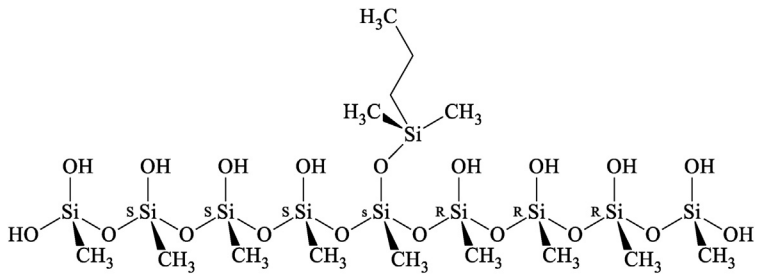


FIGURE 8.2.3 Model small molecule potentially representing the unit for an alkyl (propyl) bonded phase on silica.

TABLE 8.2.5 Various values for f_n parameter for the calculation of log K_{ow}^{model} of model molecules for stationary phase.

Fragment	Connection	f_n
$-OSi(CH_3)_2OH$	Replace $-CH_3$ in siloxane chain with $-OSi(CH_3)_2OH$	−0.85
$-OSi(CH_3)\cdot(OH)_2$	Replace terminal −OH in siloxane chain with $-OSi(CH_3)(OH)_2$	−0.2
−OH	Replace nonterminal $-CH_3$ in siloxane chain with −OH	−1.05
$-CH_3$	Replace nonterminal −OH in siloxane chain with $-CH_3$	+0.4
$-CH_3$	Replace terminal −OH in siloxane chain with $-CH_3$	+0.4
$-CH_2-$	Insert additional $-CH_2-$ in the alkyl chain	+0.396
$-Si(CH_3)_3\cdot(TMS)$	Replace −H from silanol with TMS	+2.18
Phenyl	Attach phenyl to the end CH_3 group of alkyl chain	+1.61
−O−	Replace $-CH_2-$ with −O− in the alkyl chain	−1.68
−CONH−	Replace $-CH_2-CH_2-$ in the alkyl chain with −CONH−	−2.68
$-CONH_2$	Attach $-CONH_2$ to the end CH_3 group of alkyl chain	−1.7
−NHCONH−	Replace $-CH_2-CH_2-CH_2-$ in the alkyl chain with −NHCONH−	−3.17
$-C\equiv N$	Attach −CN to the end CH_3 group of alkyl chain	−1.0
−OH	Attach −OH to the end CH_3 group of alkyl chain	−1.44
Diol	Attach −OH terminal and −OH vicinal	−2.51
$-NH_2$	Attach $-NH_2$ to the end CH_3 group of alkyl chain	−1.49
$-NH_3^+$	Attach $-NH_3^+$ to the end CH_3 group of alkyl chain	−4.27
$-NHCONH_2$	Attach $-NHCONH_2$ to the end CH_3 group of alkyl chain	−1.93
$-NHCONH_3^+$	Attach $-NHCONH_3^+$ to the end CH_3 group of alkyl chain	−5.21
$-NH_3^+$	Attach $-NH_3^+$ to the end CH_3 group of alkyl chain	−4.27
$-N(CH_3)_3^+$	Attach $-N(CH_3)_3^+$ to the end CH_3 group of alkyl chain	−5.35
$-N(CH_3)_2^+-$	Replace $-CH_2-$ with $-N(CH_3)_2^+-$ in the alkyl chain	−6.20
$-SO_3^-$	Attach $-SO_3^-$ to the end CH_3 group of alkyl chain	−3.28

$-CN < -OH < -NH_2$ (not including the capability of NH_2 to be protonated). Addition of an NH_3^+ group to an alkyl chain produces a significant decrease in log K_{ow}^{model} (−4.27) indicating the role of protonation in the change of hydrophobicity. However, the polarity generated by the presence of two opposite ionic groups e.g., $-SO_3^-$ and $-N(CH_3)_3^+$ cannot be estimated based on the values from Table 8.2.5, because the effect of two charges is counterbalanced by the proximity of the two ions of opposite charges. For example, in an HILIC zwitterionic phase, with the group $-N(CH_3)_2^+-(CH_2)_3-SO_3^-$, the total contribution to log K_{ow}^{model} is −3.34, while the calculated one should have a larger negative value. Also, for the groups that can be both neutral and anionic such as amine, or urea, the polarity of the phase depends on the mobile

phase pH, and log K_{ow}^{model} can vary between the neutral and the charged value.

Unfortunately, the previous propyl model does not have any quantitative significance. For example, the addition of 5 $-CH_2-$ groups to the model (to simulate an octyl group) will generate a log $K_{ow}^{model} = -0.26 + 5 \times 0.396 = 1.72$. The simulation of an octadecyl group (by adding 15 CH_2 groups to the initial model molecule) generates a log $K_{ow}^{model} = -0.26 + 15 \times 0.396 = 5.68$. The significant difference between the results for an octyl as compared to an octadecyl fragment would suggest a significant difference in the polarity of the two types of stationary phases for reversed phase chromatography. In reality as described by log k' for hydrophobic compounds such as ethylbenzene, the differences in the retention properties of the two types of columns (C8 and C18) are not consistently different. Fig. 8.2.4 shows the variation of hydrophobic character of several columns with different number of carbons in the stationary phase as described by the value of log k' for ethylbenzene (log k'_{EB}) in a mobile phase acetonitrile/aqueous 60 mM phosphate buffer 50/50 v/v at pH = 2.8 [170].

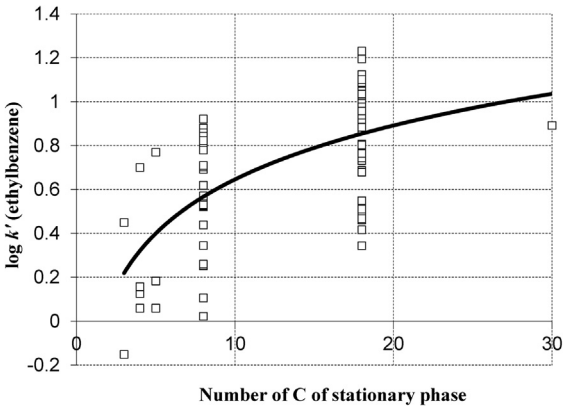


FIGURE 8.2.4 Variation of hydrophobic character of several columns with different number of carbons in the stationary phase as described by the value of log k'_{EB} (mobile phase acetonitrile/aqueous 60 mM phosphate buffer 50/50 v/v).

From Fig. 8.2.4, it can be seen that hydrophobicity as expressed by log k'_{EB} as an average increases with the number of carbons (as expected from log K_{ow}^{model}), but a large variation in the hydrophobic character is seen from column to column with the stationary phase having the same number of carbons. Also, log k'_{EB} is not always higher as the number of carbons in the bonded chain is higher, as would indicate the calculated value for log K_{ow}^{model} based on the model previously described. This result can be easily explained considering the complexity of the solid surface of the stationary phase and the simplicity of the model of unit small molecule to describe the phase. A higher number of free silanol groups can easily modify the log K_{ow}^{model} value, and the result will be that a C8 column with less silanols is more hydrophobic than a C18 column with more silanols. As further shown in Chapter 9, the carbon load of the stationary phase is another parameter related to its hydrophobic character as predicted by the simple model of log K_{ow}. In spite of obvious discrepancies between the quantitative predictions of the model and experimental results, the polarity of stationary phase can still be estimated in term of contribution to log K_{ow}^{model} value for different fragments.

8.3 Selection of a column for an HPLC separation

General comments

The main measure in the choice of a chromatographic column is the adequacy for properly performing the intended separation. This separation must produce well-defined peaks (narrow, with no tailing) such that the peak processing can be done accurately. Peak recognition, peak area measurement, data averaging, and other data processing operations done by the computer programs controlling the HPLC instruments are always performed more accurately and reproducibly when the peak

shape is good and the peaks are narrow (see Section 3.4). The criteria of comparing the performance of a stationary phase include a number of characteristics, some related to the chemical and physical properties of the phase, and other related to external factors. Among these criteria are those listed in Table 8.3.1.

In general, the selection of a column is determined by (1) the properties of the compounds that have to be separated, (2) the specific column properties that are compound independent, and (3) the requirements of the separation, independent of analytes and mobile phase. More details about the column selection are indicated for particular types of HPLC.

(1) The column choice depends mainly on the properties of the compounds to be separated. This should be associated with the selection of the mobile phase/elution program such that a successful separation is achieved. The main goal in a separation is to obtain good resolution R values for the compounds of interest. This is achieved having large N, α, and k' values for the separation. Further discussions will be found in this book regarding ways to obtain columns with large N values, good separation characteristics, and high k' and α values for the compounds of interest.

Sometimes other special requirements are imposed on the analysis, for example, low t_R (that implies low k' values) in a separation is necessary when rapid analyses are needed. Also, as the separation time increases, the broadening of the chromatographic peaks increases (see Eq. 3.1.45 and Fig. 3.1.2). Since an improvement in the resolution R is related to the increase in the k' value, other procedures are involved that attempt to reduce both t_0 and t_R, such that the value of k' is not affected, although t_R is smaller. This can be achieved using higher flow rates, narrower, and shorter chromatographic columns. This type of columns is used, for example, in UPLC type techniques.

(2) The main property of the column that is basically independent of a specific compound can be as considered the number of theoretical plates N (although N varies to a certain extent from compound to compound, this variation is not significant and the choice of a specific molecular species for measuring N eliminates this variability). As previously discussed, columns with smaller and more uniform particles lead to higher N values. Also the columns with core-shell particles are highly recommended for their high N values.

Geometric properties of the column also contribute to the success of a separation. Longer columns lead to a higher N, but at the same time the analysis time is typically increased. Also the column backpressure is higher for longer columns. The linear variation of the retention time with the column length is shown in Fig. 8.3.1 for seven test compounds on a Zorbax SB-C18; 3.5 μm particle diameter column. The change in column diameter also may affect the separation. Eq. 8.2.6 shows the requirement in volumetric flow rate change for achieving identical linear flow rates in columns of different diameter. Columns with larger diameter offer better resilience regarding the number of samples that can be injected without affecting results, but in general have a slightly lower N (for the same length) and require larger volumes of mobile phase.

Besides the properties of a column related to the separation in itself, other characteristics, related to the column utilization, are important. For example, the column should be chemically stable and show no bleed. The bleed indicates column degradation but also can interfere with detection, in particular for specific detectors (such as MS). The resilience to a wide pH range is another desirable property. The backpressure

TABLE 8.3.1 Criteria used for selecting a good HPLC column.

No.	Criterion	a
1	Efficiency and ability to obtain separation of sample analytes, between them and from the sample matrix	V
2	Low skew/low asymmetry/low tailing	C
3	Large values for theoretical plate number N	I
4	Achieve separation within relatively low t_R (low t_R being related to low k values)	C
5	No bleed of the stationary phase	I
6	Stability to extreme operating conditions such as low pH, high pH, range of solvents (e.g., high water content for hydrophobic columns), various components in the sample matrix	D
7	Capability to maintain identical separation characteristics for a large number of injections	I
8	Stability under varying operating conditions	D
9	Rapid regeneration capability	D
10	Acceptable values for the backpressure and the capability to maintain this backpressure constant in time when identical conditions of a separation are used	C
11	Ability to tolerate high pressure and a rapid change in pressure	D
12	Reproducibility of the separation from column to column of the same type but from different batches	D
13	Capability to achieve analytes separation within a wide range of experimental conditions (have versatility)	D
14	Availability in different formats that can be chosen to fit a wide range of available HPLC instruments, and ease of transferability from format to format of separation conditions	D
15	Low price and extensive information regarding the column characteristics	D

[a] *Note:* C, *Critical;* D, *Desirable;* I, *Important;* V, *Vital.*

produced by the column is another practical parameter to consider. High backpressures are not desirable for a number of reasons, including frequent instrument failure associated with it. The HPLC separations are typically performed repeatedly, and analyzing numerous samples by the same method is common in many laboratories. The analysis of numerous samples and the associated data analysis is typically performed using computer-controlled instruments. Maintaining the same separation characteristics when numerous samples are analyzed is another important property for the chromatographic column. This will allow peak identification without human intervention and facilitates accurate data reporting.

(3) The analysis requirements such as speed, number of samples to be analyzed, desire to collect the separated analytes or not, and restrictions regarding the nature and the volume of solvents to be used in the mobile phase also play a role in the choice of the column. The type of available HPLC instrumentation regarding solvent supply system, maximum pressure delivered by the pumps, types of detectors, etc., are also factors influencing or determining the choice of the chromatographic column.

A number of other column characteristics are listed in Table 8.3.1 and labeled as desirable. The importance of these characteristics may vary. When some of them cannot be fulfilled but the separation is good and the results are reproducible, the column is still preferred to a

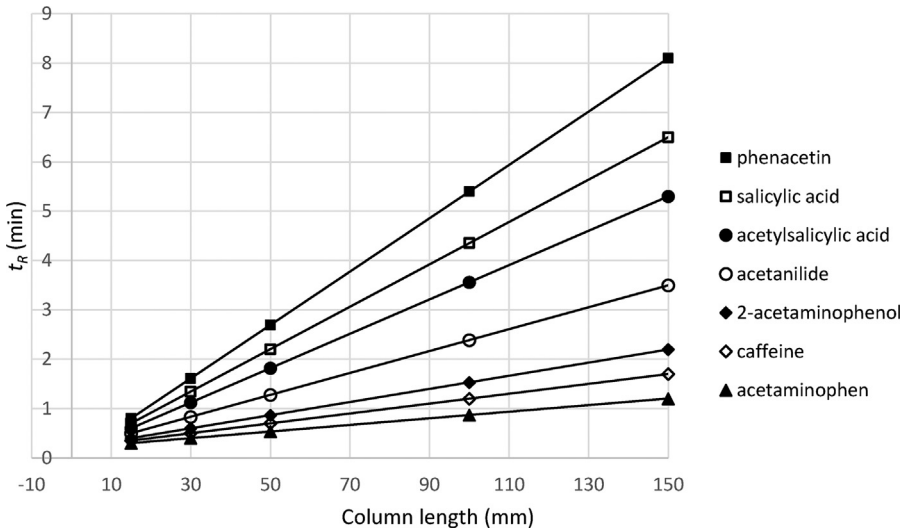


FIGURE 8.3.1 The variation of retention time for seven test compounds with the variation of the column length (Zorbax SB-C18; 3.5 μm particle diameter) (as shown in Ref. [171]).

similar one that offers a certain better property in the desirable group but does not offer, for example, such a good separation. At this point, the choice of the column is more related to individual preferences and needs.

The use of guard columns and cartridges

Some of the column qualities, in particular the capability to maintain identical separation characteristics for a large number of injections, can be improved with the use of guard columns or guard cartridges (e.g., SecurityGuard from Phenomenex). Even if the frits that are used in the path of mobile phase eliminate solid contaminants that may plug the analytical column, when the column is used for a longer period of time, certain strongly retained materials can accumulate on the column, be never eluted, and dramatically reduce column lifetime. By modifying the surface of the stationary phase, these retained materials can cause shifts in peak retention, loss of resolution and efficiency, as well as degradation of peak shape. Another factor contributing to column degradation can be the use of "harsh" mobile phases that can destroy in time the stationary phase (e.g., when a silica base stationary phase is subjected to $pH < 2$ or $pH > 8$ solutions). Even when common solvents are used, some dissolution of silica (for silica-based columns) may occur, leading to the formation of a void volume at the head of the column and subsequent reduced column resolution. A common procedure to protect the analytical column from these problems is to use a guard column. Guard columns are short (or very short) columns or cartridges, packed with a stationary phase having identical or slightly less retentive bonded phase as the analytical column. The guard column should have a very small dead volume such that it should not affect the column performance. At the same time, the guard column retains the noneluting contaminants and provides saturation of the mobile phase with silica by "bleeding" silica into the mobile phase instead of from the analytical column. The guard column must be replaced from

time to time, before the analytical column starts to degrade. The replacement time interval is determined by the specific experimental conditions and can be done either at a fixed schedule (such as 100 injections) or when signs such as increase in the back pressure or some loss of resolution start to be noticed.

Column protection, cleaning, regeneration, and storing

Commercially available chromatographic columns are typically received filled with a specific solvent that is specified by the vendor, and their storage is recommended to be done in a similar solvent as received. The columns for reversed phase are usually received filled with acetonitrile/water 65:/35 v/v; silica-based ion exchange columns are received filled with methanol or methanol/water; normal-phase columns are received filled with isopropanol or hexane. Other columns such as those used for ion exclusion can be received filled with water. Typically, buffers are avoided as storage solvent, although some columns are received filled with a specific solvent with buffer, such as HILIC columns that can be received in acetonitrile/water (e.g., 90:10 v/v) that contains 100 mM of $HCOONH_4$. The columns must be properly installed, and prefilter and the adequate guard columns are always recommended. The mobile phase that is selected with the goal of achieving separation must comply with specific restrictions regarding the nature of the solvent and the pH range. When using gradients, the solvents used as mobile phase must be miscible. Also switching from one solvent to another should be done considering solvent miscibility. The solvent composition must be kept within the limits recommended by the vendor. For example, the water content used with an RP column should not exceed the recommended limit in order to avoid phase collapse by dewetting. The addition of salts for the modification of pH or ionic strength must be done at the lowest possible concentration and salt precipitation in the column will destroy it. The pH range where the column is stable should be strictly obeyed. The temperature at which the column is used should not exceed the vendor specifications. For RP columns, this temperature is typically not higher than 60°C. The maximum backpressure of the column (indicated by the vendor) should never be exceeded, and the flow should go in the recommended direction (indicated on the column). Sudden pressure changes must be avoided. Also, the samples injected in the column should be (as much as possible) completely eluted.

After a period of usage, some column degradation may be noticed, with broader peaks, lower retention times, decreased separation capability, and increased column backpressure. When any of these problems appear, a column cleaning may be useful. The cleaning is typically done by rinsing the column (e.g., 10–20 column volume) with a specific solvent or sequence of solvents at 1/5 to 1/2 typical flow rate. For example, for RP columns, a set of solvents covering a wide range of polarity is recommended, starting with 95:5 water/acetonitrile v/v, followed by THF followed by 95:5 acetonitrile/water v/v. Cleaning of columns used for protein separations may be washed with 0.1% trifluoroacetic acid (TFA) in water followed by 0.1% TFA in acetonitrile/isopropanol $^1/_2$ v/v, followed by rinsing with a common mobile phase (columns should not be stored in THF). Other specific cleaning procedures may be recommended by the column manufacturer. In general, it is not recommended to use a flow in the opposite direction than recommended, in order to diminish the backpressure increase.

Columns that are not used for a certain period of time must be stored appropriately. For this purpose, the clean column must be filled with a specific solvent. For RP-type columns, a mixture of 65% acetonitrile and 35% water is recommended. Before storing, the column must be cleaned from the presence of buffers. For normal phase

columns, isopropanol is typically recommended as storing solvent. Ion exchange columns are usually stored in methanol or methanol/water. For size exclusion columns, water with 0.05% NaN_3 (to avoid bacteria growth) or with 10% methanol is typically recommended. However, column manufacturers may provide specific recommendations for column storage that must be followed.

Selection of columns for orthogonal separations

In HPLC practice, it is sometimes difficult to decide whether a peak belongs to a unique compound or belongs to a mixture of unseparated compounds. The use of detectors that allow the measurement of a compound even if present in a mixture improves the capability of analysis even if the HPLC separation is not good. This is, for example, the case of the use of UV detection for a compound with a unique absorption wavelength, or the use fluorescence detection when the compound has unique excitation/emission wavelengths or the use of MS detection. However, in many cases, the use of selective detection is not sufficient or it is not possible. In such cases, the use of two columns with very different selectivity may solve the problem. If the second column does not lead to the separation of additional peaks, it is more likely that the first separation is complete. Columns with very different selectivity are also desirable for two-dimensional separations, when segments from the eluate from one column are further separated on the second column (2-D or multidimensional separations). The HPLC separations performed on different types of columns and using different solvents that lead to a different separation are labeled as orthogonal. The "orthogonality" of one separation versus another one can be evaluated using procedures specifically developed for this purpose. These procedures are based on the use of test mixtures on the two columns and the comparison of separation results [172]. A potential procedure for testing orthogonality is to make a plot of retention times for the compounds from the test mixture for the separation on the two columns. A good correlation between the retention times indicates a poor orthogonality. Because the separation selectivity in the HILIC mode is complementary to that in reversed-phase and other modes, combinations of the HILIC, RP, and other systems are attractive for two-dimensional applications. Other criteria are based on comparing column properties such as hydrophobic character, hydrogen bonding characteristics, etc. Further discussion on column differences is given in the dedicated chapters for each type of HPLC.

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CHAPTER

9

Reversed-phase HPLC

9.1 Retention and elution process in RP-HPLC

General comments

Reversed-phase chromatography (RP-HPLC) is the most common HPLC separation technique and it is used for the separation of many types of compounds. The compounds amenable for RP-HPLC separation should have some (accessible) hydrophobic moieties in their molecule but the presence of polar groups does not preclude the use of RP-HPLC. Compounds with very different polarities can be analyzed by RP-HPLC. Based on octanol/water partition coefficient for polarity characterization, compounds with log K_{ow} in the (approximate) range between −1.75 and +5.55 can be analyzed using RP-HPLC which includes many types of molecules. Examples of four compounds with log K_{ow} values in this range are shown in Fig. 9.1.1.

For the molecules shown in Fig. 9.1.1, the partial charges on atoms are also indicated. The presence of parts of each molecule bearing partial charges shows that in such cases the RP-HPLC is without difficulty applicable as a separation procedure. Even compounds with acidic or basic character can be analyzed using RP-HPLC [1].

In RP-HPLC, the stationary phase has a predominantly hydrophobic character and the mobile phase is made from a mixture of water and an organic solvent, most common ones being methanol and acetonitrile. As a result, the mobile phase has a polar character. The RP-HPLC is usually practiced using a bonded phase on silica. The bonded phases contain nonpolar groups such as hydrocarbon chains (e.g., C8, C18, etc.), although some stationary phases used in RP-HPLC also include groups with some polarity (e.g., phenyl groups, CN groups, or polar-embedded groups in a hydrocarbon chain). Such phases with partial polar character can be used in RP-HPLC as long as the mobile phase is selected to be more polar than the stationary phase.

RP-HPLC typically generates excellent peak shape, with peaks usually narrower than in other types of chromatography [2]. This result is caused by the fact that the retention sites are not localized in RP-HPLC, whereas they are, for example, in HILIC. The RP-HPLC columns have longitudinal diffusion B/u terms in van Deemter equation (see Eq. 3.1.67) that increase very little with increasing the retention factors. Also, RP-HPLC columns generate short-range eddy dispersion and solid−liquid mass transfer resistance Cu terms that increase less than do other column when increasing retention factors. For this reason, when possible, RP-HPLC is the preferred type of HPLC compared to other alternatives.

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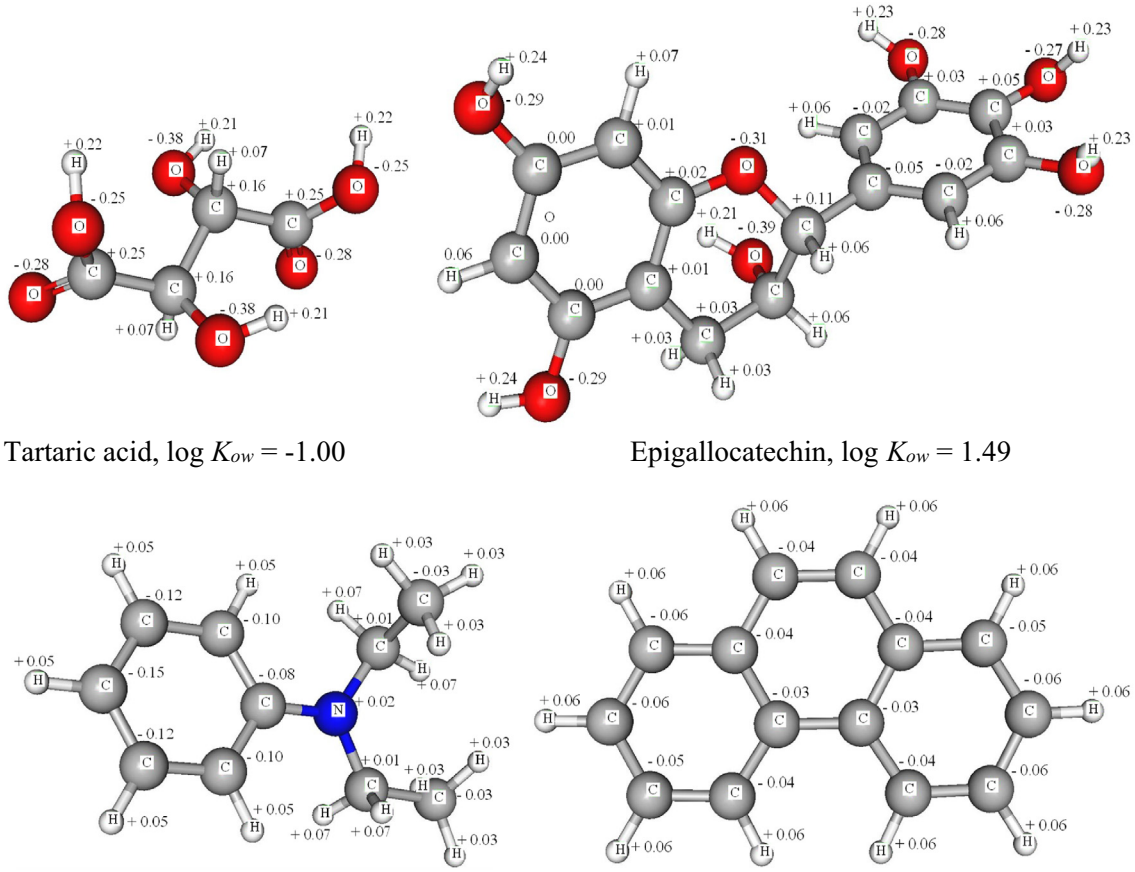

Tartaric acid, log K_{ow} = -1.00

Epigallocatechin, log K_{ow} = 1.49

N,N-Diethylaniline, log K_{ow} = 3.31

Phenanthrene, log K_{ow} = 4.46

FIGURE 9.1.1 Examples of compounds that can be analyzed using RP-HPLC showing partial charges on the atoms.

Retention/elution in RP-HPLC

The retention/elution in RP-HPLC is a complex process based on interactions between the analyte molecules, the solvent, and the stationary phase. As stationary phase is not homogeneous, for example, in case of silica-based phases containing the bonded moieties (e.g., C8 or C18) but also the silanol underivatized groups, and in case of end-capping the groups added for this process, as the mobile phase is very frequently made using a mixture of solvents (e.g., water–methanol or water–acetonitrile), and as the analytes include a wide range of functionalities, the understanding of RP-HPLC separation is not simple. This explains why the *type of equilibrium*, the *type of molecular interactions*, and the *prediction of the result* of separation in RP-HPLC are subject of numerous studies (e.g., Refs. [3–7]).

Equilibrium type in RP-HPLC separation

The equilibrium type of separation in RP-HPLC can be considered in spite of all difficulties of the problem as being of partition [8] although the process is more complex (e.g., Ref. [9]). Only

the separation on RP-phases of some polymeric molecules, such as proteins, may be better described as an adsorption process. Assuming that in RP-HPLC the equilibrium type is partition, the equilibrium constant K_X can be expressed with confidence as a function of the free enthalpy (free Gibbs energy) ΔG^0 of the process based on Eq. 4.1.10, and the retention factor $k'(X)$ can be expressed with Eq. 4.1.11.

Molecular interactions in RP-HPLC

The main type of molecular interactions in RP-HPLC can be successfully approximated with the help of solvophobic theory [10,11]. This theory shows that, during retention on the stationary phase, the molecules containing hydrophobic moieties are "expelled" from a polar solvent and are "accepted" in a hydrophobic stationary phase. In this process, new strong polar–polar bonds (including hydrogen bonds) are formed between the mobile phase molecules from where the analyte is removed. In the same time, the previous bonds of the analyte with the solvent are lost and new bonds are formed between the analyte and moieties of the stationary phase that "accept" the analyte (some interactions between the nonpolar chains of the stationary phase are lost but usually neglected). The new bonds of the analyte with the nonpolar groups C8 or C18 of stationary phase are energetically weaker than the new bonds between solvent molecules (see Table 5.1.1 for the range of values for different types of interactions). As a result, the process is energetically favorable as long as the following relation is satisfied:

$$\left|\Delta G^0_{\text{eluent}-\text{eluent}}\right| - \left|\Delta G^0_{\text{eluent}-\text{analyte}}\right| \gg \left|\Delta G^0_{\text{analyte}-\text{stationary phase}}\right| \tag{9.1.1}$$

(vertical bars indicate absolute value and are necessary because free enthalpy has negative values for the displacement of the equilibrium $X_{mo} \leftrightarrows X_{st}$ to the right). In part because of this simple equilibrium, the retention/elution process is fast and the peak shape in RP-HPLC is typically very good. This process is schematically pictured in Fig. 9.1.2 where different types of interactions are indicated.

Besides the hydrophobic interactions, the process of transfer of a molecule from the mobile phase to the stationary phase is in fact more complex. The stationary phase has other groups that are involved in additional interactions and even in fully end-capped RP-HPLC phases 30%–50% of the silanol groups are accessible to the analyte and solvent producing polar interactions. However, these other interactions can be studied as "corrections" to the basic hydrophobic ones.

Solvophobic theory offers a procedure for the estimation of free enthalpy (free Gibbs energy) ΔG^0 of the retention process. From the value of ΔG^0, the equilibrium constant K_X can be calculated and then the retention factor $k'(X)$, when phase ratio Ψ is known (based on Eq. 3.1.20 $k' = K\Psi$). The partition process is assumed to take place between an immobilized liquid S acting as stationary phase and the liquid mobile phase L. When no volume changes occur during the process the free enthalpy ΔG^0 is taken as equal to the free energy of the process ΔA^0 and Eq. 4.1.9 can be written in the following form:

$$K(X) = \exp\left(-\frac{\Delta A^0_{X,S} - \Delta A^0_{X,L}}{RT}\right) \tag{9.1.2}$$

In Eq. 9.1.2, the free energy $\Delta A^0{}_{X,S}$ result from the change in energy necessary for placing the molecular species X into the solvent formed by molecules S. Likewise, the process of placing the molecular species X in solvent L generates the free energy $\Delta A^0_{X,L}$ (exothermic processes take place with negative free energy). Based on solvophobic theory, the value of $\Delta A^0_{X,S}$ and of $\Delta A^0_{X,L}$ can be estimated starting with Eq. 5.1.77. After taking the logarithm of formula 9.1.2

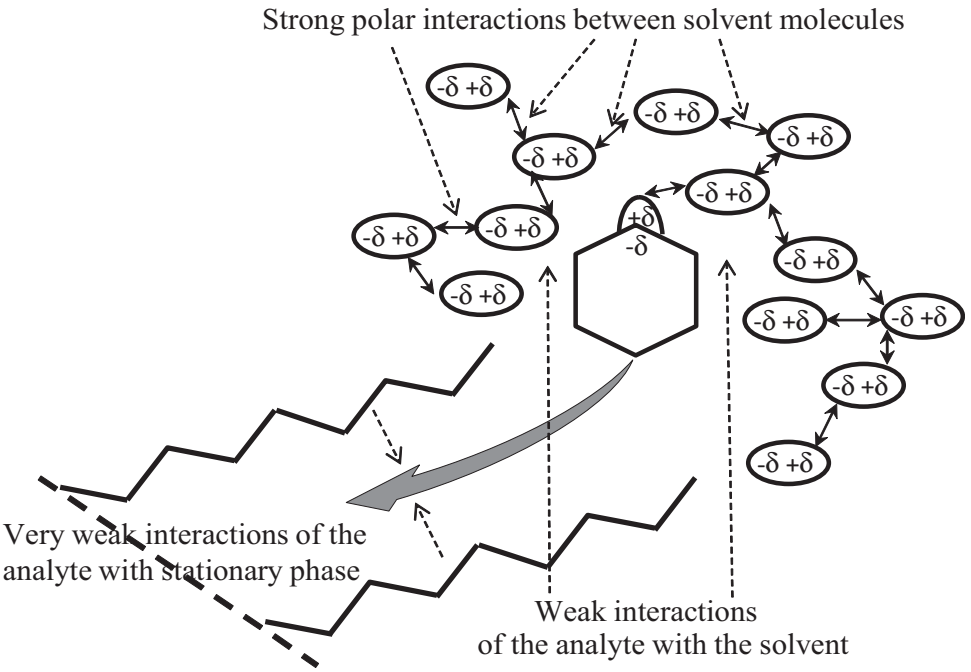

FIGURE 9.1.2 Schematic description of solvophobic effect where the removal of a weak polar molecule from the polar solvent and placing it in the stationary phase is energetically favorable.

(and omitting the symbol Δ and index 0 for the simplification of notation), the following formula is obtained:

$$RT\ \ln K(X) = A^{cav}_{X,L} - A^{cav}_{X,S} + A^{es}_{X,L} - A^{es}_{X,S} + A^{disp}_{X,L} - A^{disp}_{X,S} \tag{9.1.3}$$

From the expressions for $A^{cav}_{X,S}$ given by formula 5.1.65, $A^{es}_{X,S}$ given by formula 5.1.66, and $A^{disp}_{X,S}$ given by formula 5.1.69 and of analogous formulas given for $A^{cav}_{X,L}$, $A^{es}_{X,L}$, and $A^{disp}_{X,L}$, the following result is obtained:

$$A^{cav}_{X,L} - A^{cav}_{X,S} = \mathcal{N}(\gamma'_L - \gamma'_S)\mathscr{A}_X + \frac{\mathcal{N}}{V_X^{2/3}}\left[(\kappa^e_L - 1)\gamma'_L V_L^{2/3} - (\kappa^e_S - 1)\gamma'_S V_S^{2/3}\right]\mathscr{A}_X \tag{9.1.4}$$

$$A^{es}_{X,L} - A^{es}_{X,S} = -\frac{\mathcal{N}^2 m_X^2 V_X}{8\pi\varepsilon_0}\cdot\frac{\mathscr{D}_L - \mathscr{D}_S}{(V_X - \mathcal{N}\mathscr{D}_L\alpha_X)(V_X - \mathcal{N}\mathscr{D}_S\alpha_X)} \tag{9.1.5}$$

$$A^{disp}_{X,L} - A^{disp}_{X,S} = -5.026\pi\mathcal{N}^3\cdot\left[\frac{\mathfrak{B}_{X,L} I_X I_L \alpha_L}{(I_X + I_L)V_L} - \frac{\mathfrak{B}_{X,S} I_X I_S \alpha_S}{(I_X + I_S)V_S}\right]\alpha_X \tag{9.1.6}$$

Formula 9.1.3 with the substitution of Eq. 9.1.4–9.1.6 offers the possibility to understand which parameters are influencing the value of $K(X)$. The parameters related to the analyte X are as follows: $\mathscr{A}_X$ the surface of the cavity in the solvent necessary to accommodate the molecule X (and $\mathscr{A}_X = \mathscr{A}^{vdW}_X$ when the whole

molecule is hydrophobic), V_X the molar volume of X, m_X the dipole moment, α_X the polarizability, and I_X the ionization potential. Regarding the mobile phase L, the parameters are as follows: γ'_L the superficial tension, V_L the molar volume, α_L the polarizability, and I_L the ionization potential. Similar parameters as for the mobile phase would be applicable for the stationary phase although their definitions and values are not usually available.

From Eq. 9.1.3, with all the substitutions, a formal expression for ln $K(X)$ can be written as follows:

$$\ln K(X) = \boldsymbol{a}\mathscr{A}_X + \boldsymbol{b}(V_X)^{-2/3}\mathscr{A}_X + \boldsymbol{c}_X m_X^2 + \boldsymbol{d}_X\alpha_X \tag{9.1.7}$$

Parameters $\boldsymbol{a}$, $\boldsymbol{b}$, $\boldsymbol{c}_X$, and $\boldsymbol{d}_X$ have rather complicated expressions which are indicated below:

$$\boldsymbol{a} = \frac{\mathscr{N}}{RT}\left(\gamma'_L - \gamma'_S\right) \tag{9.1.8}$$

$$\boldsymbol{b} = \frac{\mathscr{N}}{RT}\left[\left(\kappa_L^e - 1\right)\gamma'_L(V_L)^{2/3} - \left(\kappa_S^e - 1\right)\gamma'_S(V_S)^{2/3}\right] \tag{9.1.9}$$

$$\boldsymbol{c}_X = -\frac{\mathscr{N}^2 V_X}{8\pi\varepsilon_0 RT}\cdot\frac{\mathscr{D}_L - \mathscr{D}_S}{(V_X - \mathscr{N}\mathscr{D}_L\alpha_X)(V_X - \mathscr{N}\mathscr{D}_S\alpha_X)} \tag{9.1.10}$$

$$\boldsymbol{d}_X = \frac{-5.026\pi\mathscr{N}^3}{RT}\left[\frac{\mathfrak{B}_{X,L}I_X I_L\alpha_L}{(I_X + I_L)V_L} - \frac{\mathfrak{B}_{X,S}I_X I_S\alpha_S}{(I_X + I_S)V_S}\right] \tag{9.1.11}$$

In Eq. 9.1.7, $\mathscr{A}_X$ is the surface of the cavity in the solvent necessary to accommodate the molecule X, and when the whole molecule is hydrophobic, this area can be taken as $\mathscr{A}_X^{vdW}$, the van der Waals area of the analyte X. In case the molecule has polar groups, these will by solvated, and from the value of van der Waals area, $\mathscr{A}_X^{vdW}$, a part corresponding to each of these polar groups must be subtracted. Including this subtraction as values b_j specific for each polar group "j" such as −OH, −NH_2, =CO, −COOH, etc., the expression for $\mathscr{A}_X$ can be written as follows:

$$\mathscr{A}_X = \mathscr{A}_X^{vdW} - \sum_j b_j \tag{9.1.12}$$

Eq. 9.1.7 does not offer a practical means to the calculation of $K(X)$ and of $k'(X)$. Besides the difficulties in performing the real calculations due to the lack of information regarding specific values for some of the parameters, the theory cannot take into account all the possible interactions, and for this reason, attempts for direct calculations of $K(X)$ or $k'(X)$ were basically abandoned [12]. On the other hand, the theory offers several conclusions that are in full agreement with practical knowledge about RP-HPLC, as follows:

(1) The retention in RP-HPLC is larger for molecules with a larger van der Waals surface area $\mathscr{A}_X^{vdW}$ (see formula 9.1.7).

(2) The retention decreases as the molecule has more polar groups (as expected) and the larger is b_j for a particular group, the lower is the retention (see formula 9.1.12).

(3) A larger surface tension γ'_L of the mobile phase leads to a stronger retention (see formula 9.1.8)

(4) A lager dipole moment m_X of the analyte decreases the retention ($\boldsymbol{c}_X$ is negative), but overall the dipole moment plays a minor role in the retention process since m_X is usually small and $\boldsymbol{c}_X$ depends on its square value (see formula 9.1.10).

(5) Molecules with larger polarizability α_X have a decreased retention ($\boldsymbol{d}_X$ is negative in formula 9.1.11).

Some approximations can be made starting with Eq. 9.1.7. One such approximation is based

on the fact that the polarizabilities α_X of many compounds are proportional with van der Waals surface area of the molecule [13]. With the use of a proportionality constant C_t between α_X and $\mathscr{A}_X$, and with the notation $\boldsymbol{d}^*_X = C_t \cdot \boldsymbol{d}_X$, formula 9.1.7 can be simplified as follows:

$$\ln K(X) = \left(\boldsymbol{a} + \boldsymbol{d}^*_X\right)\mathscr{A}_X + \boldsymbol{b}(V_X)^{-2/3}\mathscr{A}_X + \boldsymbol{c}_X m^2_X \tag{9.1.13}$$

The value for $\boldsymbol{d}^*_X$ was found to vary only slightly from compound to compound (although it is dependent on the solvents S and L). Further simplification can be made by neglecting the term $\boldsymbol{c}_X m^2{}_X$, which is very small [13]. As a result, the expression for ln $K(X)$ can be written as follows:

$$\ln K(X) = \left[\left(\boldsymbol{a} + \boldsymbol{d}^*_X\right) + \boldsymbol{b}(V_X)^{-2/3}\right]\mathscr{A}_X \tag{9.1.14}$$

Replacing $\mathscr{A}_X$ in Eq. 9.1.14 with its expression given by formula 9.1.12, and changing the natural logarithm into decimal logarithm, the formula for log $K(X)$ can be written in the following condensed form:

$$\log K(X) = a'\mathscr{A}^{vdW}_X - \sum_j b'_j \tag{9.1.15}$$

In Eq. 9.1.15, a' is a coefficient depending only on the nature of phases S and L, and b'_j are constants with specific values for each type of polar functionality j present in the molecule X and with $b' = 0$ for hydrocarbons (since their whole molecule is hydrophobic). From Eq. 9.1.15, the retention factor for a specific separation can be written as follows:

$$\log k'(X) = a'\mathscr{A}^{vdW}_X - \sum_j b'_j + \log \Psi \tag{9.1.16}$$

The results of this theory were verified experimentally for a separation on a Zorbax SB-C18 with 5 μm particle size, and 50/50 acetonitrile/water mobile phase. However, the values for the parameters from Eq. 9.1.16 were obtained based on correlations. These values were $a' = 7.417\ 10^{-3}$, b' as given in Table 9.1.1, and the phase ratio as obtained from literature [13] $\log\Psi = -0.511$.

TABLE 9.1.1 Values for constant b' for different polar groups and an HPLC separation on a Zorbax SB-C18 with 5 μm particle size and 50/50 acetonitrile/water mobile phase.

Group	Constant b'
Alcohol	1.4860
Phenol	1.1919
Aldehyde	0.9560
Ketone	1.2107
Ester	1.2087
Disubstituted amide	1.8253
Nitrile	0.9824
Nitro aliphatic	1.0360

The calculated values for log $k'(X)$ based on formula 9.1.16 for a set of 51 different compounds were compared with the data reported in the literature for the same compounds [14]. The comparison of experimental and calculated values for log $k'(X)$ is shown in Fig. 9.1.3.

Retention results based on molecular interactions evaluation

The prediction of the result of separation based on solvophobic theory using Eq. 9.1.7 for the calculation of $K(X)$ (and consequently of ln k') is impractical. The theory cannot address the complexity of all interactions in RP-HPLC, one example being that of hydrogen bond formation (addressed, for example, only through the value of γ'_S and γ'_L, the surface tension). However, some attempts of using this theory for such calculations have been reported

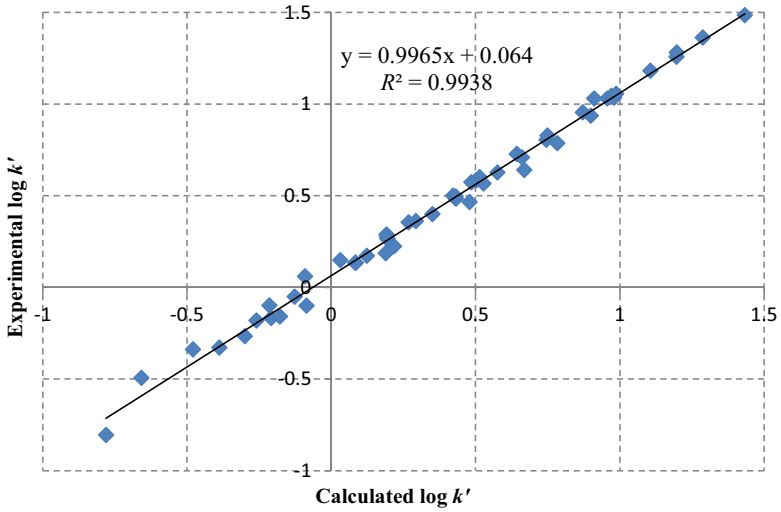

FIGURE 9.1.3 Correlation between the experimental log $k'(X)$ and those calculated based on Eq. 9.1.16 for 51 compounds on a Zorbax SB-C18 with 5 μm particle size, and 50/50 acetonitrile/water mobile phase.

in the literature (e.g., Refs. [15,16]). On the other hand, parametrization based on formula 9.1.13 leads to very good results and excellent correlation between the experimental and calculated data with formula 9.1.16 as shown in Fig. 9.1.3. This parametrization being based on a formula generated by the solvophobic theory indicates that as a basic approximation this theory offers a sound approach for explaining the retention process in RP-HPLC.

Because of the complexity of the RP-HPLC separation, more successful for the prediction of parameters describing the retention/elution (e.g., log k') are models based on empirical best fitting of experimental data for a series of stationary phases and molecules from a training set followed by interpolation or extrapolation for predictions for a new compound not included in the training set (e.g., Refs. [17,18]). In this category can be included the use of formula 9.1.16 based on solvophobic theory. The prediction of the results of retention/elution process has also been attempted using computer simulations based on molecular dynamics (MD) and on Monte Carlo (MC) approach (e.g., using Lennard–Jones interaction potential) [19–21]. The MD and MC procedures were utilized for the description of a variety of aspects related to the structure of stationary phase, interaction of stationary phase with the mobile phase (e.g., Ref. [22]), and the retention for specific molecules (e.g., Ref. [23]). Further discussion on prediction of parameters describing the retention/elution in RP-HPLC is given in Section 9.7.

Other interactions affecting RP-HPLC separations

Numerous other studies were dedicated to the evaluation of the interactions involved in retention/elution process in RP-HPLC (e.g., Refs. [24,25]). Although solvophobic interactions are important in RP-HPLC retention/elution process, or even dominant in some separation (e.g., for highly hydrophobic compounds), other interactions have some role in RP-HPLC. These interactions can be classified as steric interactions, hydrogen bonding between basic solutes

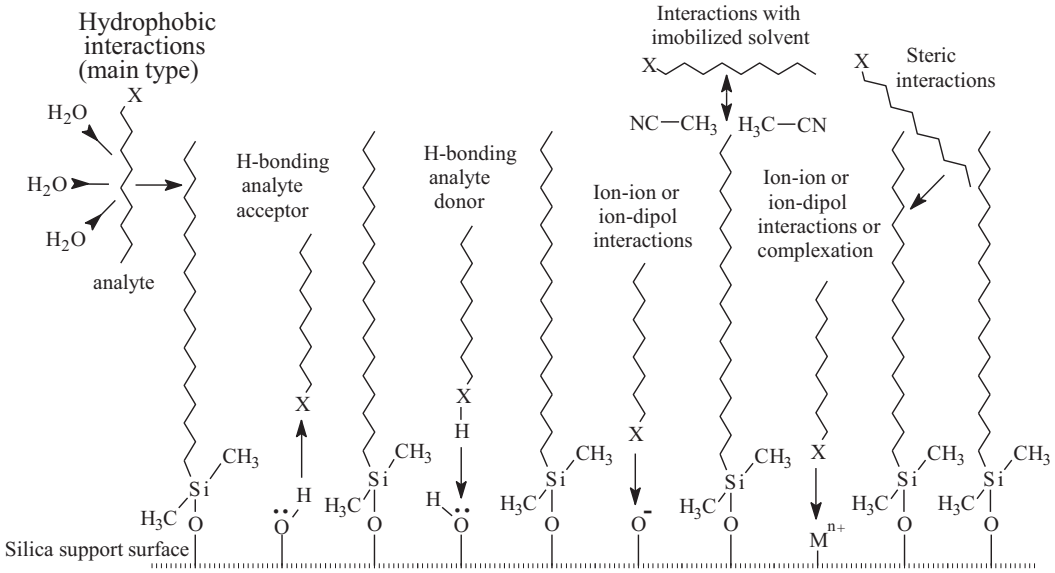

FIGURE 9.1.4 Schematic representation of different interactions present in RP-HPLC.

and the acidic groups of the stationary phase due to the silanols present in the stationary phase or embedded polar groups in the active bonded phase, hydrogen bonding between an acidic solute and basic groups of the stationary phase, and cation exchange type, and/or ion–ion interactions usually caused by impurities in the silica backbone [26]. A schematic diagram of these interactions is given in Fig. 9.1.4 [27]. The figure shows schematically only the interactions for a stationary phase not containing embedded polar groups, or being end-capped with polar substituents, cases when basically the same type of interactions are present, but with modified intensity.

The contributions of various interactions in the retention/elution process in RP-HPLC are important for the modulation of separation on this type of stationary phases. These contributions are characterized by different methods which are further discussed in this chapter. For accounting of these other effects, different models were developed (e.g., Ref. [28]), some such theories even attempting to evaluate the values for k'(or for retention time t_R). One commonly utilized procedure for evaluating the effect of various contributions to the retention process is the hydrophobic-subtraction model [29–32]. In this parametric model, various interaction types are used as modifiers of the basic solvophobic interactions which dominate the retention/elution in RP-HPLC (see Section 9.3).

9.2 Stationary phases and columns for RP-HPLC

General comments

Reversed-phase chromatography (RP-HPLC) uses a variety of stationary phases, the most common ones being octadecyl (C18 or ODS) which have octadecyl groups bonded to silica gel and octyl (C8) that have octyl groups bonded to silica gel. The same type of columns are commonly used in nonaqueous reversed-phase chromatography (NARP), as well as in ion-pair

reversed-phase chromatography. In addition to C18 and C8 stationary phases, various other type of stationary phases are used in RP-HPLC and they are further presented. Several aspects regarding the construction of RP-HPLC type columns are discussed in this section.

Specific procedures for the synthesis of stationary phases in RP-HPLC

As indicated in Section 8.1, the chemical nature of stationary phase is an essential factor assuring the chromatographic separation. Several types of stationary phases including some used in RP-HPLC were already listed in Table 8.2.3. A more detailed classification of hydrophobic phases is given in Table 9.2.1.

Preparation of various types of stationary phases was presented in Section 8.1, and most RP stationary phases are prepared using those procedures. Only the requirement for the bonded phase to have a hydrophobic character makes the preparation procedures specific for the RP type. The most common hydrophobic bonded phases are C8 and C18. Among other stationary phases listed in Table 9.2.1 are short alkyl phases such as C1, C2, and C4 and long-chain phases such as C27 or C30. These phases are used occasionally for special applications. Also considered as hydrophobic stationary phases, but having special properties, are the fluorinated phases. Phenyl phases are hydrophobic but also able to develop special $\pi-\pi$ interactions. Intermediate between hydrophobic and polar phases are the cyanopropyl type. Cyano groups can be ending a propyl handle or longer hydrophobic chains such as C8 or even longer. In such cases, a larger hydrophobic moiety is placed in the stationary phase structure, in addition to the CN group. Phases with a long hydrophobic chain interrupted with an embedded polar group are also available. As shown in Table 9.2.1, most phases are based on a silica support and are in the form of particles but some are as monoliths.

Other types of RP-phases are obtained using organic polymeric materials such as poly(-styrene-divinylbenzene) (PS–DVB) with or with no hydrophobic groups attached to the polymer base. This type of phase usually has lower column efficiencies than those based on silica gel. Polymeric stationary phases can be used as particles or as monoliths [33]. Special other phases such as graphitized carbon, silicon hydride, and hydrophobic groups bonded on zirconia (and much less common alumina and titania) are also known and have hydrophobic character [34–37].

In addition to the construction of columns with a unique hydrophobic character, numerous RP type columns having intentionally some polar characteristics are commercially available. Among these are the columns with embedded polar groups in a long hydrocarbon chain. It was demonstrated that the embedded polar groups do not necessarily decrease the column hydrophobicity, but they offer better wettability and capability to generate additional types of interactions with the analytes besides hydrophobic [38–40]. Some such columns can be used with 100% water mobile phase, which is not recommended for other columns that can develop "dewetting/phase collapse" when the mobile phase is 100% water. Commercially available columns with C8, C18 chains, or phenyl groups may be available with polar-embedded moieties such as those indicated in Fig. 9.2.1. These columns may have also end-capping with hydrophobic or with polar small groups. The solid support for these columns can be silica, but also hybrid silica with ethylene bridged moieties (e.g., Acquity UPLC BEH Shield RP18 columns from Waters that include embedded carbamate groups and ethylene bridge technology). Also the particles can be fully porous or core-shell (e.g., Ascentis Express RP-Amide from Millipore-Sigma).

Among advantages that may come from using the polar-embedded phases include better capability to separate compounds with small hydrophobic moieties and strong polar groups, a

TABLE 9.2.1 Several types of hydrophobic stationary phases.

No.	Type of phase	Phase	Interactions
1	Common n-alkyl on silica end-capped	C8, C18	Strong hydrophobic
2	Special n-alkyl on silica end-capped	C2, C4, C12, C14, C20, C22, C27, C30	Medium or strong hydrophobic
3	n-Alkyl on organic/silica end-capped (BEH type)	C8, C18	Strong hydrophobic
4	Core-shell n-alkyl on silica end-capped	C8, C18, phenyl	Strong and medium hydrophobic
5	n-Alkyl on silica not end-capped	C8, C12, C14, C18	Strong hydrophobic and weak polar
6	n-Alkyl on silica polar end-capped	C8, C18 with	Strong hydrophobic and medium polar
7	Polar embedded on silica end-capped	C8, C18, etc. with−O−, −CONH−, urea, etc.	Strong hydrophobic and weak polar
8	Polar embedded on silica polar end-capped	C8, C18, etc. with−O−, −CONH−, urea, etc.	Strong hydrophobic and polar
9	Cyclic alkyl on silica	Cyclohexyl, n-C6 linked cyclohexyl	Medium hydrophobic
10	Mixed alkyl on silica	C18-short alkyl, C4−C18	Medium or strong hydrophobic
11	Aryl on silica	Phenyl, diphenyl, C2 linked phenyl, C6 linked phenyl	Medium hydrophobic and strong π−π aromatic
12	Mixed alkyl-aryl on silica	C18-phenyl, C6-phenyl, cyclopropyl-phenyl, C14-phenyl	Strong hydrophobic and medium π−π aromatic
13	Cyano on silica	Cyanopropyl, phenylcyanopropyl	Medium hydrophobic and strong polar
14	Fluorinated phases on silica	Pentafluorophenyl, perfluoroalkyl	Medium hydrophobic
15	Other on silica	Fulerene, cholesterol	Strong hydrophobic
16	Silicon hydride	C8, C18 bonded, silicon hydride partially graphitized	Special
17	Monolith silica n-alkyl bonded	C8, C18	Strong hydrophobic
18	Graphitized carbon	Graphytized carbon	Strong hydrophobic
19	Polymeric block	Polystyrene/divinylbenzene, ethylvinylbenzene/divinylbenzene	Strong hydrophobic
20	Phases bonded on organic polymers	C18 bonded on polymethacrylate, phenyl bonded on polymethacrylate, C18 bonded on divinylbenzene, pentafluorophenyl bonded on divinylbenzene	Medium or strong hydrophobic
21	Phases bonded on zirconia	C8, C18, etc.	Strong hydrophobic

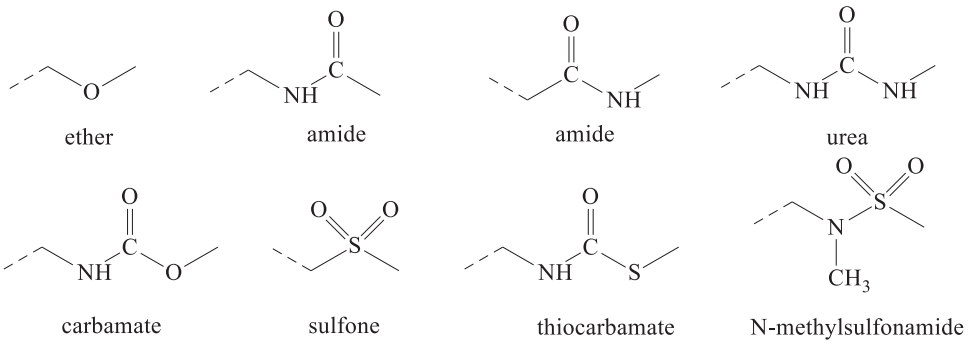

FIGURE 9.2.1 Some embedded polar groups used in hydrophobic stationary phases.

wider pH range of stability, and less effect on the separation of the free silanol groups.

Polar-embedded phases having two zones of polarity or an extended carbon linker (e.g., 5 or 6 atoms to the silica) are also known [41]. These phases reduce the possibility of interactions between the main polar group and silanol from silica surface. Bidentate embedded stationary phases were also reported in the literature [42]. The additional end-capping of polar-embedded stationary phases can be done in the same manner as for other common hydrophobic phases.

Columns with mixed phase (both hydrophobic) such as C18 and pentafluorophenyl were also developed [43]. The combination of active phase compositions provides additional flexibility in separations. Also, phase modifications that allow better separation of amines (e.g., Kintex PS C18 phase from Phenomenex) that have groups adding positive charges on silica surface were developed. Columns with mixed mode phases, e.g., C18 and alkylamine (C18 and ion exchange) are also made (e.g., InertSustain AX-C18 columns from GL Science), allowing better separation of polar acids and ideal peak shapes for basic compounds. Similar phases with mixed mode (but with proprietary phase composition) were developed for specific tasks such as analysis of amino acids [44].

An important part of preparation of stationary phases for RP-HPLC is the end-capping and this procedure was described in Section 8.1. End-capping is usually performed by derivatizing with trimethylsilyl groups (TMS) as much as possible of the remaining −OH groups on silica surface. Because of the smaller dimensions of TMS groups, they can be attached to silanol groups even after the saturation of the silica surface with the main hydrophobic groups. Besides end-capping with small hydrophobic groups such as trimethylsilyl or with bulky fragments, end-capping with small fragments containing polar groups was found to protect the silica structure and extend the range of pH where the columns can be utilized (sometimes indicated as base deactivation technology or BD). This procedure is also utilized for generating phases with some polar character in addition to the hydrophobic character of long alkyl groups. The polar end-capping groups are usually amino or hydroxy, bonded to a propyl chain. These polar-end-capped stationary phases possess hydrophobicity similar to nonend-capped ones, and they display enhanced hydrogen bond type interactions, but the acidity of the polar groups is reduced and it is better controlled compared to that of silanols. Polar end-capping makes the phases more compatible with highly aqueous mobile phases, and this type of column has useful specific applications. For example, the use of a polar end-capped C18 phase can retain more polar water-soluble compounds such as water-

soluble vitamins, being a better choice for their separation.

In column manufacturing, the utilization of a certain technology may be geared toward enhancing a specific property but it can be at the same time somewhat detrimental to another property. For example, a larger number of silanol groups in the initial silica backbone will be preferable for achieving a higher degree of derivatization, but at the same time may diminish the resilience of the stationary phase to an extended pH range. The result is the availability of a variety of columns with many different characteristics.

Basic physical properties of hydrophobic stationary phases and columns

The importance of physical properties of the stationary phase and of the column in HPLC in general was presented in Section 8.2. Some particular aspects regarding the physical properties of stationary phases and RP-HPLC columns are summarized in Table 9.2.2.

It is common that for the same stationary phase there are commercially available several column dimensions. Regarding the other physical attributes, they depend on the procedure for the synthesis of silica support, and they can vary considerably from one phase type to another. A wide variety of technologies are used in silica base manufacturing. Some examples of physical (and chemical) attributes of various hydrophobic silica-based columns are listed in Appendix 9.2.1 and for several polymeric-based hydrophobic columns are given in Appendix 9.2.2. The influence of column and stationary phase physical attributes on different aspects of separation was discussed in Sections 8.2.

Among the physical properties, surface area of the silica support is correlated to the retention capability of the HPLC column as characterized for example by the value of log k' for a test compound (see Section 9.3). This correlation is shown in Fig. 9.2.2 for the set of C18 columns listed in Appendix 9.2.1, and using as a test compound toluene in a mobile phase 80% CH_3OH and 20% aqueous buffer at pH = 6.

As shown in Fig. 9.2.2, the correlation between the retention of toluene and surface area of the stationary phase is rather poor ($R^2 = 0.3473$). This indicates that other factors are more important regarding the retention capability of the column.

TABLE 9.2.2 Basic physical properties of columns and stationary phases with hydrophobic character.

Attribute	Common range
Column internal diameter (i.d.)	2–10 mm (common i.d. in mm 2.1, 3.0, 4.6)
Column length	50–300 mm (or longer) (common length in mm 150, 250)
Particle type	Porous, core-shell, pellicular, monolithic
Particle shape (for silica)	Irregular or more common spherical
Particle size	1.7–10 μm
Particle size distribution d_{90}/d_{10}	$d_{90}/d_{10} < 1.7$ ($d_{90}/d_{10} \approx 1.1$ indicates very homogenous particles)
Surface area (for silica)	50–500 m^2/g
Particle size distribution	Narrow (1%–2% size variation) to larger distributions
Pore size	50 Å–4000 Å
Theoretical plate number (per m)	30,000–120,000 for porous particles, higher for core-sell.

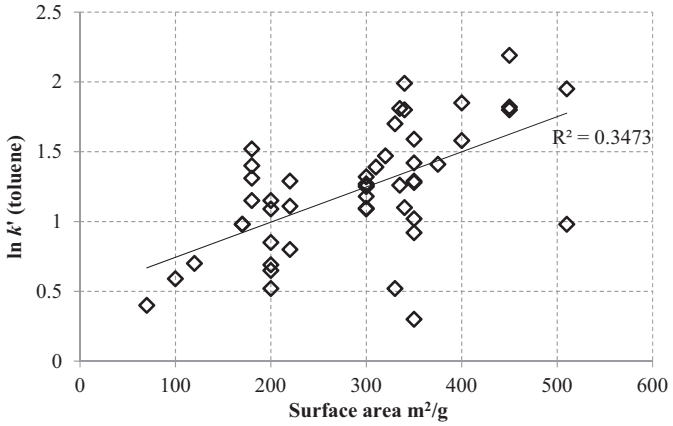

FIGURE 9.2.2 Dependence of ln k' on surface area of the stationary phase for different C18 columns (listed in Appendix 9.2.1) for toluene as a test compound and a mobile phase 80% CH_3OH and 20% aqueous buffer at pH = 6.

Basic chemical characteristics of RP-type stationary phases

The chemical nature of the stationary phase has a critical role for the stationary phase properties and its adequacy for a specific application. Several chemical characteristics of stationary phases and RP-HPLC columns are indicated in Table 9.2.3.

(1) The support type of the bonded phase is one basic chemical characteristic of the stationary phase. As indicated in Table 9.2.3 and in Section 8.1, a variety of supports are used in RP-HPLC, although the most common are silica and silica organic (ethylene bridged). Some organic polymers are also used to make RP-HPLC columns, and a few columns have other inorganic materials a support. The advantages offered by the porous silica support with a bonded phase (in porous or core-shell particles) as compared with other supports such as zirconia or polymeric materials lead to an overwhelming use of silica-based columns in RP-HPLC [45]. The silica support specific chemistry refers to the various ways the hydrated silica has been prepared, and the use of organic/inorganic silica such as ethylene bridged (BEH) type materials. The base silica can be made with a larger or a smaller number of silanols (depending on dehydration process as discussed in Section 6.1) which may lead to different silica reactivity. As a result, all silica materials (including ethylene bridged type products) are sensitive to the pH of the mobile phase, but with different degrees of stability. This stability may be limited to the range of pH from 2 to 7, or the range can be wider depending on silanol reactivity and protection. Ethylene bridged products are resilient to a wider pH range (e.g., 1−12) as compared to common silica-based columns. The preparation of silica support is an important step in column manufacturing and various procedures and thermal treatment may modify several support characteristics such as the microchannels in the porous silica structure.

(2) The purity of silica has been discussed in Section 8.1. Pure silica (*Type B*) is now very common in stationary phase construction. Metal

TABLE 9.2.3 Basic chemical characteristics of phases with hydrophobic character.

No	Property	Common range
1	Support for stationary phase	Silica, silica/organic, other inorganic, organic polymer
2	Silica purity (for silica), and metal activity	High to medium (type B or type A); the metal activity may vary from very low to high
3	Phase type	Bonded on silica, bonded on organic/silica (BEH type), graphitized carbon, polymeric, bonded on polymer
4	Reagent type	Monofunctional, bifunctional, trifunctional
5	Polymerization type	Horizontal, vertical
6	Carbon load (for silica)	3%–25% *C*
7	Coverage (for silica)	Depending on phase, 2.0 μmol/m^2–4.5 μmol/m^2
8	Uniformity of coverage	Less uniform to more uniform
9	Phase ratio	Log Ψ between −0.3 and −0.9
10	Silanol activity and end-capping (for silica)	Silanol activity low to high based on end-capping
11	pH resilience	From range 2–7 to extended range
12	Wetting characteristics	Poor to good

impurities in silica are capable of interacting through coordinative bonding in particular with amines generating additional undesired interactions with the analyte which lead to peak broadening.

(3) The nature of bonded substituents can vary, and some common bonded substituents were listed in Table 9.2.1 (as phase). The RP type columns are constructed to have a hydrophobic character. However, in the variety of RP type columns some are made with the purpose of eliminating as much as possible other types of interactions and others are intentionally made to add specific polar interactions.

Regarding the hydrophobic character, this is an essential property in RP-HPLC. Although in RP-HPLC the mobile phase composition is key in the retention process to "reject" the analyte molecules from the mobile phase and place them in the stationary phase, the role of solid phase hydrophobicity is still strongly manifested in the separation. A stationary phase with very low polarity is still more accessible to hydrophobic molecules compared to a less hydrophobic stationary phase. Besides other interactions involved in the separation, differences in the hydrophobicity of the phase contribute to differences in the response to analyte in particular for molecules having no polar groups. Also, since most molecules separated by RP-HPLC do have polar groups, a stationary phase with reduced polar interactions will act differently toward analytes as compared to less hydrophobic stationary phases. The model log K_{ow}^{model} described in Section 8.2 gives some indication about phase hydrophobicity. Several other parameters are used for describing hydrophobicity.

One important parameter related to hydrophobicity is log k' measured for hydrophobic compounds such as toluene, ethylbenzene,

butylbenzene, acenaphthene, etc (see Section 9.3). Larger log k' values for such compounds indicate a more hydrophobic phase. However, the values for k' (log k'), although directly related to column hydrophobicity, depend on both the equilibrium constant $K(X)$ (for a compound X) and also on the phase ratio Ψ, and Ψ is not related to hydrophobicity. Both $K(X)$ and Ψ are function of other stationary phase characteristics and for this reason different procedures were developed for stationary phase hydrophobicity characterization (see Section 9.3). The values for k' are still informative regarding hydrophobicity and as an example, for ethylbenzene as a test analyte X, and the mobile phase 50/50 (v/v) acetonitrile/aqueous buffer 60 mM phosphate at pH 2.8, the values for log k' for various types of RP columns are shown in Fig. 9.2.3.

The data from Fig. 9.2.3 is informative regarding several aspects of hydrophobic character of the columns. One first observation is the relatively large spread of log k' for columns with the same type of phase. As expected, the values for log k' are the lowest for columns with a bonded phase containing the CN group (these columns make the transition toward polar ones). As an average, the increase in the bonded hydrocarbon length (from C4 to C18) shows an increase in the log k' values. However, some C8 phases and even phenyl phases have larger log k' than some C18 columns. Also, two columns having very long hydrocarbon moiety (C30) do not show an increase in the log k' value compared to C18 or even C8 columns. This probably happens because the surface coverage with the bonded phase is not high in C30 columns, due to the high volume of this chain, or because not entire hydrophobic chain is exposed to the interaction with the solute molecule. Phenyl columns have log k' not very different from C8 type columns. The same remark is valid for fluorinated columns. In these columns, a fluorinated fragment (usually pentafluorophenyl) is attached to the silica surface with a short propyl or longer hexyl handle. Besides hydrophobic character, these fluorinated phases tend to have

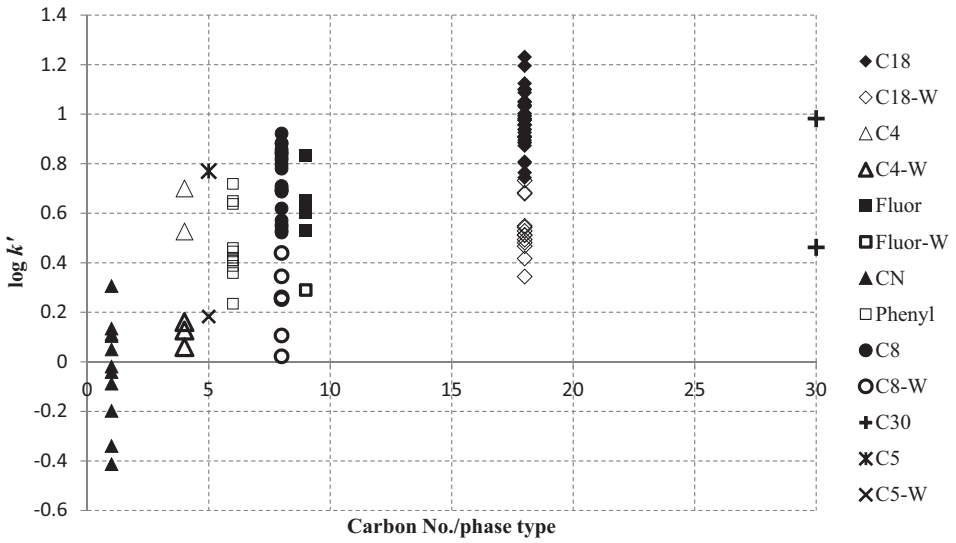

FIGURE 9.2.3 Values for log k' for various types of columns for ethylbenzene as a test analyte, and the mobile phase 50/50 (v/v) acetonitrile/aqueous buffer 60 mM phosphate at pH 2.8 (W indicates wide pore type stationary phase).

higher propensity for ion–ion interactions in addition to the hydrophobic character. The fluorinated phases offer an alternative material for separations and were proven very useful in particular separations [46,47]. Columns with bonded groups such as perfluorohexyl straight chain, perfluorohexyl branched chain, perfluorooctyl, perfluoropropyl, perfluorododecyl, pentafluorophenyl, pentafluorophenylalkyl, pentafluorophenylpropyl, etc., are available. These phases cover a wide range for the k' values indicating that the fluorinated materials offer a wide range of hydrophobicity.

(4) The reagent type as monofunctional, bifunctional, or trifunctional, or the production of a stationary phase by direct synthesis as it was discussed in Section 8.1 is common for RP-HPLC phases.

(5) Polymerization type for silica-based columns (horizontal or vertical) is another parameter used for obtaining certain desired properties of the stationary phase of the column, such as loading capacity, surface coverage of the support, resilience to a wide pH range, etc.

(6) The carbon load (as *C*%) offers a global characterization of hydrophobic phases. In general, a higher carbon load (which can reach up to 22%–24%) is an indication of a more hydrophobic character. However, with new more modern phases that have polar-embedded groups, organic/inorganic solid support, various types of end-capping, etc., it is even more difficult to predict hydrophobicity from the stationary phase construction and carbon load. For several C18 columns listed in Appendix 9.2.1, the dependence of ln k' on *C*% is shown in Fig. 9.2.4, using toluene as test compound and a mobile phase 80% CH_3OH and 20% aqueous buffer at pH = 6.

(7) The density of surface coverage, also discussed in Section 8.1 (see Eq. 8.1.21), is related to various parameters of column construction including carbon load but also the accessibility of the analytes to the active bonded phase. Depending on the density of silanol groups on silica surface (see Eq. 8.1.7), as well as the ratio of silanol density α_{OH} and that of the ligand δ_{ligand}, different column characteristics can be achieved.

(8) The uniformity of the coverage of silica surface influences the accessibility of the analyte molecules to silanol groups. In case of highly uniform coverage, more active silanol groups

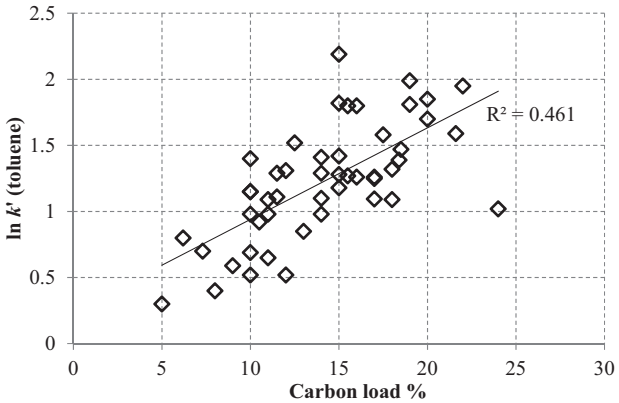

FIGURE 9.2.4 Dependence of ln k' on carbon load (*C*%) for different C18 columns (listed in Appendix 9.2.1) for toluene in a mobile phase 80% CH_3OH and 20% aqueous buffer at pH = 6.

are covered with the desired bonded phase. In case of vertical polymerization, several layers of bonded moieties can be added such that the carbon load of the phase is increased, but this does not assure that patches of bare silica are absent. A uniform coverage diminishes the silanol activity and increases the resilience to higher pH for the column.

(9) Phase ratio Ψ is an important characteristic of chromatographic columns defined by Eq. 3.1.18 as the ratio of V_{st} and V_0. Phase ratio is directly related to retention factor k' as shown by Eq. 3.1.20. Through k', other important parameters in HPLC depend on Ψ, among these being column resolution R (see Eq. 3.2.8 or 3.2.10). Also, the evaluation of thermodynamic parameters of an HPLC separation from van't Hoff plots (entropy changes evaluation) requires the value of phase ratio (see Eq. 4.6.3).

Several procedures for the evaluation of V_0 (and of dead time or void time t_0 with $V_0 = t_0 U$) and for the evaluation of V_{st} were reported in the literature (e.g., Refs. [48–51]. Formula 8.2.12 indicates one of the expressions that can be used for the evaluation of V_{st}, but the calculated value should be considered "constructional" since this value does not account for potentially immobilized solvent component on the stationary phase and therefore for the variation of the value of Ψ with the change in the mobile phase composition. The difficulties in evaluating Ψ are associated with a considerable number of theoretical and experimental studies on the subject [52–54].

An alternative procedure for the evaluation of an "effective" phase ratio Ψ is based on the correlation that exists between the retention factor k' ($\log k'$) and the octanol/water partition coefficient K_{ow} ($\log K_{ow}$) for hydrocarbons in a homologous series [50]. Based on solvophobic theory, a relation between $\log k'$ and $\log K_{ow}$ has been developed and it was shown that for a hydrocarbon the following relation can be written:

$$\log k'(X) = a \log K_{ow}(X) + \log \Psi \qquad (9.2.1)$$

(X denoting different hydrocarbons). From the linear plots of $\log k'(X)$ versus $\log K_{ow}(X)$ for a series of hydrocarbons, the values for a and $\log \Psi$ can be obtained as the slope of the line (a) and intercept ($\log \Psi$). An example is given in Fig. 9.2.5 for the evaluation of Ψ for a Luna C18(2) column, 150 × 4.6 mm with 5 µm particles (from Phenomenex, Torrance, CA,

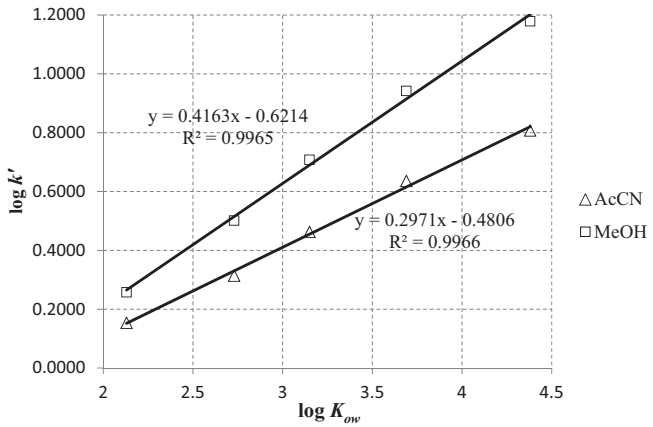

FIGURE 9.2.5 Determination of Ψ for a Luna C18 (2) column with the mobile phase water/organic solvent 30/70 (v/v) where the organic solvent is either methanol (MeOH) or acetonitrile (AcCN) and the analytes are benzene, toluene, ethylbenzene, propylbenzene, and butylbenzene.

USA), with the mobile phase water/organic solvent 30/70 (v/v) where the organic solvent is either methanol (MeOH) or acetonitrile (AcCN) and the analytes are benzene, toluene, ethylbenzene, propylbenzene, and butylbenzene, [55].

The results from Fig. 9.2.5 indicate that log $\Psi = -0.6214$ ($\Psi = 0.2391$) for mobile phase water/methanol and log $\Psi = -0.4806$ ($\Psi = 0.3307$) for mobile phase water/acetonitrile. Results for log Ψ for a series of columns obtained by the previously described procedure, with the mobile phase consisting of 65/35 acetonitrile/aqueous buffer 20 mM KH_2PO_4–K_2HPO_4 at pH 7 are indicated in Table 9.2.4, [50].

(10) Silanol activity is an important characteristic of RP type of columns influencing the separation characteristics. In many columns, the reduction of silanol activity is performed as much as possible, but some columns have intentionally some silanol activity for enhancing the interaction with polar compounds.

The silanol coverage is related to the phase resistance to the pH of the mobile phase, as well as the intensity of other interactions exhibited by the phase. The common pH range of stability for silica-based stationary phases is between 2 and 7 (or 8). This range is not entirely satisfactory in some applications. Basic pH is able to deteriorate the silica structure, and pH below 2 produces hydrolysis of the fragments that make the active phase, while pH higher than 8 leads to the dissolution of silica. Silica-based stationary phases resistant to a wider pH range are highly desirable, to allow the use of more acidic or more basic mobile phases. Lowering the pH of aqueous component in mobile phase to very acidic values, close to 1, may improve some of the retention parameters (retention time, peak shape, or selectivity), in particular for analytes having $pK_a < 2$. One reason for this effect is that at low pH values the interactions of residual silanols with amino containing solutes are suppressed and thus peak tailing is no longer observable. However, such low pH values may not be acceptable for the stationary phase stability. The end-capping of silanol groups, with the purpose of reducing secondary interactions with the analytes, also has some effect on extending the acceptable pH range for the mobile phase. This effect is due to the blocking of the access of H^+ or OH^- ions toward the silica.

For the reduction of silanol activity, a key problem with silica-based materials is related to the presence of a large number of residual silanol groups after the derivatization with the intended bonded phase. This problem remains even for ethylene-bridged (BEH) type phases,

TABLE 9.2.4 Calculated values for log Ψ for several reversed-phase columns including conventional C18, polar-embedded, and polar-end-capped.

Column	log Ψ	Ψ
Luna5 um C18(2)	−0.523	0.300
Inertsil ODS(3)	−0.504	0.313
Zorbax XDB C18	−0.642	0.228
Symmetry C18	−0.559	0.276
HyPurity Elite C18	−0.834	0.146
Zorbax SB C18	−0.679	0.209
Prodigy ODS(3)	−0.558	0.277
Keystone Aquasil	−0.594	0.255
Aqua C18	−0.544	0.285
YMC Hydrosphere	−0.611	0.245
YMC ODS-Aq	−0.547	0.284
Prontosil C18 AQ	−0.610	0.246
Metasil AQ	−0.440	0.363
Synergi Hydro RP	−0.463	0.345
Zorbax Bonus-RP	−0.652	0.223
Polaris C18-A	−0.748	0.179
Discovery RP-Amide	−0.740	0.182
Symmetry Shield RP18	−0.491	0.323
Polaris Amide C18	−0.462	0.345

although they offer some advantages regarding better resilience to a wider pH range. A number of procedures have been used for the reduction of the effect of residual silanols. Among these strategies, the most common is the end-capping using trimethylsilyl groups. This procedure was previously discussed in Section 8.1. Columns "double end-capped" are also reported as providing further coverage of silanol groups (e.g., Eclipse XDB). Repeated end-capping operations are not uncommon, although some free silanol groups still remain on silica surface. Other procedures were utilized to shield the interaction of silanol groups with the analytes, such as the use of bidentate or tridentate reagents for attaching the desired group on silica surface [56]. Also, horizontal polymerization that allows a more uniform derivatization of silica surface was shown to offer better protection of silanol surface interactions [57]. The introduction of a small percentage of groups displaying positive charges on silica surface (charge surface hybrid or CSH) also has been used for reducing peak tailing for basic compounds by diminishing the interaction with the silanol groups. The free silanols may be present inside some small pores and reaching them with the derivatization reagent may pose hindrance problems. However, even these free silanols present in very small pores remain accessible to small solvent molecules such as water that can form "water wires" as schematically shown in Fig. 9.2.6. The silanols from very small pores may still affect the hydrophobicity and interact with polar molecules through the water wires.

Besides potentially unwanted interactions with the analytes, the presence of free silanols is undesirable since they contribute to the

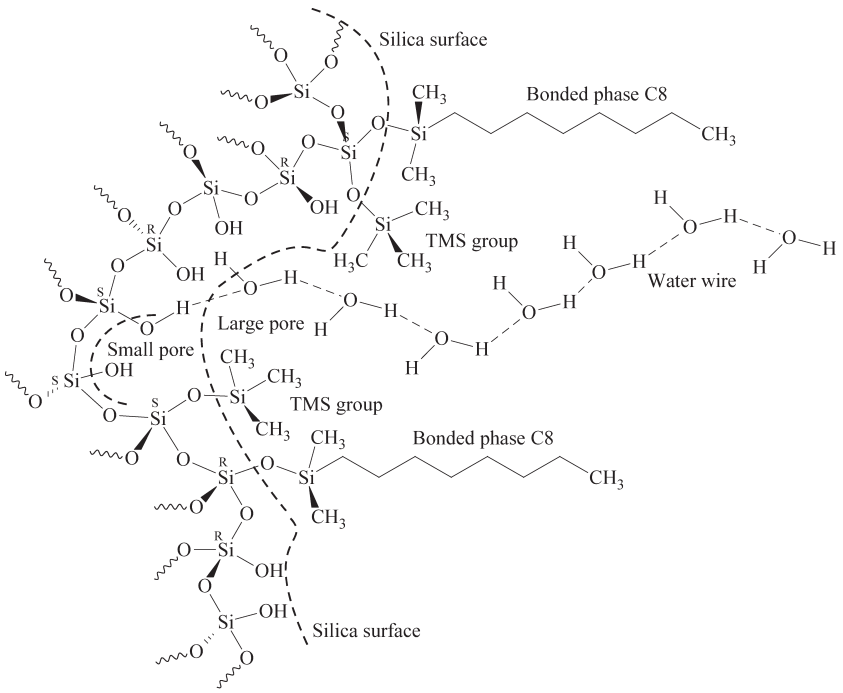

FIGURE 9.2.6 Schematic diagram showing a derivatized silica surface with C8 groups, TMS groups in a larger pore, and underivatized silanol from a small pore forming a water wire.

stationary phase instability outside common at pH range of 2−7. Stationary phases with a large number of silanol groups are less stable in mobile phases with pH higher than 7 or lower than 2. Basic mobile phases tend to dissolve silica and the access of acids to the hydrated silica support leads to instability at pH lower than 2. For this reason, significant effort has been made to increase the range of pH stability of various stationary phases.

(11) Stability of bonded phases is influenced mainly by the pH, the nature of different salts in the buffers used for the mobile phase, and temperature. The degradation of different bonded phase is usually a result of silica support dissolution (at high pH) or hydrolysis of covalently bound silane ligands (at low pH). For enhancing the resilience of a stationary phase to pH values higher than 7−8 or lower than 2, and to make it more resilient to the use of buffers containing salts (in particular phosphates), a number of strategies were employed. Such strategies are usually based on the reduction of free silanol number or blocking the access to the free silanols. This has been achieved in various ways, some previously discussed such as the reduction of the number of free silanol groups by exposing the initial hydrated silica to higher temperatures for the formation of silicagels. It has been shown that silicas made by the sol-gel process can be more resistant to dissolution than supports made by a silicate-gel (xerogel) process.

A common method for increasing the stability of silica-based stationary phase is end-capping, with double or multiple end-capping steps, which is superior to single end-capping [56,58]. Protection of stationary phases below pH = 2 relies on a special derivatization approach by which protecting groups are present close to the silica surface [59]. By steric hindrance, these groups provide a kind of shield for the area where the active phase is attached, protecting it from an acid attack from the mobile phase. This type of protection is achieved by performing end-capping with voluminous groups, such as isopropyl or isobutyl. The larger volumes of isopropyl and isobutyl groups provide some protection to the bond connecting the silica surface with the group acting as stationary phase. This type of technology is sometimes indicated as StableBond or SB with the silica base protected by end-capping the silanols with bulky groups. End-capping with small fragments containing polar groups also protect better the silica structure and extend the range of pH stability.

Other procedures to enhance stability include the use of ethylene-bridged type organic/inorganic silica (in BEH type columns), the use of charged surface hybrid (CSH) technology, the use of extra dense bonding of bonded phase (XDB technology), etc. Also, shielding of the silanol groups from the silica surface can be achieved by using silica derivatization with trifunctional reagents as previously shown in reactions 8.1.12. The resulting mass of vertical polymerized bonded phase may contain some silanol groups which are not directly bonded to the silica gel surface. This is achieved when the derivatization of silica is performed in special conditions (with a higher proportion of water). The silanol groups not connected to the silica gel surface may have lower acidity and have a protection effect to the attack of strong bases or acids.

Another procedure to protect the silica surface is the use of very long hydrophobic alkyl chains (C27, C30) for derivatization. Because of the higher degree of surface shielding, long-chain stationary phases are stable over a wider pH range than C8 or C18 bonded phases. Stationary phases with polar embedded groups (e.g., −O−, −CONH−, etc.) also seem to have a better resilience to extended range of mobile phase pH. This can be explained by the disruption of water wires and by hindering the penetration of ionized acids or bases toward the silica backbone. Depending on the specific application, the selection of a column resilient

to a wider pH range may be or may not be necessary. The selection of a fully adequate column should be done based on analysis necessities.

A different path for enhancing pH stability is the use of organic polymers for the stationary phase. Although typically much more resilient to an extended pH range than silica-based columns, polymeric columns have other disadvantages (see Section 8.1).

(12) Wettability is the ability of a liquid to maintain contact with a solid surface, and it is controlled by the balance between the intermolecular interactions of adhesive type (liquid to surface) and cohesive type (liquid to liquid). In RP-HPLC, the wettability problem occurs between the hydrophobic stationary phase and the mobile phase that has a high water content. The wettability can be estimated by measuring the maximum water concentration in common organic solvents (methanol, acetonitrile, and isopropanol) that allow the phase to remain wetted. This can be checked by taking phase in bulk form and verify the content of water in a solution when the phase does not float to the surface of the liquid. Hydrophobic columns that have as bonded phase CN or phenyl have less problems with wettability, while this problem may occur for C8 and C18 columns when no specific technology is utilized to improve wettability. All the brush phases, since they have a larger number of underivatized silanols, were shown to tolerate higher concentrations of water in the mobile phase than the bulk phases. It was also proved that the more dispersive and less polar is the solvent, the more water the solvent can contain before any of the phases become unwettable. Stationary phases that are incompletely derivatized can be wetted even by pure water. This can be explained by the high content of residual silanol on their surfaces, which allows the polar interactions with water molecules. Better wetting properties are also displayed by phases end-capped with polar groups or containing embedded polar groups in a hydrophobic bonded phase.

C18 phases are wetted by a lower content of water in organic modifier as compared to shorter chain phases such as C2, which becomes wetted with solvents with a higher content of water (for a similar derivatization degree) [60].

Dewetting of the hydrophobic phases is possible when concentration of water in the mobile phase is higher than 80%−90%. It is manifested by the loss of retention capability of the stationary phase, retention irreproducibility, increased tailing, and inability of the column to regenerate when returned to a high concentration of organic component. For separation of compounds with small hydrophobic moieties, it is sometimes necessary to use high content of water in the mobile phase. For such separations, it is recommended to use columns having good tolerance for a high level of water, such as polar end-capped columns and hydrophilic end-capped columns, column with polar-embedded groups, or combination of structures [61]. For example, the EVO type columns from Phenomenex, Cortecs and Atlantis from Waters, and InertSustain from GL Sciences are recommended to use even at 100% water in the mobile phase. Also, the columns with ethylene bridged technology have better resilience to mobile phases with high water concentration. Various columns compatible with 100% aqueous mobile phase are indicated in Appendix 9.2.3.

The study of this process [62] showed that two potential mechanisms are responsible for dewetting. One mechanism is related to the disturbance in the conformation of the alkyl chains in the presence of high water content in the mobile phase and is indicated as phase collapse. This explanation starts with the observation that short-chain stationary phases such as C4 and very long stationary phases such as C30 are less prone to dewetting. The short chains on the silica surface are not very well oriented, and the lack of effect from the water is expected

since no specific conformation is to be disturbed. The very long chain phases are in a strongly bundled form, and the water is not able to disturb easily the phase conformation, which may explain the resilience of this type of phase to higher water concentrations [63]. The interaction forces between the alkyl chains in C8 or C18 phases are easier to disturb. The use of melting point as a measure for interchain interactions indicates that $C_{18}H_{38}$ melts at 27−28°C and it is easier disturbed, while $C_{30}H_{62}$ melts at 64−67°C and forms more stable bundles. It can be assumed that water perturbs the aligned conformation of the C8 or C18 phases, reducing their capability of retaining the analyte. However, other studies indicated that the alkyl bonded phases are always in the most compact conformation, regardless of the concentration of the organic modifier in the mobile phase, and the change in conformation due to the water is not the main cause of the dewetting [64]. This suggests that dewetting is very likely caused by the exclusion of the aqueous mobile phase from the pores covered with a hydrophobic material and the inability of the mobile phase to reenter the pores. Since most of the stationary phase surface is inside the pores, the exclusion of the mobile phase from the pores is the reason of reduced active phase. The process is pictured in Fig. 9.2.7.

A dry sorbent requires a specific pressure for the solvent to reenter the pores. This pressure can be estimated with the following formula:

$$\Delta p = -\frac{4\gamma' cos\theta}{d_c} \qquad (9.2.2)$$

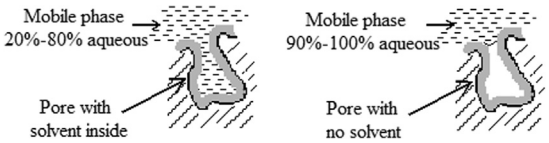

FIGURE 9.2.7 Schematic explanation of dewetting process when a mainly aqueous mobile phase does not penetrate the pores covered with a hydrophobic phase.

where Δp is the pressure required for the liquid to enter the pore, γ' is the surface tension of the liquid, d_c is the effective pore (or capillary) diameter, and θ is the contact angle between water and air on the adsorbent surface. For water, $\gamma' = 72.8$ dyn/cm, $\theta \approx 110.6$ (for a hydrophobic surface), and $d_c \approx 150$ Å, generating a pressure of about 150 bar (in an experiment using a C8 column and 0.1% CH_3COOH in water, a pressure of 270 bar was necessary to rewet the column [65]). The mechanism based on inability of the mobile phase to enter the pores is in agreement with several experimental findings and can easily explain why short-chain alkyl phases are less prone to dewetting, their "exclusion" of water being much lower than that of C8 or C18 phases. The explanation for C30 type phases may be related to the fact that such phases are less densely packed, although the carbon content (C%) is high. However, it is more likely that the dewetting is a complex process where the exclusion of the highly aqueous mobile phase from the pores of the stationary phase plays an important role in the loss of separation capability of the phase, but changes in the conformation of the alkyl chains from the silica surface under the influence of water are also likely to occur and to play a role in the modification of phase properties.

Dewetting/phase collapse can be reversible (not always 100%) by regenerating the column with a partially organic aqueous mobile phase. The process of regenerating the phase can require hours of flushing the column with partially organic phase (e.g., 60% CH_3CN and 40% H_2O).

Advances in the construction of common RP-columns

Chromatographic columns for RP-HPLC are manufactured and commercialized by numerous vendors. A large body of research on homemade columns is also reported in the

literature. Published by the vendors are available numerous catalogs and websites (e.g., Refs. [66–71]). In these publications are reported the new columns that are continuously introduced on the market, as well as many older columns that are still in use. A number of studies are published about the new trend in column technology [43,72–78]. Several newer columns commercially available are indicated in Appendix 9.2.4.

Since common columns cover a wide range of types and manufacturers, only common characteristics of these columns are further commented. Older type of common columns were typically limited to C8, C18, phenyl, and CN type, with fully porous particles having 5 μm diameter. Some older columns did not have monodisperse particles (had irregular shape particles) and silica purity was not as high as *Type B*. Several characteristics are now common for RP-HPLC columns, having the following characteristics: (1) pure silica (*Type B*), (2) monodisperse spherical particle shape, (3) smaller particle size (3 μm, or even 1.6–1.7 μm diameter), (4) core-shell particles, (5) bonded phase generated by using di- or trifunctional reagents, (6) wider pH range utilization, (7) wettability to 100% water, (8) polar-embedded groups in the hydrophobic chain, (9) wider range of hydrophobic phases capable of developing additional interactions beside hydrophobic, (10) better end-capping, (11) very low column bleed, (12) longer utilization time (up to 1000–1500 injections without degradation), (13) availability of columns with wide pores adequate for the analysis of polymers such as proteins, and (14) other improvements in column construction.

(1) The utilization of *Type* B silica of high purity became common. Advantages of pure silica include the elimination of undesired metalanalyte type of interaction and better resilience to pH outside the range 2–7. Also pure high strength silica (HSS type) capable to be used at pressures up to 15,000 psi (1034 bar) has been developed.

(2) Technology for producing spherical monodisperse hydrated silica particles allows improvement of column efficiency, and this type of particles become standard [79,80], while irregular particles of a range of dimensions are not anymore common.

(3) Smaller particles (3 μm, or even 1.6–1.7 μm diameter) that provide higher efficiency (higher N/m) are common. Although smaller particles generate higher backpressure, the newer UPLC instruments are capable of overcoming pressures as high as 1300 bar.

(4) Core-shell particles became frequently utilized [81]. For example, one series of core shell columns is the Kinetex (from Phenomenex), available in C8, C18, XB-C18 (which has butyl side chains for protecting against silanol access), phenylhexyl, pentafluorophenyl (PFP), with particle sizes of 2.6 and 1.7 μm [46,82]. Another series of core-shell columns is Ascentis Express (from Supelco), available in C18, phenyl-hexyl, C8, and pentafluorophenyl, and one more series is Accucore from Thermo Scientific that offers C18 and PFP columns as well as a column end-capped with polar groups (Accucore aQ). Agilent offers Poroshell series of core-shell columns. The newer columns bring significant advantages such as higher efficiency (larger number of theoretical plate N values), and core-shell type columns using 1.7 μm size particles can reach as much as $N/m = 300{,}000$. The recommended sample load is typically lower for core-shell stationary phases compared to fully porous ones. However, the frequent use of more sensitive detection based on MS or MS/MS requires lower samples load which is totally adequate for core-shell columns.

(5) The use of di- or trifunctional reagents (see Section 8.1) for silica surface derivatization is

now common. This type of reagent allows a more uniform derivatization of silica surface, with fewer remaining underivatized silanol groups, higher phase stability, and higher carbon load.

(6) Progress has been made in extending the pH range of utilization for silica-based columns. This is achieved by different procedures, one being the use of trifunctional derivatization reagents. However, significant advantages are offered by the use of organic/inorganic ethylene bridged support (BEH type columns or with TWIN technology) that is now more frequently used. For example, X-Terra Shield C18 column from Waters is stable in the range of pH from 2 to 12, and Gemini-NX C18 from Phenomenex is stable in the range of pH from 1 to 12 (both using ethylene bridged silica). Various other new technologies were introduced for extending the range of pH for the columns. Among these are the CSH technology and end-capping with more voluminous groups (e.g., *tert*-butyl). CSH technology with particles incorporating a low level of surface charge also improves sample loadability and peak symmetry [83]. CSH technology can be applied to organic/inorganic ethylene bridged phases bringing further improvements for pH stability of the column. The uniform distribution of bonded groups on silica surface also seems to improve the stability at a wider pH range [84].

(7) Wettability to 100% water in the mobile phase is another quality achieved. This is done using embedded polar groups and using polar end-capping such as in the Synergy Hydro-RP and Aqua type columns from Phenomenex, SunShell RP-AQUAfrom Nacalai, Aquasil C18 from Restek, etc.

(8) Polar-embedded hydrophobic columns are more common and they are useful for the extention the area of application to less hydrophobic compounds. Attempts to classify these special RP columns are based on various parameters, some presented in Section 9.3 such as methylene selectivity, polar selectivity, configurational selectivity, or even zeta potential (electrokinetic potential in colloidal dispersions) [39,85].

(9) A variety of columns capable of producing additional interactions in addition to strong hydrophobic are also offered. For example, fluorinated bonded phase columns became more common [86].

(10) Better end-capping of the silica surface is also common in many RP-type chromatographic columns. The columns may be made with different types of end-capping. The covering or encapsulating of the silica surface with polymeric materials is also used in some phases to reduce the activity of silanols. The results were phases showing minimal interaction and no or little tailing with strongly basic compounds (from where the name base deactivated).

(11) One other typical characteristic of newer columns is very low bleed important for sensitive detectors such as MS and MS/MS. Progress made in silica surface derivarization with bi- and trifunctional reagents, and better end-capping techniques assure that the access of mobile phase to the silica base is limited and the stability of stationary phase is higher.

(12) Also, newer columns display extended lifetimes with up to 1500 injections without significant degradation of the stationary phase. This is a result of better derivarization techniques, higher silica purity and using of additional techniques for protecting the access of mobile phase to the covalent connection between the silica surface and the bonded phase (see Section 8.1).

(13) For better accessibility to the active phase on the inert support, a series of new large pore

RP stationary phases were developed. As an example, a recent study has shown that the separation selectivity for oligonucleotides increased several times by using stationary phase packing with 300 Å compared to 100 Å pores [87].

(14) Other improvements to the column technology such as better and more uniform packing of the stationary phase in the body of the column, special type of frits at the end of the column that allow a uniform distribution of mobile phase in the column body, etc., are common.

Availability of special types of hydrophobic columns

A number of more special columns are commercially available. Among these types of columns, the following can be mentioned: (1) columns with linear aliphatic bonded phase with unusual number of carbons (C3, C4, C5, C27, C30), (2) column with silica coated with a polymer, (3) monolithic columns, (4) silica hydride columns, (5) graphitic columns, (6) columns with zirconia support, (7) organic polymer type columns. Various procedures to make these types of stationary phases were presented in Section 8.1.

(1) Besides the common C8 and C18 bonded phases, columns with bonded alkyl chains (on silica support) with different numbers of carbons were also commercialized. Two distinct groups of such columns were made, one with very short carbon chain and the other with long carbon chain. In the group with very short carbon chain are C3 columns (L56 in USP classification), such as Zorbax StableBond 80A C3, Zorbax StableBond 300A C3, C4 columns (L26 in USP classification) such as Accucore 150-C4, ACE 5 C4, Aeris WIDEPORE XB-C4, BioBasic 4, Biobond C4, Epic C4, Genesis C4 EC 120A, Ultra C4, ProntoSIL 300 C4, and C5 columns such as Discovery BIO Wide pore C5, Jupiter 300 C5, Luna C5. These columns are designed to offer a lower hydrophobic interaction and some have wide pores for utilization in large molecules separations such as proteins.
The other group includes columns with extralong aliphatic chain among these some having C27 chains such as Cogent UPHOLD C27 (from MicroSolv), CAPCELL CORE AQ (from Shiseido), and columns with C30 chains (L62 in USP classification) such as Accucore C30, Develosil C30-UG-5, ProntoSIL 120-3-C30, ProntoSIL 200 C30, ProntoSIL 300 C30, and ProntoSIL 300 C30 EC. The long alkyl chain stationary phases were found useful for the separation of small only partly hydrophobic molecules. These phases are more retentive for polar and nonpolar analytes than are most polar-embedded and even high-coverage C18 phases. Long chain phases may offer greater pH stability than do C8 and C18 phases. They are also more resistant to phase collapse under high aqueous conditions than are C18 phases. This behavior may be explained by the resistance of long chains to conformation changes in the presence of water at the column temperature, or it may be related to a lower density of C30 chains on the silica surface for similar C% content as the C18 phases [88]. A column with cholesterol groups bonded on silica is also commercially available (Cogent UDC Cholesterol from MicroSolv).

(2) Columns with different type of silica coating technology were developed with the goal of reducing free silanols activity. As an example, EnviroSep type phases (from Phenomenex) contain a silica and polymer support and a hydrophobic bonded phase. Other columns are made using a variety of technologies. For example, Capcell Pak type columns (Shiseido Co. Ltd.) involve a surface coating of the silica

base support with a silicone polymer that shields the silanol groups, the bonded phase being connected to the coated surface.

(3) Silica monoliths can also be considered special columns. The C18 bonded phase on monoliths is, however, a relatively common type, such as in Onyx Monolithic C18 or Chromolith Performance RP-18 columns, which are available in various formats. These columns have a typical dual porous structure with mesopores of about 130 Å and macropores of about 2 μm diameter. The nature of the bonded phase is similar to that for spherical particle C18 columns, and the silica surface is end-capped. This type of column allows a reduction in elution time up to nine times due to the capability to use higher flow rates without having problems with the column backpressure. Due to a rapid mass transfer of the solutes between the bonded phase and the mobile phase, the decrease in the number of theoretical plates at higher flow rates, as predicted by van Deemter equation (see Eq. 3.1.70), is not as intense. Shorter retention times also lead to better resolution [89]. An example of a separation of a monolith type column is given in Fig. 9.2.8 for the separation of norfloxacin, one of its metabolites, and of ciprofloxacin. The column utilized for the separation is a Chromolith Performance RP-18e (Merck KGaA), 100 mm × 4.6 mm. Two overlaid chromatograms are shown in Fig. 9.2.8, corresponding to a blank sample (A) and a sample collected from a same human volunteer using norfloxacin (B). Sample preparation was based on deproteinization of 200 μL human plasma with 50 μL acrylonitrile that also contained ciprofloxacin as internal standard. The separation was performed in gradient

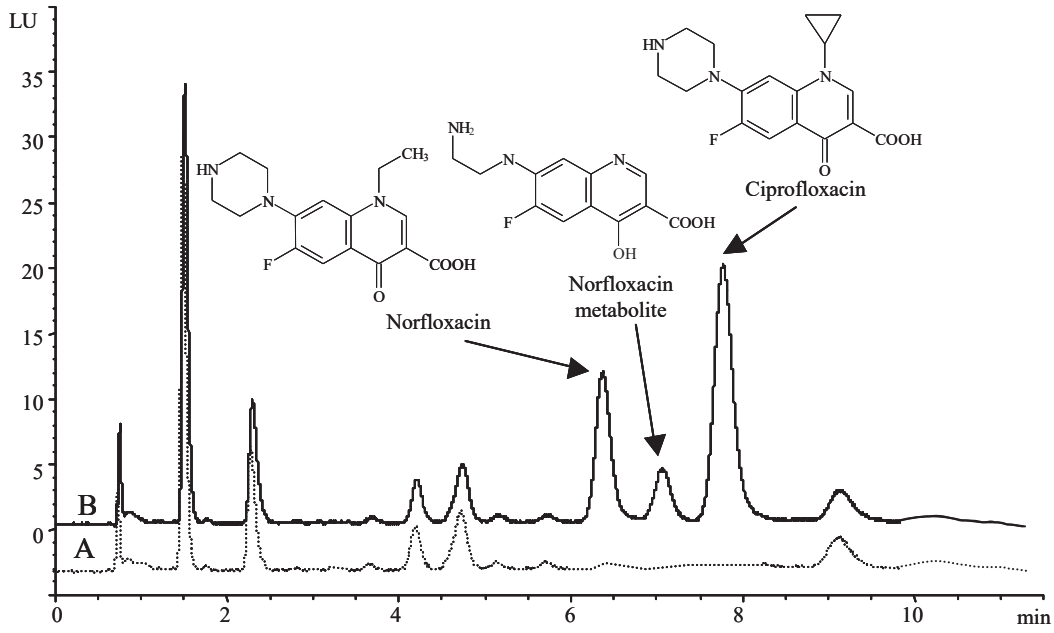

FIGURE 9.2.8 Separation on a Chromolith Performance RP-18e (Merck KGaA), 100 mm × 4.6 mm column of norfloxacin, one of its metabolites, and ciprofloxacin (added as internal standard); (A) blank plasma sample, (B) sample from human volunteer using norfloxacin [90].

conditions with the mobile phase made of 1% triethylamine in aqueous solution brought to pH = 4.0 with phosphoric acid and methanol as organic modifier. The detection was done with a fluorescence detector (FLD) set at 268 nm excitation and 445 nm emission [90].

(4) Among other special stationary phases used in HPLC are those based on silica hydride. Bare silica hydride can be used for direct phase separations (in organic-normal phase HPLC) or for aqueous normal phase separations. Bonded phases on hydride silica (*Type C* silica) with C18, C8, cholesterol groups, etc., are commercially available (e.g., Cogent Bidentate C18, Cogent Bidentate C8, Phenyl Hydride, Cogent UDC Cholesterol, etc.). Stationary phases with dimensions such as 4 μm particle diameter and pore size of 10 nm are available. These types of columns can be used in reversed-phase type conditions [91].

(5) Porous graphitic carbon can also be used as stationary phase. Unlike silica-based packing materials, carbon-based stationary phases have the advantages of being more resistant to hydrolysis, while the lack of swelling or shrinking makes them more useful than the polymeric materials. The efficiency of columns packed with these materials is comparable with modified silica-based columns [92,93].

(6) Hydrated zirconia can be used as a support for RP-bonded phases having the advantage of a wider pH stability compared to silica. The display of additional interactions besides hydrophobic of RP-phases with zirconia directed efforts to obtain alternative supports still using zirconia resistance to high pH. One such alternative is the carbon-clad zirconia which allows the use of superheated water as mobile phase [94]. Zirconia type columns have their niche of utilization although still not with a widespread use.

(7) Organic polymer-based hydrophobic stationary phases are sometimes used in RP-HPLC, having the best resilience to a wide pH range for the mobile phase. Some polymeric phases can operate at pH values as low as 1 and as high as 13.5. Also, the polymeric substrate does not have a potential layer of unwanted polar groups as do the silica base stationary phases. Polymeric materials typically show lower theoretical plate numbers N for the same dimensions as the silica base particles, although phases with $N/m = 80{,}000$ are available. Another disadvantage of polymeric supports is caused by the variation in the swelling of the polymeric particles when they are used in various solvents. This variation in swelling affects the volume occupied by the stationary phase in the column and can lead to the formation of void spaces and loss of efficiency. Organic polymeric materials are also less resilient to high pressures, and this makes polymeric columns less adequate for the new developments toward UPLC. For these reasons, the use of polymeric columns, particularly for RP-HPLC, is limited. The stationary phase from these columns may consist of the polymer in itself or may have a specific bonded phase (such as C8, C18) on the polymeric support [33].

Besides stationary phases made with particles of rigid organic polymers, polymeric monoliths are also used as chromatographic media [95]. Polymeric phases with hydrophobic bonded groups such as C18 or pentafluorophenyl are commercially available (e.g., Jordi phases from MicroSolv). Among different polymeric materials used as stationary phase in HPLC, several molecular imprinted polymers were also available. The polymeric structure has been typically based on methacrylic acid and styrene cross-linked with ethyleneglycol dimethacrylate that were polymerized in the presence of a template molecule (e.g., nortriptyline [96]). More information on monolithic stationary phases can be found in dedicated books, such as [97].

9.3 Parameters used for the characterization of RP-HPLC phases and columns

General comments

Separation process in HPLC depends on the nature of analyte, mobile phase, stationary phase, as well as on the separation external conditions such as temperature, flowrate of the mobile phase, etc. For specific selections of the analyte and mobile phase (and external conditions), each stationary phase may perform differently and such differences can be used for the stationary phase characterization. A considerable number of studies were dedicated to stationary phase characterization [98–102]. Three types of column characterization are basically utilized: (1) characterization based on physico-chemical column characteristics (column efficiency, pH range of utilization, pore size, carbon load, reagent type used for derivatization, etc.), (2) tests for verifying specific properties using key analytes and specific mobile phase, (3) fitting to specific retention models using multilinear regression.

The characterization of a stationary phase based on physico-chemical properties has been discussed in part in Section 8.2, and is further commented in this section. Other aspects related to the stationary phase characterization in RP-HPLC using specific testes or fitting models are presented in this section.

Efficiency of columns (theoretical plate number)

The efficiency of a chromatographic column is characterized by the height equivalent to a theoretical plate H (HETP given by Eq. 3.1.53), and by the related parameter theoretical plate number N (given by Eq. 3.1.55) expressed for the length of the chromatographic column, or per 1 m (N/m). An additional related parameter to characterize efficiency is the effective plate number $\boldsymbol{n}$ (given by Eq. 3.1.58). The value of N depends on column construction (stationary phase properties and uniformity of stationary phase packing), but also on the nature of the analyte and on the nature and flowrate of the mobile phase used for its measurement, as described in Section 3.1 (van Deemter equation). Previous discussions on how the stationary phase construction influences the value of N were given in Sections 8.1 and 8.2. Several characteristics of stationary phase such as particles or monolith, fully porous particle or core-shell (superficially porous), particle shape (irregular or spherical), diameter of particle d_p, uniformity of particle dimensions, surface area of stationary phase, type of porosity (tortuosity τ), pore size diameter, as well as the type of bonded phase, carbon load (C%), etc., influence the value of N. Since N depends on so many aspects, it is common to describe its value as obtained by measurement using a test compound such as toluene. Some values of N (per m) for various hydrophobic columns (5 μm particles) are listed in Table 9.3.1.

The values for N for more modern chromatographic columns such as those with porous particles with the diameter d_p smaller than 2 μm can reach values of N/m as high as 300,000. Also, columns with core-shell particles have higher values for N/m in the range of 200,000. Monolithic columns can also be characterized by their theoretical plate number N/m between 100,000 and 150,000.

Retention capability of columns used in RP-HPLC

The retention capability of an analytical column is characterized by the retention factor k' (see Eq. 3.1.20) and it was also discussed in Section 9.2 related to the chemical characteristics of hydrophobic columns. The nature of the stationary phase influences the value of log k' through both the value of phase ratio Ψ and

TABLE 9.3.1 List of theoretical plate height N per m for several C18 columns (5 μm particles) that are commercially available (for toluene with mobile phase 10% H_2O and 90% CH_3OH).

Column	N/m	Column	N/m
ACE C18	111,000	Nucleosil C18	101,000
ACE C18-300	103,500	Nucleosil C18AB	87,000
ACE C18-HL	102,000	Partisil ODS	47,500
μBondapak C18	36,000	Partisil ODS2	41,000
Capcell Pak AG C18	51,000	Partisil ODS3	52,000
Develosil ODS-HG	85,500	Prodigy ODS2	48,000
Develosil ODS-MG	66,000	Prodigy ODS3	62,000
Develosil ODS-UG	92,000	Resolve C18	45,500
Exsil ODS	93,000	SunFire C18	91,500
Exsil ODS1	114,000	Symmetry C18	92,000
Gemini C18	75,500	Vydac 218TP	63,000
Hichrom RPB	97,500	Waters Spherisorb ODS1	100,500
Hypersil BDS C18	76,500	Waters Spherisorb ODS2	91,500
Hypersil GOLD	91,000	Waters Spherisorb ODSB	92,000
Hypersil HyPurity C18	73,000	YMC J'Sphere ODS H80	64,500
Hypersil ODS	94,500	YMC J'Sphere ODS M80	58,000
Inertsil ODS	73,500	YMC ODS A	99,500
Inertsil ODS3	60,500	YMC ODS AM	83,500
Inertsil ODS2	32,000	YMC Pro C18	105,000
Kromasil C18	99,000	Zorbax Extend C18	80,500
LiChrosorb RP-18	74,000	Zorbax ODS	85,500
LiChrospher RP-18	80,000	Zorbax Rx-C18	90,500
Luna 5 C18(2)	88,000	Zorbax SB-C18	103,000
Novopak C18	60,000	Zorbax XDB-C18	96,000

that of the constant K for the analyte equilibrium between the stationary and mobile phase. Various approaches were reported in the literature regarding the measure and prediction of k' of Ψ and of K. The values of k' and of K depend not only on the column but also on the analyte X for which k' and K are measured, and also on the mobile phase composition. The value of Ψ also has some variation depending on the mobile phase composition. In addition, all these parameters depend on temperature. As a result, in order to use k' for the characterization of a hydrophobic column, the values of these parameters must be indicated for a specified mobile phase and a selected analyte. As indicated in Section 9.1, the main retention process in RP-HPLC is through hydrophobic interactions and as a result hydrophobicity is the main character of columns used in RP-HPLC. This property can be characterized by the value for log k' for a compound with no polar groups, which has only hydrophobic interactions with the stationary phase, and no (or minimal) other additional interaction types. Among test compounds for hydrophobicity are ethylbenzene (EB), toluene, butylbenzene, acenapththene, etc.

For many columns, the experimental values for k' (or log k') are reported for a specific analyte and a specific mobile phase. Some such log k' values for acenaphthene when the mobile phase is 65% CH_3OH/35% aqueous buffer

20 mM K_2HPO_4/KH_2PO_4 at pH = 7.0 (v/v) are given in Appendix 9.3.1 [103]. More k' values for the test compound ethylbenzene (indicated as H_y) and k' for amitriptyline (indicated as *CFA*) in a mobile phase 80/20 methanol/aqueous buffer 60 mM phosphate v/v at pH = 7.0 are indicated in Appendix 9.3.2 [104]. Other values for k' for butylbenzene (BB) in 65% AcCN and 35% H_2O buffer 20 mM phosphate at pH = 7 are given in Table 9.3.4, and for the test compound toluene with a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6, the values for log k' are given in Appendix 9.2.1. The results from Appendix 9.2.1, for example, show that lower log k' values are common for columns with polar-embedded groups or containing a combination of groups (such as AMT RP-Amide).

In Appendix 9.3.1 are listed the values for the selectivity log α for the separation of amitriptyline/acenaphthene. It can be seen from the values in Appendix 9.3.1 that log k' is not correlated with log α and this is also shown in Fig. 9.3.1 where the plot of log k' for acenaphthene versus log α for amitriptyline/acenaphthene is given.

In an HPLC separation, both k' and α are important for achieving a good resolution R (see Eq. 3.2.8 and 3.2.10) and in the selection of a column for a specific separation both these values must be considered. For acenaphthene log $K_{ow} = 3.92$ and for amitriptyline log $K_{ow} = 4.92$, indicating that although both compounds are hydrophobic there are differences in this character which leads to separation. In addition amitriptyline has a polar group (with a partial charge $\delta = -0.31$ on the nitrogen atom) involving in the separation other interactions besides hydrophobic. Also, the polarizabilities of the two molecules are different with $\alpha = 17.81\ \ 4\pi\varepsilon_0(\text{Å})^3$ for acenaphthene and $\alpha = 33.76\ \ 4\pi\varepsilon_0(\text{Å})^3$ for amitriptyline. As indicated in Section 9.1, the separation in RP-HPLC, although based mainly on hydrophobic interactions, is affected by other interactions when the analyte has polar groups.

Methylene selectivity and general selectivity for hydrophobic columns

Selectivity parameter α is also used for column characterization. As indicated by Eq. 3.2.2, α depends only on equilibrium constant K of the two compounds X and Y that are separated, eliminating the dependence on phase ratio Ψ that is part of k'. Also, α is part of the expression for resolution R of a chromatographic column and as a result its value is important for column characterization and selection for a specific separation (e.g., Ref. [105]). The value of parameter α depends on the nature of analytes X and Y, as well as on the mobile phase used for the separation. For this reason, the values for α must be indicated for a specific set of test compounds and a specific mobile phase. The use of such set of selected conditions can provide values for a "standard" parameter $\alpha(X/Y)$ that can be used for the comparison of columns selectivity related to a specific class of compounds.

One set of selected conditions for measuring α is designed to characterize mainly the hydrophobic character of the phase, and it is applicable for the characterization of separation between nonpolar compounds. In this set of conditions, the values for α are compared between nonpolar compounds (usually aromatic hydrocarbons) that are in a homologous series and differ by one or more CH_2 groups (one example being the pair toluene and ethylbenzene). This selectivity is indicated as *methylene selectivity* $\alpha(CH_2)$. Methylene selectivity $\alpha(CH_2)$ characterizes mainly the hydrophobic character of a stationary phase and its capability to separate nonpolar molecules. Numerous studies were dedicated to the evaluation of $\alpha(CH_2)$ [38,106–111].

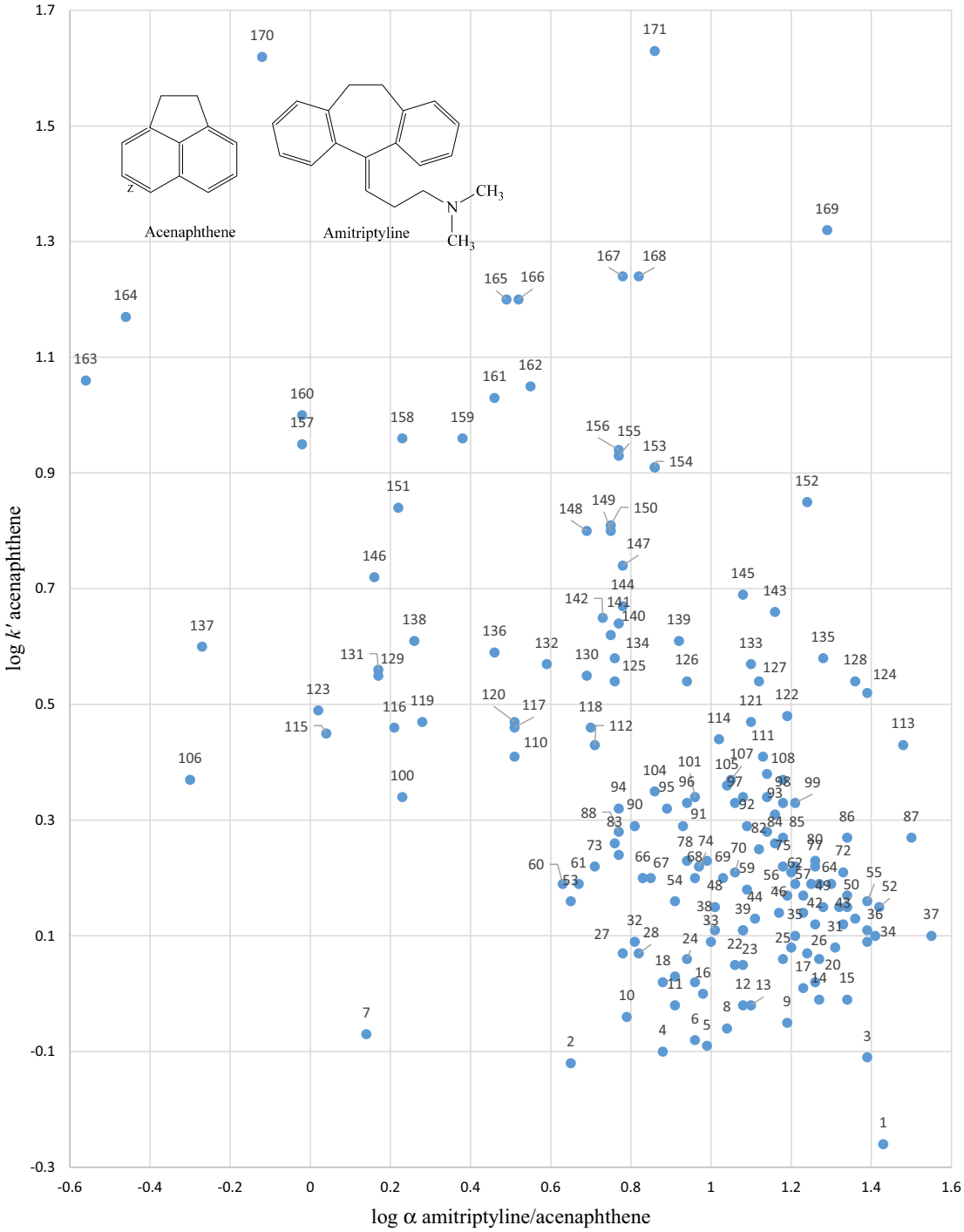

FIGURE 9.3.1 Plot of log k' for acenaphthene versus log α for amitriptyline/acenaphthene separation with a mobile phase 65% CH_3OH/35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH = 7.0 (v/v). The numbers in the graph correspond to column number in Appendix 9.3.1.

For the measurement of methylene selectivity, the mobile phase composition is not specifically recommended, but must be the same for the homologous series and the values of $\alpha(CH_2)$ are different when the mobile phase composition changes. The formula for $\alpha(CH_2)$ is the following:

$$\alpha(CH_2) = \frac{k'(R - CH_2 - R)}{k'(R - R)} \qquad (9.3.5)$$

A linear dependence for log $\alpha(CH_2)$ on the number of CH_2 units was noticed for certain homologous series such as the one from benzene to amylbenzene. This finding is not unexpected. This linearity was verified for various columns [38,109]. Based on this observation, the values for log $\alpha(CH_2)$ can be obtained from the slope of the trendline of the graph of log k' versus the number of methylene groups in an alkylbenzene by the following formula (see Fig. 9.3.2):

$$\begin{aligned} \log\alpha(CH_2) &= \log\frac{k'(R - CH_2 - R)}{k'(R - R)} \\ &= \log k'(R - CH_2 - R) \\ &- \log k'(R - R) = \tan\beta \end{aligned} \qquad (9.3.6)$$

In Fig. 9.3.2, an example of calculation of log $\alpha(CH_2)$ for a C18 monolithic column (Phenomenex Chromolith Performance RP-18) with the mobile phase 60% methanol 40% water (v/v) is illustrated. The slope of the graph of log k' versus the number of methylene groups in an alkylbenzene ($n = 0$ for benzene, $n = 1$ for toluene, etc.) gives the value for log $\alpha(CH_2)$. For the indicated phase system, log $\alpha(CH_2) = 0.2822$.

The variation of $\alpha(CH_2)$ with the mobile phase composition for several columns is shown in Fig. 9.3.3. The columns evaluated are LiChrospher 100 RP (LiChr.), Purospher RP (Puro.), Symmetry-Shield RP (Symm.), and two mobile phase systems, acetonitrile/water (ACN) and methanol/water (MeOH), at different concentrations [105]. All columns had 5 μm particle size, the column dimensions were 125 × 4 mm (LiChr. and Puro.), 150 × 3.9 mm (Symm), and except for LiChr. C8 all columns were end-capped.

When a column is selected for an application requiring the separation of hydrophobic compounds, the preferred characteristic should be a high $\alpha(CH_2)$ for obtaining a better separation. However, for compounds with polar groups

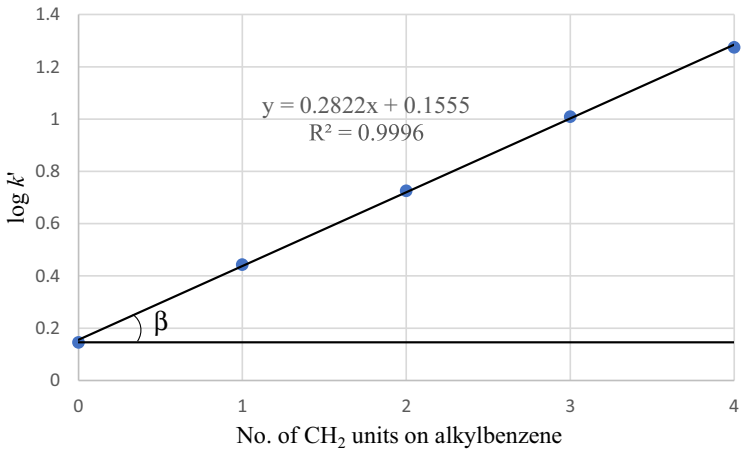

FIGURE 9.3.2 Calculation of log $\alpha(CH_2)$ for a C18 monolithic column (Phenomenex Chromolith Performance RP-18) with the mobile phase 60% methanol 40% water (v/v) for the series benzene to butylbenzene (log $\alpha(CH_2) = 0.2822$).

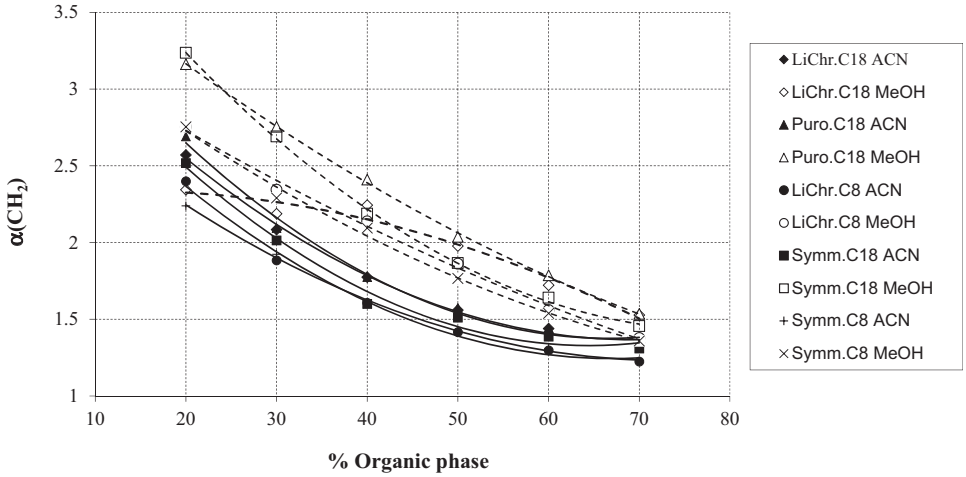

FIGURE 9.3.3 Variation of $\alpha(CH_2)$ for several columns and two mobile phase systems (acetonitrile/water and methanol/water) at different concentrations of the organic phase.

and hydrophobic moieties, methylene selectivity may not be a good indicator for the best separation capability.

Other conditions for measuring α for a set of column are sometimes utilized. In this case, the selectivity can be considered not particularly related to hydrophobicity, although the hydrophobic component remains in the value of α indicated now as "general." For example, it is useful to have some test conditions in which besides hydrophobicity it is possible to assess polar interactions, $\pi-\pi$ interactions, as well as steric selectivity of the stationary phase. For this purpose, specific pairs of test compounds have been recommended, as well as specific mobile phase compositions that provide α values relevant for such separations. Such tests can provide information regarding how strong are the hydrophobic interactions but in addition other types of interaction such as polar, $\pi-\pi$, and steric etc., are influencing the result. Depending on the analytes and mobile phase such interactions are more or less manifested for a specific column. One such pair test of compound is amitriptyline and acenaphthene and a mobile phase 65% CH_3OH and 35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH = 7.0 with the values for log α given in Appendix 9.3.1 and represented graphically in combination with log k' values in Fig. 9.3.1. Other tests designed to describe selectivity α, for specific compounds, but in addition to describe other column properties will be further discussed.

Peak asymmetry for RP-HPLC columns

Peak asymmetry (A_S) in HPLC (see Eq. 3.1.78) is dependent on the analyzed compound, mobile phase composition, and on the stationary phase of the chromatographic column. The main cause of peak asymmetry in RP-HPLC is the presence of interactions of the analyte with the solid phase which do not affect equally all the molecules of the same analyte. This is caused by the analyte having more than one molecular form in the mobile phase and/or by the stationary phase interacting selectively with only a part of the analyte molecules. An illustration of this process indicating two mechanisms of interaction with the same type of molecules can be seen in Fig. 9.3.4.

Not every compound in an RP-HPLC separation shows peak asymmetry. In order to put in

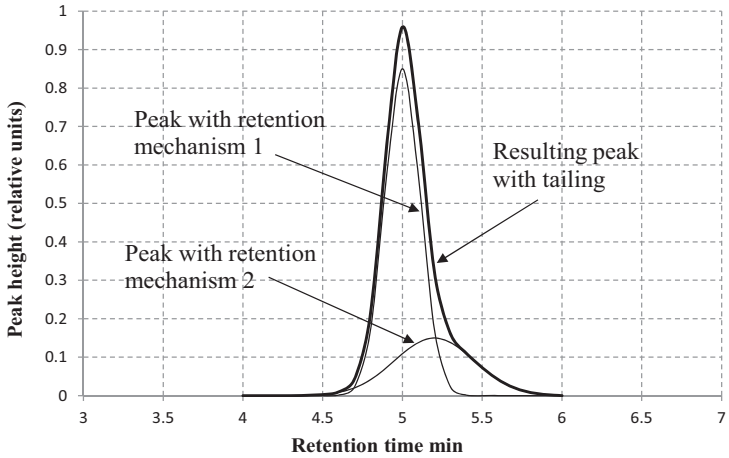

FIGURE 9.3.4 Illustration of the formation of asymmetrical chromatographic peaks as a result of two mechanisms of retention that do not affect equally all molecules of one analyte.

evidence the column potential to generate asymmetric peaks, it is necessary to use specific test compounds. A column showing significant asymmetry for such compounds may show no asymmetry, for example, for a hydrocarbon. Two common test compounds used to put in evidence asymmetry are quinizarin and amitriptyline. Quinizarin is typically used for the evaluation of metal activity in a column, and amitriptyline, which is a basic compound ($pK_a = 9.4$), is used for the characterization of silanol activity based on its peak shape (other procedures to evaluate silanol activity are also known [112]).

Since quinizarin is a metal chelating reagent and its retention behavior is indicative of the presence or absence of metal ions in the stationary phase, low metal activity is characterized by symmetric peaks of quinizarin, and high metal activity is characterized by strong tailing. Amitriptyline has besides the hydrophobic interaction with the stationary phase, an additional interaction with the silanol groups. Peak asymmetry generated by quinizarin and amitriptyline is not correlated, since each compound has a different type of additional interaction with the stationary phase. Values for *As* measured for amitriptyline and for quinizarin for several common columns, in a mobile phase 80% CH_3OH and 20% aqueous potassium phosphate buffer with conc. 5 mM and with pH = 7, are shown in Table 9.3.2 [113].

Hydrophobic subtraction model for selectivity characterization

A more complete description of column separation capabilities is based on an empirical procedure known as *hydrophobic subtraction model* or HSM [29–32,114]. This procedure is based on fitting to a specific retention model using multilinear regression. The procedure starts with Eq. 3.2.2 for the selectivity α, which indicates that α depends on the equilibrium constants for the two analytes (solutes) to be separated. When the mobile phase is kept unchanged, and selecting one of the solutes to be ethylbenzene (EB), the selectivity can be considered as dependent only on the chemical nature of the analytes (solutes) to be separated and the chromatographic column properties. Taking into consideration different type of interaction mechanisms for a separation on an RP-HPLC

TABLE 9.3.2 Values for asymmetry As for various columns as measured for amitriptyline and for quinizarin [113].

	Column	As amitriptyline	As quinizarin
1	ACE C18	1.03	1.07
2	Xterra MS C18	1.12	1.12
3	Intersil ODS3	1.26	1.24
4	Luna C18(2)	1.52	1.46
5	Hypersil Elite C18	1.61	1.09
6	Discovery C18	1.78	1.37
7	Waters Spherisorb ODS1	1.88	2.27
8	Intersil ODS2	1.93	1.27
9	Symmetry C18	2.07	1.31
10	Hypersil BDS-C18	2.21	1.26
11	Partisil ODS2	2.74	3.07
12	Zorbax XDB-C18	3.05	1.07
13	Novapak C18	3.05	2.42
14	Zorbax SB-C18	3.07	1.43
15	Nucleosil C18	3.19	2.41
16	Vidac Selectapore 300M	6.50	1.34
17	Cosmosil C18 AR-II	8.30	1.16
18	μ Bondapack C18	8.99	2.56

column, the following expression can be written for log $\alpha(X,EB)$:

$$\log \alpha(X,EB) = \log\left(\frac{k'_X}{k'_{EB}}\right) = \eta'(X)H^* - \sigma'(X)S^* + \beta'(X)A^* + \alpha'(X)B^* + \kappa'(X)C^* \quad (9.3.7)$$

The contribution of each term in Eq. 9.3.7 has a specific meaning, and $\eta' H^*$ accounts for column capability to separate the analyte from ethylbenzene based only on hydrophobic interactions, $\sigma' S^*$ accounts for steric interactions, $\beta' A^*$ accounts for hydrogen bonding between a basic solute and the acidic groups of the stationary phase, $\alpha' B^*$ accounts for hydrogen bonding between an acidic solute and basic groups of the stationary phase, and $\kappa' C^*$ accounts for cation exchange type and/or ion−ion interactions. Parameters η', σ', β', α', and κ' depend on solute X properties (and mobile phase composition), and H^*, S^*, A^*, B^*, and C^* depend only on column properties. The values for H^*, S^*, A^*, B^*, and C^* can therefore be utilized for comparing different columns and consequently a possibility to establish when two different columns will have similar separation capabilities and can replace each other [115].

The values for both η', σ', β', α', and κ' and H^*, S^*, A^*, B^*, and C^* were obtained for a specific mobile phase that consisted of 50/50 acetonitrile/aqueous buffer containing 60 mM phosphate at pH = 2.8 or at pH = 7.0. The values for parameters η', σ', β', α', and κ' and for parameters H^*, S^*, A^*, B^*, and C^* were obtained initially based on a large number of measurements for the retention factors k'_X on a specific set of compounds X and a set of columns. The procedure is well explained in the literature (e.g., Ref. [29]). The basic idea was to initially isolate a group of compounds that are separated exclusively based on hydrophobic interactions, for which it can be assumed that σ', β', α', and κ' are all zero. In such case, Eq. 9.3.7 would be reduced to the following:

$$\log \alpha = \log\left(\frac{k'_{comp}}{k'_{EB}}\right) = \eta'_{comp} H^* \quad (9.3.8)$$

Eq. 9.3.8 applied to two different columns will generate the following expression:

$$\log \alpha_{col.2} = \left(\frac{H^*_{col.2}}{H^*_{col.1}}\right) \log \alpha_{col.1} \quad (9.3.9)$$

An arbitrary value $H^*_{col.1} = 1$ must be selected at this point (in the initial study, the selected column for $H^*_{col.1} = 1$ was HP Zorbax SB C18

(nonend-capped) [29]). Using Eq. 9.3.9, the values for H^* for a number of columns can be obtained, using k' values for ethyl benzene and other compounds retained only based on hydrophobic interactions. For all these hydrophobic compounds, the values η'_i can be obtained (where i indicates different compounds).

Using a large number of compounds with different structure, some with polar groups that have besides hydrophobic interactions other types of interactions with stationary phases, a deviation from Eq. 9.3.8 can be noticed. The values for these deviations can be written in the following form:

$$\Delta_{i,col.j} = \log \alpha_{i,col.j} - \eta'' H^*_{col.j} \qquad (9.3.10)$$

(where η'' is the average of η'_i values). The test compounds for all tested columns are further grouped based on cross-correlations such that the compounds from each group manifest as much as possible one specific type of interaction and very weak interactions of other types. For example, differences in retention caused by steric differences can be evaluated using two aromatic hydrocarbons: one with a twisted structure and the other with a planar one. The two compounds should have practically identical hydrophobicity, but in the same time different volumes. The selectivity factor α for these compounds is a measure for the steric selectivity. For example, the selectivity factor can be evaluated for the following pairs: triphenylene/ortho-terphenyl [116], benzo[a]pyrene/tetrabenzonaphthalene [117], or benzoic acid/sorbic acid [118,119]. From averages of Δ values for each group, the values for S^*, A^*, B^*, and C^* were calculated. Once H^*, S^*, A^*, B^*, and C^* are established for each column, using multiple regression, the values for η', σ', β', α', and κ' are generated for the whole set of test compounds, and further small adjustments to H^*, S^*, A^*, B^*, and C^* are made using multiple regression of log α versus the values of η', σ', β', α', and κ'.

One addition to the previous summary description of establishing the values for H^*, S^*, A^*, B^*, and C^* is related to the dependence on pH of one group of interactions, namely that for cation exchange type and/or ion–ion interactions. The group of test compounds used for the determination of C^* are made of strong bases, and their retention (values for log $\alpha_{i,col.j}$ and therefore of $\Delta_{i,col.j}$) depends on the pH of the mobile phase. For this reason, C^* is indicated by two values, $C^*(2.8)$ which is the value obtained with the mobile phase at pH = 2.8, and $C^*(7.0)$ which is the value obtained with the mobile phase at pH = 7.0.

A considerable number of studies were later dedicated to the task of establishing H^*, S^*, A^*, B^*, and C^* for a variety of columns [88, 120–128]. The values for the parameters H^*, S^*, A^*, B^*, and C^* are reported for about 753 different alkyl-silica columns (PQRI approach from Product Quality Research Institute) and are listed in Appendix 9.3.3 [104]. The parameters were finally measured for the mobile phase of 50/50 acetonitrile/aqueous buffer containing 60 mM phosphate and using the following test compounds: thiourea, amitriptyline, 4-butylbenzoic acid, N,N-diethylacetamide, 5-phenyl-1-penthanol, ethylbenzene, N,N-dimethylacetamide, 5,5-diphenylhydanoin, toluene, nortriptyline, acetophenone, mefenamic acid, p-nitrophenol, anisole, 4-hexylaniline, cis/trans chalcone, benzonitrile, and berberine [121,129].

The hydrophobic subtraction model provides a useful procedure for the column comparison. A similarity parameter F between two columns can be calculated for each column pair by the following formula:

$$F_{col.1-col.2} = \Big[\left(H^*_{col.1} - H^*_{col.2}\right)^2 + \left(S^*_{col.1} - S^*_{col.2}\right)^2 + \left(A^*_{col.1} - A^*_{col.2}\right)^2 + \left(B^*_{col.1} - B^*_{col.2}\right)^2 + \left(C^*_{col.1} - C^*_{col.2}\right)^2\Big]^{1/2} \qquad (9.3.11)$$

Column comparison based on Eq. 9.3.11 can be done by accessing the USP site (http://apps.usp.org/app/USPNF/columnsDB.html). The closer to zero is the value of F, the more similar are the columns. The F values vary between 0 (identical columns) and 35. For large F values, the columns can be considered "orthogonal" [114].

The utilization of Eq. 9.3.7 for the calculation of α for two specific compounds on a given column with known H^*, S^*, A^*, B^*, and C^* is limited by the need to know all the parameters η', σ', β', α', and κ' for both compounds. In comparison with the enormous number of chemicals, only a very limited number of compound parameters are reported in the literature (see, e.g., Ref. [31]). Since the importance of different components in a column selection is not equal, it was suggested to add to each contribution weighing factors f such that Eq. 9.3.11 becomes the following:

$$F_{col.1-col.2} = \Big\{ \big[f_H\big(H^*_{col.1} - H^*_{col.2}\big)\big]^2 + \big[f_S\big(S^*_{col.1} - S^*_{col.2}\big)\big]^2 + \big[f_A\big(A^*_{col.1} - A^*_{col.2}\big)\big]^2 + \big[f_B\big(B^*_{col.1} - B^*_{col.2}\big)\big]^2 + \big[f_C\big(C^*_{col.1} - C^*_{col.2}\big)\big]^2 \Big\}^{1/2} \quad (9.3.12)$$

The weighing factors for the columns listed in Appendix 9.3.3 have the following values: $f_H = 12.5$, $f_S = 100$, $f_A = 30.3$, $f_B = 142.8$, and $f_C = 83.3$ [32].

Various other parameters and tests for RP-HPLC column characterization

Several other column characterization procedures have been developed and they serve for column comparison and selection [38,130,131]. One such procedure (USP approach from United States Pharmacopeia) uses a standard mixture of compounds known as Standard Reference Material (SRM) 870 and some information from column manufacturer for column characterization. The SRM 870 material contains uracil (27.1 μg/g), toluene (1430 μg/g), ethylbenzene (1730 μg/g), quinizarin (90.8 μg/g), and amitriptyline hydrochloride (2740 μg/g) in a methanol solution. The USP parameters are as follows: H_y given as retention factor k' for ethylbenzene, chelating tailing factor CTF for quinizarin, retention factor k' for amitriptyline CFA, tailing factor for amitriptyline TFA, and the bonding density BD in μmol/m^2 of the stationary phase [129]. The test is performed using as mobile phase composition 80% methanol and 20% aqueous phosphate buffer of pH = 7. The values for the parameters H_y, CTF, CFA, TFA, and BD are reported for about 117 C18 columns (USP approach) and are listed in Appendix 9.3.2 [104]. A similar distance between columns as given by Eq. 9.3.11 can be developed by using the parameters measured by USP test H_y, CTF, CFA, TFA, and BD.

Various other tests and analytes are reported in the literature for comparing different column characteristics [125,132–135]. For example, metal activity in a stationary phase can be evaluated using other reagents besides quinizarin. The measurement of the effect of metals is important since metallic ions can increase the acidity of adjacent silanols, and can become adsorption sites for compounds that are able to form complexes. Overall, they can interfere in the retention process. For measuring metal activity on the surface of stationary phase, acetylacetone and also the couple of 2,2′-dipyridyl and 4,4′-dipyridyl were used. The retention behavior of 2,2′-dipyridyl and 4,4′-dipyridyl in the presence of metal ions on the silica surface is rather different: 2,2′-dipyridyl can form complexes with metallic species in particular with iron, whereas 4,4′-dipyridyl cannot form complexes. Therefore, the peak of 2,2′-dipyridyl will exhibit a posttailing with an asymmetry depending on the concentration of metallic ions from the silica

surface, while the peak of 4,4′-dipyridyl will remain symmetric. Although these dipyridyl derivatives are basic compounds that can be involved in strong interactions with dissociated silanol groups from silica surface, this inconvenience was solved by using buffered (pH = 7) mobile phase [136].

Another type of test for comparing columns is related to the evaluation of stationary phase polarity. The ability of a reversed-phase column to retain polar compounds depends not only on the extent of hydrophobic interactions between the column and the hydrophobic moiety of the compound but also on the two participants' polarity. For example, the selectivity difference between a conventional C18 and a polar-embedded phase results from their polarity differences. In general, higher polarity of the embedded group in a stationary phase results in a longer retention of polar compounds. One simple test to characterize the polarity of the stationary phase is based on using a mixture containing uracil, pyridine, phenol, N,N-dimethylaniline, *p*-butylbenzoic acid, and toluene in a mobile phase made of acetonitrile/aqueous buffer 60/40 (v/v) (the buffer consisted of 50 mM KH_2PO_4/KH_2PO_4 pH = 3.2). With this mixture, a column polarity index (*Po*) was defined [135]:

$$Po = \frac{k'_{p-\text{butylbenzoic acid}} \cdot k'_{\text{phenol}}}{\left(k'_{\text{toluene}}\right)^2} \qquad (9.3.13)$$

This test shows that the polarity of polar-embedded stationary phases with the same hydrocarbon chain varies in the following order:

amide > carbamate > sulfonamide > alkyl.

Another test for measuring polarity uses butyl paraben and dipropyl phthalate [137]. Retention studies on amide-embedded stationary phases showed that dipropyl phathlate elutes before butyl paraben, whereas the elution order on carbamate-embedded group phases is opposite. The order of polarity for the polar-embedded phases using this test is the following:

amine > amide > carbamate > ester > alkyl.

The possibility to use ionic compounds in a 100% aqueous mobile phase for the characterization of RP columns was also suggested [138]. In this test, various naphthalene disulfonic acids and naphthalene trisulfonic acids were employed as test compounds. These compounds are practically completely ionized in aqueous solution, and when using only water as a mobile phase, they are excluded from the pores of the hydrophobic packing material, having a retention time shorter than compounds such as uracil typically used for measuring the column dead time (or volume). This effect is attributed to ionic repulsion between the slightly negatively charged stationary phase surface (as a result of silanol groups ionization) and the sulfonic acid anions. The test was performed using as mobile phase a solution 0.4 M of Na_2SO_4. With this mobile phase, the retention times of the test compounds increase compared to pure water mobile phase, on all tested hydrophobic columns. However, the dead time t_0 for the columns as measured, e.g., using uracil, cannot be used for calculating a capacity factor, and the differences in retention must be compared versus the least retained test compound (1,5-naphtalenedisulfonic acid for disulfonic acids and 1,3,5,7-naphthalene-tetrasulfonic acid for trisulfonic acids). The test provides information on hydrophobicity of the column and peak asymmetry.

Besides the nature of the column (and considering the nature of the test compounds), the retention factor, the methylene selectivity $\alpha(CH_2)$, and also other interactions are significantly influenced by the mobile phase used in the test [105,110,139]. However, in the previous discussion, the nature of the mobile phase was not explicitly discussed, except that the mobile phase must be kept the same for a pertinent comparison between columns. Since the selection of the mobile phase strongly affects the value of capacity factor, it can be concluded

that Eqs. 6.4.14 and 6.4.9 are incomplete, and a coefficient dependent on solvent should be included in these expressions. Because the nature of each interaction is dependent on the composition of the mobile phase the interaction intensities (e.g., by the dissociation or suppressing dissociation of a given analyte), are affected by the mobile phase and each interaction type has a different solvent dependent parameter. This would lead from Eq. 9.3.7 to a formula for the capacity factor of the type:

$$\begin{aligned}\log \alpha(X, EB) &= \log\left(\frac{k'_X}{k'_{EB}}\right) \\ &= \eta'(X)H^*_{col} - \eta''(X)H^*_{mo} \\ &- \sigma'(X)S^*_{col} + \beta'(X)A^*_{col} \\ -\beta''(X)A^*_{mo} + \alpha'(X)B^*_{col} &- \alpha''(X)B^*_{mo} \\ &+ \kappa'(X)C^*_{col}\end{aligned} \quad (9.3.14)$$

In Eq. 9.3.14, $\log k'_{EB}$ depends the column and on the mobile phase, H^*_{col}, S^*_{col}, A^*_{col}, B^*_{col}, C^*_{col} depend only on the column, while H^*_{mo}, A^*_{mo}, and B_{mo} are parameters related only to a specific mobile phase and account for polar/polarizability, hydrogen bonding for a basic solute, and hydrogen bonding for an acidic solute, respectively. The sign of respective coefficients depends on the effect they have on separation (decreasing $\log k'$ when the solvent interactions are stronger).

The combination of the idea of choosing a specific analyte and a specific mobile phase for putting in evidence a specific character of the stationary phase led to the development of several tests that allow the characterization a hydrophobic column from the point of view of hydrophobicity, polar interactions, hydrogen bonding, ion exchange interactions, and steric interactions. Several such tests are listed in Table 9.3.3.

Some other tests and parameters were reported in the literature for column characterization [131,157,158]. One such parameter characterizing hydrophobicity is obtained as $\log k'_w/S$ where $\log k'_w$ is obtained from the intercept of the plot of $\log k'$ as a function of acetonitrile concentration S for a given column and a set of 8 compounds [135]. The results generated with different tests are important for column characterization. As an example, such results are given in Table 9.3.4 for several conventional C18 columns, polar end-capped columns, and polar-embedded columns [38] using *Test 17* from Table 9.3.3.

Tests for the evaluation of aging of the chromatographic column

Stability of bonded phases can be evaluated periodically by measuring peak performances of test compounds. Also, for some columns, the resilience to aging is reported after the column stability has been evaluated in an aging study when the column is subjected to different stress conditions with the aim of observing the performance modifications in time. One such test consists in continuously passing through the column a mobile phase containing acetonitrile and phosphate buffer with a specific pH and at a specified temperature (40–60°C), followed by column performance tests (for k', α, N). More detailed studies of column aging including both evaluation of chromatographic parameter changes and material characterization with FTIR, 1H-NMR, and solid state 13C- and 29Si-NMR of hydrolyzed bonded phases were also reported [159].

9.4 Selection of the column in RP-HPLC

General comments

Reversed-phase HPLC is the most utilized type of HPLC used for the analysis of organic compounds in various matrices. Currently, there are a large number of RP-HPLC columns, some sources indicating a number exceeding 600 columns commercially available (e.g., Ref. [160]).

TABLE 9.3.3 Various tests for the characterization of hydrophobic columns.*

No.	Test conditions	Parameters measured	Ref
1	Test analytes: 1. uracil (t_0), 2. thiourea (t_0), 3. amylbenzene, 4. butylbenzene, 5. triphenylene, 6. *o*-terphenylene, 7. caffeine, 8. phenol, 9. benzylamine Eluent: MeOH−H_2O 80:20 v/v Eluent: MeOH−H_2O 30:70 v/v Eluent: MeOH-0.02 m phosphate buffer (pH = 7.6) 30:70 v/v Eluent: MeOH-0.02 m phosphate buffer (pH = 2.7) 30:70 v/v	- Hydrophobicity $\alpha(CH_2) = k'(3)/k'(4)$ - Alkyl ligands amount = $k'(3)$ - Steric selectivity = $k'5)/k'(6)$ - Hydrogen bond capacity = $k'(7)/k'(8)$ - Ion exchange capacity (IEC) for pH > 7 = $k'(9)/k'(8)$ - IEC for pH < 3 = $k'(9)/k'(8)$	
2	Test analytes: 1. thiourea (t_0), 2. aniline, 3. phenol, 4. *N*,*N*-dimethylaniline, 5. *p*-ethylaniline, 6. toluene, 7. ethylbenzene, 8. *m*-toluidine Eluent: MeOH−H_2O 55:45 v/v; $T = 40°C$. $\lambda = 254$ nm	- Hydrophobicity $\alpha(CH_2) = k'(7)/k'(6)$; - Silanol activity *As*(4), *As*(5), *As*(8) - Retention factors for basic solutes	[140]
3	Test analytes: 1. uracil (t_0), 2. triphenylene 3. o-terphenyl Eluent: MeOH−H_2O 55:45 v/v; $T = 40°C$. $\lambda = 254$ nm	- Shape selectivity = $k'(2)/k'(3)$;	[139]
4	Test analytes: 1. uracil (t_0), 2. benzene, 3. anthracene Eluent: AcCN-H_2O 65:35 v/v; $T = 40°C$. $\lambda = 254$ nm	- Hydrophobicity = $k'(3)/k'(2)$	[141]
5	Test analytes: 1. uracil (t_0), 2. anthracene, 3. *N*,*N*-diethyl-*m*-toluamide, 4. nitrobenzene, 5. benzene, 6. toluene Eluent: 100% AcCN, $T = 40°C$	- Silanol activity = $k'(3)/k'(2)$ - Silanol index $k'(4)$ - Hydrophobicity $\alpha(CH_2) = k'(6)/k'(5)$ - Theoretical plate *N* from $W_b(6)$	[141]
6	Test analytes: 1. nortriptyline, 2. nicotine, 3. amphetamine, 4. pyridine, 5. benzylamine,6. codeine, 7. quinine, 8. procainamide, 9. diphenhydramine Eluent: AcCN, MeOH, THF at pH 3.0 and neutral pH	- Retention factors - Asymmetry factors - Column efficiency	[142,143]
7	Test analytes: 1. naphthalene, 2. acetonilidine, 3. phenol, 4. benzonitrile Eluent: MeOH−H_2O and AcCN-H_2O	- Various characterization of columns for RP HPLC	[144]
8	Test analytes: 1. amitriptyline, 2. naphthalene, 3. acetylnaphthalene, 4. dipropylphthalate, 5. butylparabene Eluent: Neutral pH, 35% 20 mm K_2HPO_4 −KH_2PO_4 buffer pH = 7.0 and 65% MeOH, acidic pH, 20% 50 mm H_3PO_4−KH_2PO_4 buffer pH = 3.0 and 80% AcCN	- Estimation of the surface silanols - Markers of packing - Hydrophobicity - Determination of the packing polar selectivity	[99,100,145]
9	Test analytes: 1. aniline, 2. phenol, 3. benzene, 4. toluene	- Hydrophobicity $(k'(3) + k'(4))/2$ - Silanophobicity (silanol index)	[146,147]

10	Test analytes: 1. benzo[a]pirene (BaP), 2. phenantro[3,4-c]- phenantrene (PhPh), 3. tetrabenznaphthalene (TBN) Eluent: AcCN-H_2O 85/15, flow rate 2 mL/min $\lambda = 254$ nm	- Column selectivity: - Monomer column $\alpha(3/1) > 1.7$ - Polymer column $\alpha(3/1) < 1$ - Oligomer column $1 < \alpha(3/1) < 1.7$	[148,149]
11	Test analytes: Big molecules such as fullerenes Eluent: *n*-hexane	- Molecular shape recognition	[150]
12	Test analytes: Macromolecules such as polymers	- Silanophilic interactions	[151,152]
13	Test analytes: 1. bronopol, 2. acetophenone, 3. benzene, 4. toluene Eluent: AcCN-H_2O 80:20	- Column classification according to selectivity to bronopol	[153]
14	Test: Analytes: 1. bronopol Eluent: 100% water	- Column classification according to selectivity to bronopol	[153]
15	25 test analytes with different properties	- QSRR method based on structure−retention relationships	[154,155]
16	Test analytes: 1. naphthalene sulfonic acids Eluent: 0.4 m Na_2SO_4	- Surface coverage homogeneity - Silanol activity	[101,156]
17	Test analytes: 1. butylbenzene, 2. amylbenzene, 3. propylbenzene, 4. ethylbenzene, 5. toluene, 6. benzene, 7. caffeine, 8. phenol, 9. benzylamine, 10. p-hydroxybenzoic acid, 11. sorbic acid, 12. benzoic acid, 13. salicylic acid, 14. p-toluic acid, 15. triprolidine, 16. chlorpheniramine, 17. diphenyhydramine, 18. tricyclic antidepressants such as desipramine and amitriptyline. Eluent: 65% AcCN, H_2O buffer 20 mM phosphate, pH = 7 Eluent: 15% AcCN, H_2O buffer 20 mM phosphate, pH = 7 Eluent: 30% AcCN, H_2O buffer 20 mM phosphate, pH = 7 Eluent: 30% AcCN, H_2O buffer 20 mM phosphate, pH = 2.5 Eluent: 75% AcCN, H_2O buffer 20 mM phosphate, pH = 2.5	- Hydrophobicity $k'(1)$, $k'(2)$ - Methylene selectivity $\alpha(CH_2)$ for benzene to amylbenzene - Hydrogen bonding capacity, $\alpha(7,8) = k'(7)/k'(8)$ - Silanol activity pH = 2.5, $\alpha(9,8)_{pH=2.5} = k'(9)/k'(8)$ - Silanol activity pH = 7, $\alpha(9.8)_{pH=7} = k'(9)/k'(8)$ - Retention acids $k'(i)$, i = 10−14 - Peak asymmetry *As*(15), *As*(16), *As*(17) at pH = 2.5, *As*(18) - Theoretical plate *N* from $W_b(5)$	[38]

* *Note: As an example,* k′*(3) indicates* k′ *for compound No.3, α(7,6) indicates α for the pair of compounds No.7 and No.6, etc.*

TABLE 9.3.4 Comparison on several test parameters for conventional, polar end-capped, and polar-embedded columns [38]. The columns were evaluated using *Test 17* from Table 9.3.3 with parameters $k'_{BB} = k'_{butylbenzene}$, $\alpha_{caffeine/phenol} = \alpha(7,8)$, $\alpha_{benzylamine/phenol\ pH\ =\ 2.5} = \alpha(9,8)_{pH\ =\ 2.5}$, and $\alpha_{benzylamine/phenol\ pH\ =\ 7} = \alpha(9,8)_{pH\ =7}$.

	Parameter and described property					
		k'_{BB}	$\alpha(CH_2)$	$\alpha_{caffeine/phenol}$	$\alpha_{benzylamine/phenol\ pH\ =\ 2.5}$	$\alpha_{benzylamine/phenol\ pH\ =\ 7}$
Column type	***N* plates/m**	**Hydrophobicity**	**Methylene selectivity**	**Hydrogen bonding**	**Silanol activity pH = 2.5**	**Silanol activity pH = 7**
Conventional C18						
Luna 5 mm C18(2)	116,889	9.11	0.176	0.21	0.059	0.131
Inertsil ODS(3)	96,669	8.63	0.171	0.261	0.045	0.147
Zorbax XDB C18	95,997	8.57	0.187	0.213	0.088	0.134
Symmetry C18	91,515	9.23	0.181	0.224	0.049	0.147
HyPurity Elite C18	87,816	2.96	0.155	0.31	0.061	0.378
Zorbax SB C18	84,568	6.74	0.179	0.283	0.079	0.412
Prodigy ODS(3)	108,384	9.82	0.184	0.21	0.051	0.13
Polar end-capped						
Keystone Aquasil	109,457	4.41	0.147	0.725	0.131	1.659
Aqua C18	87,666	8.67	0.176	0.251	0.094	0.149
YMC Hydrosphere	109,430	6.25	0.167	0.27	0.055	0.14
YMC ODS-Aq	90,049	7.83	0.171	0.262	0.094	0.169
Prontosil C18AQ	92,800	7.04	0.173	0.303	0.079	0.254
Metasil AQ	88,846	7.77	0.158	0.217	0.073	0.162
Synergi Hydro-RP	113,187	10.07	0.174	0.24	0.063	0.203
Polar embedded						
Zorbax Bonus-RP	110,156	3.86	0.147	0.201	0	0.167
Polaris C18-A	105,481	4.38	0.165	0.2	0.102	0.121
Discovery RP-Amide	115,577	3.34	0.15	0.166	0.05	0.097
SymmetryShield RP18	109,530	6.15	0.152	0.164	0.013	0.098
Supelco ABZ+	95,551	1.88	0.143	0.182	0	0.23
Experimental urea	54,458	6.25	0.157	0.133	0	0.8
Polaris amide C18	87,744	5.11	0.139	0.123	0	0.08

Part of these commercial columns and some of their characteristics are listed in Appendix to Chapter 9. There is not a general procedure of selecting a stationary phase and a corresponding RP-HPLC column, but some principles are helpful in this choice. The choice refers to the column chemistry (the nature of the bonded phase, the bonding type and its density, the end-capping, and the carbon load), the particle physical characteristics, and the column dimensions. The choice is influenced by the nature of the analytes and also by the sample matrix. The selection is judged by the chromatographic performance such as the column resolution and efficiency, suitability for the analysis, reproducibility, and time stability. The present section is dedicated to some criteria that should be considered in the selection of a stationary phase and column in RP-HPLC. In practice, a common procedure "trial and error" could solve the selection, but this process can be time consuming and costly.

Sample nature in RP-HPLC and its influence on column selection

The utilization of RP-HPLC covers a wide range of molecules. The variety of chromatographic columns of reversed-phase type and changes in the mobile phase allowed the analysis of small molecules and also of macromolecules such as peptides and proteins (e.g., Refs. [161–163]).

Before making a selection of an RP-HPLC column, several aspects of the sample must be considered. Among these can be listed: (1) sample contains nonpolymeric analytes, polymeric ones, or a mixture, (2) sample has a "dirty" matrix, (3) sample has a low level of analytes, (4) sample contains compounds with basic character, (5) sample contains acidic compounds, (6) molecules of the analytes have very low hydrophobic character, (7) molecules of the analytes have very high hydrophobic character, (8) sample contains a mixture of compounds with a wide range of log K_{ow} values.

(1) The difference in the type of column when analyzing nonpolymeric or polymeric compounds is related to the pore size of the column that must be selected. Specific columns with the stationary phase having large pore must be selected for the analysis of polymers (biopolymers). For proteins and macromolecules in general, log K_{ow} is not a good criterion for polarity description, but even peptide nucleic acids that are very acidic can be separated using RP-HPLC [164].

(2) For the analysis of samples that have a high content of matrix (and possibly with a complex composition), the HPLC core analysis has a more difficult task since it must separate the analytes and also the matrix components. When the matrix has basically a different chemical character than the analytes, it is possible to select the column and the mobile phase composition such that the matrix either elutes very close to the dead time of the column or is eluted only when the mobile phase has a high organic content (is a "strong" eluent). Even when the analytes are well separated from the matrix components, the peak shape of the analytes may be affected (see Section 6.3). In some cases, a sample preparation step for cleanup is unavoidable. For samples with "dirty" matrix, it is highly recommended to use a precolumn for the protection of the analytical column and to make as small injections as possible (depending on detector sensitivity). Also, for sample with a "dirty" matrix, it is recommended to use columns with a larger diameter (e.g., 4.6 mm) and larger particle size (e.g., 5 μm).

(3) For samples that have a low level of analytes, columns with smaller diameter and small particle size are usually recommended. Such columns generate narrower peaks that allow an increase in sensitivity of the HPLC detection (better signal to noise, see Section 3.4).

(4) For the case of basic compounds, the pH of the mobile phase is necessary to be relatively high to keep the analytes from becoming ionized. In such cases, columns with higher resilience to pH above 7 or 8 must be selected (e.g., columns using ethylene-bridge technology). Also, column with low tailing characteristics must be selected for basic compounds (e.g., with high purity silica support). Many amines have high propensity to form coordinative bonds with trace transitional metals (e.g., Fe^{2+}, Fe^{3+}, Ni^{2+}, etc.) and some columns show tailing of amines peaks (see USP test for tailing character in Section 9.3).

(5) For acidic compounds, the columns must be resilient to low pH. In order to keep the organic acids in molecular form (not ionized), the mobile phase should have a low pH (2.5−3) and the column must be selected to be resilient to this pH.

(6) Molecules with high polarity and low hydrophobic character (log K_{ow} −1.25−0.0) require a mobile phase with high water content or even 100% water and a column providing high hydrophobicity and possibly able to develop additional types of interactions (with polar-embedded groups or polar end-capping). Such columns should have excellent wetting capabilities (like those polar end-capped or with embedded polar groups), and at the same time a high carbon load (high *C*%) and possibly high surface area of silica support.

7) Some molecules have high log K_{ow} indicating high hydrophobicity (above 4.5). For such compounds, the retention may be too strong on C18 or C8 columns and such compounds elute at long retention times even if the mobile phase has a high content of organic component. In such cases, columns with a low *C*% load are used, and if separation of other compounds potentially present in the sample is acceptable, short columns are recommended. For compounds with very high hydrophobicity (log $K_{ow} > 5$) the alternative is to use NARP.

(8) When the sample contains a mixture of compounds with very different log K_{ow} values, it is common to select a column capable of separating the compounds with low log K_{ow}, and then by using gradient, to increase the organic component content in the mobile phase in order to elute all the analytes (and the matrix) from the column.

Column choice from several possibilities

A large proportion of published analytical methods use C18 columns, some are using C8 columns, and fewer methods using other RP columns. The popularity of C18 columns is explained by the fact that these columns are adequate for many types of analysis. However, a considerable variety of C18 columns (as well as of C8 columns) is available, the columns differing in physical construction, carbon load, range of pH resilience, wettability, etc. For this reason, even by limiting the choice to a C18 column, there are still many parameters that require a selection.

Since it is always easier to modify an already developed method than to develop a radically different one (or to reinvent the method), it is always advisable to search for reported analyses similar to the one intended to be implemented. It is common that small modifications to a reported method can make it adequate for a specific new task. In some instances, the columns recommended in the literature for a specific analysis are not available, and the PQRI or USB approaches offer the capability to select similar columns with the one initially recommended (see Ref. [104]). Besides general literature references, computer programs have been developed for guiding the selection and optimization of an HPLC separation, using computer simulation [165]. Such a computer program (e.g., DryLab4 [166]) starts with input from 2 or more (up to

12) experimental chromatograms and allows to virtually modify various parameters such as mobile phase composition (isocratic or gradient), flow rate, column dimensions, and predicts retention times and optimum conditions for a separation. Also the program can assist regarding the transferring of a method from one column to an equivalent one [167]. Since the change of a selected column that was proven inadequate represents some waste of resources (time, cost), it is preferable to select from the beginning a good column. The variety of columns commercially available on the market makes this selection a rather complicated task. However, the process is somewhat simplified by the fact that for many analyses not only a unique selected column can be fully adequate for a successful HPLC analysis. Similar results can be obtained with several different selections. As an example, the results for the separation of several polycarboxylic and/or hydroxy organic acids with low K_{ow} values are indicated in Fig. 9.4.1 (the chromatogram obtained on a Synergy Hydro-RP with detection on an MS/MS instrument) and Fig. 9.4.2 (the chromatogram obtained on an InertSustain AQ-C18 column with MS detection). As shown in these figures, both columns can be successfully used for organic acids separation, and the MS capability allows the measurement of specific ions such that the interferences are eliminated in spite of some peak overlapping.

Optimization of column selection between the fully adequate columns remains optional, depending on the goal of the analysis. The selection of the column must always be made in the context of analysis requirements such as goal of the analysis, e.g., quantitation, sample

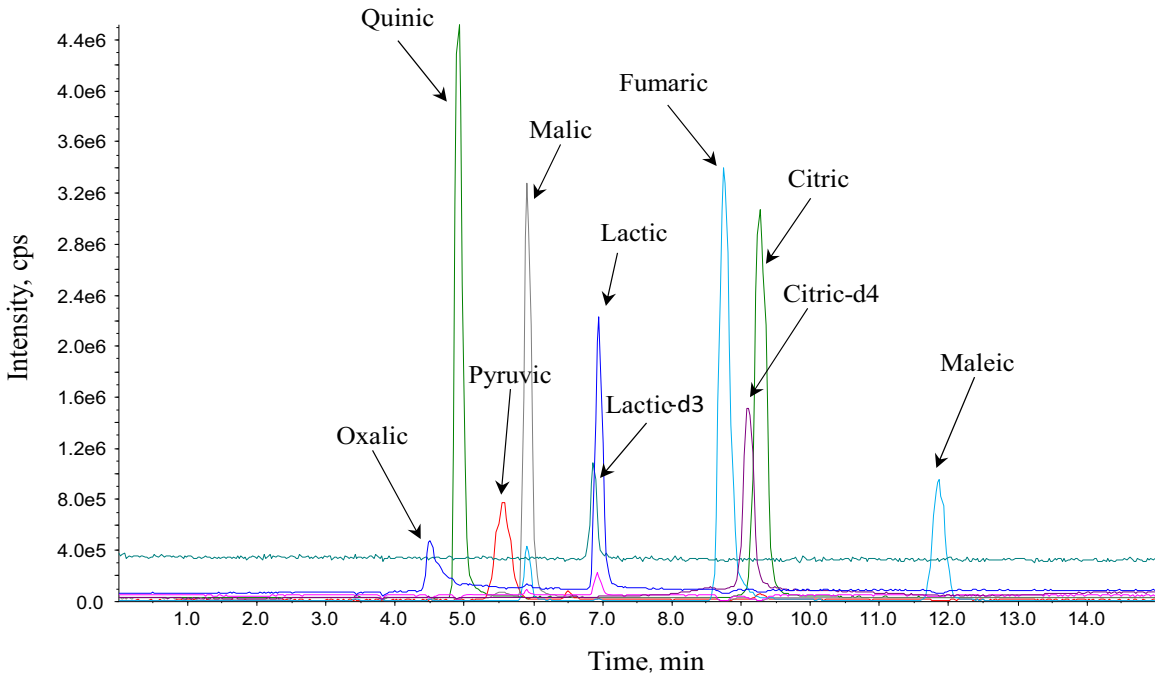

FIGURE 9.4.1 Separation of a standard mixture of organic acids containing about 10 µg/mL for each analyte and about 5 µg/mL of deuterated standards on a Synergy Hydro-RP column in isocratic conditions 95% H_2O + 5% CH_3OH and 0.1% HCOOH 0.3 mL/min flow, 1 µL injection (MS/MS detection).

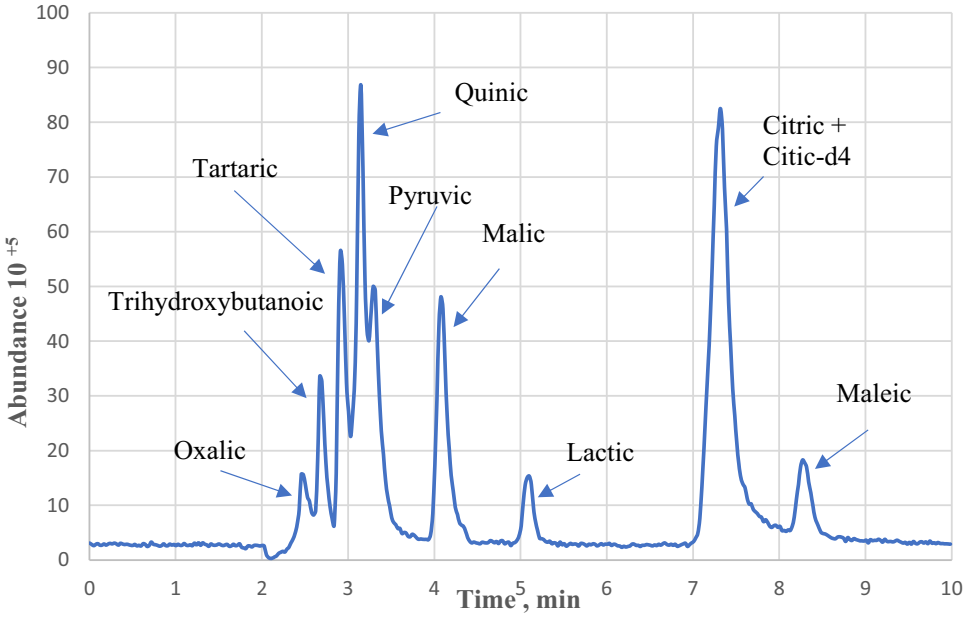

FIGURE 9.4.2 Separation of a standard mixture of organic acids containing about 12.5 μg/mL for each analyte on an Inert-Sustain AQ-C18 column in isocratic conditions 95% H_2O + 5% CH_3OH and 0.02% HCOOH 0.3 mL/min flow, 1 μL injection (MS detection).

complexity, relative concentration of analytes in the sample, sample size availability, number of samples to be analyzed, requirements for time length of the chromatographic run, etc.

For a long period of time, the most frequently utilized columns in RP-HPLC have 100 or 150 mm length, 2.1, 3, or 4.6 mm diameter, with uniform fully porous spherical particles of 1.8, 3.0, or 5.0 μm diameter [168]. In the last several years, columns with core-shell particles are utilized and the tendency is to use more narrow columns (2.1 mm diameter), smaller particles (1.7, 1.8, 3.0 μm), and shorter length (50 mm, 100 mm). Many different physical parameters from those indicated can be selected, depending on the analysis requirements. For faster analyses, 50 mm length column can be selected, and for separation demanding higher N values, 250 mm column length may be necessary. For the analysis of cleaner samples, core-shell particles with 1.7 or 2.6 μm diameter can be better, such columns having typically a higher N. This choice depends also on the maximum backpressure that can be sustained by the HPLC or the UPLC system since smaller particles produce higher backpressure (see Eq. 8.2.9). Depending on the sample load, wider columns are recommended for more concentrated samples, but for small amount injections, columns with 2.1 mm diameter are fully adequate. Even columns with 1.5 mm diameter can be used with good separation results [169].

For trace analysis, columns with small dimensions are typically recommended, and core-shell columns offer advantages for high N at relatively short column length. Also, for the analysis of large molecules, it is recommended to select stationary phases with larger pores (200 Å or larger, depending on the molecule Mw). In many analyses, the need for higher efficiency and resolution is solved by using columns packed with small particle size which

may lead to high backpressure in the system. To avoid these problems, the use of monolithic columns, especially silica-based monolithic columns, can be an alternative choice to the packed columns [170].

The RP columns frequently show similarities among themselves regarding the separation properties, and the replacement of one column with an equivalent one is not usually a problem. The main characteristics to consider in column selection are the following: (1) the column efficiency (N/m), (2) resilience to a wider pH range, (3) wettability, (4) peak tailing in case of the separation of basic compounds, (5) better separation when more difficult analytical tasks are encountered, (6) loading capacity of the column, (7) use of special RP-HPLC columns, and (8) selection of column for polymers when the analytes are macromolecules.

(1) Column efficiency becomes critical when the analytes of interest have peaks in close proximity to other peaks. In such situations, when the modifications in the mobile phase are not capable of improving the peaks of interest separation, a column with better N may solve the problem. This can be achieved using columns with smaller particles, core-shell particles, longer columns, etc. The selection depends on the backpressure sustained by the HPLC system (HPLC or UPLC), limitations in the length of run time, etc.

(2) Columns resilient to a wider pH range are frequently necessary for the analysis of compounds capable of existing in more than one form at different pH values of the mobile phase (see Section 9.3). In such cases, the pH of the mobile phase is usually recommended to have a more extreme pH such that only one form of the analyte is present in solution (e.g., a high pH for the analysis of basic compounds capable to exist in forms like B and BH^+ or low pH for acidic compounds capable to exist in forms like AH and A^-). Depending of the pK_a (or pK_b) of the analytes, the pH of the mobile phase may need to be lower than 2 or higher than 7 or 8. In such cases, the column pH range of stability must be selected carefully. When a wider pH range for the mobile phase is necessary in a separation, the columns with silica technology should be preferred to those based on organic polymers. The ethylene bridge silica type columns offer the same mechanical characteristics as silica-based columns and do not have the typical backpressure restrictions of organic polymeric columns.

(3) In some separation, it is useful to have a very high content of water in the mobile phase. This high content may be utilized only at the beginning of the chromatographic run when gradient elution is employed or may be necessary for an extended range or even for the whole chromatographic separation. In such situations, the wettability of the chromatographic column is very important, since a mobile phase with 100% water may ruin some columns (see Section 9.2). Columns using polar end-capping, polar-embedded groups, and those with ethylene bridge organic/inorganic silica support are usually more resilient to high water content.

(4) In the analysis of some basic compounds (amines), a common problem is the peak tailing of the analytes of interest. Peak tailing is a common characteristic described by the manufactures, and when necessary, columns with low tailing should be selected (usually made with silica of very high purity or with special phase surface treatment).

(5) When several alternatives for selecting an analytical column are available, it is recommended to start with the simplest and most straightforward choice. If the separation is possible using RP-HPLC or HILIC, the RP-HPLC should be preferred. When the analytes have differences in the hydrophobic moiety,

simple C18 or C8 columns are recommended. Phenyl type columns are typically offering a better separation when aromatic compounds should be separated. Cyano columns also offer better separation between compounds with lower polarity from those with higher polarity. If a simple hydrophobic column or a column with embedded polar groups or polar end-capped is the alternative and no restriction regarding wetting or better separation is present, the simple hydrophobic column is likely to offer a more robust analytical method. However, columns with embedded polar groups, polar end-capped, with phenyl, or cyano groups may offer better separation characteristics, since they produce additional types of interactions that may help the separation. However, multiple types of interaction may not be necessary in all separations.

(6) One problem may be related to the column providing good separation, but with peak shape problems such as fronting or too much tailing. Fronting typically indicates either overloading of the column or nonideal equilibria in the chromatographic process. Overloading can be corrected by diminishing the sample volume, concentration, or both but can also be corrected by selecting columns with more stationary phase, e.g., with larger diameter and with fully porous particles instead of core-shell particles. For correcting the nonideal equilibrium in the chromatographic process, the column and/or the mobile phase must be changed.

(7) Special types of columns, such as microcolumns, are used for some applications. For example, a Separon SGX C18 microcolumn, 150 × 1 mm, was utilized for the analysis of doxycycline from biological samples [171]. Various techniques have been reported to make microcolumns for RP-HPLC [172]. However, the utilization of micro- and nanocolumns usually offers lower resolution compared to conventional ones.

(8) Several aspects regarding the column (and mobile phase) selection are specific for polymer analysis. For the easy access to the pores of stationary phase, the pore size for polymer analysis should be at least three times the hydrodynamic diameter of the macromolecule (often pores of 300 Å diameter or even larger are used) [173]. This parameter is related to the molecular weight of the analyte. The free access of the analyte to the bonded phase is very important since the retention on the stationary phase depends on the accessible surface area of the packing. The estimation of steric hindrance for a molecule at a pore entrance has been reported in the literature [174]. The theory indicates that for relatively small molecules, such as small peptides ($Mw < 1000$ Da), the stationary phase with 80−120 Å pore size is well suited. For larger molecules with the Mw higher than 1000−2000 Da (and up to 10,000), stationary phases with pore size around 300 Å are necessary. The larger pores are necessary for allowing the interaction of the analyte with the stationary phase and avoid size exclusion effects. For large proteins (Mw 10,000−100,000 Da, or higher), even larger pores are necessary, up to 1000 Å. Larger pores are typically associated with smaller surface areas for the phase. The columns with wider pores (300 Å) have typically lower k' for hydrophobic molecules (e.g., ethylbenzene), but in case of proteins they offer a better separation [175].

Besides large pores utilization for protein analysis, another alternative is the use of nonporous C18 columns (e.g., Presto FF-C18 column from Imtakt). Such columns have lower loading capacity but large proteins (of about 150 kDa) were separated on such columns [176].

Since the interaction of large molecules with the hydrophobic bonded phase is probably mainly based on an adsorption process (not on distribution), monomeric type bonded phases or phases obtained by horizontal polymerization

display a similar capacity factor as vertical polymeric bonded phases (see Section 8.1). Monomeric bonded phases and those with horizontal polymerization typically show better reproducibility in protein/peptide separations. For this reason, monomeric phases are preferred to phases obtained with vertical polymerization, although these may have a larger carbon load.

The diffusion of proteins in the phases is in general slower than that of small molecules. When the diffusion coefficients of the analyte in the mobile phase D and in the stationary phase D_s are small, several terms contributing to the plate height (H_C, H_T, H_S) are large, and for this reason, the resulting peaks of the proteins may be significantly wider than for small molecules (see Eqs. 3.1.63–3.1.65) As shown from these formulas, a lower flow rate u in the column has the effect of diminishing plate height components H_C, H_T, and H_S, but longer separation times result in this case. For this reason, columns with a high theoretical plate number N (as measured for a test small molecule compound) are preferred for protein separation to compensate for peak broadening inherent to large molecules (although the N value for the proteins is much smaller than obtained with the test compound). Columns with small particles or with core-shell that have high N values can be useful for protein separation. The flow rate is typically maintained at a constant value in the range 0.5–2 mL/min. Some problems in protein separation, such as peak tailing, are corrected by increasing the acid content in the mobile phase. In this way, the silanol activity of the column is reduced and the tailing of basic compounds is diminished. The acids used in the mobile phase for proteins and peptides, in particular TFA, may act as an ion pair to the proteins with basic character (pI > 7). The ion pair formed with TFA has a stronger retention (larger $\log k'$) than expected for the free compound. Protein degradation during separation must be avoided, in particular when the physiological property of the protein must be preserved.

Different proteins may have a wide range of molecular shapes and also of polarities, and their retention on an RP column may vary considerably [177]. The most common hydrophobic phases used for protein separations are C8 and C18. Also, columns that have lower hydrophobicity but additional interactions with polar groups from the protein molecule may be better suited for some separations. Phenyl and cyano columns may show some special selectivity and are used in specific applications where the differences in hydrophobicity between different proteins are not sufficient for their separation. The same is true for columns end-capped with polar groups or not end-capped and having a larger proportion of free silanols. However, some proteins with higher hydrophobicity can be separated more easily using short-chain bonded phases such as C1 to C3 since they may be retained too strongly on C8 or C18 columns.

9.5 Mobile phases in reversed-phase liquid chromatography

General comments

Polar mobile phases used in RP-HPLC typically contain water with a certain proportion of one or more organic solvents (besides buffers and additives). As organic solvents, methanol or acetonitrile is usually added to water. Other solvents with solubility in water can also be used as organic component. In RP-HPLC, solvents with high polarity have a low elution strength (see Section 7.1). Although not useful for a direct calculation, Eq. 7.1.6 that uses Hildebrand solubility parameter δ for the estimation of the equilibrium constant for an analyte X provides useful directional guidance and it indicates the following value for $K(X)$:

$$K(X) = e^{\mathbb{K}(\delta_{mo}-\delta_{st})} \tag{9.5.1}$$

In Eq. 9.5.1 $\mathbb{K} = [V_X(\delta_{st} + \delta_{mo} - 2\delta_X)]/\mathrm{RT}$ and has a positive value for nonpolar or weakly polar compounds with δ_X relatively small and close to δ_{st}. As a result, $K(X)$ is larger when δ_{mo} is larger (see Appendix 6.1.1 for δ_{mo} values). Polar solvents such as water have large values for δ indicating that they have a weak eluting strength. The less polar solvents having lower δ values decrease the value of $K(X)$ and therefore diminish the retention of the analyte X which can be eluted. In many RP-HPLC separations, gradient elution is used. In such cases, δ_{mo} is approximated based on Eq. 7.1.27. For many separations, the logarithm of retention factor (and implicitly of the retention time) depends linearly on the content of organic component ϕ as shown by Eq. 7.1.20 that has the following form:

$$\log k'_{\phi}(X) = a - b\phi \qquad (9.5.2)$$

The linearity of $\log k'$ as a function of ϕ is illustrated in Fig. 9.5.1 for a sample of scopoletin (7-hydroxy-6-methoxychromen-2-one) separated on a C18 column (Gemini 5u C18, 150 mm × 2.0 mm) in isocratic conditions at various contents of organic solvent in the mobile phase. The mobile phase consisted of an aqueous acetate buffer at pH = 4.4 and methanol as the organic phase. The detection was done by UV absorption at λ = 340 nm.

The linear decrease of $\log k'$ when organic component in the mobile phase increases is common for many other separations in RP-HPLC (as also discussed in Section 7.1). However, in some cases, deviations are present in this type of dependence as illustrated for three compounds in Fig. 9.5.2 for the increase in methanol concentration with separation on a μBondapak C18 column that has a rather high silanol activity and besides the hydrophobic mechanism displays

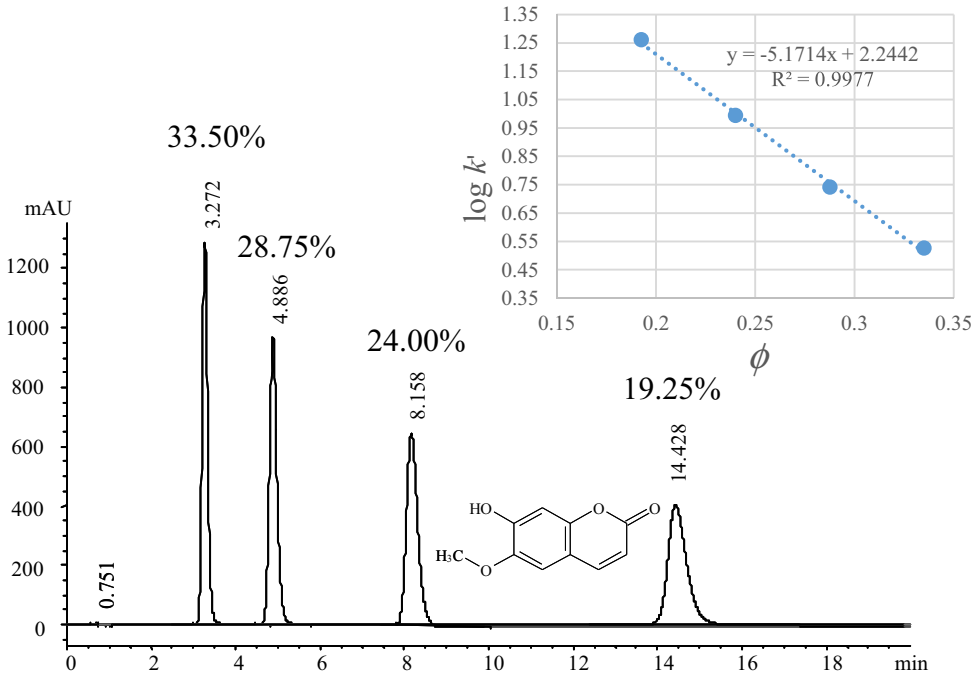

FIGURE 9.5.1 Separation of scopoletin on a C18 column with mobile phase an aqueous acetate buffer at pH = 4.4 and methanol. Different contents of methanol % in the mobile phase led to different retention times. The detection was done at λ = 340 nm. The variation of $\log k'$ with the methanol content (as ϕ) is also shown.

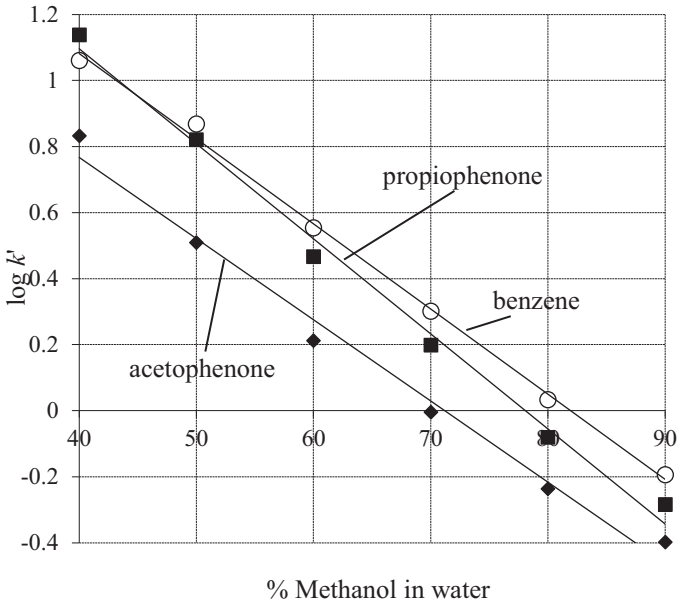

FIGURE 9.5.2 Separation of three analytes on a μBondapak C18-column with mobile phase water/methanol.

more polar interactions. These interactions are basically absent for benzene (which show excellent linearity) but present for acetophenone and propiophenone.

Also, in some cases, the dependence on organic phase concentration can be better described by a quadratic equation as shown in Fig. 7.1.11 (see Chapter 7), for the separation of chlorobenzene and 2,4-dinitrophenol on a Lichrospher 100 RP-10 column with the mobile phase acetonitrile/water.

Water and mobile phases with high water content

Water is always a component of the mobile phase in RP-HPLC (and only NARP type of HPLC does not use water in the mobile phase). The percentage of water in the mobile phase for RP-HPLC can vary significantly and water is used in both isocratic and gradient elution. For gradient elution in RP-HPLC, the higher content of water is used at the beginning of the gradient with the content of the organic component increasing toward the end of the elution process when even pure organic solvent can be used as mobile phase. Water with no added organic solvent (100% water mobile phase) may be used as mobile phase on stationary phases specifically designed for this purpose (e.g., Synergy Hydro RP from Phenomenex, Inertsil ODS-4 from GL Science, Acquity UPLC HSS T3 and Atlantis T3 from Waters, etc.). The 100% water as mobile phase is sometimes used for the separation of highly hydrophilic compounds. Many RP columns may suffer dewetting in mobile phases with a very high content of water, and they must be used only with a certain proportion of organic solvent (e.g., higher than 10% in organic component) in the mobile phase.

Water has a high polarity ($\delta = 21$, experimental log $K_{ow} = -1.38$, $P' = 10.2$, $\pi^* = 1.09$ as shown in Appendix 6 and 7). Also it has a high hydrogen-bond donor capability and an average hydrogen-bond acceptor capability. With these

characteristics, water is a very weak eluent in RP-HPLC, and the compounds with some hydrophobic character are strongly retained on the stationary phase from mobile phases with high water content. Only by increasing the content in organic solvent these compounds can be eluted within acceptable t_R values. Besides acting as a polar solvent with "weak" elution character in RP-HPLC, water is an ideal solvent for buffers and ionic additives in the mobile phase. Also, water is a good solvent for polar samples including amino acids, carbohydrates, proteins, and many other compounds. In addition, the pH of a water solution can be directly measured using a pH meter. The purity of water is very important for HPLC, in particular when high water content is necessary in the mobile phase and when sensitive detection is utilized (e.g., MS). Water is also used in superheated water chromatography, at higher temperatures water becoming less polar [178].

For most detection techniques, water is an adequate solvent. It has a very low UV cut-off value (<190 nm), does not interfere with fluorescence, and for evaporative detection techniques, it can be volatilized. For MS detection, the mobile phase of pure water is not recommended, but a level of as little as 5% organic modifier (e.g., methanol or acetonitrile) in water can be used successfully to achieve good sensitivity (additives such as HCOOH are typically used in the mobile phase for MS detection). Often the best sensitivity in ESI is achieved when the analyte is ionized already in a liquid phase by using an acidic mobile phase for basic analytes, such as amines (pH two units below pK_a of the analyte), and basic conditions for acidic analytes, such as carboxylic acids and phenols (pH two units above pK_a of the analyte) [179]. On the other hand, when no water is present in the mobile phase, the ionization in ESI is not favored and in this case APCI ionization is necessary.

One aspect related to water used as mobile phase, in particular when it contains specific additives such as acetates or phosphates, is the potential growth of microorganisms. This is posing risks to the system contamination and column longevity [180]. Microorganisms growth can be prevented by preparing fresh aqueous component of mobile phase or by addition of traces of NaN_3.

Alcohols used in mobile phase

One group of solvents very common in RP-HPLC is that of alcohols, with methanol the most frequently utilized. Alcohols are typically utilized in mixture with water. The alcohols polarity is lower than that of water (for methanol $\delta = 12.9$ and $\log K_{ow} = -0.52$), but they have a relatively high capability to form hydrogen bonds, both as donor and as acceptors, usually with higher hydrogen-bond acceptor capability compared to water. Besides methanol, the alcohols more frequently utilized are isopropanol and ethanol. For the decrease of mobile phase polarity (making the mobile phase "stronger" for RP), it is sufficient in many separations only to increase the content of the organic phase (ϕ), not changing the chemical nature of the alcohol. However, if this does not provide enough "strength" for the mobile phase, alcohols with higher number of carbon atoms in the molecule can be used (C2, C3, C4) as stronger eluents [181–183]. As the length of alcohol hydrocarbon chain increases, a higher solubility is shown for less polar solvents such as hexane, cyclohexane, etc. This allows the use of mixtures of alcohol/hydrocarbon as solvent phase in NARP when very hydrophobic analytes have to be separated. Alcohols in general have a low UV cut-off value. Methanol (and other alcohols) presence in an aqueous mobile phase decreases the solubility of inorganic salts, acids, and bases typically used for adjusting the pH. The amount of buffers and/or additives allowed should be selected such that they are completely soluble in the mobile phase even when the organic content of the mobile phase increases (see Chapter 7).

Acetonitrile

Acetonitrile is another very common solvent used in RP-HPLC [184]. This solvent has medium polarity ($\delta = 11.8$ and $\log K_{ow} = -0.17$), a weak hydrogen-bond acceptor, and very weak hydrogen-bond donor capability. Compared to methanol, acetonitrile has slightly higher values for P' and π^* but a higher K_{ow}. It typically acts as a stronger eluent compared to methanol. As an example, the correlation between $\log k'$ for a set of 30 various compounds on a Lichrosphere 100 RP-18e with 5.0 μm particles (from Merck) in three different concentration (20%, 40%, and 60%) of organic component (methanol or acetonitrile) in water is shown in Fig. 9.5.3 ($\log k'$ data from Ref. [105]).

The results from Fig. 9.5.2 indicate a relatively good correlation between $\log k'$ values of the mixtures of solvent/water (solvent methanol or acetonitrile) with higher values for $\log k'$ when the solvent is methanol, and with the slope of correlation increasing as the organic content increases.

Although acetonitrile has $\log K_{ow} = -0.17$, pure acetonitrile can elute from a hydrophobic chromatographic column even molecules with $\log K_{ow}$ as high as 7. Mixtures of water/acetonitrile are common solvents in RP-HPLC, and the eluting strength of this mobile phase increases upon acetonitrile addition. From the $\log K_{ow}(X)$ of the analyte X, an empirical formula can be given, which provides guidance regarding the volume fraction of water in acetonitrile for achieving elution within a 25 min window from a common 150 mm RP column:

$$\phi = \frac{7 - \log K_{ow}(X)}{7.5} \tag{9.5.3}$$

Eq. 9.5.3 does not provide an exact value for the necessary organic content (compounds with low $\log K_{ow}$ will elute close to the dead volume

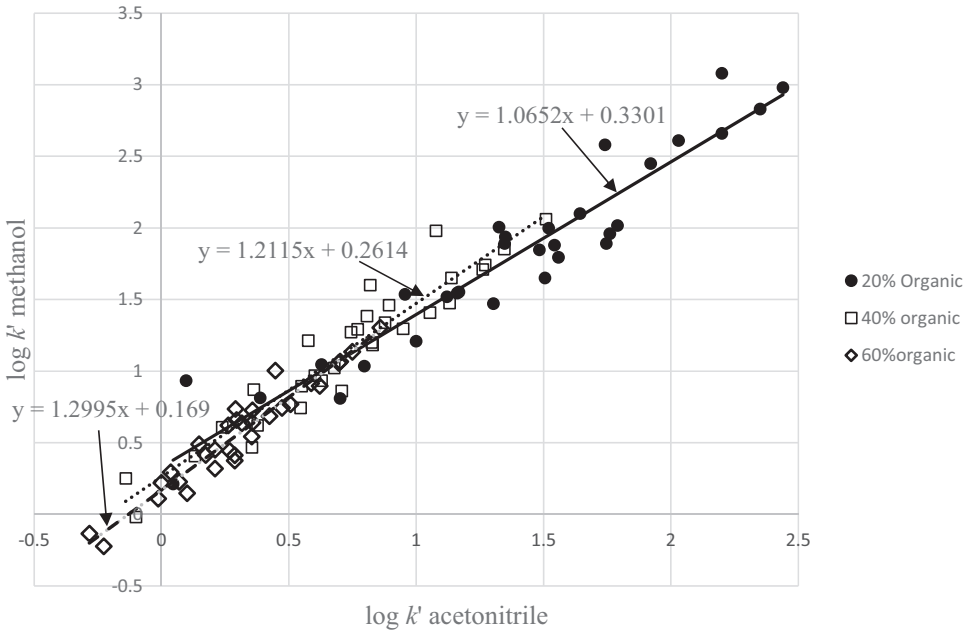

FIGURE 9.5.3 Variation of $\log k'$ for methanol/water compared to $\log k'$ for acetonitrile/water for 30 various compounds [105].

of the column, while those with large log K_{ow} toward the end of the time window), but it indicates the approximate value for the necessary composition for acetonitrile/water mixtures. Acetonitrile presence in an aqueous mobile phase decreases the solubility of inorganic additives.

The UV cut-off of acetonitrile is very low (see Appendix 7.3.1), which is an important characteristic when UV detection is utilized. For MS detection, acetonitrile is adequate in water/acetonitrile mobile phases. The response intensity in MS is usually dependent on the mobile phase composition, and the use of methanol or acetonitrile as organic modifier in the mobile phase can generate very different results. In a number of cases, acetonitrile favors detection in positive mode by increasing sensitivity, and methanol favors detection in negative mode ionization. However, only direct experiments may select the optimum solvent for MS detection.

Other solvents used in RP-HPLC

A variety of other solvents and solvents mixtures can be used in RP-HPLC as mobile phase. Among these are ethers, ketones, and esters. Ethers are compounds typically used in RP-HPLC for further decreasing the polarity of the mobile phase compared to short-chain aliphatic alcohols or acetonitrile. Tetrahydrofuran (THF), dioxane, tert-butyl methyl ether, and diethyl ether are among the ethers used in RP-HPLC. Ethers also have very low donor hydrogen bonding capability and medium acceptor hydrogen-bonding capability. Consequently, they are able to form complexes with the phenolic analytes, which can modify the nature of interaction between analyte and stationary phase. In some cases, by using THF, there has been observed that the retention of weakly acidic analytes increases with the increase of THF content in the mobile phase, in contrast to the behavior observed for AcCN or MeOH [185]. Except for NARP where ethers may be more frequently used, in conventional RP-HPLC, the ethers, when used, are typically at relatively low proportions. These compounds have a UV cut-off value around 210–215 nm.

Ketones are not very different in their solvent capabilities compared to ethers. However, ketones typically have relatively high UV cut-off values (around 330 nm) and therefore are less amenable for UV detection, which is one the most common detection techniques. For this reason, the use of ketones as modifiers of the mobile phase in RP-HPLC is less common. However, since some ketones may have strong eluting properties in RP-HPLC, they can be used, for example, for the elution of hydrophobic compounds from graphitic columns. Acetone has a lower hydrogen-bond basicity than acetonitrile and methanol, and its elutropic strength on C18 stationary phase is higher than for the mentioned solvents (see Section 7.1). Experimentally, it has been shown that there is a good correlation between the retention when acetone and acetonitrile mobile phases are used for RP stationary phases except for the phenylhexyl-silica stationary phases [186]. Also, acetone is considered as a greener alternative to replace acetonitrile in reversed-phase liquid chromatography, but its applicability with UV-based detectors is limited [187].

Esters, such as methyl or ethyl acetate, are sometimes used as mobile phase modifiers in RP-HPLC, but due to their low solubility in water a third component (methanol) is necessary for mixture miscibility. However, their low water solubility, potential to hydrolyze in acidic or basic media, and their relatively high UV cut-off value make these solvents less useful in RP-HPLC [188].

Hydrocarbons and halogenated hydrocarbons as solvents have applications in NARP, but due to their immiscibility with water, they are basically useless in conventional RP-HPLC. Attempts to use of deep eutectic solvents (DESs) and ionic liquids, mainly as additive in

mobile phase, are known in specific applications [189–191].

9.6 Selection of mobile phase in RP-HPLC

General comments

The selection of mobile phase in RP-HPLC is performed with the goal to obtain a good separation and peak shape quality, either using isocratic condition or, more frequently, using gradient. A very large body of information regarding separation methods is available in books, peer-reviewed journals, and on the web. This information may be sufficient for adopting a separation method. A common procedure for selecting the mobile phase is based on selecting the mobile phase similar to the one used for a similar (although not identical) set of analytes and reported in the literature. When such information is not available, the selection of the mobile phase must be done by trial experiments considering several factors: (1) nature of the analytes (2) isocratic or gradient separation, (3) column selected for the separation, (4) the need of buffers and additives, (5) detection type utilized, (6) flow rate, (7) mobile phase temperature, (8) solvent consumption, (9) and environmental issues [180,192].

(1) The nature of sample is the main element determining the selection of the mobile phase. The composition of the mobile phase includes the water and organic components content, the pH generated by the addition of buffers and/or additives, and the content of specific additives utilized for the modification of the ion strength of the mobile phase or for enhancing ionization in MS detection. The initial composition of a mobile phase can be estimated based on the evaluation of log K_{ow} values of the analytes. The value of log K_{ow} being proportional with retention factor log k', as indicated by Eq. 4.1.24, can be assumed that the compounds with low log K_{ow} elute at short retention times and those with large log K_{ow} at longer retention times. From such estimation, it can be decided if a mobile phase with strong eluting capability (high in organic component) or with low eluting capability must be selected. The second guidance is provided by the basic or acidic nature of the analytes. It is usually preferred for RP-HPLC to keep the analytes in nonionic form, and this is achieved by selecting the mobile phase pH. For acidic compounds, it is preferred to keep the pH of the mobile phase at a value of 2 units below the pK_a of the acids in the sample, and in case of basic compounds at a value 2 units higher than the pK_a of the bases. Although for nonionizable compounds the pH of the mobile phase is less important, the pH of the mobile phase for compounds that can be ionized affects significantly the separation. As an example, in Fig. 9.6.1, the change of retention time and peak shape for the separation of nicotine on a Xterra RP 18 column with the mobile phase water + buffer and acetonitrile at different pH of the aqueous component of the mobile phase is illustrated. As shown in Fig. 9.6.1, the change in pH from 9.9 to 4.4 changes the retention time from 10.35 min to 3.43 min. The peak shape is also modified and the high pH of the mobile phase (when nicotine is in free base form) has the effect of narrowing of the peak. Otherwise, the peak areas are not changed.
Although some guidance regarding the mobile phase selection can be obtained from values of log K_{ow} and pK_a, very frequently the selection of a specific mobile phase for an HPLC analysis is a trial-and-error process. However, there are several general procedures to narrow the search. In Section 9.7, a discussion regarding the prediction of log k' in RP-HPLC provides some help in this choice. The use of Eq. 7.1 18, $\log k\prime_{\phi_2}(X) = \log k\prime_{\phi_1}(X) - S(X)(\phi_2 - \phi_1)$, also provides guidance regarding the choice of a specific mobile phase composition. Eq. 7.1.18

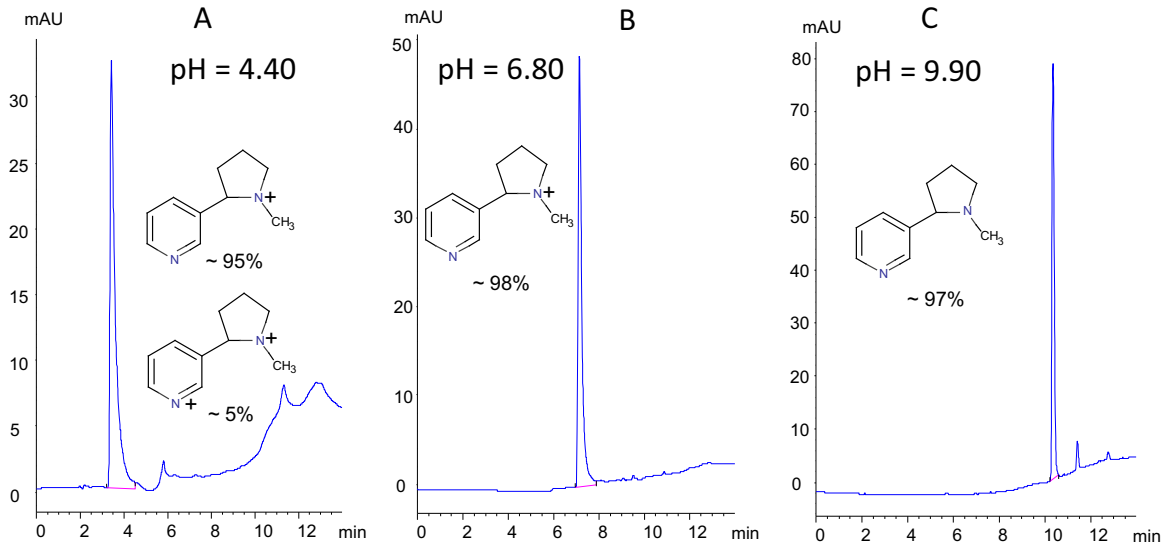

FIGURE 9.6.1 Nicotine (at 100 μg/mL) separated on a Xterra RP 18 column with acetonitrile/aqueous buffer mobile phase at different pH values (Note: the pH refers at the value measured for the aqueous buffer).

indicates the modification in $\log k'_{\phi_2}(X)$ for a content ϕ_2 in the mobile phase as compared to $\log k'_{\phi_1}$ in a mobile phase with the composition ϕ_1. When a shorter retention time is desired, a higher organic content ϕ_2 is selected. Some computer programs assisting in the optimization of separation based on mobile phase selection (e.g., DryLab4 [166]) make use of Eq. 7.1.18 for this purpose. Regarding the nature of the organic modifier, the discussion in Chapter 7 and in Section 9.5 describes various properties of the solvents and their selection for a successful separation.

The nature of the sample also imposes several other requirements for the mobile phase, including solubility, absence of potential reactions such as hydrolysis, requirements to keep the analytes in a specific form such as free base for basic compounds or free acid for the acidic compounds, etc.

(2) The decision to use gradient in an HPLC separation may be made after several trials using isocratic mode [193], but when the analytes in the sample have very different log K_{ow}, it is usually recommended to use gradient. The gradient is also used to optimize a separation even for compounds with close log K_{ow} values. As shown in Fig. 7.7.5A and B, the variation of log k' as a function of organic content in the mobile phase can be different for different compounds, and such differences can be used to obtain a better separation. One factor when using gradient is related to the pH of the mobile phase that is an important parameter for many separations. If the intention is to keep a constant pH during the separation and change only the water/organic components in the mobile phase, all the components of the mobile phase must have the same pH (obtained using buffers or additives as indicated in Chapter 7). Changes in the pH of the mobile phase can also be intended and they can easily be achieved using gradients. Another factor when selecting gradient separation is related to the selected detection. Gradients cannot be used with some detectors, in particular with refractive index (RI)

detection. Gradients can be used with MS detection, although in some cases a better reproducibility of results is obtained when using isocratic separation.

(3) Depending on the selected column, the water content in the mobile phase should not exceed a specific concentration unless the column can be used with 100% water. Also, a specific range of the pH of the mobile phase must be selected. It is common that the pH range for column stability is indicated by the manufacturer and the mobile phase should not have a pH outside the recommended range. In case the pH of the mobile phase must be different than the one recommended for a specific column, the nature of the column must be changed.

(4) In certain separations, certain buffers and additives are necessary. The importance of the pH of the mobile phase for certain separation was previously emphasized. Regarding additives, the most common ones include HCOOH and $HCOONH_4$ used to facilitate ionization in MS detection. Also, the content of certain additives contributes to the quality of RP-HPLC separation, in particular related to the peak shape. The addition of low levels of inorganic salts such as KH_2PO_4, for example, leads to narrower peak shape. However, inorganic salts have very limited solubility in organic solvents and their concentration should not exceed (see Appendix 7.4.5). The selection of a mobile phase composition must consider the type and the concentration of buffers and of the necessary additives.

(5) Depending on the type of detection, specific characteristics for the mobile phase must be selected. As previously indicated, some restrictions regarding mobile phase are imposed to the use of gradients. However, other aspects are important such as UV cut-off discussed in Section 7.3, volatility of mobile phase components to be amenable for MS detection, etc.

(6) Selection of the flow rate of the mobile phase was presented in Section 7.5 and it is determined by several factors such as the backpressure produced by the selected column and the quality of the separation (with optimal value for column efficiency N (as described in Section 3.1). These aspects were previously discussed related to the column construction (see Section 8.2). Some restrictions regarding the flow rate are also imposed by the type of detector to be used. For UV or RI detection, flows of 1.0 mL/min are common, but for MS or MS/MS detection, such flows may be too high. In such situation, the flow may need to be limited to 0.2–0.5 mL/min and at these flows the retention time for some analytes may be too long as compared to the use of the same column at 1 mL/min flow. In such cases, an equivalent column should be selected, having narrower diameter (and possibly smaller particles).

(7) The influence of temperature of the mobile phase to a separation was presented in Section 4.6 and also discussed in Section 7.5. For common separation, the temperature of the mobile phase is either close to room temperature or in some separations is elevated at temperatures up to about 70°C usually for decreasing the mobile phase viscosity η to achieve lower backpressures. Advantageous changes in the separation can sometimes be obtained using temperatures different from ambient.

(8) Solvent consumption related to solvent cost can be a criterion for the selection of a specific mobile phase. For example, the price of methanol is typically three times lower than that of acetonitrile, and for larger solvent utilization, it is recommended to use methanol instead of acetonitrile when the separation and detection are not affected. Also the mobile phase selection must take into consideration the environmental issues. Solvents and additives harmful for the environment should be avoided as much as possible. The use of green solvents (see Section

7.5) is becoming a more common practice in HPLC in general and RP-HPLC in particular. When the separation performances are not altered, the harmful solvents may be replaced with safer solvents with environmental, health, and safety benefits, such as for specific applications with 75% ethanol and 25% methyl acetate (v/v) [194]. Prior to the experimental trials, useful information provided by web sources, such as GreenScreen for Safer Chemicals, can be used as the first filter to select safer solvent candidates from thousands of chemicals, based on their safety ratings [195,196].

9.7 Prediction of parameters describing the separation in RP-HPLC

General comments

The characterization of a separation is done using various parameters presented in Section 3.1. There are two broad approaches that are used for prediction of some of these parameters. The first is the use of models (expression) connecting the predicted parameter with physico-chemical properties of the column, mobile phase, and analyte. The second approach is to use statistical modeling based on database information and correlating certain measurable characteristics of the column, mobile phase, and analyte obtained on a large number of measurements with a specific parameter to predict. In this section, only the first type of approach is presented.

Considerable effort has been made for the prediction of retention factor k' and selectivity α based on physico-chemical properties of the analyte, of the column, and of the mobile phase involved in the separation [3,154,197,198]. From such theoretically estimated values, other parameters such as the retention time t_R and the resolution R in a separation can be obtained. The contribution of analyte, column, and mobile phase properties to the evaluation of a parameter for the separation is suggested in the diagram given in Fig. 9.7.1.

In the diagram from Fig. 9.7.1, each of the axes (column, mobile phase, analyte) can be further characterized by a number of parameters previously discussed in this book. For example, $\alpha(CH_2)$ and PQRI parameters such as H^*, S^*, A^*, B^*, and C^* characterize the column (see Section 9.3). The mobile phases are characterized by parameters such as Hildebrand solubility δ, polarity P', solvatochromic parameters $E_T(30)$, π^*, α, and β (see Section 7.1), and other physico-chemical parameters such as viscosity η, dielectric constant ε, surface tension γ', A^{SASA}, dipole moment m, etc. The analytes have their own physico-chemical characterizing properties, including MW, pK_a (pK_b), partial charges, K_{ow} (log K_{ow}), molecular surface area $\mathscr{A}^{vdW}$, solvatochromic parameters π^*, α, and β, dipole moment m, polarizability α, ionization potential I (see Section 6.1), etc. Additional parameters such as pH, temperature, and mobile phase flow rate were also considered among the factors that influence separation [199]. Because of the complexity of the task, in spite of numerous attempts to calculate k' and α based on some of the previously listed parameters, in most instances the results remained affected by deviations from the correct experimental value [200,201]. In cases of good agreement with the experiment, the results were limited to a small group of analytes. Nevertheless, important

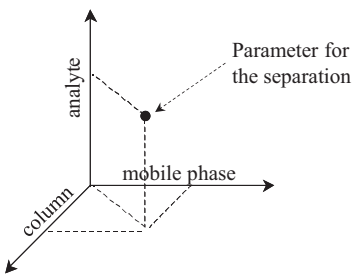

FIGURE 9.7.1 Illustration of the dependence of a separation parameter on column, mobile phase, and analyte.

information can be obtained from the predicted values for retention factor and selectivity, either for comparison purposes between the analytes of a sample or for directing the separation in the desired direction.

Estimation of retention factor k' for similar systems at different mobile phase compositions

For a separation, the simplest expression for the value of k' related to the mobile phase composition for a given analyte X and a specific chromatographic column is given by Eq. 7.1 20, written below once more:

$$\log k'_{\phi}(X) = \log k'_{w}(X) - S(X)\phi \qquad (9.7.1)$$

This expression for $\log k'_{\phi}(X)$ has been justified on thermodynamic concepts in Section 4.1, which are valid for partition equilibria that are typical for RP-HPLC separations. In Section 4.2, it is indicated a different type of dependence known as Snyder–Soczewinski, but in RP-HPLC the linear solvent strength model described by Eq. 4.1.16 is more adequate. Various studies are available in the literature comparing the results of the two models on different HPLC systems [6,202].

Eq. 9.7.1 utilizes the value for $\log k'_{w}(X)$ corresponding to pure water as a mobile phase. These values are usually obtained by extrapolation from known values of $\log k'$ at decreasing values of ϕ as indicated in Section 7.1. An example of obtaining $\log k'_{w}(X)$ for two compounds (ethylbenzene $\log k'_{w}(X) = 3.258$ and methylbenzoate $\log k'_{w}(X) = 2.263$) is given in Fig. 9.7.2 for a LiChrospher 100 RP-8 column and methanol in the mobile phase. The same type of graph allows the evaluation of $S(X)$. As an example, when the organic solvent % shown in Fig. 9.7.2 is replaced with ϕ, the obtained values are $S(X) = 2.78$ for methylbenzoate and $S(X) = 3.77$ for ethylbenzene.

Besides the problem to find $\log k'_{w}(X)$ and $S(X)$ from known experimental data at several organic phase concentrations, the resulting $\log k'_{w}$ values may show differences depending on

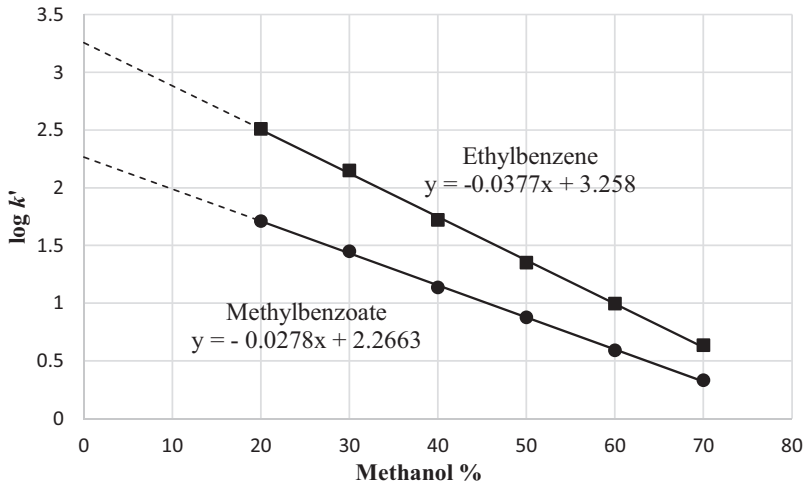

FIGURE 9.7.2 Graph showing the calculation of $\log k'_{w}(X)$ for two compounds on a LiChrospher 100 RP-8 column and methanol in the mobile phase.

the water/organic solvent system on which log k' is measured. This is exemplified in Table 9.7.1 where the values for log k'_w are given for toluene separated on five different columns using methanol/water or acetonitrile/water as a mobile phase [105]. The calculation of log k'_w was performed based on six organic phase concentrations (20%–70%) using a quadratic least squares deviation fit.

When a value $\log k'_{\phi_1}(X)$ (at ϕ_1 phase composition) and not $\log k'_w(X)$ is known, the expression for $\log k'_{\phi_2}(X)$ is obtained using Eq. 7.1.19 written once more below:

$$\log k'_{\phi_2}(X) = \log k'_{\phi_1}(X) - S(X)(\phi_2 - \phi_1) \quad (9.7.2)$$

Eq. 9.7.2 provides for many systems a good estimation of log k' at a new mobile phase composition ϕ_2 when known for another composition ϕ_1. However, for some separation systems, Eqs. 9.7.1 and 9.7.2 do not provide accurate predictions. For example, when acetonitrile/water is used as a mobile phase, the linear dependence of $\log k'_{\phi_2}(X)$ on $\phi_2 - \phi_1$ indicated by Eq. 9.7.2 or on ϕ indicated by Eq. 9.7.1 is less frequently obeyed as compared to the case of methanol/water mobile phase. A better fit with experimental data has been reported for some systems by a quadratic equation of the form 7.1.20 [203,204]. Such quadratic dependence is illustrated in Fig. 7.1.11. Eq. 7.1.20 written for two mobile phase compositions ϕ_2 and ϕ_1 and has the following form:

$$\log k'_{\phi_2}(X) = \log k'_{\phi_1}(X) - S_1(X)(\phi_2 - \phi_1) - S_2(X)(\phi_2 - \phi_1)^2 \quad (9.7.3)$$

The procedure of evaluating $\log k'_{\phi_2}(X)$ based on Eqs. 9.7.1, 9.7.2 or 9.7.3 can give good results in predicting log k', but it is limited to one compound, one column, and one solvent system (only the % composition being changed) and requires k' values for water (k'_w) or for other concentration ϕ_1 $\left(\log k'_{\phi_i}(X)\right)$ of the solvent. Equations of the type 9.7.3 were also developed for ternary mobile phase compositions [205]. Also, in order to obtain better fit with experimental data, more elaborate expressions were developed, e.g., of the following form [206]:

$$\ln k'_{\phi}(X) = \ln k'_w(X) + 2\ln[1 + S_2(X)\phi] - \frac{S_1(X)\phi}{1 + S_2(X)\phi} \quad (9.7.4)$$

Since a considerable number of HPLC separations are performed in gradient conditions, direct calculation of log k' based on Eqs. 9.7.1, 9.7.2, or 9.7.3 is not very useful except for the estimation of log k' at specific points of different organic composition. This can be used only for the estimation of the expected separation results

TABLE 9.7.1 Calculated values for log k'_w for toluene (quadratic fit), using data for two different mobile phase systems.

Column	log k'_w from AcCN/H_2O	log k'_w from MeOH/H_2O
LiChrospher 100 RP-18e (125 × 4 mm, 5 μm)	3.62	3.94
Purospher RP-18e (125 × 4 mm, 5 μm)	3.76	4.76
LiChrospher 100 RP-8 (125 × 4 mm, 5 μm)	3.38	3.35
SymmetryShield RP-C18 (150 × 3.9 mm, 5 μm)	3.77	4.48
Symmetry-Shield RP-C8 (150 × 3.9 mm, 5 μm)	3.32	4.28

and not for a direct calculation of log k'. Better predictions for log k' during gradient separations can be obtained using Eq. 3.5.17 (for linear gradients) when the value k'_0 is known (see Section 3.5).

The linear type dependence of log k' on ϕ has been utilized in commercial programs such as Drylab [166] that was applied for various types of molecules and RP-HPLC columns (e.g., Ref. [207].

Evaluation of retention factor k' from octanol/water partition constants K_{ow} or D_{ow}

Various studies were reported with attempts to evaluate retention factors $k'(X)$ or the selectivities $\alpha(XY)$ using octanol/water partition values $K_{ow}(X)$ [135,154,181,208]. Values for log $K_{ow}(X)$ are readily available for many compounds. The use of K_{ow} for the estimation of k' has its origin in several studies on liquid–liquid extraction, which show that partition coefficients $K_A(X)$ and $K_B(X)$ for a compound X in two systems, (a) solvent A/water and (b) solvent B/water, are given by Eq. 4.1.21. The extension of Eq. 4.1.21 to two systems, (a) stationary phase/mobile phase and (b) octanol/water, generated Eq. 4.1.25, once more written below:

$$\log k'_X = a \log K_{X,ow} + b \qquad (9.7.5)$$

Eq. 9.7.5 is applicable for compounds that are present in a single form in solution. For ionizable compounds, the octanol/water partition constant $K_{X,ow}$ must be replaced with the distribution coefficient $D_{X,ow}$, this distribution coefficient being pH dependent as indicated in Section 4.5. In such case, Eq. 9.7.5 becomes the following:

$$\log k'_X = a \log D_{X,ow} + b \qquad (9.7.6)$$

For a given column and mobile phase, the knowledge of the retention factor log $k'(X)$ (notations $k'(X)$ or k'_X used interchangeably) for at least two compounds allows the estimation of coefficients a and b and in principle the calculation of log k' for any other compound (not changing column or mobile phase). The use of a larger set of compounds with known log k' for the calculation of a and b (by a least squares deviation technique) leads to more reliable results. The experimental verification of Eq. 9.7.5 has been done in several studies (e.g., Refs. [135,154,208]) some described in Section 4.1. For example, for 72 mono and disubstituted aromatic compounds, a specific column, and mobile phase system, a good correlation can be obtained between the calculated log k' by Eq. 9.7.5 and experimental log K_{ow} values, as it was shown in Fig. 4.1.3 ($R^2 = 0.9355$). Those results were obtained for a C18 stationary phase with water/methanol 50/50 (v/v) as a mobile phase [208]. Another example of the dependence between log k' and log K_{ow} (experimental) is further illustrated in Fig. 9.7.4 for two sets of compounds listed in Table 9.7.2 [158]. One set of compounds is missing groups having strong polarity, while the other contains compounds that are more polar (containing phenol, alcohol, or ester groups). These compounds were separated on a Symmetry-Shield RP-C8 column, 150 × 3.9 mm with 5.0 μm particles end-capped, using methanol/water 60/40 v/v mobile phase.

As shown in Fig. 9.7.4, the correlation between log k' and log K_{ow} is very good for each set, and the correlation performed on all compounds taken together also generates a good correlation with $R^2 = 0.9688$ (trendline not shown).

For a different stationary phase, a graph similar to that shown in Fig. 9.7.4 is given in Fig. 9.7.5. In this case, the chromatographic column was a LiChrospher 100 RP-8, 125 × 4.0 mm with 5.0 μm particles not end-capped, with the same mobile phase as in the separation used for the graph shown in Fig. 9.7.4.

In the case of the separation of the LiChrospher 100 RP-8, the correlation for each set of

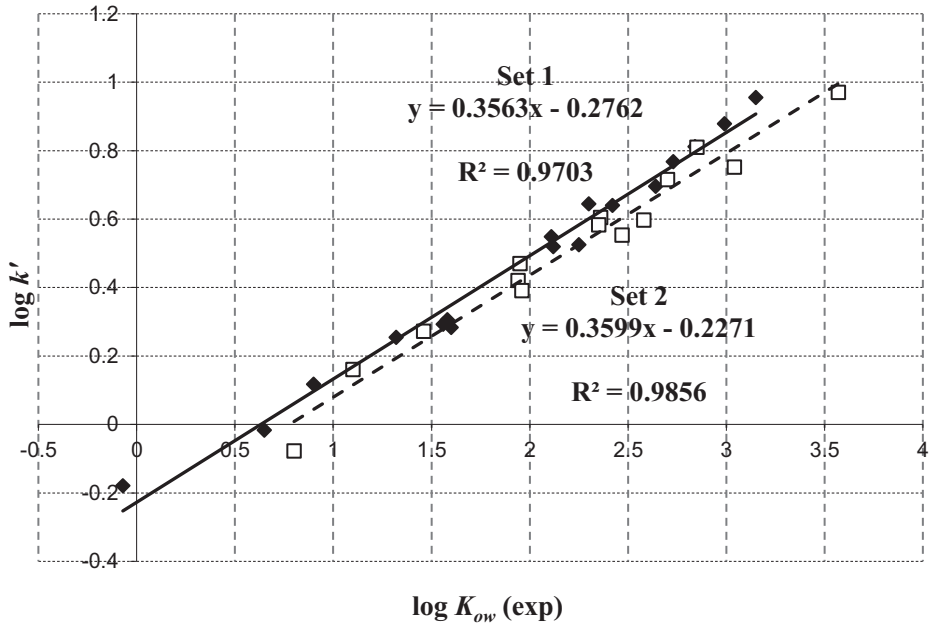

FIGURE 9.7.4 Correlation between log k' and log K_{ow} for the two sets of compounds listed in Table 9.7.1 separated on a Symmetry-Shield RP-C8 column (end-capped) using methanol/water 60/40 v/v mobile phase.

compounds is still very good, but more scatter is noticed for all compounds taken together with all compound correlation giving $R^2 = 0.8168$ (trendline not shown). The comparison of Figs. 9.7.4 and 9.7.5 shows that the stationary phase is important for the values of the parameters a and b in Eq. 9.7.5 and also indicates that different classes of compounds behave

TABLE 9.7.2 Example of compounds showing good linearity for log k' versus log K_{ow}, as described by Eq. 9.7.5 in a separation on a Symmetry-Shield RP-C8 column [158].

Set 1 of compounds		Set 2 of compounds	
Aniline	Benzyl cyanide	p-Cresol	α-Naphthol
Methylbenzoate	α-Naphtylamine	2,6-Dimethylphenol	Methylparaben
Toluene	o-Nitrotoluene	Hydroquinone	Ethylparaben
Ethylbenzene	Acetophenone	Phenol	Propylparaben
Ethylbenzoate	Dimethyl phthalate	o-Cresol	Butylparaben
Chlorobenzene	Pyridine	3,5-Dimethylphenol	
Bromobenzene	Anisole	β-Naphthol	
Caffeine	N,N-Dimethylaniline	Benzyl alcohol	
o-Toluidine		p-Ethylphenol	

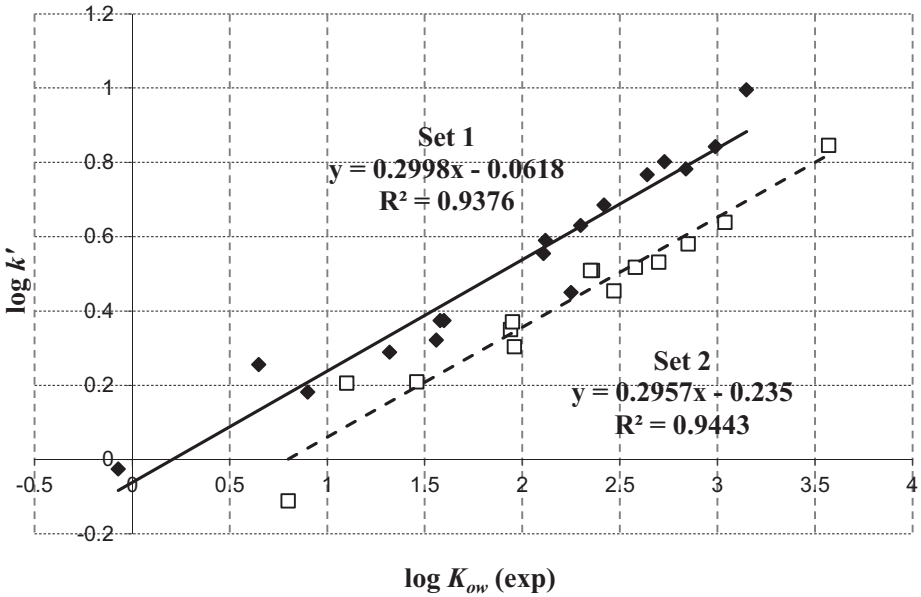

FIGURE 9.7.5 Correlation between log k' and log K_{ow} for the two sets of compounds listed in Table 9.7.1 separated on a LiChrospher 100 RP-8 column (not end-capped) using methanol/water 60/40 v/v mobile phase.

differently depending on the stationary phase and the structure of the evaluated compounds. The more polar compounds (from Set 2) interact differently with the end-capped column Symmetry-Shield RP-C8 column than with the not end-capped column LiChrospher 100 RP-8.

In general, the complexity of the chromatographic processes makes it impossible to use a single parameter like K_{ow} to describe a wide range of separations and other correlations between log k' and log K_{ow} were not that good. For example, the correlation reported in Ref. [135] for 76 very different compounds gave only an $R^2 = 0.5539$. Also, the dependence shown by Eq. 9.7.6 for ionizable compounds typically leads to weaker correlations. This can be justified by the influence of various types of interactions besides hydrophobic ones, which are more important in the case of ionizable compounds as compared to nonionizable ones.

The nature of the mobile phase also affects parameters a and b in Eq. 9.7.4. An exemplification of this effect is further discussed for a number of aromatic compounds listed in Table 9.7.3, with the separated on a Lichrospher 100 RP-18 column 250 × 4.0 mm with 5 μm particles. The two mobile phases used for the separation were methanol/water and acetonitrile/water. In Table 9.7.3 are given the values for log K_{ow} as well as the values for the retention factor log k'_w when the mobile phase is water. The values for K_{ow} were obtained using the computer package MarvinSketch 5.4.0.1 (ChemAxon Ltd. [209], see Section 6.1 for a discussion regarding various sources for K_{ow} values).

For pure water as mobile phase, the correlation between log k'_w and log K_{ow} is given in Fig. 9.7.6. The figure shows one trendline for all compounds, and also the trendlines for the data corresponding only to compounds with phenolic groups ($R^2 = 0.8985$) and without phenolic groups ($R^2 = 0.9906$).

The results from Fig. 9.7.6 are in good agreement with the predictions of Eq. 9.7.5 (for the

TABLE 9.7.3 Compounds investigated for the correlation of their log k'_w with log K_{ow} (log K_{ow} estimated using MarvinSketch package).

Compound	log k'_w	log K_{ow}	Compound	log k'_w	log K_{ow}	Compound	log k'_w	log K_{ow}
Phenol	1.60*	1.67	2,6-Dichlorophenol	2.80	2.88	Chlorobenzene	3.20	2.58
4-Nitrophenol	2.04*	1.61	4-Chloro-3-methylphenol	3.19	2.79	Naphthalene	3.66	2.96
3-Nitrophenol	2.09*	1.61	2,4-Dichlorophenol	3.14	2.88	p-Xylene	3.58	3
2-Methylphenol	2.17*	2.18	3,5-Dichlorophenol	3.44	2.88	Propylbenzene	4.10	3.38
2-Chlorophenol	2.21*	2.27	2,4,6-Trichlorophenol	3.54	3.48	Biphenyl	4.31	3.62
2,4-Dinitrophenol	2.10	1.55	Pentachlorophenol	4.85	4.69	Butylbenzene	4.49	3.82
2-Nitrophenol	2.13	1.61	Benzene	2.20	1.97	Anthracene	4.79	3.95
3-Chlorophenol	2.65	2.27	Nitrobenzene	2.16	1.91	Pyrene	5.13	4.28
4-Chlorophenol	2.59	2.27	Toluene	3.16	2.49	Chrysene	5.79	4.94
2,4-Dimethylphenol	2.44	2.7	Ethylbenzene	3.48	2.93			

Note: * indicates measured values in water, the other log k'_w being extrapolated to water.

particular system with water mobile phase $a = 1.247$, $b = 0.0597$). Further verification of Eq. 9.7.5 can be made for various mobile phases. The correlations between log k' and log K_{ow} for a mobile phase 50% methanol in water and for a mobile phase 50% acetonitrile and water are shown for the compounds listed in Table 9.7.3 in Fig. 9.7.7.

From Fig. 9.7.7, it can be seen that a relation of the form 9.7.4 is still valid for various mobile phases, but parameters a and b are changed, and they are different as the mobile phase changes. The variation of parameters $a(\phi)$ and $b(\phi)$ with the modification in mobile phase composition is illustrated in Fig. 9.7.8 for the separation using the Lichrospher 100 RP-18 column with mobile phase methanol/water or acetonitrile/water.

Based on the shape of graphs shown in Fig. 9.7.8, the estimation of log k' from log K_{ow} when the mobile phase composition is changing should be obtained from an equation of the following form:

$$\log k'_X = a(\phi) \log K_{ow,X} + b(\phi) \tag{9.7.7}$$

with:

$$a(\phi) = a_0 + a_1(\phi) + a_2(\phi)^2 \tag{9.7.8}$$

$$b(\phi) = b_0 + b_1(\phi) + b_2(\phi)^2 \tag{9.7.9}$$

In Eqs. 9.7.8 and 9.7.9, the parameters, a_0, a_1, and a_2 and b_0, b_1, and b_2, are independent of the compounds separated. This indicates that Eq. 9.7.7 can be used for the estimation of log k'_X when $K_{ow,X}$ is known for a range of compounds and the coefficients involved in the calculation of $a(\phi)$ and $b(\phi)$ are also known. These coefficients can be obtained from best fit curves, but this requires values for k'_X at several mobile phase concentrations. Formula 9.7.7 extends the applicability of calculation of log k'_X to a range of solvent compositions (besides a variety of compounds). However, the use of Eq. 9.7.5 and/or 9.7.7 for any compound must be done with caution. Larger deviations from the correct value of calculated log k'_X are seen when the compounds used for the calculation of parameters a and b are obtained from compounds very different from the one with unknown retention factor. Since K_{ow} values are

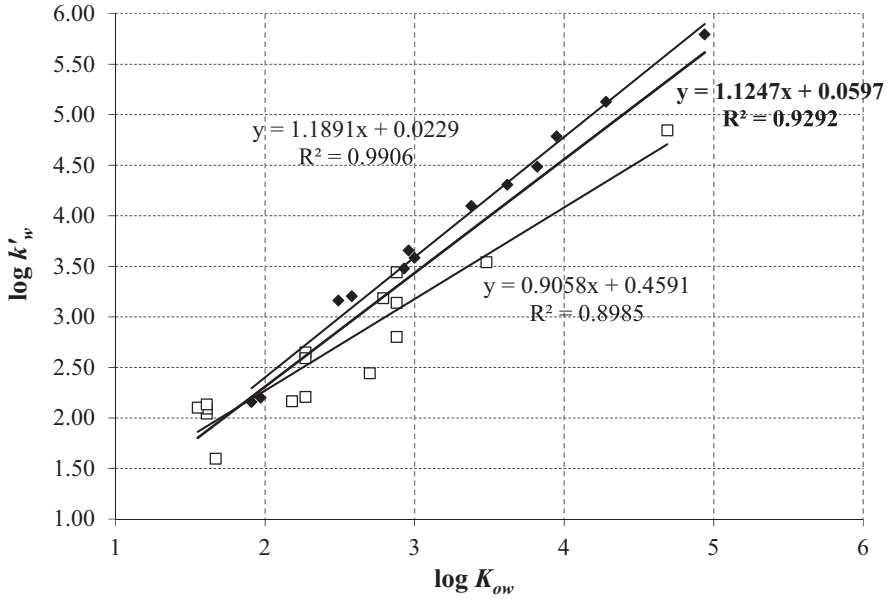

FIGURE 9.7.6 Correlation between log k_{w} and log K_{ow} on a Lichrospher 100 RP-18 column with mobile phase water. Trendline equations listed on figure for phenolic compounds (–□– $R^2 = 0.8985$), other aromatic (–◆– $R^2 = 0.9906$), and all compounds ($R^2 = 0.9292$).

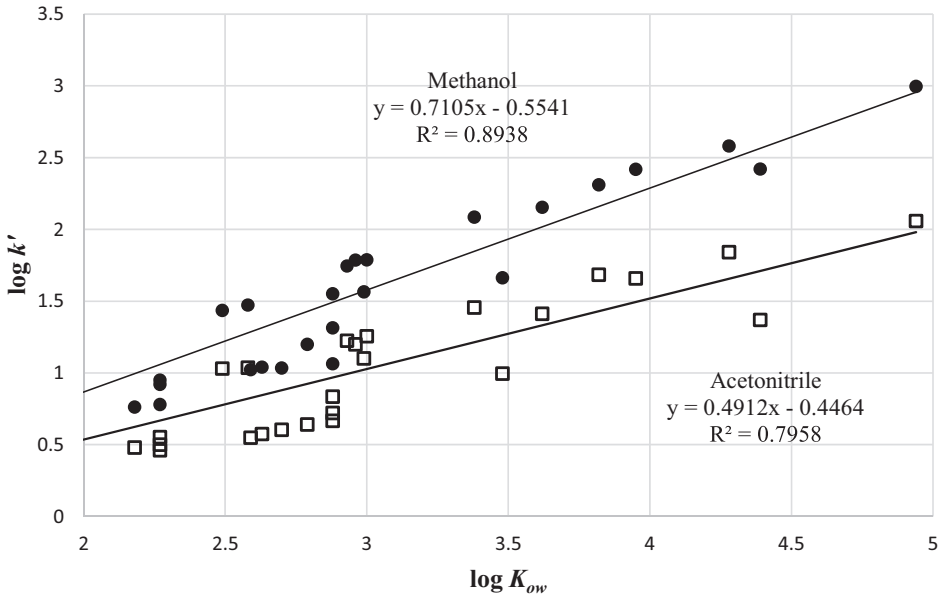

FIGURE 9.7.7 Correlation between log k' and log K_{ow} on a Lichrospher 100 RP-18 column with mobile phase 50% methanol in water and 50% acetonitrile in water.

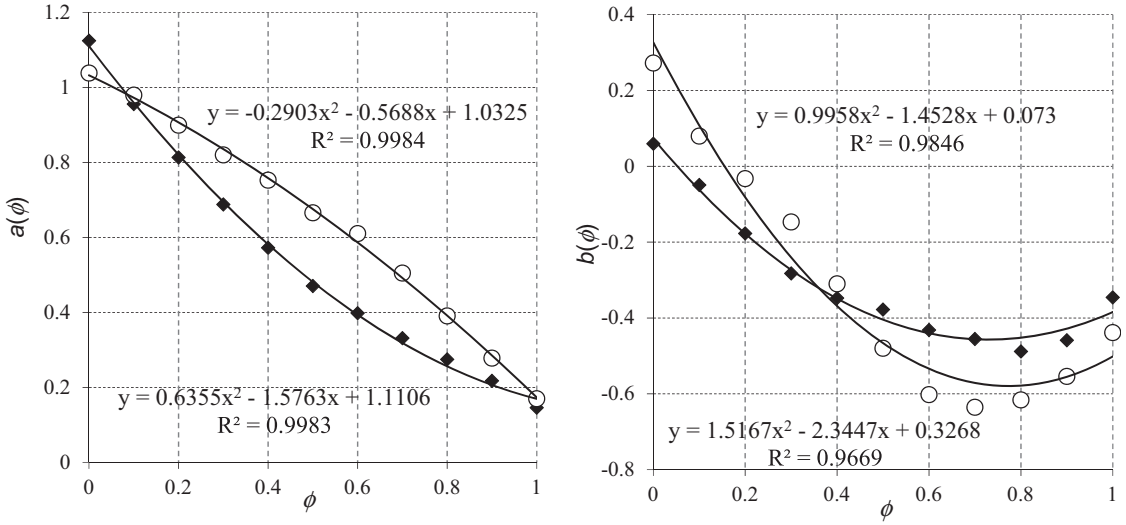

FIGURE 9.7.8 Variation in parameters $a(\phi)$ and $b(\phi)$ in Eq. 9.7.7 when the composition of the mobile phase changes for methanol/water (–○–) and for acetonitrile/water (–◆–).

readily available, it is very convenient to use K_{ow} for the description of chromatographic processes, in spite of various shortcomings discussed in this section.

Calculation of retention factor from van der Waals molecular surface of the analyte

Van der Waals molecular area of the analyte $\mathscr{A}^{vdW}(X)$ is a parameter shown to influence the value of the free energy for the equilibrium, in particular for RP-HPLC, but also for IP separations, and to a certain extent for HILIC and NPC. Unfortunately, as shown in Section 9.1, the detailed calculations of retention factors k'_X using formulas of the type 9.1.16 as a function of $\mathscr{A}^{vdW}(X)$ are dependent on parameters a' and b'_j that are difficult to estimate. Since Eq. 9.1.16 predicts a dependence between log $k'(X)$ and $\mathscr{A}^{vdW}(X)$, empirical correlations are possible to be established between the two values. Such correlations were already described in Section 9.1 (see Fig. 9.1.3.). The results obtained for the calculation of log $k'(X)$ using Eq. 9.1.16 for the compounds listed in Table 9.7.1 (not including caffeine) are further illustrated in Fig. 9.7.9 and 9.7.10. In Fig. 9.7.9 is shown the correlation between the experimental values reported in the literature and the calculated values using Eq. 9.1.16 for the separation on a Symmetry-Shield RP-C8 column, 150 × 3.9 mm with 5.0 μm particles end-capped with methanol/water 50/50 v/v as a mobile phase. In Fig. 9.7.10 is shown the same correlation for the mobile phase methanol/water 70/30 v/v.

The results shown in Figs. 9.7.9 and 9.7.10 indicate very good agreement between the experimental and calculated values, considering the variety of compounds evaluated and the limited number of parameters used in the calculation. For the use of Eq. 9.1.16, the values for a' and b'_j must be known for each column and each mobile phase, but the values for a' and c_n are known, and $\mathscr{A}^{vdW}(X)$ can be calculated for any compound (e.g., using MarvinSketch package). This type of calculation can be of considerable utility when the values for log k'_X are known for two or more other compounds for a given

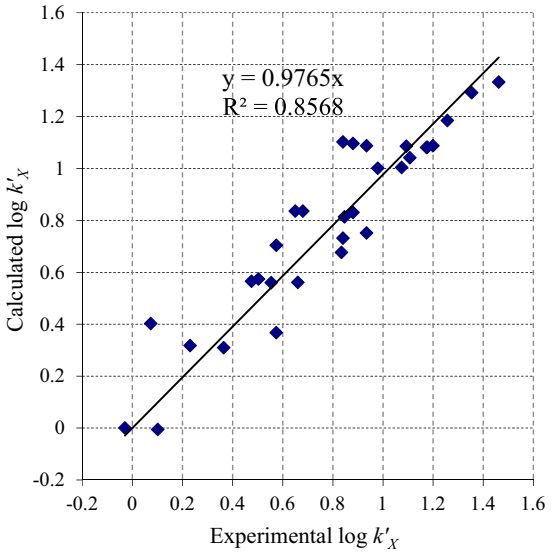

FIGURE 9.7.9 Calculated versus experimental log k'_X values for methanol/water 50/50 v/v on a Symmetry-Shield RP-C8 column.

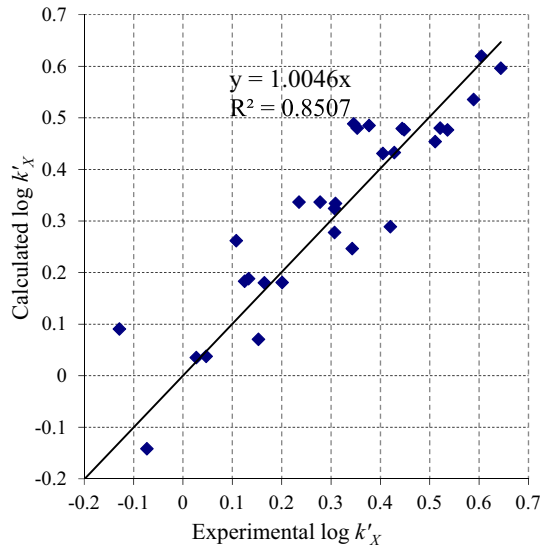

FIGURE 9.7.10 Calculated versus experimental log k'_X values for methanol/water 70/30 v/v on a Symmetry-Shield RP-C8 column.

mobile phase and a given column. These known log k'_X values together with the corresponding $\mathscr{V}^{vdW}(X)$ can be used for the calculation of a' and b'_j, and further for the estimation of log k'_X for any other compound separated in the same conditions (the same column and the same mobile phase). For the compounds listed in Table 9.7.1, the average relative standard deviation of calculated log k'_X versus experimental value was found to be about 17% for methanol/water 50/50 v/v mobile phase and about 13% for methanol/water 70/30 v/v mobile phase case. Similar to the case of the use of log K_{ow} for the calculation of log k'_X, the use of a larger set of compounds with known log k'_X for the calculation of a' and b'_j (by a least squares deviation technique) leads to more reliable results.

Prediction of log k' based on solute, mobile phase, and stationary phase characteristics

A considerable number of attempts were made to develop a general model for the characterization of any free energy A^0 related property of a separation. For HPLC, this energy-related property is typically log k'. Most prediction procedures are based on two similar approaches, one being the linear solvation energy relationship (LSER approach) and the other the hydrophobic subtraction model (HSM). As discussed in Section 9.1, the interactions in RP separations must include (1) hydrophobic interactions, (2) steric interactions, (3) hydrogen bonding between a basic solute and an acidic column groups (column acidity), (4) hydrogen bonding between an acidic solute and a basic column groups (column basicity), and (5) cation exchange and/or ion–ion interactions. Since the contribution of these interactions is additive, a general formula was developed indicated as

LSER [198,210] with the following general expression:

$$\log k' = c + eE + sS + aA + bB + vV \quad (9.7.10)$$

Parameters c, e, s, a, b, and v are solute dependent and are obtained from regressions, while parameters E, S, A, B, and V are dependent on the analyte [211,212]. In Eq. 9.7.10, E is the excess molar refraction, S is the dipolarity/polarizability, A is the hydrogen bond acidity, B is the hydrogen bond basicity, and V is the McGowan characteristic volume (see Section 6.1). E and V are calculated terms, and S, A, and B are usually measured experimentally using chromatographic methods and by applying regression techniques to experimental retention factors for a known set of analytes on a specific stationary phase. A positive value of an analyte descriptor indicates that the analyte interacts more strongly with the stationary phase than with the mobile phase, while a negative descriptor indicates the opposite.

A number of further developments followed the use of Eq. of the type 9.7.10 [18,197,213]. For this purpose, a relation of the type 9.7.10 for the prediction $\log k'$ was investigated such that each term in Eq. 9.7.10 can be estimated not from purely empirical correlations, but based on solvent and solute solvatochromic characteristics [18,213]. One such approach, for example, suggests the following expression for the calculation of $\log k'_X$:

$$\begin{aligned}\log k'(X) = \log k'_0 + r\frac{(\delta^2_{mo} - \delta^2_{st})}{100}V_X \\ + e(\pi^*_{mo} - \pi^*_{st})\pi^*_X + a(\beta_{mo} - \beta_{st})\alpha_X \\ + b(\alpha_{mo} - \alpha_{st})\beta_X\end{aligned} \quad (9.7.11)$$

In Eq. 9.7.11, the index X indicates the solute, and mo and st stand for mobile and stationary phases. Several parameters such as Hildebrand solubility parameter δ, molar volume V, and solvatochromic (normalized) parameters π^*, α, and β were previously described in Section 7.1. The coefficients r, e, a, and b are numbers generated from correlations, and $\log k'_0$ is a reference value [214].

For a specific stationary phase (and assuming that its properties do not change with the mobile phase), Eq. 9.7.11 can be reduced to the following:

$$\log k'_X = c_{st,X} + r_X\delta^2_{mo} + e_X\pi^*_{mo} + a_X\beta_{mo} + b_X\alpha_{mo} \quad (9.7.12)$$

where $c_{st,X}$ depends on the stationary phase through k_0 (reference) and indirectly on δ_{st}, π^*_{st}, α_{st}, and β_{st} and also on the solute X, while r_X, e_X, a_X, and b_X depend on the solute only. The other parameters, δ_{mo}, π^*_{mo}, α_{mo}, and β_{mo}, depend only on the mobile phase. A linear correlation has been demonstrated to exist between δ^2 and π^* for some solvents (not including polar solvents) [215]. Therefore, Eq. 9.7.12 can be further simplified to the following:

$$\log k'_X = c_{st,X} + e^1_X\pi^*_{mo} + a_X\beta_{mo} + b_X\alpha_{mo} \quad (9.7.13)$$

(where e^1_X is an adjusted coefficient). Another simplification is possible, since the retention in RP-HPLC does not depend strongly on the interaction of the solute hydrogen bond acidity a_X with the solvent hydrogen bond basicity β_{mo}, such that Eq. 9.7.13 can be written in the following form:

$$\log k'_X = c_{st,X} + e^1_X\pi^*_{mo} + b_X\alpha_{mo} \quad (9.7.14)$$

The terms depending on π^*_{mo} and α_{mo} can be described together with a certain approximation by E^N_T since this parameter incorporates both types of interactions described by parameters π^* and α (see Eq. 7.1.16). For this reason, the following expression can be expected to be valid for the retention factor:

$$\log k'_X = c_{st,X} + p_X E^N_{T,mo} \quad (9.7.15)$$

The application of Eq. 9.7.15 is of limited use for a true calculation of the retention factor k, since the value for p_X and for c_{stX} must be known for a calculation. However, such values are available in the literature for several series of

compounds [18,211,214]. The results expressed by Eq. 9.7.15 show the usefulness of parameter E_T^N of the mobile phase for solvent characterization, showing that compounds with larger E_T^N lead to larger k' values and therefore to larger retention times in RP-HPLC. This can be a criterion for selecting a specific solvent (or solvent mixtures) as mobile phase in RP-HPLC.

Eq. 9.7.15 can be used for the estimation of the variation in k'_X when the mobile phase is changed. Using the notation $k_X(mo_1)$ and $k_X(mo_2)$ for the retention factors in two different mobile phases (mo_1) and (mo_2), from Eq. 9.7.15, the following Eq. can be obtained:

$$\log k'_X(mo_1) - \log k'_X(mo_2) = p_X\left[E_T^N(mo_1) - E_T^N(mo_2)\right] \tag{9.7.16}$$

Eq. 9.7.16 indicates that when changing a solvent (e.g., from methanol to acetonitrile) the value for log k' changes proportional to the change in E_T^N of the two solvents.

Rel 9.7.15 also points out a certain disagreement with rel 9.7.1 regarding the linear variation of log k'_X with the mobile phase composition. In Section 7.1 (see Fig. 7.1.7), it was shown that the variation of E_T^N for a solvent mixture is not linear. Substituting such a variation for $E_{T\,mo}^N$ in Eq. 9.7.15, a disagreement with Eq. 9.7.1 is obtained. However, both Eqs. 9.7.1 and 9.7.15 being approximations, it can be concluded that for specific systems they may provide useful information.

From the success of column characterization as described in Section 9.3 in hydrophobic subtraction model, a similar formula with 9.3.7 has been developed for the estimation of log k' as follows (HSM model):

$$\log k' = h_0 + hH + sS + aA + bB + cC + vV \tag{9.7.17}$$

In Eq. 9.7.17, parameter h is related to solute's polarizability and dipolarity (with some contribution from polarizability), s to molecular shape, a to hydrogen bond donating ability, b to hydrogen bond accepting ability, c to cation exchange capability, and v to molecular size [197,203]. The parameters h_0, H, S, A, B, C, and V are dependent on both the stationary phase and mobile phase, but for a specific mobile phase they depend only on the stationary phase. Solutes capable of interacting by a unique type of interaction (in addition to hydrophobic) were identified and their contribution to the retention factor was measured and averaged for a number of columns obtaining solute characterizing parameters [32]. For each column c_1, it was then possible to generate parameters for column characterization. This result was expressed by formula 6.1.41 or 9.3.7, written below:

$$\log k'(X) = \log k'(EB) + \eta'(X)H^*_{c_1} - \sigma'(X)S^*_{c_1} + \beta'(X)A^*_{c_1} + \alpha'(X)B^*_{c_1} + \kappa'(X)C^*_{c_1} \tag{9.7.18}$$

Comparing formula 9.7.17 with 9.7.18, $h_0 = \log k'(EB)$, solute parameters h, s, a, b, and c are represented by $\eta'(X)$, $\sigma'(X)$, $\beta'(X)$, $\alpha'(X)$, and $\kappa'(X)$ and column parameters H, S, A, B, and C are represented by H^*_{c1}, S^*_{c1}, A^*_{c1}, B^*_{c1}, and C^*_{c1}. The term vV from Eq. 9.7.17 related to molecular size was not relevant for Eq. 9.7.18. The success of Eq. 9.7.18 for the characterization of the chromatographic column with a set of test compounds and a well-specified mobile phase has been remarkable [17,121]. However, the main role of expression of the type 9.7.18 is not for solute characterization and cannot be used for the prediction of log k' without having the values $\eta'(X)$, $\sigma'(X)$, $\beta'(X)$, $\alpha'(X)$, and $\kappa'(X)$ for a specific solute X. These values are available for the set of "test compounds" but not for other analytes that were not in the test set.

Besides expression of the form 9.7.10 and 9.7.17 used for the prediction of log k', other procedures based on molecular properties were suggested [216]. One of these procedures is based on

the additive fragment concept where the calculation uses a relation of the following form:

$$\log k' = \sum f_n + \sum F_m \qquad (9.7.19)$$

where f_n is a constant for the particular fragment and F_m is a correction factor for a specific structural feature in the molecule. The procedure can also be extended to the calculation of a log k'(new) for a compound similar in structure to another compound that has a log k'(known) value using the following expression:

$$\log k'(\text{new}) = \log k'(\text{known}) \pm \sum f_n \pm \sum F_m \qquad (9.7.20)$$

where the fragments f_n and the corrections F_m are subtracted and/or added appropriately to change the known compound into the new one. Although this type of procedure provides good results, it requires information on f_n and F_m values, which are available for a limited range of compounds [216–218].

Evaluation of the energies of interaction in the separation system

For PR-HPLC, the solvophobic theory [11,146,219,220] offers a method for such calculation of the energies of interaction in the separation system. The role of the solvent (mobile phase) in the separation results from its physical parameters including surface tension γ', dielectric constant ε, molar volume V, polarizability α, ionization potential I, etc., that must be used for the calculation of k' (see Eq. 9.1.4, 9.1.5 and 9.1.6). The problem with such calculations is that particular parameters for solute, solvent, and stationary phase are not always available. This is in particular the case for the stationary phase and for mixtures of solvents used as mobile phases. For this reason, approximations and results for systems with restricted values for some parameters were reported in the literature [147]. The formula developed in Section 9.1 for explaining the retention is useful mainly for the understanding of the role of various solvent parameters in influencing the separation.

Other approaches

Numerous other attempts to predict separation parameters in RP-HPLC and in HPLC in general are reported in the literature. These include statistical approaches such as so-called design of experiments (DoE) [221] and quantitative structure–retention relationships (QSRR) [222]. The overall use of quantitative structure–activity relationship (QSAR) for the prediction of chromatographic properties is well represented in the literature (e.g., Refs. [3,154]).

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CHAPTER

10

Other HPLC separations performed on hydrophobic stationary phases

10.1 Nonaqueous RP-HPLC

General comments

Nonaqueous reversed phase HPLC (NARP) is a type of HPLC very similar with "standard" RP-HPLC with the difference that the mobile phase in NARP does not contain water although it is more polar than the stationary phase [1]. This requirement is imposed by the high hydrophobic character of the analytes typically separated by this technique. These compounds usually have log K_{ow} higher than about 5.5 and are not soluble in water or even in solvent mixtures containing water. For this reason, the presence of water in the mobile phase is not acceptable. Also, the interaction of such analytes with hydrophobic stationary phases is so strong that their elution from the HPLC column is possible (within acceptable retention times) only with a very "strong" mobile phase entirely composed of organic solvent(s). These solvents can be those also used in RP-HPLC, such as methanol, acetonitrile, and tetrahydrofuran, but other solvents or cosolvents that can be considered very strong in RP-HPLC are typically used [2]. These are nonpolar solvents that are not miscible with water. Their eluotropic strength usually increases with temperature. The type of retention/elution in NARP is the same as in standard RP-HPLC, but the absence of water from the mobile phase may cause a lower importance of the cavity free-energy component in the separation mechanism, as presented in Section 9.1. The standard enthalpy change ΔH^0 calculated from van't Hoff plots is usually larger as calculated for "standard" RP type separation. For example, the value of ΔH^0 calculated for the retention of tocopherol on C18 stationary phase with methanol 100% as mobile phase is higher than ΔH^0 calculated for retinol or calciferol on the same stationary phase and mobile phases with 5%–10% water in methanol as mobile phase [3]. In NARP, the same type of columns as in RP-HPLC are used, with C18 or C8 types being the most common [4,5].

Mobile phase composition in NARP

The solvents used in NARP are frequently different from those used in common RP-HPLC by having lower polarity P' and higher log K_{ow} values. For further decreasing the polarity of the mobile phase compared to a mobile phase containing short chain aliphatic alcohols or acetonitrile, there are used solvents such as acetone, ethers, esters, hydrocarbons, or chlorinated hydrocarbons. For example, tetrahydrofuran, dioxane, *tert*-butyl methyl ether, and diethyl ether are

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among the common ones used in NARP. Ethers have very low donor hydrogen-bonding capability and medium acceptor hydrogen-bonding capability. These compounds have a UV-cut-off value around 210–215 nm. Ketones can also be used in NARP. As previously indicated (see Section 9.5), they have solvent capabilities compared to ethers but high UV cut-off values (around 330 nm) [6]. Among the esters used in NARP, a common one is ethyl acetate. Esters also have a relatively high UV cut-off.

Hydrocarbons are an important group of solvents with applications in NARP. Mixtures of alcohols and hydrocarbons are common components of the mobile phase added with the purpose of lowering the polarity of the mobile phase. Hydrocarbons such as hexane, heptane, or cyclohexane are good solvents for highly hydrophobic analytes such as triglycerides, or carotenoids. Halogenated hydrocarbons have medium polarity (except for CCl_4) but low hydrogen-bonding capability. They are very good solvents for many compounds with nonpolar character and are useful in NARP. Among the solvents used more frequently in this technique are methylene chloride, chloroform, and carbon tetrachloride. Other chlorinated solvents are seldom utilized. However, for some solubility reasons, special chlorinated solvents such as chlorobenzene can be used, and in this case, the separation is usually performed at temperatures higher than 70°C when a less-volatile solvent must be used as an eluent. Some concerns related to the use of chlorinated compounds as mobile phase may come from environmental issues and from potential hydrolysis with the formation of HCl.

Utility of NARP compared to RP-HPLC

NARP type separation is successfully used in case of polyaromatic hydrocarbons, triglycerides, terpenes, hydrophobic vitamins, and carotenoids including xanthophylls such as β-cryptoxanthin, zeaxanthin, astaxanthin, canthaxanthin, or lutein. In such separations, the mobile phase contains acetonitrile or methanol as main solvent and a cosolvent that is a hydrophobic solvent. Ternary mobile phase compositions are also necessary for specific separations, such as that of geometric isomers of carotenes, when mixtures of acetonitrile/methanol/dichloromethane can be employed for attaining a desired resolution [7]. Mixtures of acetonitrile/methanol/dichloromethane are also useful in separating retinol, α-tocopherol, α-carotene, β-carotene, lycopene, zeaxanthin, and other carotenoids from complex samples [8].

As an example of NARP separation, α-tocopherol and α-tocopheryl acetate, which are basically insoluble in water ($C_{X,w} = 1.9 \cdot 10^{-6}$ mg/L), can be analyzed in tobacco and cigarette smoke [9] and in liquids used in electronic cigarettes (e-liquids) [10] using a Zorbax Eclipse XDB-C18 column, 4.6 × 250 mm 5 μm. The mobile phase contains as a more polar solvent acetonitrile ($P' = 5.8$, $\log K_{ow} = -0.17$) and isopropanol ($P' = 3.9$, $\log K_{ow} = 0.25$) as a less polar one and the separation is performed using gradient elution. The chromatogram obtained using UV detection for a standard containing 6.16 μg/mL α-tocopherol and 6.03 μg/mL α-tocopheryl acetate, Uvitex-OB as internal standard, and BHT as antioxidant is shown in Fig. 10.1.1.

For the detection done by MS/MS in ESI-MRM positive mode and the same separation as shown in Fig. 10.1.1 but with addition of 0.1% HCOOH in the mobile phase, the obtained extracted ion chromatograms are shown in Fig. 10.1.2.

The use of MS detection in ESI ionization mode when the mobile phase is nonaqueous may pose problems in some analyses generating very little or no signal, mainly when nonionizable solvents such as hydrocarbons are used. In cases when ESI is not adequate for detection, APCI can usually be utilized.

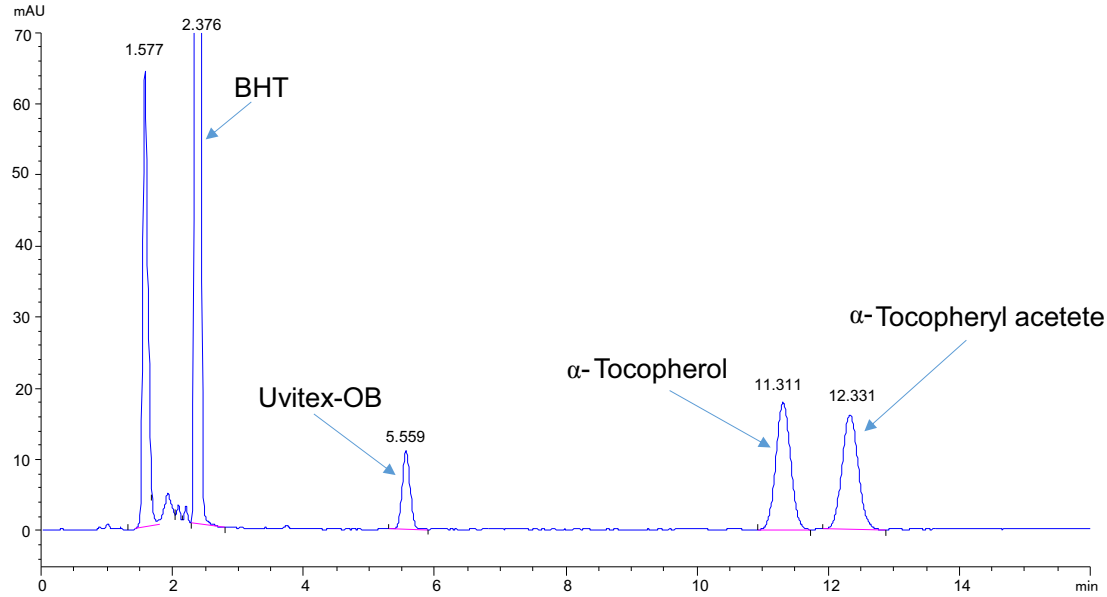


FIGURE 10.1.1 Chromatogram showing separation of α-tocopherol and α-tocopheryl acetate on a Zorbax Eclipse XDB-C18 column with acetonitrile/isopropanol mobile phase using UV detection at 210 nm.

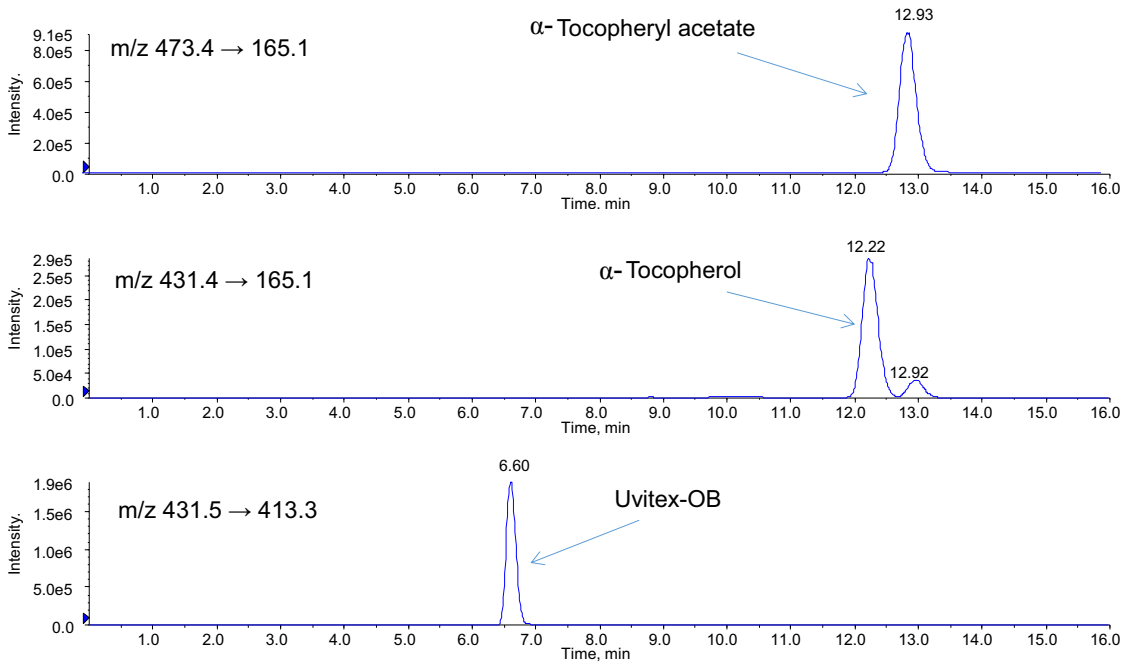


FIGURE 10.1.2 Extracted ion chromatogram showing separation of α-tocopherol and α-tocopheryl acetate on a Zorbax Eclipse XDB-C18 column with acetonitrile/isopropanol mobile phase +0.1% HCOOH using MS/MS detection in ESI-MRM positive mode.

10.2 Ion pair liquid chromatography

General comments

Ion pair liquid chromatography (IP) is a frequently utilized HPLC type applied for the separation of analytes (organic or inorganic) that contain ionizable or strongly polar groups, which make these compounds to have a poor retention on hydrophobic columns (e.g., C18 or C8). Among these analytes are organic acids, amino acids, amines, etc. IP-LC makes these compounds amenable for RP-HPLC type separation, using hydrophobic stationary phases (e.g., C18, C8). This can be achieved with the aid of an ion-pairing agent (*IPA* or hetaeron), which is added to the mobile phase usually at concentrations between 10 and 100 mM/L. The ion-pairing agent *IPA* is an ion species selected such that it has opposite charge to the analyte and is able to form molecular association with it (or with any targeted solute). Compounds used as *IPA* can be, for example, quaternary amines (with various chain length of the substituent) in case of analyzing acids or strong organic acids (e.g., sulfonic acid with various chain substituents) in case of analyzing amines. Two examples of *IPA*, one for acidic compounds analysis and the other for basic compounds analysis, and the formation of ion pairs are shown below:

$$R{-}COO^- + C_6H_{13}(CH_3)_3N^+ \rightarrow R{-}COO^- \,{}^+N(CH_3)_3C_6H_{13} \quad (10.2.1)$$

$$R{-}NH_3^+ + CH_3(CH_2)_7{-}SO_3^- \rightarrow R{-}NH_3^+ \,{}^-O_3S{-}(CH_2)_7CH_3 \quad (10.2.2)$$

The use of *IPA* with amphoteric character was also reported in the literature [11].

IPA molecules must contain a hydrophobic moiety, which allows its interaction with the nonpolar stationary phase. After adding *IPA*, the separation on hydrophobic stationary phase becomes similar to that in RP-HPLC, and the increase in the hydrophobic character of the complex leads to stronger retention, while the increase in the hydrophobic character of the mobile phase leads to the faster elution of the analytes. Although ion pairs are typically separated on RP type chromatographic columns, separations on silica with nonpolar solvents as a mobile phase were also reported for the separation of enantiomers [12]. This type of separation is a normal phase (NP) type presented in Section 14.5.

The effect of adding *IPA* in the mobile phase is shown in Fig. 10.2.1 for the retention of a basic polar compound, acyclovir (log $K_{ow} = -1.56$), on a common RP stationary phase without *IPA* and with addition of *IPA* (sodium 1-heptansulfonate). Without the addition of *IPA* into the mobile phase, acyclovir elutes close to the void time of the column. The separation was done on a C18 150 × 4.6 mm i.d. × 5 μm column. The mobile phase was made of solutions A: 15% CH_3OH + 85% aq. solution with 0.1% H_3PO_4 and B: 15% CH_3OH + 85% aq. solution with 0.1% H_3PO_4 and 10 mM/L $C_7H_{15}SO_3Na$ as hetaeron (*IPA*). The flow rate was 1 mL/min, T = 25°C, and fluorescence detection was used, excitation −262 nm, emission −380 nm [13]. The effect of *IPA* is that of enhancing the retention of the analytes with the opposite charge to *IPA*, decreasing the retention of analytes with the same charge with *IPA*, and having a negligible effect on the retention of uncharged analytes. Since the interactions between the *IPA* and the analyte depend on the ionization state of the two participants, and the ionization state of both *IPA* and analyte depends on the pH, IP-LC is a technique where the mobile phase pH plays an important role.

By increasing the retention (retention factor k') for polar and ionic compounds, the addition of *IPA* to the mobile phase found numerous applications in HPLC separations. The versatility and advantages offered by RP-type columns and the simplicity of just adding *IPA* to the mobile phase make IP technique very useful. Only for specific separations, some backpressure increase can be noticed due to the increase in mobile phase viscosity caused by the addition of the hetaeron.

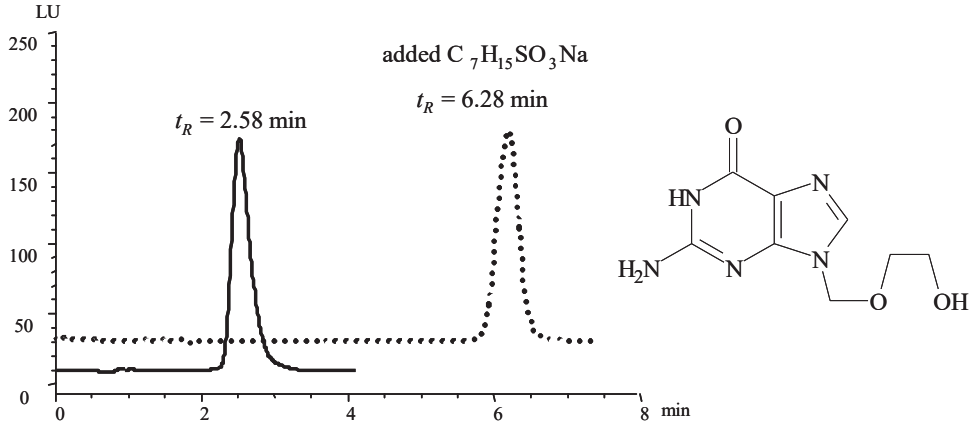


FIGURE 10.2.1 Chromatographic peaks belonging to acyclovir on a C18 column with and without $C_7H_{15}SO_3Na$ as hetaeron (*IPA*).

Ion-pairing mechanisms

There are two limiting model theories explaining the mechanism of retention in IP: (1) Partition model where the mechanism assumes the formation of ion pairs in solution, with the ion pair formed between the ionic or partially charged molecules of the analyte and the *IPA* with an opposite charge, followed by the retention of the preformed ion pair on the stationary phase and (2) Electrostatic model, which assumes that *IPA* is first bound to the hydrophobic stationary phase by its hydrophobic moiety, and once adsorbed it interacts with the components of the mobile phase by ionic/polar forces [14]. Both mechanisms indicate similar dependencies on pH, *IPA* and analyte concentration, and polarity of *IPA* and analyte [15,16]. Other more complex treatments of the retention process in IP were developed that attempt to include in the equilibria both the formation of ion pairs in solution as well as after the *IPA* is adsorbed on the stationary phase [17–20].

Partition model in IP

Partition model for the IP separation considers that the formation of ion pairs takes place first in solution. Once the ion pair is formed, the separation can be considered as based on the interaction of the ion pair with the hydrophobic stationary phase in a polar solvent. For a basic compound and a strong acid *IPA*, the formation of the complex in the solution is favored by higher stability constant *K* of the complex, higher basicity of the analyte, higher concentration of *IPA*, and a lower pH. For an acidic analyte, the same parameters will influence the formation of the ion pair, but the *IPA* should be an ionizable base, and the equilibrium is favored by a stronger acidity constant and a higher pH.

The association mechanism of the analyte and the *IPA* is rather complicated. According to partition model, two steps characterize the partition process of ion pair between a partially aqueous mobile phase (*mo*) and stationary phase (bonded organic liquid phase) (*st*) that is immobilized on the stationary phase:

$$A^+ + IPA^- \rightarrow A^+IPA^- \qquad (10.2.3)$$

$$\left(A^+IPA^-\right)_{mo} \rightleftarrows \left(A^+IPA^-\right)_{st} \qquad (10.2.4)$$

Similar equilibria take place for an analyte A^- and an IPA^+. Examples of A^+ can be compounds containing mainly amino groups (from primary $-NH_2$ to quaternary $-NR_4^+$) and examples of A^- can be compounds

containing dissociated functional groups such as $-O^-$ (phenolic), $-COO^-$, $-SO_3^-$, $-O-SO_3^-$, and $-S^-$. Compounds with zwitterionic character such as amino acids can also be included in the types of analytes A that can be separated by ion pair chromatography.

The partition of ion pairs between two immiscible phases is further exemplified for an amine (indicated as A), but can be equally discussed for any other compound capable of ion pair association. The formation from an amine of ammonium cations in aqueous phase is dependent on the concentration of H^+ in this phase, and therefore, pH plays a major role in the formation of ion pairs. The counterion in this case must be a compound that is able to form anions IPA^-. Examples of IPA^- are compounds containing sulfonic groups or strong carboxylic acid groups (e.g., perfluorinated organic acids) and a hydrocarbon chain. In order to provide hydrophobicity to the ion pair, IPA^-, the hydrocarbon chain denoted here by R must provide hydrophobic character. The IPA^- will have the form $R-X^-$ (e.g., a totally dissociated sulfonic acid $R-SO_3^-$). The schematic representation of partition of these species is given in Fig. 10.2.2.

As shown in Fig. 10.2.2, there are two species that can be present in the organic (stationary) phase, A and $[AH^{+-}X-R]$. Although the partition of A is significantly lower than for the ion pair $[AH^{+-}X-R]$, the distribution process of A cannot be neglected. Therefore, two partition constants can be assigned to the distribution of two species between *mo* (partially aqueous) and *st* (organic) phases:

$$K(A) = K_A = \frac{C_{A,st}}{C_{A,mo}} \tag{10.2.5}$$

$$K\left(AH^{+-}X-R\right) = K_{AH^{+-}X-R} = \frac{C_{AH^{+-}X-R,st}}{C_{AH^{+-}X-R,mo}} \tag{10.2.6}$$

The distribution coefficient for the equilibrium based on ion-pairing contribution $D_{\text{total}}(A)$ can be written as follows:

$$D_{\text{total}}(A) = \frac{C_{A,st} + C_{AH^{+-}X-R,st}}{C_{A,mo} + C_{AH^{+-}X-R,mo}} \tag{10.2.7}$$

In Eq. 10.2.7, it can be taken into account that the expression of K_b for the compound A is given by the following relation (see Eq. 4.5.11):

$$K_b = \frac{C_{AH^+}C_{OH^-}}{C_A} = \frac{C_{AH^+}K_w}{C_A C_{H^+}} \tag{10.2.8}$$

With the use of Eqs. 10.2.5 and 10.2.8, the dependence of the distribution constant can be written in the following form:

$$D_{\text{total}}(A) = \frac{1}{K_w + K_b 10^{-pH}}\left(K_w K_A + K_b K_{AH^{+-}X-R} 10^{-pH}\right) \tag{10.2.9}$$

With the replacement of the first product in Eq. 10.2.9 with the formula for D_Y given by Eq. 4.5.18 in which Y is substituted with A, the resulting expression for the total distribution

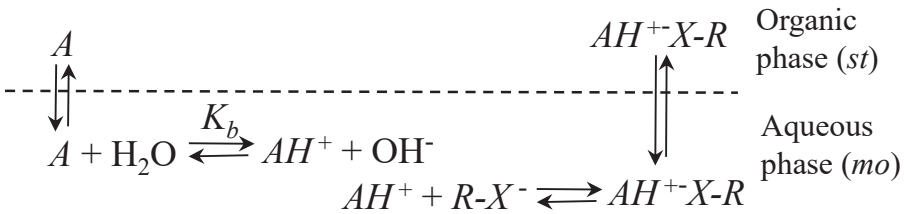


FIGURE 10.2.2 Representation of the partition process of a basic compound A (ionized as AH^+) as ion pairs formed with $R-X^-$ as *IPA*.

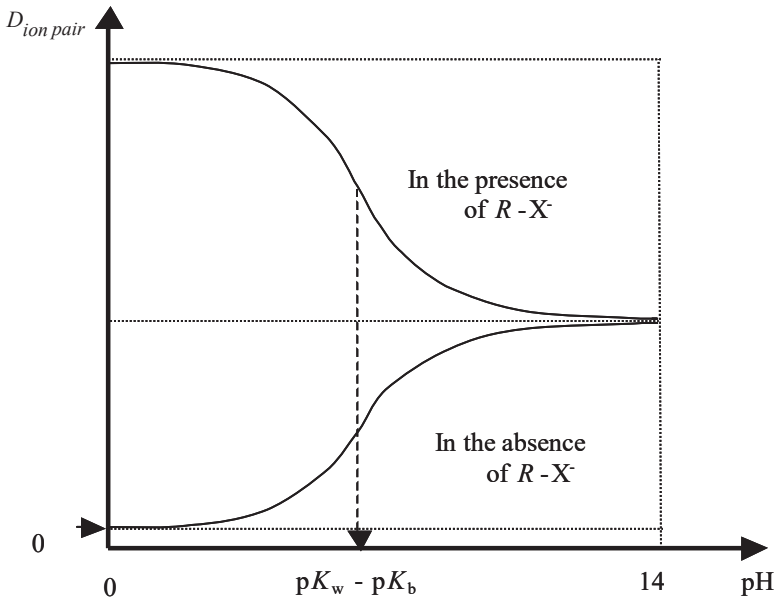


FIGURE 10.2.3 The dependence of the distribution coefficient of a basic analyte (A) on pH for two situations: in the presence and in the absence of the ion-pairing agent, $R{-}X^-$.

coefficient is given by the following (where $D(A)$ is the distribution coefficient in the absence of IPA):

$$D_{\text{total}}(A) = D(A) + K_{AH^{+-}X-R}\frac{K_b 10^{-pH}}{K_w + K_b 10^{-pH}} \tag{10.2.10}$$

Eq. 10.2.10 indicates that for a very basic pH of the aqueous phase (i.e., $[H^+]$ value close to 10^{-14}), the second term of the above expression becomes 0, indicating that at this pH region the ion pair between analyte and IPA^- does not play a role, and the partition is based only on the free analyte A. This partition is very small (according to the initial assumption), and therefore, very little retention in the organic phase of the species A will take place. On the contrary, for very acidic aqueous phase (pH close to 0), $K_b >> K_w$ (for an analyte with a strong basic character) and the value of distribution coefficient of the process becomes almost identical to the partition constant of the ion pair formed between the analyte and IPA^-. A comparison of two graphs showing the variation of $D_{\text{ion pair}}$ as a function of pH in the presence and the absence of IPA in aqueous phase is shown in Fig. 10.2.3.

For the organic phase being immobilized as stationary phase, the higher value for $D_{\text{ion pair}}$ indicates larger retention factors and longer retention times. Eq. 10.2.10 indicates a formal dependence of distribution $D_{\text{ion pair}}$ on the pH value. However, the pH also influences the concentration of A and $[A{-}H^{+-}X{-}R]$ in a solution, without the influence of the extraction equilibrium. For understanding this influence, two equilibria in solution should be considered:

$$A + H_2O \rightleftarrows AH^+ + OH^- \tag{10.2.11}$$

$$AH^+ + R - X^- \rightleftarrows AH^{+-}X - R \tag{10.2.12}$$

The first equilibrium is governed by the basicity constant K_b given by Eq. 10.2.8. The second equilibrium depends on the stability of the $[AH^{+-}X{-}R]$ association, and is governed by

the stability constant K given by the following expression:

$$K = \frac{C_{AH^{+-}X-R}}{C_{AH^{+}}C_{R-X^{-}}} \quad (10.2.13)$$

From the initial concentration of the analyte $C_{init} = C_A + C_{AH^{+-}X-R}$, the concentration of the complex $C_{AH^{+-}X-R}$ can be obtained. For this purpose, the expression for the concentration C_A is replaced using Eqs. 10.2.13 and 10.2.8. After a few calculations, the following expression is obtained:

$$C_{AH^{+-}X-R} = \frac{KK_bC_{init}C_{R-X^{-}}10^{-pH}}{K_w + KK_bC_{R-X^{-}}10^{-pH}} \quad (10.2.14)$$

Eq. 10.2.14 indicates that when the term $K \cdot K_b \cdot C_{R-X^{-}}$ $[H^+]$ is large, K_w can be neglected and the (molar) concentration of the complex $[AH^{+-}X-R]$ is basically equal with the initial concentration of the compound A. The term is high when the constant K of the complex formation is high, when the compound A has a high basicity constant K_b, when the concentration of IPA $C_{R-X^{-}}$ is high, and also when the pH is low. The equilibria Eqs. 10.2.11 and 10.2.12 are further influenced by the distribution, and Eqs. 10.2.13 and 10.2.14 describe the process in the absence of the organic phase. However, the conclusions regarding the parameters increasing the formation of ion pairs remain valid even in the presence of the organic phase (hydrophobic stationary phase).

A similar discussion with that for A can be made for strong acidic analytes HX such as those containing carboxyl or sulfonyl groups. These analytes are found in aqueous phase more in the dissociated X^- form than not dissociated. The ion-pairing agent in such cases should be a cation or IPA^+. Common IPA^+ compounds are quaternary amines with a hydrophobic moiety R, and common examples of such compounds are quaternary ammonium salts $R{-}N(CH_3)_3^+$ with a long (e.g., C6, C7) hydrophobic chain R. The ion pair formed between these ionic species and IPA can be written as $[R{-}N(CH_3)_3^+X^-]$. These associates lead to the retention of the ionic X^- on a hydrophobic phase. For these cases, the equivalent result with Eq. 10.2.10 will indicate a stronger retention of the ion pair at higher pH.

The distribution process for the ion pair expressed by Eq. 10.2.10 indicates that two effects must be taken into consideration following the ion pair formation. One is the distribution equilibrium for the free analyte, and the other is the distribution of the ion pair. Both analyte and ion pair can be considered hydrophobic components, and the theory developed for RP chromatography will apply for understanding their retention. Larger hydrophobic areas of the analyte and the complex analyte-*IPA*, more polar solvents and with higher superficial tension γ', and stationary phases with higher hydrophobic character will contribute to larger k' values for the analytes. Since the ion pair contains the hydrophobic moiety of the *IPA*, larger retention factors will be expected for the ion pair with *IPA* having larger hydrophobic moiety, e.g., a longer hydrocarbon chain for an alkyl sulfonic acid in case of basic analytes, or for a quaternary amine in case of acidic analytes (amino acids are typically separated in IP using strong acids as *IPA*).

Electrostatic model in IP

Electrostatic model for the IP separation also considers two stages, but the first process is the retention of *IPA* by hydrophobic interactions on the stationary phase, and the second is the retention of the analyte by the ionic/polar forces between the ionic/polar groups of the *IPA* and those of the analyte. In support for this mechanism is the observation that the column used in IP requires for good reproducibility an equilibration time when the mobile phase containing *IPA* is flushed through the column (conditioning of the stationary phase). This step would suggest that the adsorption of *IPA* on the stationary phase is initially necessary.

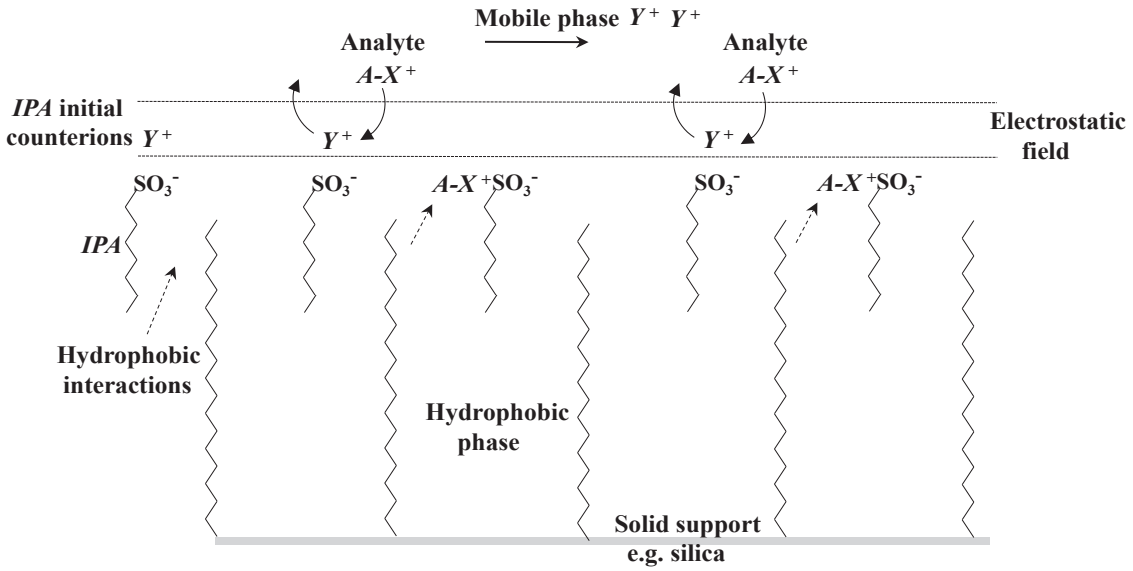


FIGURE 10.2.4 Illustration of the formation of electrical double layer during ion-pairing mechanism (the analyte is represented by $A{-}X^+$, *IPA* is an alkyl sulfonate).

Electrostatic model assumes the formation of two parallel layers of charges (double electrostatic layer) when electrically charged molecules of *IPA* are adsorbed on the stationary phase surface. The adsorption of *IPA* is assumed to take place by hydrophobic interactions, and since the concentration of *IPA* in the mobile phase is high (compared to any analyte), the surface of the stationary phase can be assumed covered with *IPA*. The ionic ends of *IPA* are creating the first adsorbed layer of charges [21]. In this theory, only the first layer of charges is compact, while the second layer is diffuse, and the electrical potential created by the first layer decreases exponentially away from the adsorbing surface (based on Gouy–Chapman theory of electrical double layer (e.g., Ref. [22])). The secondary diffuse layer is due to the *IPA* counterions (initial ones or provided by the solutes). Both layers are assumed to be under dynamic equilibrium. The counterions of opposite signs to those of the adsorbed *IPA* are electrostatically attracted toward the charged surface, and those with the same electrical charges are repelled from the surface [23]. This process is illustrated in Fig. 10.2.4.

Considering that ion-pairing agent is a hydrophobic anion, IPA^-, the analyte is represented by the cation $A{-}X^+$, and the stationary phase is considered a ligand L, the main equilibria within this mechanism are the following:

$$L + IPA^- \rightleftarrows L\,IPA^- \tag{10.2.15}$$

$$L\,IPA^- + A\,-X^+ \rightleftarrows L\,IPA^{-+}X\,-A \tag{10.2.16}$$

The electrostatic model is, however, also an approximation. Experimentally, it can be proven that the analyte has its own interaction with the stationary phase, and some retention of the analyte takes place without *IPA* in the mobile phase. The effect of *IPA* is only that of enhancing the retention. The interactions between the analyte and the stationary phase can be considered as "mediated" by the addition of *IPA*.

Although the direct interaction of the solute $A{-}X^+$ with the stationary phase independent of *IPA* can be small, this process must be taken

into consideration when discussing the retention process. Assuming that the process takes place involving free energies ΔA that are independent on temperature $\left(\frac{\partial A}{\partial T} = 0\right)$, based on Eq. 5.1.23 the theory can be developed in terms of energies E involved in the system. The change in energy E to bring a charged solute to a surface of the stationary phase that involves *IPA* can be divided into two contributions:

$$E_{AX^+} = E_{\text{hydrophobic}} + E_{\text{el}} \tag{10.2.17}$$

In Eq. 10.2.17, $E_{\text{hydrophobic}}$ represents total variation in (free) energy of the adsorption of analyte AX^+ in the absence of the electric field created by the adsorption of *IPA* on the stationary phase surface. The contribution to the (free) energy caused by the electrostatic interaction between the charged analyte AX^+ and the electric field of the stationary phase surface is given by E_{el}. Taking into account the relation between retention factor k' and equilibrium constant K for the partition of the species AX^+ between mobile and stationary phase, i.e., $k' = \Psi \cdot K$ (where Ψ is the phase ratio), the expression for k' can be written as follows:

$$\begin{aligned} k' &= \Psi \exp \frac{-\left(E_{\text{hydrophobic}} + E_{\text{el}}\right)}{RT} \\ &= \Psi \exp \frac{-E_{\text{hydrophobic}}}{RT} \exp \frac{-E_{\text{el}}}{RT} \end{aligned} \tag{10.2.18}$$

The first factor in Eq. 10.2.18 represents the retention factor of the species AX^+ in the absence of *IPA* in the mobile phase. The contribution to the retention factor due to the electrostatic interaction between charged analyte AX^+ and the electric field created by *IPA* adsorbed onto the stationary phase is represented in this equation by the term $\exp(-E_{\text{el}}/RT)$ [24]. As a result, the expression for k' can be written as follows:

$$k' = k(0) \exp \frac{-E_{\text{el}}}{RT} \tag{10.2.19}$$

The electrostatic contribution that represents the work involved in the transfer of an ionic analyte with charge z to the charged surface of the stationary phase (E_{el}) is given by the following formula:

$$E_{\text{el}} = z F \Delta \overrightarrow{\mathbb{E}} \tag{10.2.20}$$

where F represents the Faraday constant and $\Delta \overrightarrow{\mathbb{E}}$ is the difference in electrostatic potential between the bulk of the mobile phase and the stationary phase surface. With this formula, Eq. 10.2.19 can be written in the following form:

$$k' = k(0) \exp \frac{-zF\Delta \overrightarrow{\mathbb{E}}}{RT} \tag{10.2.21}$$

Eq. 10.2.21 indicates an important contribution of $k(0)$ to the separation process, which corresponds to experimental finding that the structure of the analyte strongly influences the separation. The electrostatic potential $\Delta \overrightarrow{\mathbb{E}}$ created by the adsorption of *IPA* on stationary phase surface (perpendicular on the surface) and its value can be obtained based on Gouy–Chapman's theory. The full expression for $\Delta \overrightarrow{\mathbb{E}}$ is reported in various publications (e.g., Ref. [24]), and for IP that involves small charges and small electrical fields, an approximation of $\Delta \overrightarrow{\mathbb{E}}$ formula is the following:

$$\Delta \overrightarrow{\mathbb{E}} = \frac{z_{IPA} F x_{IPA}^{st}}{\kappa \varepsilon_0 \varepsilon_{mo}} \tag{10.2.22}$$

where x_{IPA}^{st} represents molar fraction of the charged species (*IPA*) on the surface of stationary phase (*st*), ε_0 is the electrical permittivity of vacuum, ε_{mo} is the dielectric constant of the surrounding medium (mobile phase), and κ is a variable known as *Debye length* and is given by the following formula:

$$\kappa = F \left(\frac{\sum_j z_j^2 C_j}{\varepsilon_0 \varepsilon_{mo} RT} \right)^{1/2} \tag{10.2.23}$$

The sum in Eq. 10.2.23 is made over all the ionic species in the solution. The electrostatic contribution to the change in free energy depends basically on the electric charge of the analyte and *IPA* involved into the partition process. Thus, the sign (+ or −) for the E_{el} can be positive when the analyte and *IPA* have the same sign charges and negative when they are of opposite charges.

The electric field created on the surface of stationary phase due to the adsorption of *IPA* could influence further adsorption of *IPA* because the adsorbed molecules of this species may repel other molecules coming from the mobile phase, resulting in an equilibrium between adsorbed and free molecules of *IPA*. Therefore, as the surface concentration of *IPA* increases, the area accessible for additional molecules on the surface decreases, and these molecules will find it more and more difficult to find an adsorption site on the stationary phase surface. On the other hand, there is an equilibrium process between adsorption and desorption of *IPA* on the surface, determined by its concentration in mobile phase and its affinity toward hydrocarbon chains of the hydrophobic stationary phase. This affinity can be estimated based on Langmuir model for adsorption that gives the molar fraction x_{IPA}^{st} of the species adsorbed on the surface of stationary phase by Eq. 4.2.17 (see Section 4.2). Based on Eq. 4.2.17 and with the appropriate expression for the equilibrium constant K_X (in this case $K_X = K_{IPA}$), it can be established that x_{IPA}^{st} is given by the following formula:

$$x_{IPA}^{st} = x_{IPA,max}^{st} K_{IPA} C_{IPA} \exp\frac{-z_{IPA}F\Delta\mathbb{E}}{RT} \cdot \left(1 + K_{IPA} C_{IPA} \exp\frac{-z_{IPA}F\Delta\mathbb{E}}{RT}\right)^{-1} \tag{10.2.24}$$

In Eq. 10.2.24, K_{IPA} is the adsorption constant for *IPA*, C_{IPA} is the concentration of *IPA* in the mobile phase (equivalent to C_{RX^-} in Eq. 10.2.13), and $x_{IPA,max}^{st}$ is the maximum value for the *IPA* molar fraction on the surface. Expression 10.2.24 can be simplified since the denominator of the ratio is practically 1, and can be written in the following form:

$$x_{IPA}^{st} = x_{IPA,max}^{st} K_{IPA} C_{IPA} \exp\frac{-z_{IPA}F\Delta\mathbb{E}}{RT} \tag{10.2.25}$$

This equation shows that the surface molar fraction of *IPA* is determined by *IPA* concentration in mobile phase C_{IPA} and by the adsorption constant K_{IPA} (which at its turn is determined by *IPA* hydrophobicity).

Further use for the value of x_{IPA}^{st} as given by rel 10.2.25 is in obtaining an expression for k' in ion pair chromatography. The first step for this goal is to express the potential $\Delta\mathbb{E}$ as a function of retention factors k' and $k'(0)$. By applying the natural logarithm to Eq. 10.2.21, the following expression is obtained:

$$\Delta\mathbb{E} = -\frac{RT}{zF}\ln\frac{k'}{k'(0)} \tag{10.2.26}$$

From Eq. 10.2.26 introduced in formula 10.2.25 of x_{IPA}^{st}, the result is the following:

$$x_{IPA}^{st} = x_{IPA,max}^{st} K_{IPA} C_{IPA} \left(\frac{k'}{k'(0)}\right)^{\frac{z_{IPA}}{z}} \tag{10.2.27}$$

On the other hand, $\Delta\mathbb{E}$ is given by Eq. 10.2.22, which can be written using Eq. 10.2.27 in the following form:

$$\Delta\mathbb{E} = \frac{z_{IPA}F}{\kappa\varepsilon_0\varepsilon_{mo}} x_{IPA,max}^{st} K_{IPA} C_{IPA} \left(\frac{k'}{k'(0)}\right)^{\frac{z_{IPA}}{z}}. \tag{10.2.28}$$

Eqs. 10.2.26 and 10.2.28, both giving an expression for $\Delta\mathbb{E}$, imply that the following formula is valid:

$$\frac{-RT}{zF}\ln\frac{k'}{k'(0)} = \frac{z_{IPA}F}{\kappa\varepsilon_0\varepsilon_{mo}} x_{IPA,max}^{st} K_{IPA} C_{IPA} \left(\frac{k'}{k'(0)}\right)^{\frac{z_{IPA}}{z}}. \tag{10.2.29}$$

Eq. 10.2.29 can be rearranged in the following form:

$$\left(\frac{k'}{k'(0)}\right)^{\frac{-z_{IPA}}{z}} \ln\left(\frac{k'}{k'(0)}\right)^{\frac{-1}{zz_{IPA}}} = \frac{F^2}{RT\varepsilon_0\varepsilon_{mo}} \frac{x^{st}_{IPA,max}}{\kappa} K_{IPA} C_{IPA} \tag{10.2.30}$$

Some transformation of the first term in Eq. 10.2.30 is further necessary. Using the approximation $\ln(x+1) \approx x$ valid for small values of x, the following formula can be obtained:

$$\ln\left[\left(\frac{1}{zz_{IPA}} \ln\frac{k'(0)}{k'} - 1\right) + 1\right] \approx \frac{1}{zz_{IPA}} \ln\frac{k'(0)}{k'} - 1 \tag{10.2.31}$$

After taking the logarithm of Eq. 10.2.30 and introducing the approximation Eq. 10.2.31, the following expression can be written:

$$-\frac{z_{IPA}}{z} \ln\frac{k'}{k'(0)} - \frac{1}{zz_{IPA}} \ln\frac{k'}{k'(0)} - 1 = \ln\frac{F^2}{\varepsilon_0\varepsilon_{mo}RT} + \ln\frac{x^{st}_{IPA,max} K_{IPA} C_{IPA}}{\kappa} \tag{10.2.32}$$

Eq. 10.2.32 can be further modified into the following equation that describes the dependence in IP electrostatic model of the retention factor k' on the main experimental parameters [25]:

$$\ln k' = \ln k'(0) - \frac{zz_{IPA}}{z^2_{IPA}+1} \cdot \left(\ln C_{IPA} + \ln\frac{x^{st}_{IPA,max} K_{IPA}}{\kappa} + \ln\frac{F^2}{\varepsilon_0\varepsilon_{mo}RT} + 1\right) \tag{10.2.33}$$

According to this equation, the retention factor k' is dependent in IP on several parameters that are further discussed in detail (natural logarithms can be replaced in Eq. 10.2.33 with logarithms in base 10).

(1) In Eq. 10.2.33, the retention factor $k'(0)$ of the ionic species $A{-}X+$ in the absence of *IPA* can be for some compounds sufficiently high to play an important role. The value of $k'(0)$ depends on the hydrophobicity of $A - X^+$ and the phase ratio of the chromatographic column Ψ. The contribution of $k'(0)$ to the total value of k' can be a key factor in the separation. On columns that have additional interactions besides pure hydrophobic ones, polar compounds can be retained stronger or weaker depending on their structure even in the absence of the ion pair agent.

(2) There is a linear dependence between $\ln k'$ (or $\log k'$) and $\ln C_{IPA}$. When changing $\ln c_{IPA}$, the charges $zz_{IPA}/(z^2_{IPA}+1)$ will give the slope of the change in $\ln k'$. For example, if the ionic analyte and *IPA* have charges +1 and −1, respectively, the slope of the dependence of $\ln k'$ on $\ln C_{IPA}$ is 0.5. This effect is exemplified in Fig. 10.2.5 for three biguanidines where the linear dependence of $\ln k'$ on $\ln C_{IPA}$ is shown and the slopes of the dependence lines are very close to 0.5 value (Experimental conditions: C18 column; 50% MeOH in mobile phase; 50% aqueous solution containing 5−50 mM $C_6H_{13}SO_3Na$ as *IPA* at pH = 2) [26].

(3) The influence of the hydrophobic nature of *IPA* in the value of retention factor is expressed in Eq. 10.2.33 by the factor K_{IPA}. The hydrophobic character of *IPA* is determined by its structure and the moiety with hydrophobic character. For example, for a series of alkylsulfonates ($C_nH_{2n+1}SO_3^-$) used as *IPA*s, the value of K_{IPA} increases with the increase of the number of C atoms (n) in its molecule. As an example, the variation of $\log k'$ with the length of the alkyl substituent for several alkylsulfonates used as *IPA* in the analysis of four polar compounds belonging to biguanidine class is shown in Fig. 10.2.6. The separation was done on a C18 column with the mobile phase composition: 40% MeOH, 60% aqueous component containing individual *IPA* as 10 mM/L, and pH = 2.0, adjusted with H_3PO_4 [26].

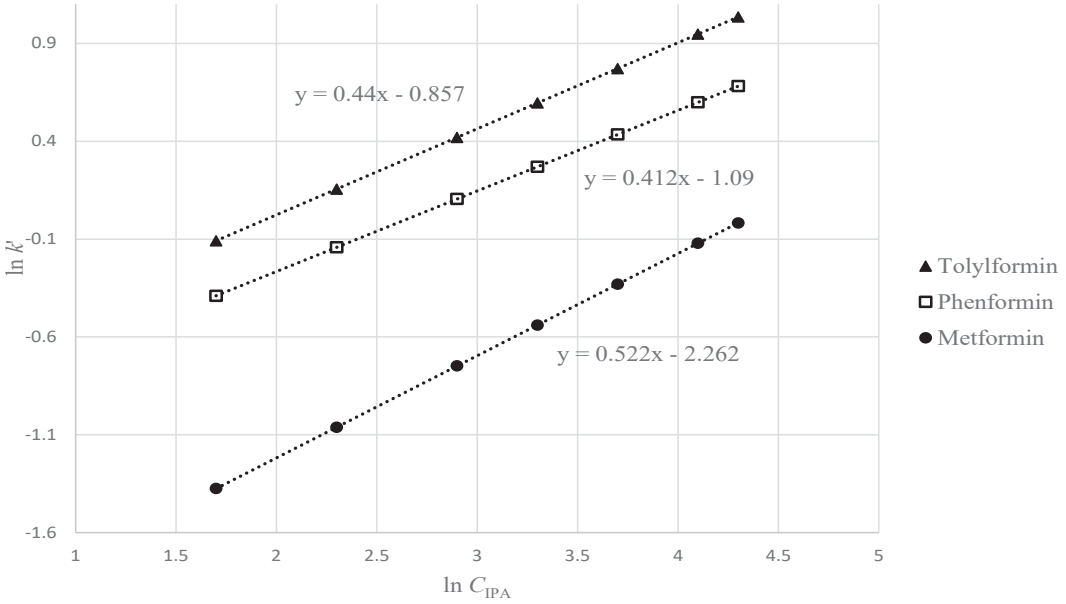


FIGURE 10.2.5 Dependence of the retention factor for three biguanidines (tolylformin, phenformin, and metformin) on the concentration of sodium hexanesulfonate in mobile phase.

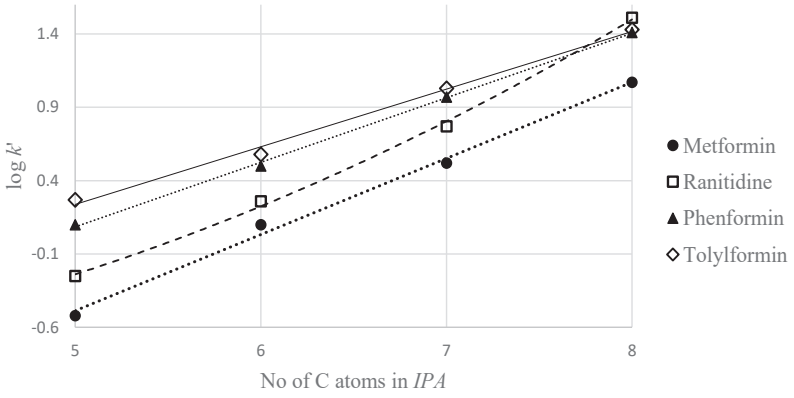


FIGURE 10.2.6 Dependence of log k' on the number of C atoms in alkylsulfonates used as *IPA* for separation of four polar compounds.

In the selected example, the dependence of log k' on the length of alkyl chain is linear for three of the compounds and quadratic for one, but this type of dependence is not predictable or universal. The only conclusion is that log k' increases with the increase in the hydrophobicity of the organic moiety of *IPA*. The increase in this hydrophobicity is also associated with the decrease in the solubility of *IPA* in water, which is a limiting factor in selecting the length of the alkyl chain. As shown in Eq. 10.2.33, a linear dependence exists between log k' and log C_{IPA}, and therefore, a higher concentration of *IPA* is desired for increased retention.

The assumption that K_{IPA} does not depend on the analyte may be only an approximation, since formation of some association solute/*IPA* in the solution is still possible, and the adsorption of the ion pairs from solution can be different depending on the analyte. Also, in the separation process, different interactions besides the hydrophobic ones may play a role. In the case of partially polar stationary phases such as phases containing silanol groups, polar groups for end-capping, or polar imbedded groups, the polar interactions may contribute to the separation.

(4) The effect of the nature and concentration of the organic modifier in mobile phase (e.g., methanol) is found implicitly in the value of $k'(0)$ and K_{IPA}. The influence of the organic modifier typically follows the same trend as described for RP-HPLC. For many IP separations, dependencies of log k' on volume fraction ϕ of the organic modifier have the form given by Eq. 9.5.2.

(5) The influence of the electrolyte concentration in the mobile phase is included in the expression of inverse Debye length κ, and the value of the retention factor decreases with the increase of the electrolyte concentration in the mobile phase. However, other effects (see chaotropic and salting-out effects) may compensate for this decrease.

(6) The influence of pH on the value of retention factor is found in the term $\ln k'(0)$, and when the analyte *i* becomes more dissociated by modifying the pH, its retention increases. It is common that ionic compounds have different ionization forms depending on pH. As an example, the % in a solution of the four possible forms of metformin (N,N-dimethylimidodicarbonimidic diamide) at different pH values is shown in Fig. 10.2.7.

As expected, the formation of ion pairs of each ionized form of the analyte will be different leading to a different k' value. The variation of k' for the case of metformin separated on a C18

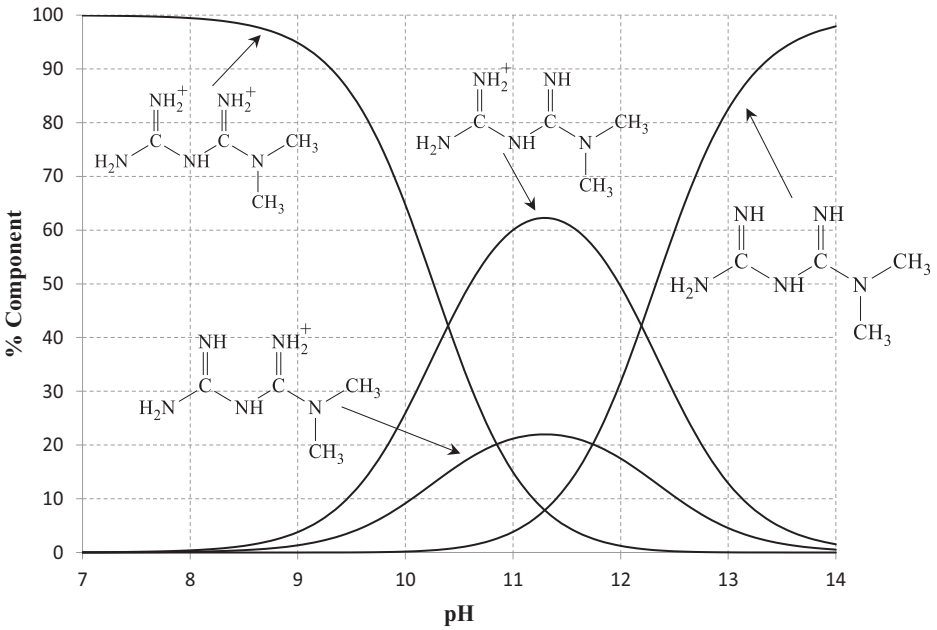


FIGURE 10.2.7 Different forms of metformin in a solution, depending on pH.

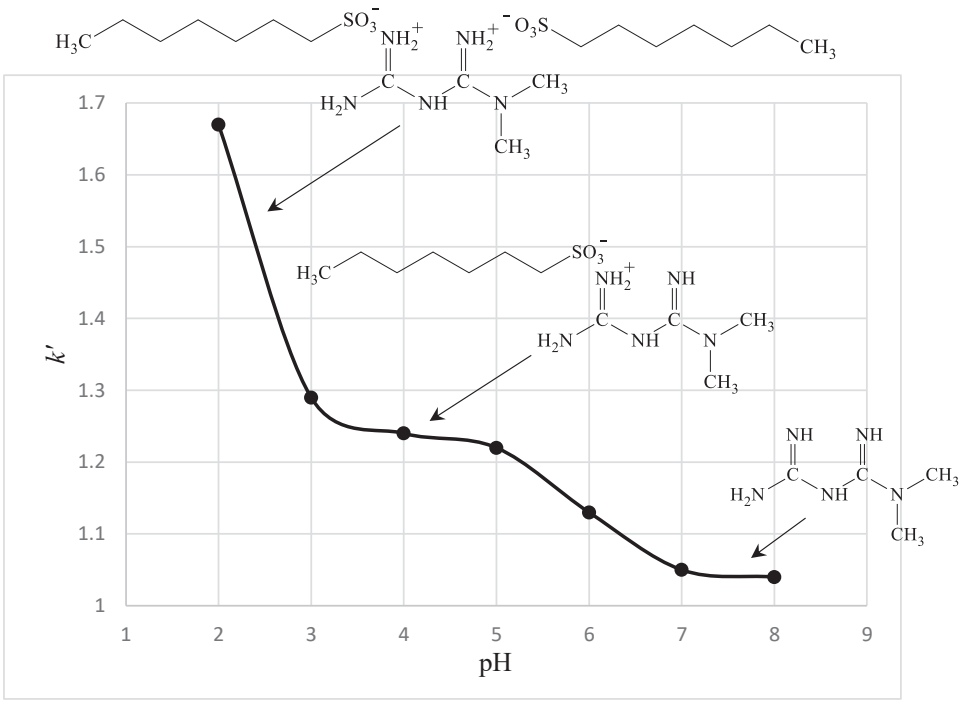
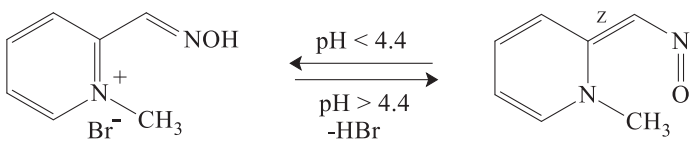


FIGURE 10.2.8 Dependence of the retention factor k' of metformin on the pH of the aqueous mobile phase in ion-pairing mechanism with sodium heptanesulfonate as IPA.

column with a mobile phase made of 35% MeOH +65% aqueous solution of 10 mM sodium heptanesulfonate +0.1% H_3PO_4 and the pH adjusted with 10% KOH solution is shown in Fig. 10.2.8 [26].

For strong acidic/basic or for ionic analytes, the influence of pH on the separation is less important, unless the analyte is involved in a structure modification that is influenced by pH. For example, the retention of pralidoxime in an IP separation, with sodium octanesulfonate as *IPA*, is strongly influenced by pH. This ionic compound has a structure that participates in the following equilibrium (reaction 10.2.34):



(10.2.34)

The retention of pralidoxime at neutral or basic pH of the mobile phase on a C18 column (Eclipse XDB C18) takes place according to a simple RP mechanism, even in the presence of sodium octanesulfonate (*IPA* reagent). In acidic conditions, in the presence of *IPA*, the retention takes place following the ion pair formation. This can be verified by studying at different pH values the variation of log k' for this compound as a function of the concentration of the organic modifier (MeOH%) in the mobile phase. For IP type retention (low pH), a linear dependence on the proportion of MeOH is noticed, while for RP retention (high pH), the dependence is not linear, proving the difference in the retention mechanism.

(7) Expression 10.2.33 also accounts for the fact that when i and *IPA* have the same charge ($z_i = z_{IPA}$), the value of the retention factor decreases with the increase of *IPA* concentration in mobile phase since a positive quantity is subtracted from $\ln k'(0)$.

As seen from the discussion based on electrostatic model and that based on formation of ion pairs in solution, both models describe parameters that influence the IP retention process, but some dependences are more obvious from one model (e.g., pH dependence from ion pair formation in solution) and other dependences more obvious from electrostatic model.

Stationary phases in ion pair chromatography

Ion pair chromatography allows in fact the increase of hydrophobic character of species to be separated, as compared to the initial analytes, making them amenable for separation on RP-HPLC type stationary phases. As an example, the direct analysis of amino acids can be performed using IP chromatography without derivatization or using other types of HPLC more adequate for the separation of polar or ionic compounds (e.g., HILIC or ion exchange). This is the case of the analysis of 19 amino acids from plant material performed on a Dionex Acclaim RSLC Polar Advantage II, 2.1 × 250 mm with 2.2 μm particles column (a polar-embedded RP column) using gradient obtained with two solutions, A: 1% acetonitrile/0.5% heptafluorobutyric acid/0.02% trifluoroacetic acid (TFA) in water and B: 0.1% TFA in acetonitrile with detection by MS/MS [27]. The procedure has a limit of detection LOQ between 0.8 and 18 ng/g. Extracted ion chromatograms obtained by this procedure for the amino acids (in the elution order) from a standard mixture containing about 0.05 μmol/L of compounds are given in Fig. 10.2.9 (actual procedure used two additional internal standards).

Chromatographic columns used in IP are selected based on the same rules discussed in Chapter 9 for RP-HPLC. The use of polar imbedded columns as described in the previous example may help in improving retention for compounds having in part a polar character.

Mobile phase in ion pair chromatography

The selection of mobile phase is very important in ion pair chromatography and it contains two components with different roles. One is the solvent, and the other is the ion-pairing agent (*IPA*) or the hetaeron. In practice, it is common to have *IPA* dissolved in water, possibly with other additives such as a buffer, to which is added the necessary organic modifier for the separation. The restrictions to the range of pH values for the buffer depend on the stationary phase and are identical to those for reversed phases, usually within the interval 2−9. IP can be performed either in isocratic or in gradient conditions. For gradient, all the components in the mobile phase can be modified, including organic modifier, *IPA* concentration, as well as the pH of the mobile phase [23].

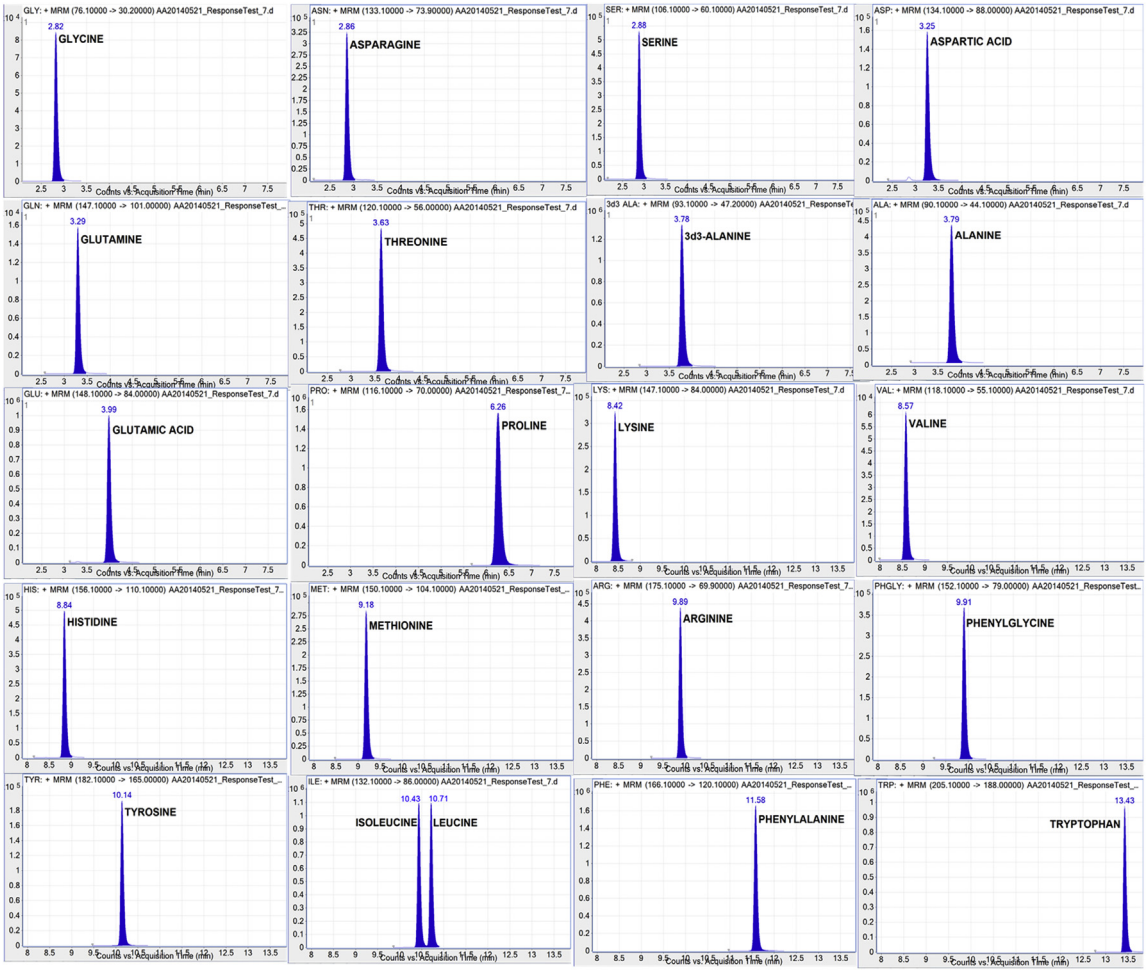


FIGURE 10.2.9 Extracted ion chromatograms for 19 amino acids + one I.S. (in the elution order) from a standard mixture containing about 0.05 μmol/L of compounds.

As the applicability of ion pair chromatography is extended from acidic and basic organic molecules to ionic species, even including inorganic ions, a large variety of ionic compounds were used as *IPA* able to form molecular association with such analytes. The *IPA*s are always selected to have the opposite charge to the analyte. The most common *IPA*s for acidic analytes are quaternary amines. The counterion of the quaternary amine can be sulfate, chloride, bromide, iodide, hydroxide, or dihydrogenphosphate. A number of alkyl substituents (hydrophobic chains) at the nitrogen can be chosen for these *IPA* molecules, and the most common ones are trimethylalkyl, withy alkyl ranging from C4 to C10. For basic compounds, typically used *IPA*s are sulfonates $R{-}SO_2{-}O^-$ and sulfates $R{-}O{-}SO_2{-}O^-$, with the R group alkyl, aryl (with alkyl side chain), and in some cases with other substituents [28]. Also, fluorinated

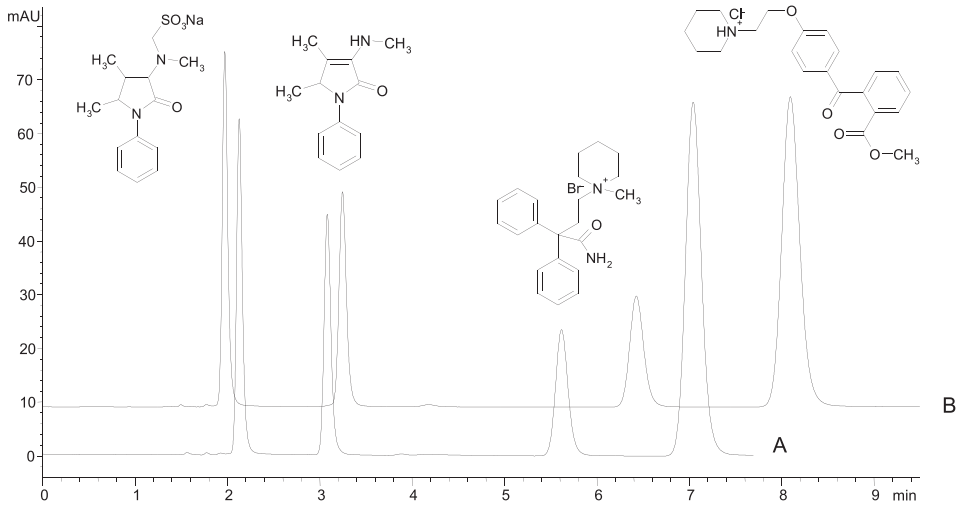


FIGURE 10.2.10 Comparison between retention of target compounds when the aqueous mobile phase (pH = 3) contains (A) both sodium hexane sulfonate and 1-butyl-1-methyl-pirrolidinium tetrafluoroborate (ionic liquid) at 10 mM/L and (B) only 10 mM/L sodium hexane sulfonate (Elution order: metamizole as Na salt; 4-methylaminoantipyrine; fenpiverine bromide; pitofenone hydrochloride) [31].

carboxylic acids $CF_3(CF_2)_nCOOH$ with $n = 0$ (TFA), $n = 1$, 2, 3, and 4, are common *IPA* for basic compounds. Among the used *IPA*s are also the sulfonated benzenes substituted with alkyl chains (C4–C8). The counterions for these reagents are usually either H^+ or Na^+ ions, but other counterions are possible.

Besides the typical *IPA*s, numerous other *IPA*s were reported in the literature [28]. For the separation of inorganic ions, for example, crystal violet can be used as *IPA*, the eluting order obtained on a hydrophobic column being $Cl^- < NO_2^- < Br^- < NO_3^- < I^- < SO_4^{2-} < S_2O_3^{2-}$, which is the same as that observed in anion exchange LC [29].

Several studies reported in the literature indicate the use of ionic liquids in IP mechanism (playing either the role of *IPA* or that of additive) [30]. Both the cationic and the anionic participants of the ionic liquid (IL) contribute to peak shape and to the improvement of resolution. The ion-pairing process can be affected such that changes in the retention time are seen upon the addition of ionic liquids in an *IPA* separation. This type of effect is illustrated in Fig. 10.2.10, which shows the separation of four pharmaceutical compounds (metamizole as Na salt, 4-methylaminoantipyrine, fenpiverine bromide, and pitofenone hydrochloride) on a C18 column with water as a mobile phase (pH adjusted to 3) and sodium hexane sulfonate at 10 mM/L as *IPA* (trace B in Fig. 10.2.10). The addition of 1-butyl-1-methyl-pirrolidinium tetrafluoroborate (an ionic liquid) at 10 mM/L in the separation produced the change shown in trace A in Fig. 10.2.10 compared to trace B. In the separation shown in trace B, sodium hexane sulfonate does not affect the retention of metamizole as Na salt, while the addition of the ionic liquid increases its retention. The effect of the addition of the ionic liquid on the other analytes is a shortening of the retention time [31].

The organic modifiers in IP are very similar to those used in RP-HPLC. Methanol and acetonitrile are the most utilized organic components of the mobile phase, although other solvents

were reported in the literature to be useful for IP separations [32]. The effect of the organic modifier is similar to that of the organic component in RP separations. However, the change of organic content in the mobile phase also causes changes in the mobile phase dielectric constant, which can influence the strength of the ionic interactions between charged analyte and *IPA*. According to the electrostatic model, the effect of the nature and concentration of the organic modifier in mobile phase is found implicitly in the values of $k'(0)$ and K_{IPA} in Eq. 10.2.33. For most of the situations, this dependence of log k' on % organic modifier is linear (see Eq. 9.5.2), but for some compounds, the dependence is polynomial. Two examples are illustrated in Fig. 10.2.11 where three biguandines separated by IP mechanism follow a linear regression between log $k'$and the content of methanol in the mobile phase, while the fourth analyte (ranitidine) follows a quadratic regression. The column used for the separation shown in Fig. 10.2.11 was a Zorbax Eclipse XDB 150 mm length, 4.6 mm i.d. and 5 μm particle size, the *IPA* agent was 10 mM $C_8H_{17}SO_3Na$ in water adjusted to pH = 2 with H_3PO_4, the flow rate was 1 mL/min, and the injection was 20 μL of a solution containing 200 μg/mL of each analyte.

The dependence of retention factor log k' on the content of the organic modifier in mobile phase can be significantly deviating from linear. As an example, bile acids sodium taurocholate, sodium taurodeoxycholate, and sodium taurochenodeoxycholate can be used as *IPA* for the analysis of pralidoxime, obidoxime, and pyridostigmine [33]. The dependence of log k' for pralidoxime on the content of methanol in the mobile phase for these three different bile acids used as *IPA* is shown in Fig. 10.2.12.

Dependencies with a U shape of log k' as a function of organic phase composition C_o% have been encountered in other ion pair separations [34]. This nonlinear dependence of log k' with increase in organic mobile phase content is caused by the different effects of the solvent on the values of various terms in Eq. 10.2.33, such as K_{IPA}, x_{IPA}^{st}, and κ (Debye length given by Eq. 10.2.23 and modified through ε_{mo}).

The pH of the mobile phase plays an important role in the formation of ion pairs and in their

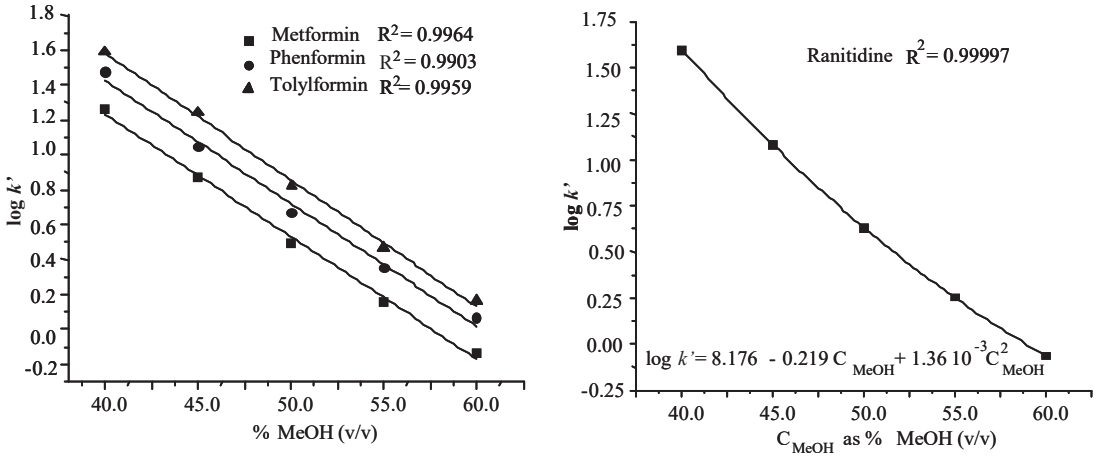


FIGURE 10.2.11 Linear and polynomial dependences of the capacity factor log k' on the methanol percentage (v/v) in mobile phase composition for IP mechanism applied to the retention of four polar compounds.

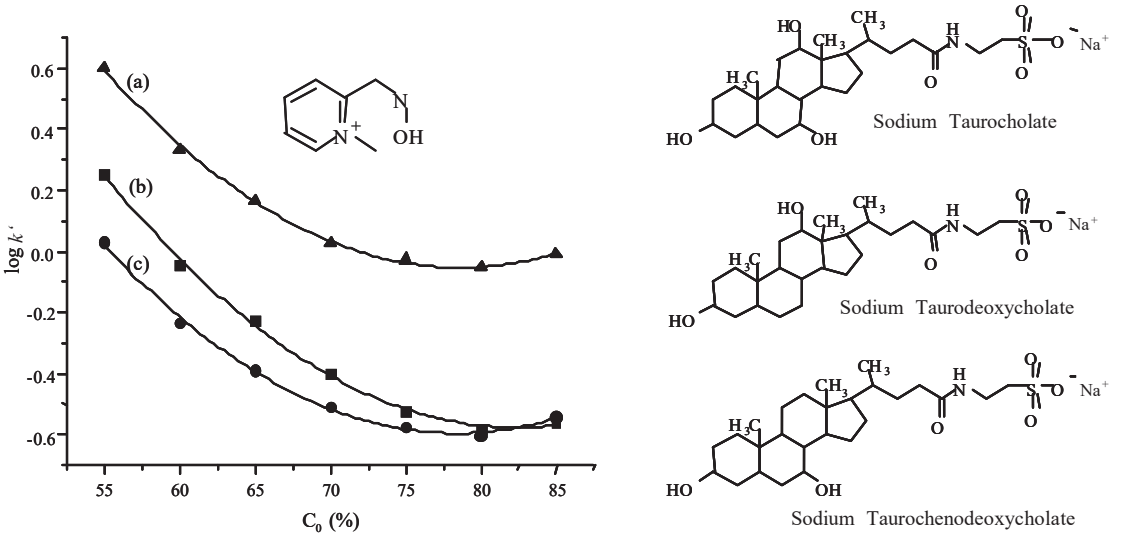


FIGURE 10.2.12 Dependences of log k' on methanol concentration (C_o) in mobile phase for pralidoxime cation (2-[(hydroxyimino)methyl]-1-methylpyridin-1-ium), and different ion-pairing agents: (A) sodium taurochenodeoxycholate; (B) sodium taurodeoxycholate; (C) sodium taurocholate.

stability. The retention factor k' in IP is strongly dependent on pH as previously indicated. For this reason, the mobile phase pH must be properly adjusted for achieving the optimum separation. This adjustment can be done using buffers or by simple addition of acids or bases. For example, in the separation of amino acids described in Fig. 10.2.9, TFA is used for pH adjustment. Other additives have been used in the mobile phase for improving IP separation. Among these can be mentioned the addition of EDTA or of potassium tetrakis (1H-pyrazolyl) borate as chelating agents [23].

Detection based on UV absorption is widely used in IPC. Therefore, ion pair agents must have a low UV cut-off wavelength. The UV absorption of sodium alkanesulfonates and quaternary ammonium salts is low for typical UV range of detection in HPLC. A higher absorption takes place when ion liquids containing pyridinium or imidazolium moieties are used. Fluorescence is also frequently used, and most ion-pairing reagents do not affect it negatively.

A number of ion pair reagents used in IP-LC separations of highly polar analytes are not suited for use in LC-MS. These reagents are nonvolatile and reduce the detection sensitivity. When MS detection is employed, volatile IP reagents such as aliphatic amines or perfluorinated organic acids at the lowest possible concentration in mobile phase are recommended. TFA, pentafluoropropanoic acid, and heptafluorobutanoic acid have commonly been used in the analysis of polar basic compounds. These ion-pairing agents form relatively stable ion pairs with basic compounds, decreasing the secondary interactions between free silanol groups of the stationary phase and resulting in decreased peak tailing, improved resolution, and better retention. However, acidic ion-pairing agents may suppress ionization in the MS source. Alkylamines (triethylamine, N,N-dimethyl-n-butylamine, N,N-dimethylhexylamine, or tri-n-butylamine) are commonly used as ion-pairing reagents in RP-LC with negative ion ESI/MS detection of acidic compounds, such as nucleoside

mono-, di-, and triphosphates and sulfonates, sulfates, sulfonated dyes, and halogenated acids [35].

The evaporative light scattering detector is another example of detector that requires volatile mobile phases, and the choice of *IPA* and buffers with this detection must avoid the nonvolatile additives such as alkylsulfonates.

Chaotropes in ion pairing

The addition of chaotropes in the mobile phase for IP is also practiced in certain separations [36]. These are used in place of usual ion pairing agents and include agents such as BF_4^-, ClO_4^-, or PF_6^-. Chaotropes have the capacity to disrupt the polar intermolecular forces such as those with water. These reagents are likely to form strong associations with polar components, such that the salts analyte/chaotrope have lower interaction with the mobile phase and can be retained on hydrophobic stationary phases. It is also possible that the solvation shell that typically surrounds the ionic analytes is disrupted by the addition of chaotropes that interact with the water. This solvation shell can suppress the ability of the analyte for hydrophobic interaction with stationary phase leading to a decrease of the retention [37–39]. If the mobile phase contains counteranions (chaotropes) with high polarizability and a low degree of hydration, they can disrupt the solvation shell of the positively charged basic analytes, and thus, they enhance the ability of the ion associated complex to interact with stationary phase.

Chaotropic effect of different anions (BF_4^-, ClO_4^-, or PF_6^-) on the retention of positively charged basic analytes is however different from the salting-out effect. The addition of salts changes the characteristics of the polar solvents (e.g., water) by increasing the polar interactions inside the solvent, decreasing the number of solvent (water) molecules available to interact with the charged part of the solute, and diminishing the interaction of the solvent with the solute. Some weakly hydrated anions such as SCN^- are more effective to the partition to an organic phase as an ion pair with a cation than strongly hydrated anions, such as Cl^- [40]. For proteins that were initially soluble in water, salting-out produces an increase in the hydrophobic interactions between the proteins and leads to their precipitation. Certain ions have a stronger salting-out effect than other regarding protein solubility, and a specific order was established (Hofmeister series) [41]. The Hofmeister effect is stronger when the charge delocalization and polarizability are higher. Anions appear to have a larger effect than cations, and are usually ordered as follows: $F^- \approx SO_4^{2-} < H_2PO_4^- < HCOO^- < CH_3SO_3^- < Cl^- < NO_3^- < CF_3COO^- < BF_4^- < ClO_4^- < SCN^- < PF_6^-$. For the cations, the series is as follows: guanidinium $> Ca^{2+} > Mg^{2+} > Li^+ > Na^+ > K^+ > NH_4^+$. The chaotropicity of an inorganic additives is related to its position in the Hofmeister series [36,42].

10.3 Hydrophobic interaction chromatography (HIC)

General comments

Besides RP-HPLC, several types of chromatographic techniques involve in the separation hydrophobic interactions. One such technique is hydrophobic interaction chromatography (HIC). In HIC, involved in the interaction process are the hydrophobic moieties of a molecule with solvent-accessible nonpolar groups (hydrophobic patches), the surface of the hydrophilic stationary phase, and the polar solvent. The molecules typically separated by this technique are certain biopolymers. The stationary phase in HIC can be either silica or an organic polymer with a hydrophilic coating on which are attached hydrophobic ligands such as short chain alkyl groups or phenyl groups. The modification of mobile phase polarity in HIC by addition of

inorganic salts enhances the adsorption of hydrophobic areas of the analyte at the hydrophobic areas on the stationary phase. The interactions involved in this type of separation are more complex, but the hydrophobic interactions play an important role in the separation. In protein separation, HIC is a useful technique since it typically does not modify the protein conformation. Besides analytical applications, HIC is widely used for the purification of proteins [43].

Retention mechanism in HIC

The retention of proteins on HIC stationary phases is influenced by kosmotropic salts, such as $(NH_4)_2SO_4$, added to the mobile phase, and also by pH. The effect of kosmotropic salts is to reduce protein solubility in mobile phase, facilitating the reversible interactions between hydrophobic patches of proteins with hydrophobic moieties of the stationary phase (e.g., butyl-silica, phenyl-silica, or methyl-, butyl-, hexyl-, or phenyl-Sepharose [44]). The presence of ions in the bulk of mobile phase disrupts the H-bond between protein and surrounding water molecules, and thus the protein can interact stronger with the stationary phase surface. Therefore, the partition equilibrium of proteins between mobile and stationary phase is influenced by the salt concentration in the mobile phase. For this reason, HIC is also known as "salting-out chromatography" [45]. The retention of protein on stationary phase surface is favored by high salt concentration, while the elution of proteins takes place at low salt concentration. In practice, the separation of proteins by HIC is performed in gradient mode, with an elution program operating with a high salt concentration at the beginning and a low salt concentration in the last part. Experimentally, it has been shown that the number of water molecules disrupted from the protein structure modified by pH change is larger for aromatic stationary phase surfaces (e.g., phenyl Sepharose) than for aliphatic surfaces (e.g., butyl-Sepharose) [46].

In HIC, the dependence between the logarithm of the retention factor of proteins (k') and salt concentration in mobile phase is linear and can be used to predict the gradient program used in applications [47]. Van't Hoff plots for temperature dependence of the retention in HIC are usually nonlinear, showing the complexity of the retention mechanism [48]. Enthalpy change (ΔH), entropy change (ΔS), and heat capacity change (ΔCp) assigned to the adsorption process of proteins on the stationary phase surface are temperature dependent, and could be calculated by Eqs. 4.6.5, 4.6.19, and 4.6.20, and show that other processes may occur simultaneously with adsorption of proteins on stationary phase [49,50].

10.4 Micellar, microemulsion, and other types of HPLC performed on hydrophobic phases

Micellar liquid chromatography

Micellar liquid chromatography (MLC) is a technique applied on hydrophobic stationary phases with the mobile phases and aqueous solutions of a surfactant at a concentration above the critical micelle concentration (CMC) [51,52]. The increase of surfactant concentration above CMC leads to the increase of the concentration of micelles in the mobile phase, whereas the concentration of unassociated surfactant molecules remains constant. The unassociated molecules of surfactant are also involved in the adsorption equilibrium on the stationary phase surface that has consequently new retaining characteristics. The analyte participates to a more complex partition process, when its molecules are involved in the partition between mobile phase and stationary phase (primary equilibrium) and in the partition between mobile phase bulk and micelles (secondary equilibrium). These equilibria are

affected by the nature of analyte and surfactants, by the concentration of surfactants, the nature and concentration of organic additives (e.g., acetonitrile, alkanols), ionic strength in the mobile phase, temperature, and pH [53].

MLC is similar to ion pair chromatography where the *IPA* agent is a surfactant. Surfactants have a hydrophobic moiety and a polar group acting as amphiphilic molecules. Separation efficiency of MLC is not always very good, but the technique is superior to ion pair LC and ion exchange LC for the separation of charged molecules and mixtures of charged and uncharged ones. In addition, the technique uses mainly water as a mobile phase and can be considered a "green" technique [54]. Surfactants are characterized by specific parameters such as the CMC, Kraft point, and/or cloud point. CMC is the concentration of the surfactant above which the micelles are formed. A suitable surfactant for MLC should have a low CMC. A high CMC would require to work at a high surfactant concentration, which would create viscous solutions giving undesirable high system pressure [55]. Kraft point is the temperature at which the solubility of a surfactant is equal to CMC. Below the Krafft point temperature, the solubility of a surfactant is low and the solution contains practically no micelles. Chromatographic work in MLC should be performed above the Krafft point to avoid surfactant precipitation. Another surfactant parameter characteristic for a nonionic surfactant is the cloud point defined as the temperature above which phase separation occurs for a surfactant solution. Chromatographic work should be conducted below the cloud point (e.g., for 1%−6% aqueous solutions of Brij-35, it is 100°C). Three examples of surfactants used in MLC are given in Table 10.4.1.

Retention behavior in MLC is controlled by solute partitioning from the bulk solvent into micelles and into stationary phase as well as on direct transfer from the micelles in the mobile phase into the stationary phase [56]. The retention of more polar compounds is determined by their partitioning from the bulk aqueous phase into micelle and hydrophobic stationary phase [57,58]. Separation of a variety of strong polar or ionic molecules can be achieved using MLC (e.g., Ref. [59]). The main problem in MLC is the decrease of column efficiency due to slow mass transfer from the stationary phase which can be attributed to the poor wetting of the stationary phase with a purely aqueous mobile phase as well as to the adsorption of monomer surfactants that change the characteristics of the alkyl-bonded stationary phases [60]. To enhance the efficiency in MLC, three main approaches have been adopted: (1) addition of small concentrations of organic modifiers to the micellar mobile phase, (2) increase of the column temperature, and (3) decreasing the flow rate. The addition of propanol, butanol, pentanol, methanol, or acetonitrile improves MLC efficiency. The technique is sometimes indicated as hybrid micellar chromatography (hybrid MLC) [61]. These modifiers increase the elution strength and often improve the shape of the chromatographic peaks. Also, the addition of triethylamine to a micellar mobile phase in combination with organic modifier enhances the efficiency over organic modifier added alone. The increase in the temperatures

TABLE 10.4.1 Characteristics of some common surfactants used in MLC separations.

Surfactant	Type	CMC (mM)	Kraft or cloud point (°C)
Sodium dodecyl sulfate (SDS)	Anionic	8.2	10
Cetyltrimethylammonium bromide (CTAB)	Cationic	0.83	26
Polyoxyethylene 23 lauryl ether (Brij-35)	Nonionic	0.06	100

favors the increase in the kinetics of mass transfer and enhances the column efficiency such that it becomes comparable with conventional RP-HPLC. The decrease in the flow rate has the role to allow longer time for the equilibrium.

Microemulsion liquid chromatography

Microemulsion liquid chromatography (MELC) uses an oil-in-water microemulsion or a water-in-oil microemulsion as the mobile phase [62]. The two variants of MELC are used in two different HPLC mechanisms: water-in-oil microemulsion for normal-phase separation and oil-in-water microemulsion for reversed-phase separation [63]. The microemulsions are typically obtained from water and a water-immiscible solvent such as cyclohexane, octane, octanol, or ethyl acetate. A surfactant and a cosurfactant are also necessary for emulsion stabilization. Similar to MLC, the stationary phase adsorbs the organic phase and the retention characteristics of the phase are modified. The separation process is complicated by an additional partition of the analytes from both mobile and stationary phases into the microemulsion droplets such that the retention of specific analytes is different from direct RP-HPLC (or NPC). MELC type separation can be used in both isocratic and gradient elution modes [64]. Retention, efficiency, and selectivity in MELC, under isocratic or gradient elution modes, can be modified by changing the concentration of the microemulsion components and the ratio between the aqueous and oil phases [65].

Other liquid chromatography types on hydrophobic phases

Some less common HPLC types can also be performed on hydrophobic phases. One such type is surface-bubble-modulated liquid chromatography, which contains a stationary gas phase in a column packed with alkyl-bonded silica. This technique has a hybrid stationary phase consisting of a gas phase, the alkyl-bonded layer, and an aqueous/alkyl chain interface [66].

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CHAPTER

11

Hydrophilic interaction liquid chromatography

11.1 Retention and elution process in HILIC

General comments

The compounds with a high polar character (very low or negative log K_{ow}) are poorly retained on hydrophobic stationary phases. The compounds that are polar, acidic, or basic can be separated using ion pair chromatography (IPC) (see Section 10.2), but another alternative leading to excellent separations is the use of hydrophilic interaction liquid chromatography (HILIC). In HILIC, the stationary phase is polar and the mobile phase is less polar than the stationary phase [1]. Compounds with a wide range of polarities can be analyzed by this technique. Based on octanol/water partition coefficient for polarity characterization, compounds with log K_{ow} in the (approximate) range between −5.55 and +0.55 can be analyzed using HILIC which includes various types of molecules. Examples of four compounds with log K_{ow} values in this range are shown in Fig. 11.1.1.

Polar compounds can also be analyzed using other types of chromatography performed on polar phases such as normal phase chromatography (NPC) or aqueous normal phase chromatography (ANPC or ANP). The main difference between HILIC and NPC is the utilization of different types of mobile phase, organic polar with some water in HILIC, and organic with no water in NPC. The total absence of water in NPC and the presence of water in HILIC may lead to a difference in the retention mechanism. Both techniques base their separation on polar interactions, but in HILIC the hydrophobic interactions still have some role in the separation, while this is (almost) absent in NPC. ANP is different, being practiced on hydride columns with a wide range of solvent polarities. High polarity phases are also those used in ion exchange, but the separation process in ion exchange is different (and is discussed in Section 13.1). The separation on ANP phases is not very well understood.

The combined partition and adsorption equilibriums for HILIC separations are also reflected in the equation showing the dependence of log k' on temperature. For a partition process, this dependence is given by van't Hoff type equation (Eq. 4.6.2) written once more below:

$$\log k' = a' + \frac{b'}{T} \qquad (11.1.1)$$

For HILIC separations, the temperature dependence of log k' showed deviations from Eq. 11.1.1 and better fits with experimental data were obtained, for example, by the addition

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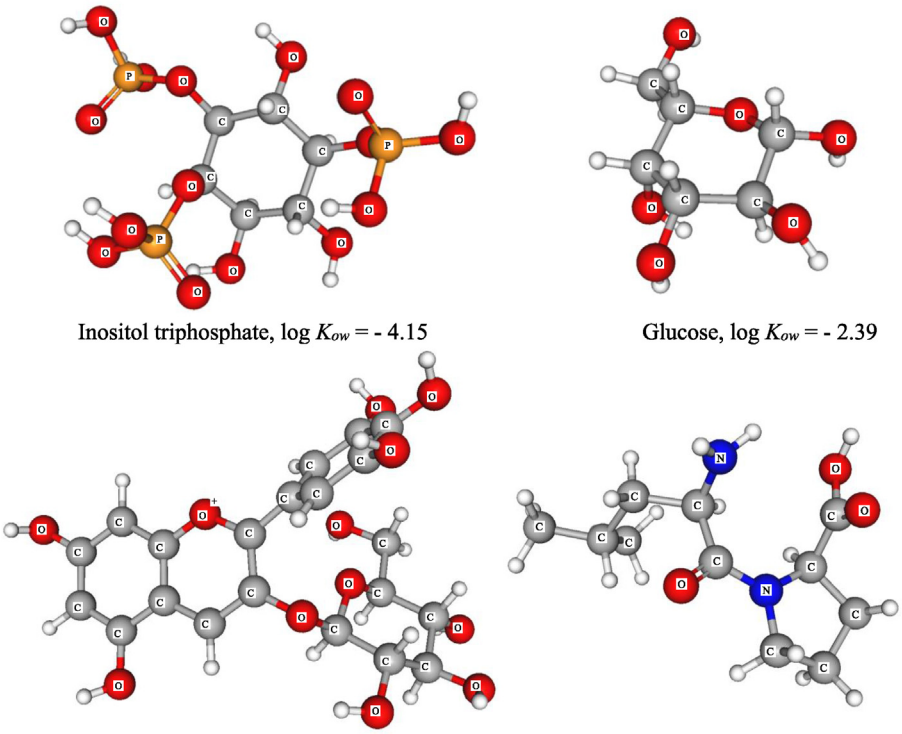


FIGURE 11.1.1 Examples of compounds that can be analyzed using HILIC.

of a quadratic term (c'/T^2) to Eq. 11.1.1 [2,3]. This effect is explained by multiple retention mechanisms in the separation system or due to the existence of the multiple forms of the analyte [4,5].

Equilibrium type for retention/elution in HILIC

The equilibrium type being adsorption or partition in HILIC is more difficult to assess than for RP-HPLC. Both types, adsorption or partition, or a combination of them may offer models for retention [6]. The results indicated that the relative importance of adsorption and partitioning equilibria depends in a complex way on analyte properties and experimental parameters and cannot be generally predicted. Some insight on clarifying if the equilibrium in HILIC is adsorption or distribution can be obtained by studying the dependence of log k' on water content in the mobile phase (see Section 4.1 and 4.2). In HILIC, the retention factor log k' decreases when the water content in the mobile phase increases and this variation will be analogous with the variation of log k' in RP-HPLC versus the volume fraction of an organic component. Using the notation ϕ_w for the volume fraction of water in the mobile phase, a typical variation of log k' (which decreases) as a function of log ϕ_w (which increases) in HILIC is shown in Fig. 11.1.2.

As shown in Section 4.2 (see Eq. 4.2.14), linearity between log k' and log ϕ_w is expected for adsorption equilibrium following Soczewinski equation and this is the case for a specific interval of water concentrations as shown in Fig. 11.1.2 [7,8].

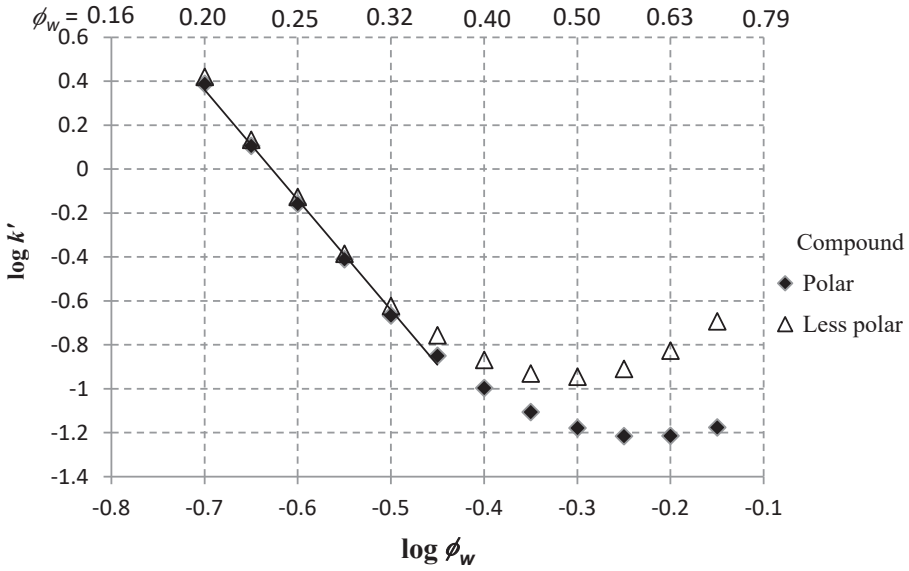


FIGURE 11.1.2 Variation of log k' with the amount of water (ϕ_w is volume fraction of water) into the mobile phase in an HILIC separation for a polar compound and a less polar one, where the partition process and the adsorption process can be both present.

However, the linear interval of dependence of log k' on log ϕ_w is not valid for the whole water concentration interval and partition can be considered as also involved in the process. Additional work regarding the adsorption of water on HILIC columns indicated considerable deviation from linearity for its adsorption Langmuir isotherms [9].

Description of molecular interactions in HILIC

The types of molecular interactions for the separations on polar stationary phases HILIC include the polar interactions, hydrogen bonds formation, charge transfer, and also ionic interactions. In HILIC, added to these types of interactions are the hydrophobic interactions, which also may play a role in the retention process [10,11]. Based on thermodynamic concepts, the expression for log $K(X)$ in HILIC is given by a formula identical to rel. 4.1.10, which can be written in the following form:

$$\log K(X) = -\frac{\Delta A^0}{2.302\,RT} \qquad (11.1.2)$$

As indicated in Section 9.1, the free enthalpy ΔG^0 (see Eq. 4.1.10) can be taken as equal to the free energy of the process ΔA^0 when no volume changes occur during the process.

For the evaluation of ΔA^0, the basic molecular interaction including solvophobic ones described in Section 5.1 will offer a start to understand the retention/elution in HILIC. In order to avoid the assumption that the separation in HILIC is based on partition (as it is done in RP-HPLC), formally, the retention process in HILIC can be described by an equilibrium of the following type:

$$X + L \rightleftarrows XL \qquad (11.1.3)$$

where X is the analyte, L is the bonding component of the stationary phase, and XL is the union of the analyte and the bonding component. The evaluation of the change in free energy ΔA^0 for the process is given by the following formal expression:

$$\Delta A^0 = A_{gas} + \sum_{i=X,L,XL} A_{i,S} \qquad (11.1.4)$$

In Eq. 11.1.4, A_{gas} represents the energy of binding the analyte X with component L in the absence of solvating medium (i.e., in gaseous state) involving various types of interaction contributing to the retention process. As discussed in Section 5.1, the interactions of van der Waals type in a medium with dielectric constant ε are given by E_T expressed by Eq. 5.1.46 (in the absence of ions). To this energy, that of hydrogen bonding, ionic interactions, and steric effects (indicated below as E_{other}) must be added if present, such that the value of A_{gas} is given by the following formula:

$$A_{gas} = E_T + E_{other} \qquad (11.1.5)$$

An exothermic process (favoring the displacement of the equilibrium 11.1.2 to the right) takes place with negative values for the free energy A and the values for E_T and E_{other} are negative.

For the evaluation of $A_{i,S}$ that describes the interaction energies with the solvent S, the solvophobic theory can be used. For each component from the equilibrium 11.1.2 present in solution, an expression of the form given by Eq. 5.1.60 is valid. As a result, the total value for ΔA^0 can be expressed as follows:

$$\Delta A^0 = E_T + E_{other} + A^{cav}_{XL,S} + A^{vdW}_{XL,S} - \left(A^{cav}_{X,S} + A^{vdW}_{X,S} + A^{cav}_{L,S} + A^{vdW}_{L,S}\right) - RT\ln\left(RT/p_0V_S\right) \qquad (11.1.6)$$

Retention results based on molecular interactions in HILIC

Eq. 11.1.6 does not provide a base for the calculation of log k' in HILIC but offers guidance regarding the contributions to retention in these types of HPLC. The larger (in absolute value) are the energies E_T and E_{other}, the larger (in absolute value) will be ΔA^0, and the larger will be the equilibrium constant $K(X)$. From Eq. 5.1.46, it can be seen that the values for E_T are higher when the dipole moments and polarizabilities of the interacting molecules are higher and when the dielectric constant ε of the solvent is lower. The polar molecules are also frequently capable of establishing hydrogen bonds, electron donation or withdrawal interactions, and ion exchange type interactions (expressed in E_{other}). These interactions may play a major role in the values of ΔA^0 and should be considered when evaluating the potential of an analyte to elute ahead of another. For this reason, the separation process in HILIC can be considered as taking place based on polar interactions, hydrogen bond formation, and ion exchange type interactions. Also, the geometry of the molecule may be important for determining the strength of the interaction of a molecule with the polar phase and steric hindrance affecting how close a polar group may come to the interacting ligand from the stationary phase. Due to the fact that polarity of the analyte molecule is affected by the position of the group in the molecule, by the donor withdraw electronic effects, as well as by the steric hindrance, separation of isomers is in general more successful when the separation is based on polar interactions.

For the interactions with the solvent S, the cavity and van der Waals free energies have their own contribution to the separation process with the A^{vdW} playing a more important role as

compared to its role in RP-HPLC and with A^{cav} a less important role. In HILIC separations, the log k' values for numerous polar compounds are for different stationary phases in the following order: silica > amino > diol > cyano, which corresponds to the stationary phase polarity. Since more polar solvents lead to larger $A^{vdw}{}_{X,S}$ (in absolute value), this type of solvent will have the effect of a decrease in log k' (or retention time of the analyte). In the theory developed for RP-HPLC separation, the evaluation of the contribution of van der Waals interactions between the solute X and the solvent S was expressed by the term $\boldsymbol{c}_X m_X^2$ and $\boldsymbol{d}_X\ \alpha_X$ in Eq. 9.1.7. For the case of interactions with polar molecules, these terms play a more important role as the molecules separated in HILIC are more polar. This is in agreement with the fact that more polar solvents will decrease the retention time of the solute. The same result is obtained from rel. 5.1.56, which indicates that the analytes with a larger A_i^{SASA} have a stronger interaction with the solvent. The analysis of the values for $A^{cav}{}_{X,S}$ indicates that the solvents that are more polar will lead to higher absolute values for this term and therefore smaller log k' values (faster elution).

For HILIC, the solvent molecules having polar character, including water as a mobile phase component, will have a considerably higher $A^{cav}{}_{X,S}$ in comparison with NPC, showing in this respect similarity with RP-HPLC. For this reason, hydrophobic interactions may be considered as having contribution to HILIC separation. This effect is more pronounced when the interaction among the solvent molecules is stronger than the one between the solvent and solute molecules. Since HILIC separations imply the presence of water in the mobile phase, various studies showed that the adsorbed water on the stationary phase plays an important role in HILIC separations; it is estimated that the occupied volume of water-rich layer represents 4%–13% of the pore volume of a silica stationary phase when using mobile phases with 95%–70% acetonitrile [7,12]. The change in the mobile phase composition from a high content of water to a low (or very low) content of water, when using a polar stationary phase, may lead to a shift from HILIC type separation to an NPC type separation. For example, for a separation on a silica column for the mobile phase containing up to about 70% acetonitrile (ACN) the separation can be considered as HILIC type and when the content of ACN is between 70% and 100% can be considered NPC with the diminishing/disappearance of hydrophobic interactions.

Among the types of stationary phases used in HILIC are the zwitterionic ones. Separations on these columns are sometimes indicated as zwitterion chromatography or ZIC. In ZIC, since the stationary phase contains dissociable groups, it is likely that ion–dipol (or ion partial charge) interactions are also contributing to the values of ΔA^0. Since such interactions are in general stronger than van der Waals forces not involving ions, it is possible that they dominate the retention process [13,14].

The previous analysis of the interaction energies determining the value for log k' in HILIC shows that the expected elution in this type of separation should be opposite to that in RP-HPLC. Relatively good negative correlation has been reported regarding log k' values for a series of compounds separated by both NPC (which is very similar to HILIC) and RP-HPLC [15]. However, since the factors that contribute to the value of $K(X)$ in NPC (and HILIC) are not directly related with the factors that were determining the expression of ΔA^0 in RP (see rel. 9.1.3), the R^2 value for such correlations is still modest.

Comparing HILIC/NPC on the one hand with RP-HPLC on the other, the theory as well as the practical results indicate that more polar phases in HILIC/NPC retain more strongly the more polar analytes, while the

more hydrophobic phases in RP-HPLC retain more strongly the more hydrophobic compounds. Regarding the mobile phase, the more polar mobile phases lead to a faster elution in HILIC/NPC, while in RP-HPLC the less polar solvents lead to a faster elution.

11.2 Polar stationary phases and columns

General comments

Polar stationary phases are used in hydrophilic interaction chromatography (HILIC) and also in NPC, as well as in ANPC or ANP. However, in HILIC are fewer available columns as compared to RP-HPLC. This lack of column availability is continuously being eliminated, and various HILIC columns are now commercially offered [16].

HILIC chromatography can be performed on silica, but bonded phases containing terminal polar groups such as aminopropyl, diol bonded, amide bonded, peptide bonded, etc., are now common phases for HILIC. Cyano (e.g., cyanopropyl) phases can also be included in the polar phases category (see log K_{ow} in Table 8.2.5), although they are used in RP chromatography with a mobile phase more polar than the bonded phase of the column. Some cyano RP phases were indicated in Section 9.2. NPC is currently practiced mainly on silica, although other polar stationary phases were used in the past, such as alumina, magnesia, etc. The polar phases are typically characterized by their polar groups (such as silanol in the case of silica) and by their surface that is wettable with polar solvents such as water. Several types of polar bonded moieties on silica and on polymeric substrates used in HILIC and NPC chromatography are indicated in Table 11.2.1 [17].

The polarity of the phase is the highest for silica, followed by phases with weak ion exchange character (anion, cation, zwitterions) such as amine phases and followed by diol, amide, and cyano.

TABLE 11.2.1 Several types of polar stationary phases used in HILIC and NPC chromatography.

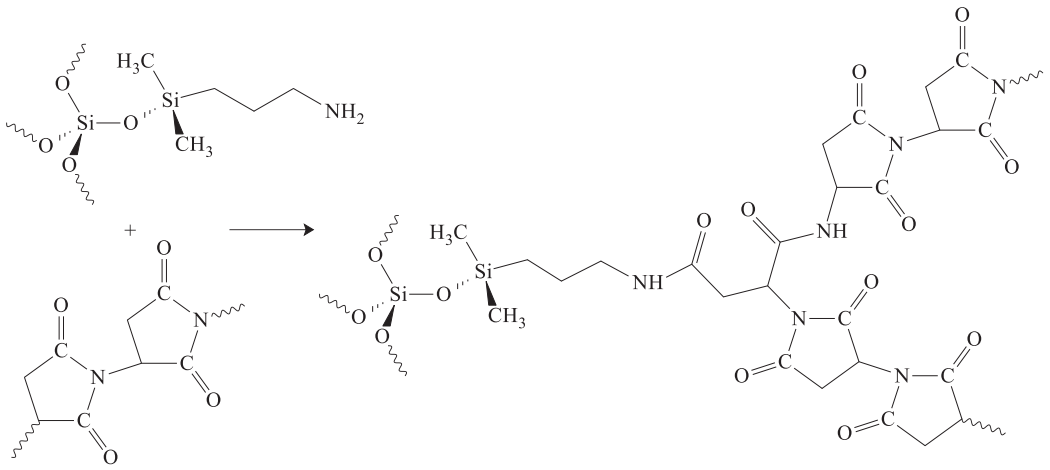
Type of phase	Phase
Silanol	Bare silica
Diol on silica	Diol, ether embedded, and diol
Amide on silica	Amide terminal, polyamide
Weak polar on silica	Cyano, perfluorinated (also used in RP mode)
Weak anionic on silica	$-C_3H_6-NH_2$, diethylamine, triazole, etc.
Weak cationic on silica	Sulfonylethyl, etc.
Zwitterionic on silica	Amino-sulfonic, amino-carboxylic
Mixed mode on silica	Both polar and hydrophobic groups
Weak anionic polymeric	Different polar groups on porous polymers

As discussed in Section 11.1, the interactions between the stationary phase and the solute (analyte) in HILIC and NPC are stronger for more polar compounds. Since the polarity of the isomers (not chiral isomers) is frequently very different, for example, because the polar groups are positioned in different parts of the molecule, the interactions with the stationary phase are different from isomer to isomer. This explains the efficiency of polar phases in isomer separation. Besides the polar group position, other effects such as steric hindrance and additional effects such as propensity to form hydrogen bonds or having electron donation or withdrawal interactions may affect the differences in retention.

Specific procedures for the synthesis of polar phases

Many polar stationary phases and columns are obtained by the general procedures indicated

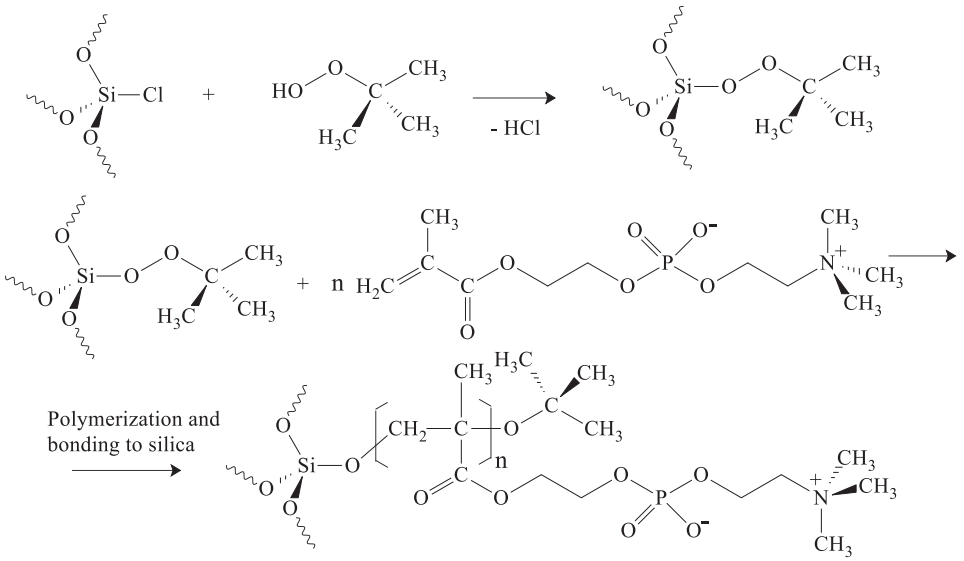
in Section 8.1 also used for obtaining hydrophobic phases, the difference being that the active phase has a polar character. For silica derivatization, for example, reagents such as γ-aminopropylmethyldiethoxysilane, dihydroxypropyltriethoxysilane, or γ-glicidoxypropyltrimethoxysilane were used, as indicated in Table 8.1.5. Such procedures are used, for example, for making common polar stationary phases such as those containing diol or amino groups. Other procedures were specifically developed to generate polar phases. One such procedure starts with the preparation of a bonded polysuccinimide on silica by reacting this compound with aminopropyl silica, as shown schematically in reaction 11.2.1.



(11.2.1)

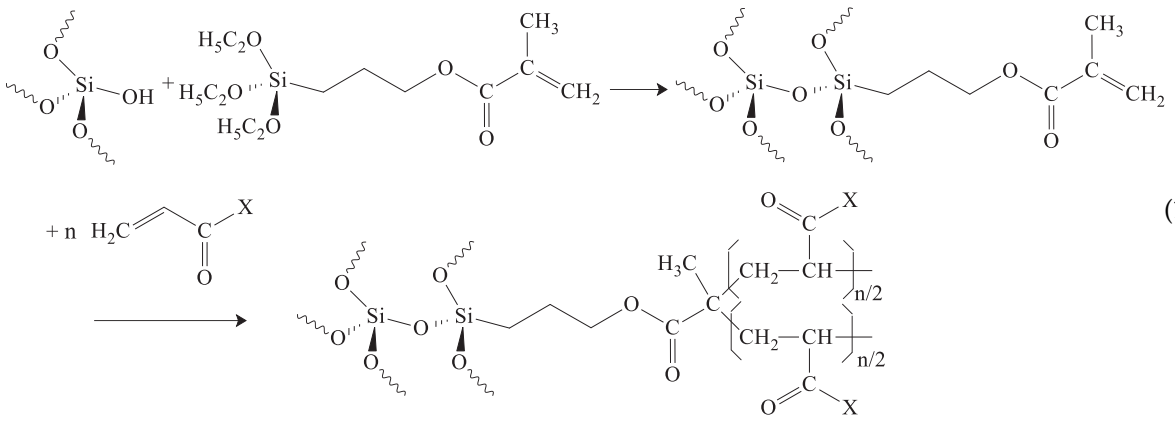
Parts of the poly(succinimide) rings are opened to form amide bonds with the aminopropyl moieties. It creates in this way a polymeric type phase bonded on silica. Only a fraction of the succinimide rings are engaged in this bonding, and the rest of the rings remain intact and susceptible to reaction with nucleophiles, which can be used to produce a variety of functional silicas [10]. The poly(succinimide)-silica material can further undergo an alkaline hydrolysis in water to form a poly(aspartic acid) bonded phase, can suffer hydrolysis in the presence of aminoethanol to form poly(2-hydroxyethyl)aspartamide bonded phase, and in the presence of aminoethansulfonic acid to form poly(2-sulfonylethyl)aspartamide bonded silica. The poly(sulfonylethyl)aspartamide silica phase displays a zwitterionic character.

Another phase with zwitterionic character can be obtained from a phosphorylcholine type vinyl type graft connected to the silica surface that was initially derivatized to have a peroxy bridge bound on the silica surface [17]. The reactions leading to this polar stationary phase are indicated below (Eq. 11.2.2):



(11.2.2)

Monolithic columns used for HILIC separations can also be obtained by the polymerization of vinyl monomers having various *X* functional groups, such as amide, carboxylic, sulfonic, amine, or quaternary ammonium in the presence of silica previously derivatized with methacryloyl or methacrylamido groups. The polymerization takes place using an initiator, such as 2,2′-azobisisobutyronitrile or ammonium peroxydisulfate [18]. This type of reaction is schematically indicated below reaction 11.2.3:



(11.2.3)

Besides vinyl monomers with −C(O)X groups, 4-vinylbenzensulfonic acid can be used as reagent in reaction 11.2.3 to generate cation exchange moieties with $-SO_3H^-$ groups. Synthesis of other active phases may include macrocycles such as perhydroxyl-cucurbit [6] uril. The bonding of perhydroxyl-cucurbit [6] uril can be done starting with silica gel derivatized with propylisocyanate groups [19]. The isocyanate further reacts with the OH groups of perhydroxyl-cucurbit [6] uril and generates a stable stationary phase that can be utilized for HILIC separations. Some of the stationary phases with polar groups previously described found commercial applications and others did not.

Physical properties of polar stationary phases and columns

The physical properties of polar stationary phases and columns are very similar to those for RP type stationary phases. These include all the parameters listed in Table 9.2.2. Column body construction, as well as stationary phase physical properties, influences the separation on HILIC phases in the same way as they influence the separation in RP-HPLC and the discussions from Sections 8.1 and 8.2 remain valid for HILIC columns. For example, column efficiency (measured by N/m) depends on type of particles in the column (fully porous, core-shell, monolithic), particle diameter, and other column construction (e.g., packing uniformity). Similar to RP-phases, HILIC phases are available on porous particles and core-shell particles. Also, similar to RP-columns, HILIC columns having the support organic/inorganic silica are available (e.g., XBridge HILIC from Waters). In the selection of the HILIC column, particle dimensions, particle surface area, particle size distribution, etc., are important parameters that should be considered for a successful separation.

Chemical characteristics of polar stationary phases

The nature of stationary phase in HILIC plays a more important role than in RP-HPLC. The phases used in HILIC separation have basically three different properties: 1) polarity (including hydrogen bonding), 2) ion exchange character, and 3) hydrophobicity. Because of various mechanisms of interaction displayed by the HILIC columns, such as polar (including hydrogen bonding), ion exchange, hydrophobic, plus other types such as steric hindrance, the properties of the stationary phase in HILIC are more difficult to classify. In addition, the role of mobile phase (its pH) may influence the nature of polar stationary phases.

1) Regarding the polarity, guidance can be obtained from the values of log K_{ow}^{model} indicated in Table 8.2.5. It can be considered that polarity varies in the following order: bare silica ≈ tertiary amine > primary amine > amide > zwitterionic > imide > urea > diol > cyano. This order can change significantly depending on the silica base structure, coverage of the silica support, use of other support materials (polymeric or zirconia), etc.

2) The ion exchange properties are not necessarily in the same order as polarity and, for example, amine columns display stronger ion exchange characteristics compared with bare silica or phases with diol, amide, or CN groups. Zwitterionic columns display ion exchange properties, but the two groups seem to suppress this character as a whole.

3) The phases with different structures have different polarities, but beside polarity, the hydrophobicity of the spacer (handle) connecting the polar group with the silica base plays an important role in the separation. For this reason, the carbon load $C\%$ of the HILIC columns should also be considered for column

characterization. The nature of the spacer (handle) connecting the polar group to the silica support plays an important role regarding this property. The spacer may be a propyl group, but also longer such as a hydrocarbon chain with 10 carbon atoms. It also may include phenyl groups or even embedded polar groups such as ether in an alkyl chain. However, the retention on HILIC columns cannot be described by the retention factor k' of hydrophobic compounds such as toluene or ethylbenzene. Series such as uracil - 5-methyluracyl or uridine - methyluridine are typically used for retention evaluation.

Other chemical characteristics of the stationary phase show similarities with RP phases. These include 1) silica purity, 2) metal activity from silica, 3) coverage of support with the bonded phase, 4) pH resilience, 5) preparation procedure using mono-, bi-, or trifunctional reagents 6) carbon load, 7) phase ratio, etc. Even end-capping and silanol activity are properties to be considered for HILIC columns (for NPC columns, water adsorption on the stationary phase play an important role). The nature of the bonded phase in HILIC is different from RP-HPLC (except for some cyano phases). Some HILIC columns and many other columns on which NPC is practiced have bare silica as a stationary phase. The silanol groups on silica may have various properties depending on the preparation procedure of hydrated silica material. On these materials, the role of silanol groups is critical for the separation. However, for HILIC columns, the silanol activity may interfere with the polar interactions generated by the bonded polar groups (the interaction with polar solvents such as water is also affected by the polar bonded group). For this reason, some polar bonded stationary phases are also end-capped with small hydrophobic groups (e.g., TSKgel NH2-100 is end-capped with TMS groups).

The carbon load of HILIC columns can vary from 0% as in the case of bare silica columns to as much as 20% or more, similar to that in RP-HPLC columns. Regarding the phase ratio Ψ, unlike RP-HPLC, its value increases with the proportion of water in the eluent [20,21].

The pH range of stability of HILIC columns is also similar to that for RP-HPLC columns. Standard columns made from bare silica or with a bonded polar phase are typically stable between pH 2–8. Special phases may have an extended pH range of stability, and this is indicated by the manufacturer for specific columns.

In HILIC, because the mobile phase always contains water, the wetting characteristic of the stationary phase is not an issue. For NPC, water adsorbed on the stationary phase plays an important role in the separation. However, because in NPC the mobile phase does not contain water, the mobile phase slowly removes the water from the stationary phase surface. This affects the separation and leads to lack of reproducibility in NPC separations. For this reason, it is important in NPC to keep the silica surface wet with water. This can be achieved either by adding a very small proportion of water in the mobile phase or by conditioning the stationary phase surface before use with a solvent containing water.

One additional characteristic of HILIC (and NPC) columns is the rate with which the column is equilibrated in a specific mobile phase. The HILIC separation process is rather complex, and the mobile phase plays an important role in the separation. The equilibration of the stationary phase with a specific mobile phase takes place by a complex diffusion and adsorption process. Since the water adsorbed on the stationary phase surface plays an important role in column properties, the HILIC (and NPC) columns require in general a longer time for equilibration as compared to RP-HPLC columns.

Bare silica stationary phases

Silica stationary phase can be used either in HILIC mode or in NPC mode. In NPC mode, the organic mobile phase has no water but this does not imply the total absence of water from the silica surface. Anhydrous silica still contains a layer of water (possibly monomolecular) on its surface. The HILIC mode is typically preferred to NPC since the layer of water on the silica surface is much thicker than when working in NPC mode, and the separations are less susceptible to silica dryness caused by mobile phase removal of the water layer during the flow of the anhydrous solvent [7,22]. Some other disadvantages of the use of bare silica as stationary phase in NPC mode are diminished in HILIC, although peak tailing may still be a problem.

For both NPC and for HILIC applications, silica of high purity (*Type B* purity) is commonly used. Silica phases based on *Type B* (purity) specifically developed for work in HILIC mode were developed and are commercially available. Among these are the columns indicated in Appendix 11.2.1. The properties of silica columns can vary significantly from one brand column to another. Silica phases have usually an acidic character, which differs from the acidic character of residual silanol for RP columns [23].

HILIC stationary phases with a bonded surface

The development of silica base stationary phases with a bonded material that has polar groups is the cause of a considerable expansion of applications using HILIC columns. The polarity of bonded phase HILIC columns is lower than that of bare silica. However, their reproducibility and rapid regeneration when changing solvent corrected some of the main problems of bare silica columns. HILIC columns are widely used for the separation of important classes of polar organic compounds such as carbohydrates, amino acids, and peptides [24]. Also, due to the use of solvents containing both water and an organic solvent, HILIC columns are frequently recommended to be selected when MS or MS/MS detection is utilized, since these solvents provide good ionization media and lead to good sensitivity. A variety of types of stationary phases can be used for HILIC separations, and some, which are commercially available, are discussed below. Most of these columns are silica based. They include mainly columns made using porous particle materials, but several are made using core shell (fused core) support.

1) Neutral HILIC stationary phases have the polar group amide, diol, cyano, imide, and also hydroxy (specifically bonded on cyclodextran, or as polyvinyl alcohol). These phases are less polar than bare silica. The columns containing cyano groups have the lowest polarity, and this type of column can also be used in RP-HPLC when the mobile phase is more polar than the stationary phase. For this reason, several cyano columns were also discussed in Section 9.2. The polar groups are bonded on silica typically at the end of a hydrocarbon chain serving as a "handle" (see Section 8.1). The length of the aliphatic chain contributes to the hydrophobicity of the phase. One other phase recommended for HILIC is a fluorinated stationary phase (Epic HILIC FL with undisclosed structure), although fluorinated phases are typically considered of RP type.

A common polar group in HILIC stationary phases is the amide. Amide polarity is higher than cyano (see Table 6.2.4 with −CN contribution to log K_{ow}^{model} of −1 and −$CONH_2$ contribution of −1.7). Another common polar group is hydroxy. According to Table 8.2.5, one

hydroxy group has a polarity contribution between cyano and amide, but a diol group brings an even stronger polar contribution (from Table 8.2.5, the −OH contribution to log K_{ow}^{model} is −1.05 and the diol contribution is −2.51). Some diol phases may have a specific structure where ether groups are also present in the connecting chain (handle) to the silica surface (cross-linked diol). Polyethylene glycol bonded to silica is also used as a polar stationary phase. The retention for neutral HILIC stationary phases is based on polar interactions and hydrogen bonding between the hydroxy or amide groups on the stationary media and the polar groups of the analytes. HILIC columns with amide groups were proved to be useful for sugars, amino acids, and peptides analyses. Dihydroxypropyl (diol) groups are able to form stronger hydrogen bonds compared to the amide groups, and these columns are used when stronger polar interactions are necessary for the separation.

More complicated structures that contain OH groups can be used as neutral HILIC stationary phases. Among these are phases with bonded cyclodextrin or bonded perhydroxyl-cucurbit [6] uril groups that contain numerous OH functionalities and can act as HILIC stationary phases. An example of a schematic structure of a phase containing various polar groups including a lactose moiety, triazole, and a phenyl spacer is shown in Fig. 11.2.1 [25].

Other materials such as those made from silica bonded polysuccinimide [26] or polyhydroxyethyl-aspatamide can be used as neutral phases for HILIC. Phases containing a sulfur atom embedded in the chain bearing on OH group, such as mercaptoethanol silica and thioglycerol silica, were also reported [16]. Several neutral HILIC columns that are commercially available are listed in Appendix 11.2.2. As indicated in Appendix 11.2.2, organic polymers are also used as a support for phases with neutral polar groups, such as methacrylic polymers with 2,3-dihydroxypropyl groups [27], sorbitol bonded to a methacrylate polymer covering silica, etc.

2) Anion exchange type stationary phases have the polar group amine or triazole. These phases are in fact weak anion exchange stationary phases that can be used in HILIC mode being applied for the separation of neutral molecules. The propyl amino type stationary phase has been in use for a long time and applied in particular for the separation of carbohydrates. Besides amino groups attached to an aliphatic hydrocarbon chain, the amine group can also be attached to an aromatic ring. Several commercially available HILIC columns with weak anion exchange properties are listed in Appendix 11.2.3.

3) Cation exchange HILIC is cation exchange column that can be used in HILIC mode for the separation of neutral molecules. Among such columns some are weak cation exchangers and

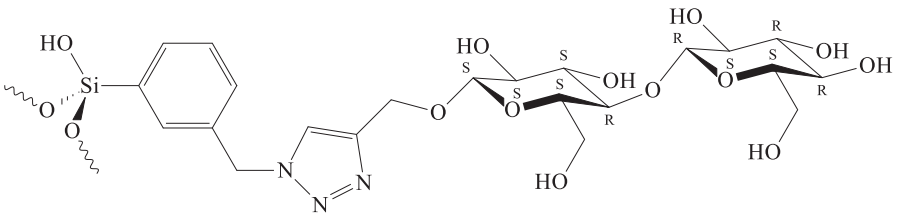


FIGURE 11.2.1 Example of a schematic structure of a phase containing various polar groups and a phenyl spacer.

some strong cation exchangers (with sulfonic groups). However, these columns can be successfully used in HILIC mode. Some columns with cation exchanger properties are listed in Appendix 11.2.4.

4) Zwitterionic HILIC (Zic-HILIC) stationary phases contain both an anionic and a cationic group in their structure. Among these group typical ones are quaternary amine (embedded) and sulfonic terminal and quaternary amine (embedded) and carboxyl terminal. Some other zwitterionic structures have been made such as phases containing polypeptides bonded to silica. Among the commercially available HILIC columns with a zwitterionic structure, several are listed in Appendix 11.2.5.

Zic-HILIC stationary phases can be divided on two types, depending on the position of charged groups toward the stationary phase surface. In one type, the cation type groups are placed toward the surface of the phase, and the anion group toward the silica base, the two groups being separated by a hydrophobic spacer. In the other type of phase, the anion type groups are toward the surface of the phase and the anion group toward the silica base. For example, the stationary phases, Obelisc R and Obelisc N (see Appendix 11.2.5), differ in the type and proximity of their charged groups as well as the hydrophobicity of their long chains. Obelisc R has cationic groups close to the silica surface separated from anionic groups by a hydrophobic chain, while Obelisc N has anionic group close to the surface separated from cationic groups by a hydrophilic chain. The two different charged structures interact differently with polar analytes and lead to different chromatographic results [28]. The functional group sulfonylalkylbetaine and functional group phosphorylcholine (see also Eq. 11.2.2) are schematically indicated in Fig. 11.2.2.

The functional group sulfonylalkylbetaine available on Zic-HILIC column bonded on silica is also available on columns with polymeric particles indicated as Zic-pHILIC columns. The columns having phosphorylcholine group densely bonded on silica are indicated as Zic-cHILIC.

The electrostatic interactions for ZIC-HILIC columns are diminished compared to that of individual ionic groups. Even the values of log K_{ow}^{model} for a model stationary phase with zwitterionic groups cannot be estimated based on the values from Table 6.2.4, because the effect of two charges is counterbalanced by the proximity of the two ions of opposite charges. For this reason, zwitterionic phases are successfully utilized for separations where the polar and ionic interactions are not very strong. Among the commercially available HILIC columns with a zwitterionic structure, several are listed in Appendix 11.2.5.

5) The polar groups present in the bonded phases are connected with a "handle" to the support (usually silica), and this handle is typically formed from a hydrocarbon chain.

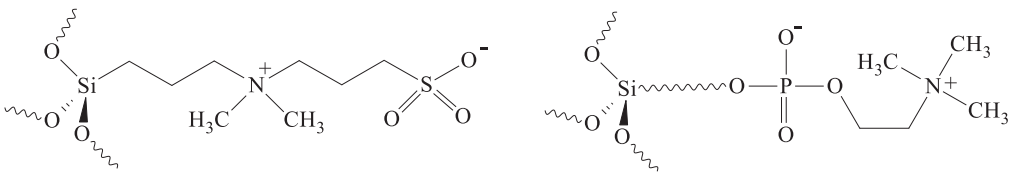


FIGURE 11.2.2 Schematic structure of functional group sulfonylalkylbetaine and functional group phosphorylcholine stationary phase.

A short chain such as propyl, although bringing some hydrophobic character to the phase, is not affecting significantly the polar character of the stationary phase, when polar groups are attached to the chain end. However, longer hydrophobic chains (such as C8 or longer) with polar groups attached at the chain end, or embedded in the hydrophobic chain, produce phases with mixed mode of action. For example, some mixed mode HILIC columns have bonded phases with nonpolar chains and groups of the type polar neutral, anion exchange, cation exchange, or zwitterionic. For example, Primesep N (Sielc) has embedded acidic groups (negatively charged, thus cation exchange) on a hydrocarbon chain bonded phase, Primesep AP (Sielc) has weak amino anion exchange groups and nonpolar moieties, and Obelisc N (Sielc) has both negatively and positively charged groups (zwitterionic) on the same long chains of bonded phase. The ion exchange groups give these columns enhanced selectivity in addition to RP character. For example, the phase in Acclaim Mixed Mode HILIC-1column (Dionex/Thermo Scientific) contains a diol group and a long (C10) hydrocarbon spacer, the column Acclaim Mixed Mode WCX-1 contains a carboxyl group at the end of a C10 spacer (and can act as an ion exchange and RP-phase), and Acclaim Mixed Mode WAX contains an amide group, a tertiary amine. The idealized structure of this phase is shown in Fig. 11.2.3.

Various other mixed mode phases were reported in the literature (e.g., Refs. [16,29]). Some of them are also presented in the section dedicated to the mixed mode HPLC (section 17.1).

Besides small particle phases, monolithic HILIC phases were also prepared. Besides bare silica ChromoLith (see Appendix 11.2.1), polymer coated monolithic silica was used as HILIC stationary phase, the coating being performed with 3-diethylamino-2-hydroxypropylmethacrylate, *p*-styrenesulfonic acid, etc. Also, monolithic organic polymers with polar functionalities such as poly(hydroxymethacrylate) were reported in the literature [30].

Silica hydride–based phases

Silica hydride stationary phases contain Si–H groups on their surface and not Si–OH groups as many silica-based columns (see Section 6.1 for the synthesis of hydride silica materials). A number of silica hydride (silica *Type C*) phases are commercially available (e.g., from Micro-Solv/Cogent) and can be used in HILIC mode. These columns include a bare hydride column (Diamond Hydride) as well as columns with a bonded phase containing polar groups such as butylamino (Cogent HPS Amino). The types of chromatography that can be practiced on bare hydride columns include ANP and HILIC [31]. Several commercially available HILIC columns with silica hydride structure are listed in Appendix 11.2.6.

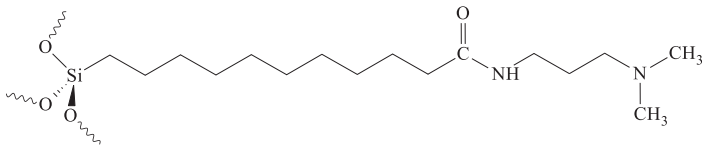


FIGURE 11.2.3 Schematic structure of a mixed mode stationary phase.

Advances in the construction of HILIC columns

New columns from different manufacturers are continuously added to the market. Several such new columns are indicated in Appendix 11.2.7. The new columns bring additional characteristics [32]. For example, SanArmor NH2 column has hydrophilic end-capping and except for SunArmor NH2; the other columns have a core-shell stationary phase [33].

11.3 Retention and separation properties of polar stationary phases

General comments

In a similar manner as described for hydrophobic phases, several parameters can be used for the characterization and comparison of polar phases [29,34–40]. These include typical parameters for chromatographic column characterization such as height of theoretical plate H, peak asymmetry As, and dead time t_0 (obtained with a very nonpolar compound such as toluene $t_0 = t_{toluene}$). The value of retention factor in HILIC was found to be significantly dependent on the mobile phase content in water [7]. For a number of columns, when tested versus polar compounds, the following formula describes this dependence of log k' on the volume fraction of water ϕ_w in the mobile phase:

$$\log k' = a - b \log \phi_w - c\phi_w \qquad (11.3.1)$$

In Eq. 11.3.1, a depends on the analyte, and b and c are parameters which depend on both the analyte and the stationary phase (The coefficients a and b in Eq. 11.3.1 have positive values such that the increase in ϕ_w leads to a decrease in log k', which is in accordance with weaker retention of polar compounds when the water content increases in the mobile phase [39,41]). However, a more detailed HILIC column characterization cannot be made using a simple parameter.

Parameters and tests for HILIC column characterization

The interactions in HILIC comprise polar (including hydrogen bonding), hydrophobic, ion exchange (ionic), and steric effects [18,38]. For this reason, the characterization of HILIC columns needs to include a number of parameters selected for the characterization of specific properties. A set of such parameters may include the following: 1) k' for uridine or $k'(Uri)$, 2) efficiency, 3) methylene selectivity $\alpha(CH_2)_{HILIC}$, 4) hydroxy group selectivity $\alpha(OH)$, 5) isomer selectivity for diastereoisomers α_{dia}, 6) selectivity for position isomerism (regioisomers) α_{regio}, 7) molecular shape selectivity α_{shape}, 8) anion exchange selectivity α_{AX}, 9) cation exchange selectivity α_{CX}, 10) acidity character α_{tt}, and 11) peak asymmetry $As(X)$. As previously indicated, mobile phase characteristics and particularly the pH must also be kept at specific values when testing and evaluating HILIC columns. The pH of the mobile phase controls not only the analyte properties, but also the ionization state of the polar groups of the stationary phase [42].

1) Similar to the use of the retention factor k' for the characterization of an RP phase using a hydrophobic compound (such as toluene or ethylbenzene), the characterization of retention of HILIC columns is typically done using k' for a polar compound, and uridine (Uri) is one of the compounds used for this purpose. The values of $k'(Uri)$ depend on the mobile phase composition, and it is common to use as a mobile phase for the measurement of $k'(Uri)$ acetonitrile/aqueous buffer 90/10, the buffer being 20 mM ammonium acetate at pH = 4.7. Since the values of k' depend on mobile phase composition, the k' values can be used only for a relative comparison of columns, with the same mobile phase and test compound utilized.

2) The number of theoretical plates N (better indicated as N/m since the columns may have

different lengths) describes column efficiency for HILIC similar to RP or other types of chromatography. However, the value for N is compound dependent, and even for the same column different N values are obtained with different test compounds. The test compounds used in RP-HPLC are also different from those used in HILIC. Among the compounds used for the evaluation of N in HILIC are uridine (*Uri*), 5-methyluridine (5*MeUri*), etc., but the choice of test compound is dependent on the type of the stationary phase [43]. The column efficiency in HILIC follows the same rules as for RP-HPLC, with smaller particles and core-shell particles leading to higher N (lower theoretical plate height H). However, typically in HILIC and in NPC, the values for N are lower than in RP-HPLC (the peaks are usually broader). This can be explained by the existence of a combination of different retention mechanisms in HILIC, which influence in different ways the analyte exchange between the mobile and stationary phase [44,45].

3) Methylene selectivity $\alpha(CH_2)_{HILIC}$ (or simply $\alpha(CH_2)$) is a parameter that quantifies the hydrophobic character (as described in Section 9.3 for RP-HPLC). HILIC separations also involve hydrophobic effects and the disruption of the interactions between the solvent molecules produced by the analyte plays a noticeable role in the energy values involved in the separation (see Section 5.1). However, for HILIC columns, the evaluation of methylene selectivity cannot be done using hydrophobic compounds such as those recommended for RP columns. Different compounds that are one CH_2 apart can be used for defining the $\alpha(CH_2)_{HILIC}$ value. A recommended comparison for the measurement of methylene selectivity in HILIC separations is the ratio of capacity factors for uridine (*Uri*) and 5-methyluridine (5*MeUri*). The value for $\alpha(CH_2)_{HILIC}$ can also be indicated as $\alpha(Uri/5MeUri)$. The mobile phase condition recommended for such evaluation is acetonitrile/aqueous buffer 90/10, the buffer being 20 mM ammonium acetate at pH = 4.7 [38]. This methylene selectivity is given by the following expression:

$$\alpha(CH_2) = \frac{k'(Uri)}{k'(5MeUri)} \tag{11.3.2}$$

The choice of nucleosides for obtaining a value for $\alpha(CH_2)$ was made because these compounds are well retained on HILIC columns. The retention of uridine and 5-methyluridine is less affected by ion exchange effects.

4) Hydroxy group selectivity α(OH) is a parameter that quantifies the hydrophilic character. A recommended comparison is the measurement of α(OH) as the ratio of retention factors for uridine (*Uri*) and 2-deoxyuridine (*2dUri*), the values for k' being obtained in the same mobile phase conditions as for $\alpha(CH_2)$ (acetonitrile:aqueous buffer 90:10, the buffer being 20 mM ammonium acetate at pH = 4.7 [38]) and given by Eq. 11.3.3.

$$\alpha(OH) = \frac{k'(Uri)}{k'(2dUri)} \tag{11.3.3}$$

The values for $\alpha(CH_2)$ and α(OH) obtained using formula 11.3.2 and 11.3.3 for the mobile phase acetonitrile:aqueous buffer 90:10 with the buffer being 20 mM ammonium acetate at pH = 4.7 were measured for several commercially available columns [38]. The list of these columns is given in Table 11.3.1, which also lists the values for the retention factor k' for uridine, values for $\alpha(CH_2)$, for α(OH), as well as other selectivities further defined.

As expected, the values for α(OH) depend on the nature of the analyte (as well as on the columns and the mobile phase), and for this

TABLE 11.3.1 List of several commercially available HILIC columns, the retention factor *k'* for uridine, and several α values [38].

Column	*k'(Uri)*	*N(Uri)*	α(OH)	$\alpha(CH_2)$	α_{dia}	α_{regio}	α_{shape}	α_{AX}	α_{CX}	α_t	*As(U)*
ZIC-HILIC (5 μm)	2.11	40,000	2.03	1.67	1.50	1.11	1.14	0.33	1.57	1.18	1.34
ZIC-HILIC (3.5 μm)	2.10	83,333	2.07	1.71	1.51	1.12	1.14	0.27	1.64	1.20	1.26
Nucleodur HILIC (3 μm) (zwitterionic)	2.20	71,429	1.55	1.28	1.46	1.08	1.14	0.34	0.95	1.00	0.88
Amide-80 (5 μm)	3.30	38,462	1.67	1.27	1.29	1.08	1.18	0.19	1.00	1.39	1.37
Amide-80 (3 μm)	4.58	111,111	1.64	1.27	1.28	1.08	1.18	0.41	2.75	1.32	0.99
XBridge amide (3.5 μm)	2.55	83,333	1.70	1.29	1.30	1.07	1.16	0.47	1.20	1.38	1.42
PolySULFONYLETHYL (3 μm)	1.58	16,129	2.13	1.48	1.21	1.06	1.24	0.06	0.35	1.00	1.11
PolyHYDROXYETHYL (3 μm)	3.92	16,393	1.92	1.36	1.31	1.07	1.21	0.34	1.31	1.14	0.99
CYCLOBOND I (5 μm)	0.70	55,556	1.21	1.13	1.24	1.10	1.20	4.73	0.63	1.01	1.73
LiChrospher diol (5 μm)	1.50	58,824	1.36	1.15	1.32	1.06	1.17	0.63	1.16	1.04	0.98
Chromolith Si	0.31	83,333	1.00	1.12	1.16	1.11	1.31	0.09	8.21	1.22	1.00
HALO HILIC Si (2.7 μm)	0.64	125,000	1.08	1.16	1.18	1.13	1.29	0.64	29.03	1.26	1.47
COSMOSIL HILIC (5 μm) (Triazole)	1.60	83,333	1.6	1.14	1.36	1.03	1.13	0.80	0.49	0.89	1.11
Sugar-D (5 μm)	1.58	58,824	1.74	1.44	1.45	1.10	1.22	1.90	0.25	0.52	1.12
NH2-MS (5 μm)	2.44	83,333	1.88	1.3	1.36	1.07	1.20	0.82	0.28	0.54	1.04

reason, various α(OH) values can be assigned for the same HILIC column and the same mobile phase, but for different test compounds. Besides the use of uridine and 2-deoxyuridine in Eq. 11.3.3, other compounds are also used for the estimation of an α(OH) [39,46]. For example, for the columns listed in Table 11.3.1, the values for α(OH) can be determined using other nucleoside/2′-deoxynucleoside pairs than those based on uracil, for example, where the base is thymine, adenine, cytosine, or guanine [38]. The values of α(OH) can be obtained similarly for pairs 2′-deoxynucleosides/2′3′-dideoxynucleoside. The formulas of some of the nucleic bases and those of the compounds used for the calculation of $\alpha(CH_2)$ and α(OH) with uracil based nucleosides for HILIC columns are shown in Fig. 11.3.1.

5) Isomer selectivity is another property that can be used for HILIC column characterization. Chiral isomers cannot be separated on phases without a chiral selector, and isomer selectivity of HILIC columns does not refer to chiral separations. However, the capability of separating structural isomers, cis-trans, and diastereoisomers is an important column characteristic. This selectivity has been suggested to be characterized by the values of α for two pairs of the following compounds [38]: 1) diastereoisomers vidarabine (*Vid*) and adenosine (*Ade*) and 2) regioisomers 2′-

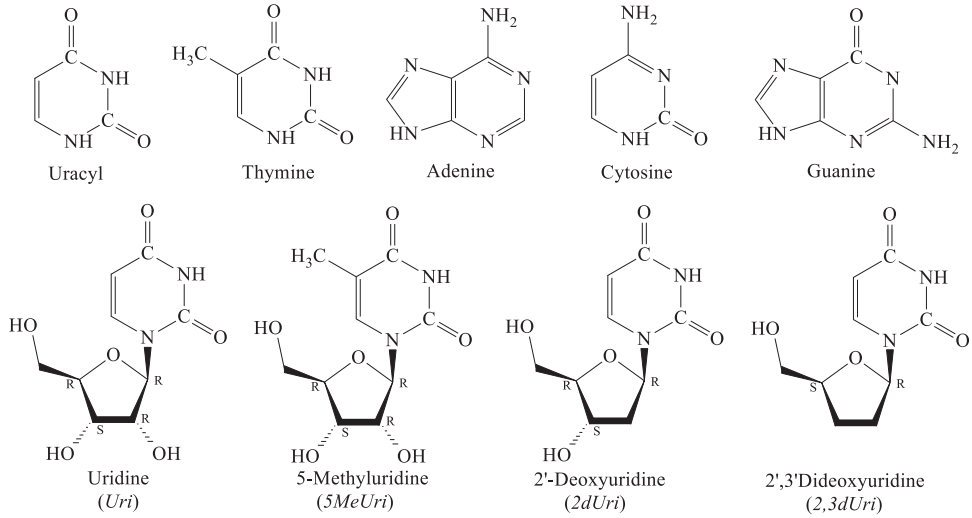


FIGURE 11.3.1 Formulas for nucleic bases and for some nucleosides based on uracil/thymine, used for the calculation of $\alpha(CH_2)$ and $\alpha(OH)$.

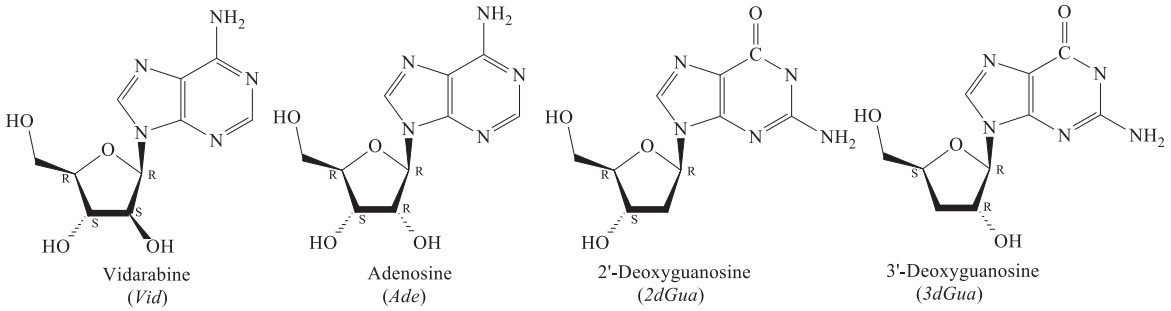


FIGURE 11.3.2 The formulas of diasteroisomers vidarabine (*Vid*) and adenosine (*Ade*) and of regioisomers 2′-deoxyguanosine (2*dGua*) and 3′deoxyguanosine (3dGua).

deoxyguanosine (2*dGua*) and 3′deoxyguanosine (*3dGua*). The formulas for these compounds are given in Fig. 11.3.2.

The expressions for selectivities $\alpha_{dia}(Vid/Ade)$ and $\alpha_{regio}(2dGua/3dGua)$ are given by Eq. 11.3.4, and their values for several columns are indicated in Table 11.3.1 (mobile phase acetonitrile:aqueous buffer 90:10, the buffer being 20 mM ammonium acetate at pH = 4.7) [38].

$$\alpha_{dia}(Vid\,/\,Ade) = \frac{k'(Vid)}{k'(Ade)}$$
$$\alpha_{regio}(2dGua\,/\,3dGua) = \frac{k'(2dGua)}{k'(3dGua)} \qquad (11.3.4)$$

6) Molecular shape selectivity α_{shape} is another parameter that can be used for the characterization of HILIC columns. The steric parameters α_{dia} and α_{regio} are related to shape of the molecule but in a more particular manner. A selectivity parameter recommended in the literature [38] for molecular shape characterization uses the two diastereoisomers 4-nitrophenyl-α-D-glucopyranoside (*NPαGlu*) and 4-nitrophenyl-β-D-glucopyranoside (*NPβGlu*). The expression for α_{shape} is given by Eq. 11.3.5. and the formulas for the two diastereoisomers are given in Fig. 11.3.3.

$$\alpha_{shape}(NP\alpha Glu / NP\beta Glu) = \frac{k'(NP\alpha Glu)}{k'(NP\beta Glu)} \tag{11.3.5}$$

For several HILIC columns, the values for α_{shape} are listed in Table 11.3.1 and were obtained using the same mobile phase (acetonitrile: aqueous buffer 90:10, the buffer being 20 mM ammonium acetate at pH = 4.7) [38].

7) Two selectivity parameters can be used for ion exchange interactions evaluation. One selectivity is used for describing anion exchange properties α_{AX} and the other for cation exchange properties α_{CX}. For these two selectivities, the pairs of compounds recommended in the literature [38] are the following: 1) sodium *p*-toluenesulfonate (*SPTS*) and uridine (*Uri*) for the characterization of anion exchange properties and 2) N,N,N-trimethylphenylammonium chloride (*TMPAC*) and uridine (*Uri*) for the characterization of cation exchange properties. The formulas used to calculate these selectivity parameters are given below:

$$\alpha_{AX}(SPTS / Uri) = \frac{k'(SPTS)}{k'(Uri)} \tag{11.3.6}$$

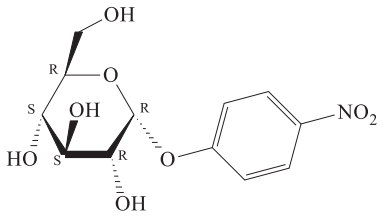
$$\alpha_{CX}(TMPAC / Uri) = \frac{k'(TMPAC)}{k'(Uri)}$$

The values for α_{AX} and α_{CX} for the several columns are given in Table 11.3.1 for a mobile phase acetonitrile:aqueous buffer 90:10, the buffer being 100 mM ammonium acetate at pH = 4.7 [38]. The chemical formulas for *SPTS* and for *TMPAC* are given in Fig. 11.3.4.

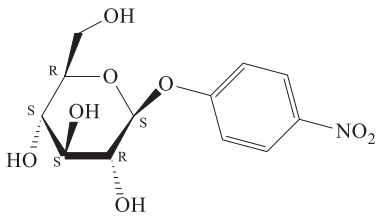
8) Another parameter used to evaluate HILIC columns where theobromine (*Tb*) and theophylline (*Tf*) are used in the test is defined by the following ratio:

$$\alpha_t(Tb / Tf) = \frac{k'(Tb)}{k'(Tf)} \tag{11.3.7}$$

This parameter differentiates columns regarding their basic, neutral, or acidic character with $\alpha_t < 1$ for basic, $\alpha_t \approx 1$ for neutral, and $\alpha_t > 1$ for

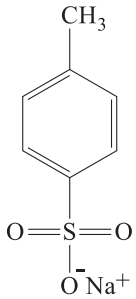


4-nitrophenyl-α-D-glucopyranoside
(*NPαGlu*)

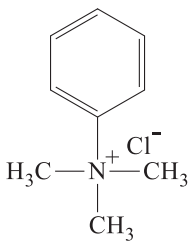


4-nitrophenyl-β-D-glucopyranoside
(*NPβGlu*)

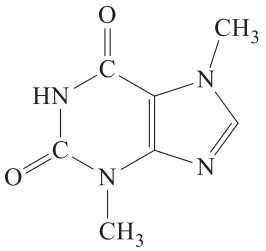
FIGURE 11.3.3 The structural formulas of diasteroisomers 4-nitrophenyl-α-D-glucopyranoside (*NPαGlu*) and 4-nitrophenyl-β-D-glucopyranoside (*NPβGlu*).



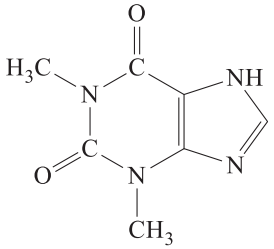
Na p-Toluenesulfonate (*SPTS*)



Trimethylphenyl ammonium chloride (*TMPAC*)



Theobromine (*Tb*)



Theophyline (*Tf*)

FIGURE 11.3.4 Formulas for sodium p-toluenesulfonate (*SPTS*), N,N,N-trimethylphenyl-ammonium chloride (*TMPAC*), theobromine (*Tb*) and theophylline (*Tf*).

acidic stationary phases [47]. The formulas for theobromine and theophylline are also given in Fig. 11.3.4.

9) Peak asymmetry *As(X)* is another parameter used for HILIC column characterization (see expression 4.2.52). Peak asymmetry is compound dependent, and among the test compounds for the estimation of *As* in HILIC separations are uridine (*Uri*), and 5-methyluridine (5*MeUri*).

Different from the case of hydrophobic columns, where the parameters for column characterization H^*, S^*, A^*, B^*, $C^*(2.8)$, and $C^*(7.0)$ were obtained using regression parameters for the calculation of α values, in the case of HILIC columns, it was found more convenient to simply characterize the columns based on measured α parameters for specific pairs of compounds. HILIC mechanism being more complex and less investigated than the mechanisms for hydrophobic columns, it would require further characterization of a multitude of columns for a similar approach as that used in RP-HPLC.

Similar to the case of hydrophobic columns, HILIC columns comparison can be done by different procedures using various α parameters. One such procedure is the use of a radar graph. Several radar plots for the columns ZIC-HILIC (5 μm) (plot A), Amide-80 (3 μm) (plot B), CYCLOBOND I (5 μm) (plot C), and for NH2-MS (5 μm) (plot D) are shown in Fig. 11.3.5.

Such graph allows the estimation of columns difference regarding a specific parameter and at the same time an overall view of similarity between the columns.

For the HILIC columns, it was also extended the hydrophobic subtraction model (See Section 9.3.) For being utilized for HILIC, Eq. 9.3.7 has been modified in the following form [48,49]:

$$\log\left(\frac{k'_X}{k'_{EB}}\right) = \eta'(X)H^* - \sigma'(X)S^* + \beta'(X)A^* + \alpha'(X)B^* + \kappa'(X)C^* + \delta'D^* \tag{11.3.8}$$

In Eq. 11.3.8, δ' and D^* account for the anion exchange activity between the analyte and stationary phases. The model was established based on retention data of 41 compounds on 8 HILIC columns using TSK gel Amide-80 as the reference column.

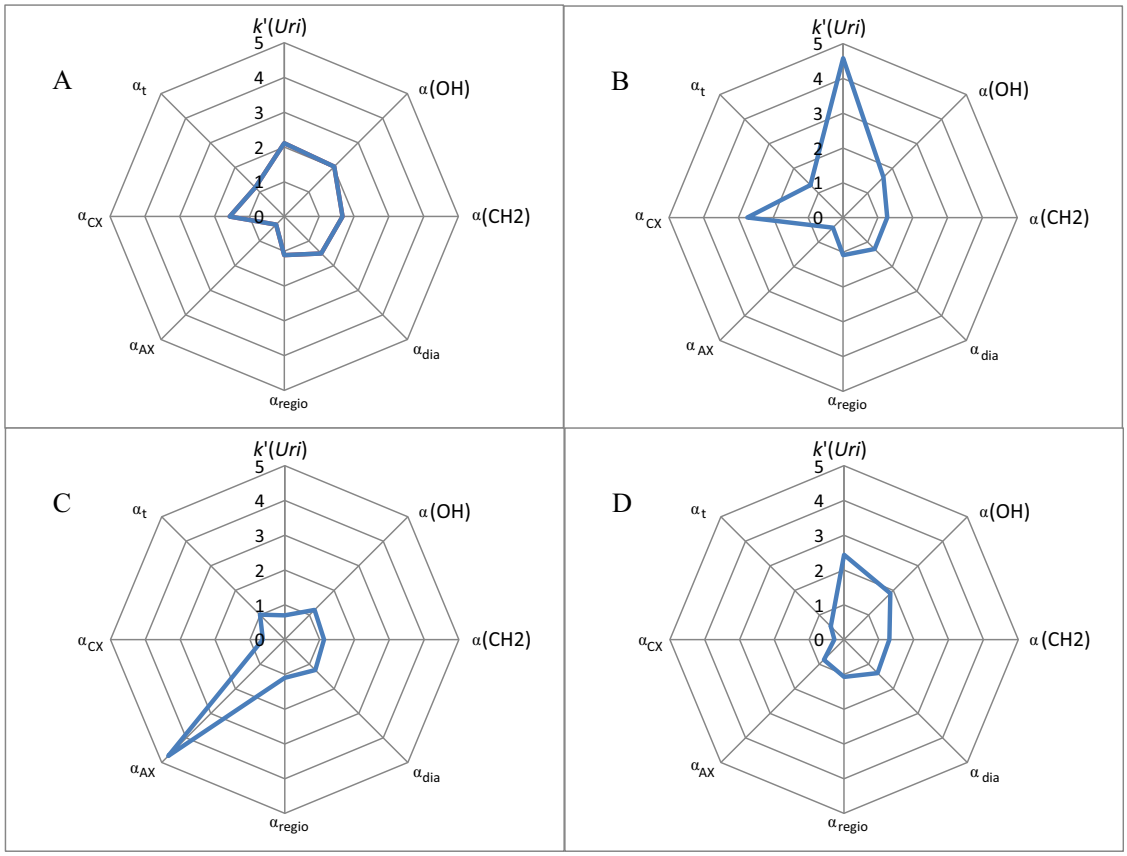


FIGURE 11.3.5 Radar plots for the columns ZIC-HILIC(5 μm) (plot A), Amide-80 (3 μm) (plot B), CYCLOBOND I (5 μm) (plot C), and NH2-MS (5 μm) (plot D).

11.4 Selection of the column in HILIC separations

General comments

Selection of an HILIC column may represent a relatively challenging subject. In HILIC (and NPC) the role of the stationary phase becomes more important and for this reason the column selection is not very simple. The interaction of the analytes with the polar groups from the stationary phase plays a crucial role in the separation, affecting the retention time, the resulting retention factor k', the peak shape and therefore the efficiency (N), and the selectivity α. Through these parameters, resolution R is affected. Mobile phase composition and pH still remain in HILIC important factors related to separation. For example, the pH of the mobile phase may influence not only the form in which the analytes are (free molecules or ions), but also may influence the polarity of the stationary phase when groups such as $-NH_2$, $>N^+(CH_3)_2$ $-N^+(CH_3)_3$, and $-SO_3^-$ are present in the stationary phase. Also, the peak shape and efficiency in HILIC may be a problem and typically they are not as good as in RP-HPLC. For these reasons, when a separation is still possible on an RP-HPLC column, this offers advantages. The development of

RP-HPLC columns having polar embedded groups, hydrophilic end-capping, and use of trifunctional reagents for the preparation of the stationary phase allows the extension of RP-HPLC separations to highly polar compounds which usually require a very low content of organic component in the mobile phase (or even 100% aqueous mobile phase). Also, for solutes that are amenable to both RP and HILIC separations, HILIC may provide a different elution order and a different selectivity compared to RP, with potential applications in orthogonal mechanism for 2-dimensional LC combining HILIC with RP type separations [42]

Another alternative to HILIC is the use of IPC that takes advantage of the excellent performances of RP-HPLC. For example, amino acids can be analyzed using an HILIC column (e.g., ZIC-pHILIC), but also using a separation on a C18 column [50]. Many compounds that are too polar to be separated on RP-HPLC such as sugars, oligosaccharides, polysaccharides, certain peptides, proteins, and nucleotides still must be separated on polar columns in HILIC mode. Also, in some applications, HILIC or NPC is necessary for the separation of certain isomers (achiral) since polar phases are more efficient than RP for isomer separations. This is explained in Section 11.1 as caused by the differences in the interactions between the stationary phase and the isomers that have the polar groups positioned in different parts of the molecule. Besides the polar group position in an analyte molecule, other effects such as steric hindrance and additional effects such as propensity to form hydrogen bonds or having electron donation or withdrawal interactions may affect the differences in retention on polar phases [1,51]. Column equilibration in a specific mobile phase must be considered in all polar separations [52].

Selection of the nature of stationary phase for the column

The stationary phases used in HILIC separations include a wide range of surface functional groups, with different polarities, such as neutral (amide and diol), cationic (amine), and anionic (sulfonic acid and silanol), as well as with zwitterionic character (e.g., sulfobetaine and phosphorylcholine). Their interactions with the analytes could be hydrophilic (ion−dipole, dipole−dipole, H-bonding), π−π interactions, hydrophobic in some cases, and based on shape selectivity [43]. The knowledge of these interactions could provide some guidance for the selection of a stationary phase for a specific purpose, although the main approach remains the time-consuming experimental trials under various elution conditions. One path for the selection of the nature of the stationary phase for an HILIC column is based on the results reported in the literature for a similar class of compounds as that of the analytes to be separated in the new method. Various written materials are available describing analytical methods practiced on HILIC columns [24,53−55].

The general rule for the selection of a phase for HILIC separation is that the more negative is the log D_{ow} value for an analyte, the greater should be stationary phase polarity required to retain it. The polarity of the phase is however difficult to estimate since it depends on the functionality but also on the silica structure, spacer length that brings hydrophobic character, end-capping of silica, as well as the pH of the mobile phase. Using as a guidance the results for log K_{ow}^{model} values from Table 8.2.5, the order of polarity for different phases is bare silica ≈ tertiary amine > primary amine > amide > zwitterionic > imide > urea > diol > cyano. However, this polarity can be changed depending on the mobile phase pH, and in an acidic medium, the amine and urea groups can be much more polar [11]. Zwitterionic phases seem to have a balance between the charged groups, such that their apparent polarity is not very high. The value of K_{ow}^{model} offers only a modest guidance for choosing an HILIC column.

Useful information for selecting an HILIC column is provided by the set of parameters described in Section 8.2. The parameters *k′(Uri)*,

α(OH), $\alpha(CH_2)$, $\alpha_{dia}(Vid/Ade)$, $\alpha_{regio}(2dGua/3dGua)$, α_{AX}, α_{CX}, and α_t are not always reported for a column, but, when available, these parameters offer a good criteria for selecting the column. When such characterizing parameters are available, it should be noticed that they are somewhat independent one from another, and a higher value of α(OH), for example, does not necessarily indicate a low value for $\alpha(CH_2)$. The hydrophobicity $\alpha(CH_2)$ is related as expected to the carbon load of the column *C%*. When $\alpha(CH_2)$ is not known, the capacity of the column to accommodate hydrophobic compounds can be estimated based on *C%*.

For compounds with anionic character, a column with anionic character is useful for the separation, while one with high cationic character is not likely to provide good separation. The same is applicable for cations that are not well separated on columns with high α_{AX} values. Acidic and basic character of stationary phases can also be estimated based on α_t values. For mixtures of polar analytes with neutral, basic, and acidic characters, neutral HILIC columns are recommended for the separation. The diol phases, for example, can be used for various separations [56].

The similarities between the column structure and analyte structure offer also guidance for column selection. For example, a number of amino acids (that have a zwitterionic character) can be separated on a Zic-pHILIC column and the separation may be favored by the interaction of amino acids with the zwitterionic stationary phase. Similarities in the phase structure and analyte structure are not always required. For example, glucose and other monosaccharides can be separated with good results on an amino type HILIC phase and not on a diol phase. As an example, the separation of several small carbohydrates in the plant materials (fructose, glucose, mannose, sucrose, maltose, xylose, sorbitol, and myo-, chiro-, and scyllo-inositols) can be separated on a YMC-Pack Polyamine II column, followed by MS/MS detection [57]. The chromatogram of a standard mixture of small carbohydrates with concentrations between 25 and 50 μg/mL is shown in Fig. 11.4.1. The mobile phase was 75% CH_3CN and 25% water in isocratic conditions. The MS/MS detection allowed the measurement without interference of the overlapping peaks since they were detected using different ions.

Selection of physical column characteristics in HILIC

Similar to RP-HPLC, the columns with an HILIC phase that are most frequently utilized have 100 or 150 mm length, and 2.7, 3, 4.6 mm diameter, with uniform fully porous spherical particles of 3 or 5 μm diameter or with superficially porous particles. For achieving a better efficiency, columns with smaller particles or core-shell particles are recommended. Smaller particles lead to higher backpressures in the HPLC system, and these pressures must be acceptable for the HPLC or UPLC pumping system. Also, phase rigidity and resilience to crushing must be considered when a column with small particles is selected. In the selection of an HILIC column for the analysis of large molecules (polymeric), similar to the case of RP-HPLC columns, it is recommended to select stationary phases with larger pores (200 Å or larger, depending on the molecule *Mw*).

Other parameters important in HILIC column selection

Similar to the case of RP-columns, other column characteristics are important in the selection of an HILIC column. Among these can be listed 1) resilience to a wider pH range, 2) low asymmetry, 3) low column bleed, 4) short

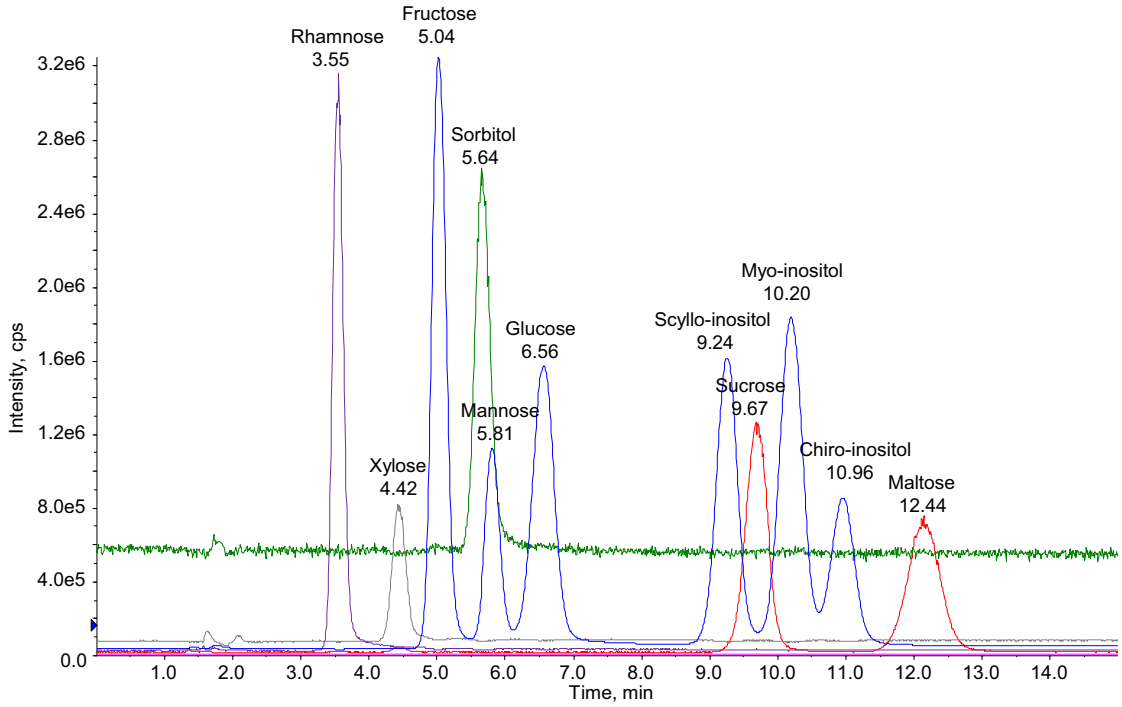


FIGURE 11.4.1 Chromatogram of a set of small carbohydrate standards with concentrations between 25 and 50 μg/mL on a YMC-Pack Polyamine II column with the mobile phase 75% CH_3CN and 25% water [57].

equilibration times, 5) reproducibility for a large number of injections, etc. All these characteristics must be evaluated during the selection process and considered in the context of analysis requirements.

11.5 Mobile phase in HILIC

General comments

The mobile phase in HILIC is characterized by its lower polarity compared to the stationary phase [58]. Typical mobile phases in HILIC are made of an aqueous buffer (10%–40% of the mobile phase) with a controlled pH and ionic strength and an organic solvent such as acetonitrile or methanol, although other solvents such as ethanol or 2-propanol are sometimes used as organic modifiers [1,59]. The solvent strength in HILIC has the following order: water > methanol > ethanol > 2-propanol > acetonitrile > acetone > tetrahydrofuran. For this reason, in HILIC separations using gradient conditions, the separation starts with a solvent low in water, and the water content is increased for the elution of the analytes that are more strongly retained [10,60]. The increase in water content leads to a decrease in retention factor k'.

One particular aspect of the mobile phase in HILIC is that compared to the mobile phase in RP, it typically contains a lower level of water (maintaining a lower polarity of the mobile phase compared to the stationary phase). This characteristic may pose problems with buffer solubility. For this reason, buffers with good

solubility in mixtures containing a higher content of organic solvents are necessary for HILIC separations. For example, ammonium formate or acetate is preferred to phosphate buffers. Some other studies suggest that trifluoroacetic acid can also be used for the control of pH of aqueous buffer. Common concentration range for the buffers is 5−20 mmol/L. The range of values of pH for the buffers that can be used in HILIC is limited by the nature of the stationary phases and is similar to that for RP-HPLC (pH = 2−8) [12,61].

Acetonitrile is the preferred organic solvent in HILIC applications, while the other solvents may lead to insufficient sample retention and broad or nonsymmetrical peak shapes. This is, for example, the case of methanol, which is less used as the organic component in HILIC separations. The relatively poor performance of methanol in HILIC may be due to its similarity to water, both methanol and water being protic solvents. Methanol can compete to the solvation of the surface of silica or of other polar stationary phases used in HILIC and provide strong hydrogen bonding interactions [16].

Double role of the mobile phase in HILIC

The mobile phase in HILIC seems to play a more important role than simply elution medium (see rel. 6.5.6). The polar solvent molecules from the mobile phase (e.g., water) can be adsorbed onto the polar sites of the surface of the stationary phase changing its interacting properties with analytes. In HILIC, this effect can be diminished when enough water is present in the mobile phase to be adsorbed on the stationary phase surface, assuring that no significant changes in surface nature take place during the separation. The same role as the water can be played in HILIC by other components of the mobile phase; for example, for aminopropyl-based stationary phases, the acidic buffer assures the protonation of amino group which participates then to the HILIC type separation.

Since the mobile phase in HILIC plays a role in the nature of stationary phase surface, its content in water or other additives must be carefully considered. For example, during gradient separations in HILIC, the content in a buffer or an added salt may be changed if the additive is present only in the aqueous phase and absent in the organic modifier. The change in the mobile phase pH or ionic strength may be avoided in cases of gradient separations with two solvents A and B, where A is mainly organic and B mainly aqueous; the same buffer/additive content should be used in both solutions. For the gradient separations with the initial mobile phase with no buffer and with the buffer content increasing during the run, changes in the retention mechanism may occur. The change in the retention mechanism can be exemplified by the variation in retention factor k' shown in Fig. 11.5.1 for the separation of several cation type oximes (HI-6, HLö-7, obidoxime, and pralidoxime) on a stationary phase containing a sulfobetaine ligand bound on silica matrix (ZIC-HILIC, Merck, 150 × 4.6 × 5; 200 Å pore size) with the change in the acetonitrile content and keeping only the aqueous phase with a content of 10 mmol/L KBr. The variation in the values of k' suggests the switch of the retention mechanism by changing the mobile phase composition and ionic strength [62].

An expected decrease in the values of retention factor k' is shown in Fig. 11.5.1 for the organic phase content decreasing from 80% to 40% (and an increase in KBr concentration). However, below 40% ACN, an unexpected increase in k' can be noticed. Between 40% and about 20% ACN, the value of k' increases as in an RP type HPLC. For even lower organic phase content, a typical decrease in k' for an HILIC separation is seen. The same separation as shown in Fig. 11.5.1 performed under constant ionic strength shows a unique NP type of retention.

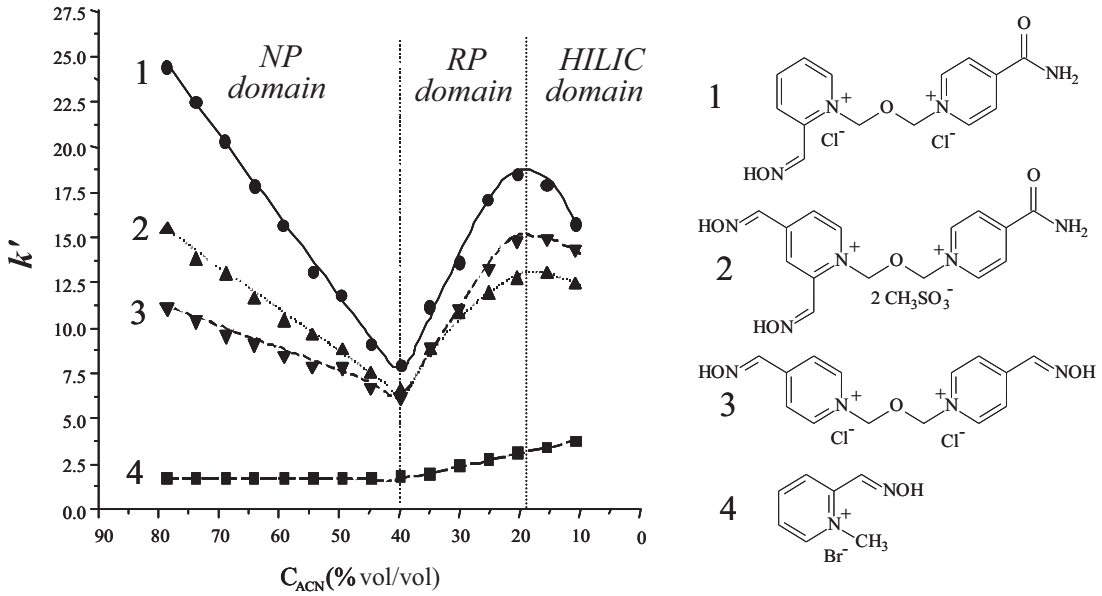


FIGURE 11.5.1 Change in the value of retention factor k' for the separation of four cationic-type oximes on a zwitterionic stationary phase upon the change in the mobile phase content in organic component (acetonitrile, ACN) and in ionic strength (KBr).

In case of separations with NP type separation practiced on a silica column, the control of water content in the mobile phase is essential to maintain a constant silica activity. Dry solvents may dissolve some of the water present on the silica surface and modify its structure such that column reproducibility is not very good, exposing to the analytes either immobilized water or silanol groups. To achieve column stability, the silica columns are usually equilibrated with a standardized solvent (ethyl acetate containing 0.06% water) [63].

The gradient elution in HILIC separations

Gradient elution is commonly used in HILIC. Although hydrophobic interactions are involved in HILIC separations, the main process is based on polar interactions. Therefore, better separations are obtained for compounds with large differences in polarity and not in the values for log K_{ow}. Gradient elution in HILIC starts from a composition of mobile phase rich in organic constituent (e.g., acetonitrile or methanol), and the concentration of the polar (aqueous) component is increased in time. Buffers are used frequently in the mobile phase, and a good practice is to have the same buffer in the two phases that make the gradient such that the pH of the mobile phase is kept constant during the run.

The high initial acetonitrile content in mobile phase will assure sufficient retention for the analytes with low affinity for the stationary phase. Running the gradient toward a high water concentration (e.g., 90%) favors the desorption of strongly retained analytes on the stationary phase. The gradient type depends on the nature of the stationary phase, organic component chosen for mobile phase composition, and on the nature of the analytes in the injected sample [64]. The equilibration of the HILIC columns to a new mobile phase composition is, however, typically slower than in RP-HPLC. For this reason,

sharp composition changes of the mobile phase in HILIC are not recommended.

The pH of the buffers for HILIC may be selected to enhance the dissociation of sample analytes (pH > 7 for acids and pH < 7 for bases). Selectivity in HILIC can be well controlled with the pH of the mobile phase, the difference in retention of different analytes in HILIC being more affected by pH as compared to RP-HPLC [16]. The hydrophobic interactions are less sensitive to pH modifications because hydrophobic surfaces are usually not changed with pH changes. On the other hand, the pH of the mobile phase can strongly affect molecular polarity.

In Zic-HILIC, the influence of the ionic strength of the mobile phase is even more important than in standard HILIC. This can be explained by the competitive equilibria involving ionic species from the salt additive and the charged analyte molecules. The competitive equilibrium of salt adsorption on stationary phase sites will shift toward desorption of charged analyte. A consequence of the addition of salts in the mobile phase is the decrease of the retention time of the amalytes which can be used in the control of highly retained analytes on polar stationary phases, under Zic-HILIC mechanism [65].

Ion-pairing additives for HILIC separations

Additives in the mobile phase that produce pairs with highly polar analytes have the opposite effect on the retention in HILIC as compared to RP-HPLC. While in RP separations the role of ion-pairing additive is to enhance the retention of analytes, in HILIC separations the ion-pairing additives decrease the retention of separated analytes. This approach can be used in case of highly retained solutes on polar stationary phases. As an example, a mixture of nucleosides and nucleotide mono-, di-, and triphosphates can be separated on an HILIC−Amide stationary phase using diethylammonium, triethylammonium, and tetrabutylammonium ions as ion-pairing reagents [66]. The pH control can be achieved with the aid of hexafluoro-2-propanol, which has also the advantage of being amenable to MS detection [67,68].

Influence of mobile phase on detection in HILIC

The conclusions regarding the influence on UV detection of the mobile phase composition used in HILIC are not different from those for RP-LC (solvents must have a cut-off UV value below the wavelength selected for detection). A particular discussion can be done in case of MS detection. The polar analytes are typically eluted with higher organic modifier content in HILIC than in RP-LC, which may improve the ESI/MS response. Often the best sensitivity in ESI is achieved when the analyte is ionized already in a liquid phase by using an acidic mobile phase for basic analytes, such as amines (pH two units below pK_a of the analyte), and basic conditions for acidic analytes, such as carboxylic acids and phenols (pH two units above pK_a of the analyte) [69]. The buffer concentrations should not exceed a level of 10 mM/L in order to avoid suppression of ionization and reduced sensitivity. Both polar and nonpolar solvents can be used in APCI.

The use of a mobile phase rich in organic volatile solvents, as practiced in HILIC, may also be favorable for enhancing the sensitivity of detection in techniques such as evaporative light scattering detection and corona charged aerosol detection, which involve an evaporative process (see Section 7.3). This has been proved for specific analytes as reported in the literature [70].

11.6 Prediction of parameters describing the separation in HILIC

General comments

Various studies were dedicated to the attempts of the prediction of several separation parameters in HILIC, similar to those dedicated to prediction in RP-HPLC [16,71]. Such studies

provide information regarding the results of HILIC separations, but the accurate values for log k' or for t_R in HILIC are usually limited to small sets of compounds where known controls are included.

The retention behavior of analytes in HILIC type separation is determined by their hydrophilic character. A good descriptor for hydrophilicity of analytes is log D_{ow}, measured at the pH of the utilized mobile phase. Unlike RP type separation, when the values of log k' are directly proportional to the values of log K_{ow}, for HILIC type separation, the values of log k' are inversely correlated to log D_{ow}. In practice, the order of elution is given by the order of D_{ow}, but the correlations between log k' and log D_{ow} are rarely characterized by acceptable correlation coefficients R^2. This can be explained by the presence of other processes than partition between the two phases [48]. Other molecular descriptors used in quantitative structure–retention relationship in HILIC separations were molecular weight, polar surface area [72], and solubility in water [73].

Estimation of retention factor k' for similar systems at different mobile phase compositions

In accordance with the combination of adsorption and partition as type of equilibria for HILIC, both Eq. 4.1.16 (used for partition) and Eq. 4.2.14 (used for adsorption) are usually combined to predict the retention factor k' for similar systems at different mobile phase compositions. This led to the use in HILIC of Eq. 11.3.1, written once more below for ϕ_w the volume fraction of water in the mobile phase:

$$\log k' = a - b \log \phi_w - c\, \phi_w \qquad (11.6.1)$$

Besides this three-parameter model (e.g., Ref. [74]), other expressions were also attempted for the evaluation of log k' such as Eq. 4.2.14 [8,75] or Eq. 9.7.4 [75].

Other estimation procedures for HILIC parameters

Other estimation procedures for the estimation of HILIC parameters were based on LSER approach, but some using additional modifications. For example, Eq. 9.7.10 was modified to the following form:

$$\log k' = h_0 + hH + sS + aA + bB + cC + vV + d^-D^- + d^+D^+ \qquad (11.6.2)$$

In Eq. 11.6.2, the additional terms d^-D^- indicate the electrostatic contribution of anionic species and d^+D^+ the electrostatic contribution of cationic species [76,77]. These terms depend on the ionization degree of the analyte or on the difference between the pH of the mobile phase and the pK_a of the compound [78]. The last two terms can be replaced by a scaled effective acid dissociation constant (denoted by P), based on dissociation constant of the acidic analyte in the mobile phase composition used for separations [79].

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CHAPTER

12

Other HPLC separations performed on polar stationary phases

12.1 Normal phase liquid chromatography

General comments

Normal phase liquid chromatography (NPC or NPLC or NP-HPLC) is a chromatographic technique performed on polar stationary phases and using a mobile phase less polar than the stationary phase similar to HILIC. The only difference between NPC and HILIC is the absence of water from the mobile phase in NPC. NPC is usually performed on bare silica and the equilibrium type of separation is considered to be mainly adsorption, although traces of water are usually adsorbed on silica surface and this layer may act as an immobilized solvent rendering the equilibrium type as partition. The molecular interactions in NPC are similar to HILIC, the variation of free energy for the separation being expressed by Eq. 11.1.6. In this expression, the solvophobic component is dominated by A^{vdW} terms having A^{cav} even smaller than in HILIC.

Other factors are also important in NPC retention process and obtaining acceptable resolution in NPC separation. For example, the retention behavior of polycyclic aromatic sulfur heterocycles (PASHs) and alkyl-substituted PASHs on the NH_2 stationary phase is well correlated with the number of aromatic C atoms. These structural characteristics and the planar shape of aromatic rings have a significant influence on retention. Nonplanarity (expressed as molecular thickness, in Å) and the position of the sulfur atom in the bay region of the structure influence the retention [1]. The interactions between analyte and stationary phase are basically a combination of dipole−dipole forces and H-bonds between amino group from stationary phase and aromatic ring from analyte molecule (see Section 5.1).

NPC compared to HILIC

A list of types of stationary phases used in HILIC and NPC is given in Table 11.2.1, and various polar phases are described in Appendix 11. However, the most common stationary phase in NPC is bare silica for which there are some useful applications, such as separation of very nonpolar compounds (for example, carotenoids or lipids) that require an organic mobile phase with no water or polar constituents when HILIC is not adequate for separation. Similar to HILIC, NPC can be used for the separation of compounds that are extremely polar and are not retained or are very weakly retained on RP-HPLC. Also, separation of certain isomers (achiral) is more efficient on NPC than RP. An alternative to NPC for the separation of

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hydrophobic compounds is the convergence chromatography [2]. This technique is a new development in SFC type separations and has been successfully utilized in the analysis of lipids, fat-soluble vitamins, steroids, and nonionic surfactants.

There are several disadvantages of silica utilization in NPC mode. The main problem is related to the reproducibility of such separations. Since the silica is always covered with a layer of water, the immobilized material acting as stationary phase can be considered to be water. The variation in the amount of water retained on the silica produces variations in the retention times. The changes in the level of water for the same column can be caused by the modification of water level on the surface of stationary phase during exposure to mobile phase solvents with different degreed of dryness or by the length of exposure of the column to a dry solvent that may dissolve some of the preexisting water forming the stationary phase. The sensitivity of column reproducibility to the water exposure is very high. Also, related to the water layer formation is the slow equilibration of the column when changing solvents and frequent irreproducibility in this equilibration process. Also, some compounds are retained irreversibly on bare silica. Although the structure of *Type B* silica is highly homogeneous, silica columns frequently produce tailing of the peaks, indicating variability in the type of interactions on the stationary phase surface.

NPC is frequently the type of separation to select when the solubility in water of the sample compounds is limited. Generally, NPC is very useful for separating isomers and compounds with similar structure that are not water soluble [3]. For example, the separation of tocopherols and tocotrienols on silica stationary phase with binary mobile phase based on n-hexane/1,4-dioxane allows a better resolution between isomers than that obtained by RP separation [4]. An approximate rule for the order of elution for different classes of organic compounds separated by NPC is the following: saturated hydrocarbons < olefins < aromatic hydrocarbons, organic halides < sulfides < ethers < nitro compounds < esters, aldehydes, ketones < alcohols, amines < sulfones < amides < carboxylic acids.

Mobile phase in NPC

Various solvents can be used in NPC. They can be alkanes, cycloalkanes (n-pentane, n-hexane, n-heptane, i-octane, cyclopentane, cyclohexane), fluoroalkanes, chlorinated alkanes (dichloromethane, chloroform, carbon tetrachloride, propylchloride), ethers (diethyl ether, di-i-propyl ether), esters (methyl acetate, ethyl acetate), alcohols (methanol, ethanol, 1-propanol, 2-propanol), amines (pyridine, propylamine, triethylamine), carboxylic acids, or their derivatives (e.g., dimethylformamide). The properties of such solvents were discussed in Chapter 7. The eluotropic strength of the polar solvents in NPLC varies with change of the stationary phase, while for the apolar organic solvents it remains almost constant for all stationary phases.

The elution can be performed in isocratic mode or using a gradient of the mobile phase composition [5]. Usually, the elution in isocratic NPC utilizes as mobile phase a solvent with a low elution strength (ε°) mixed with a second solvent having a greater ε° which is added until the separation is attained. For example, the separation of tocopherols can be achieved on aminopropylsilica or diol-bonded silica, using mobile phases containing cyclohexane with ether cosolvent. In this situation, the retention factor of the separated isomers varies in dependence of the polarity of the added cosolvent: k'(tetrahydrofuran) < k'(dioxane) < k'(t-butyl methyl ether) < k'(diisopropyl ether) [6].

Regarding the detection in NPC, this can be performed using most of the detectors presented in Chapter 2. However, the lack of water in NPC is usually a problem for electrochemical detectors

and in some cases for the use of MS detection with electrospray ionization (ESI). Highly volatile NPC solvents are well suited for atmospheric pressure photonionization (APPI). Lower vaporization temperatures can be used with easily vaporizable solvents, and this may be useful when analyzing thermolabile compounds. Many NPC solvents possess ionization energies below the 10.6 eV photons (e.g., 2-propanol 10.17 eV, n-hexane 10.13 eV, i-octane 9.89 eV, tetrahydrofuran 9.40 eV) and can be directly ionized by a krypton discharge lamp without any dopant addition. The use of low proton affinity NPC solvents (hexane, chloroform) with toluene as a dopant can enhance the ionization through charge exchange, and thereby they improve the ionization efficiency for nonpolar compounds. NPC solvents successfully applied to APPI analysis include ethanol, 2-propanol, hexane, heptane, cyclohexane, i-octane, tetrahydrofuran, ethylacetate, and chloroform [7].

The mobile phases commonly used in NPC are not compatible with ESI-MS due to their low polarity and dielectric constant. However, there are modalities to overcome this difficulty, for example, by adding postcolumn a polar component(s). This can be added through a T union or sheath liquid interface to the NPC effluent in order to generate a stable ESI spray. A more complicated strategy is the off-line collection of effluent with analytes of interest, followed by solvent evaporation and reconstitution in a solvent compatible with ESI [8]. The replacement of ESI ionization with APCI ionization is usually a better alternative.

12.2 Other chromatographic techniques based on polar interactions

Aqueous normal phase liquid chromatography

Aqueous-normal-phase chromatography (ANPC or ANP) is a technique performed on a special stationary phase (silica hydride commercially known as type-C silica, by MicroSolv Technology) and the mobile phase covers the range including the types used in reversed-phase chromatography and those used in HILIC and NPC. The most important feature of a hydride silica phase is that water does not adsorb strongly to the phase surface. Regardless of the equilibrium type based on partition or adsorption in NPC and HILIC, the water from the surface of the polar stationary phase plays an important role in the types of interaction with the analyte and in the separation in general. For silica hydride surfaces, this water is either absent or it is not strongly bound to the stationary phase, with less than one monomolecular layer of water adsorbed. The experimental average number of monomolecular water layers per the adsorbent surface is about 0.4–0.5 [9]. This allows a silica hydride phase to work as either a normal phase (NPC) with organic nonaqueous mobile phase (procedure sometimes indicated as ONP), as an HILIC phase with normal phase with partially aqueous mobile phase and with the stationary phase more polar than the mobile phase, and also as an RP phase with the mobile phase containing water and also being more polar than the stationary phase. These retention modes are summarized in Table 12.2.1.

The different types of retention displayed by hydride type stationary phases can also be manifested differently in function of the type of compounds separated on silica hydride phase. Some hydrophobic compounds behave on the silica hydride in the same way as on an RP column, with significantly decreased retention when the organic component increases in the mobile phase. This is exemplified by hypothetical compound #1 in Fig. 12.2.1. Other hydrophobic compounds show more retention than expected when the mobile phase is high in the organic constituent as exemplified by compound #2. Regarding polar compounds, some will behave similarly to a polar compound on an HILIC column, with low retention at low concentration or the organic constituent in the mobile phase (as

TABLE 12.2.1 Types of retention on silica hydride stationary phase.

Stationary phase	Mobile phase	Water content	Term	Similar to
Weak polar	Nonpolar	None	ONP	NPC
Weak polar	Some polarity but less than stationary phase	Some	ANP	HILIC
Weak polar	Polar (more than the stationary phase)	Added water	RP	RP

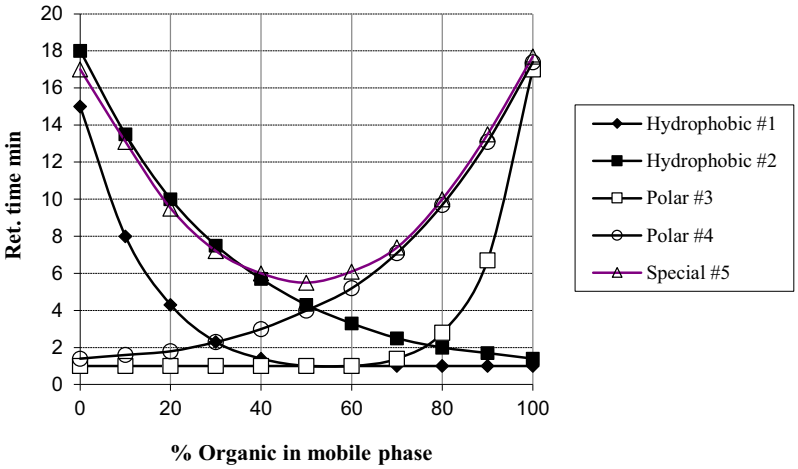

FIGURE 12.2.1 Retention of model compounds on a silica hydride column, exemplifying different types of behavior.

exemplified by compound #3). However, some compounds show unexpected high retention when the content of the polar component in the mobile phase is still high. This is exemplified by compound #4 in Fig. 12.2.1. There are also compounds with both polar groups and hydrophobic moieties in their structure, which have retention in both high organic and in low organic mobile phase, with a minimum of retention in partly organic partly polar mobile phases [10]. This type is exemplified by compound #5, whose dual retention behavior is characterized by a U-shape curve [11], with the minimum $t_R^{\min}$. Generally, the U-shape curve can be fitted by a two-degree polynomial equation written as follows:

$$t_R = a + b\phi + c\phi^2 \qquad (12.2.1)$$

The minimum of this curve can be calculated for $\phi = -\frac{b}{2c}$ and $t_R^{\min} = a - \frac{b^2}{4c}$. According to this dependence, the retention mechanism can be considered as reversed-like mechanism for the domain of ϕ between 0 and $-b/2c$, and from this minimum to 100% organic solvent the retention is a normal-like mechanism.

Since silica hydride (with hydride surface coverage up to 95% [12]) can work in such various conditions, it is difficult to assign a specific mechanism for the separation [10,13–15]. A possible mechanism for this separation relies preferentially on the adsorption of hydroxide ions from mobile phase on the water layer from stationary phase surface and less due to the residual silanols from the stationary phase surface [16]. In this way, the positively charged analytes are attracted

to the negatively charged surface of stationary phase, while other polar analytes are likely to be retained through displacement of hydroxide ions.

A new type of silica hydride is commercially known as diamond hydride surface, which contains a small amount of carbon (approximately 2%) to the silica hydride surface. The attached carbon gives hydrophobic properties to the stationary phase in addition to the ANP retention properties possessed by silica hydride stationary phases. This diamond hydride stationary phase can perform separation in both ANP and RP type separation [17,18].

Electrostatic repulsion hydrophilic interaction chromatography

HILIC type separations (polar stationary phase, less polar mobile phase but containing water) can also be performed on ion exchange (cation exchange or anion exchange) stationary phases, when the technique is sometimes indicated as eHILIC or ERLIC [19]. In this technique, in addition to water, the mobile phase always contains organic solvents. The retention/separation is based on both ion interaction and HILIC type interactions. Since electrostatic forces are about 10 times stronger than nonionic polar interactions (see Section 5.1), ions from the solution that have the same polarity with those from the stationary phase are strongly repelled from it. On the other hand, neutral polar molecules can easily interact with the stationary phase by an HILIC type separation. A cation exchange column (surface negatively charged) will show no retention for anionic species, but will show typical polar interaction with neutral molecules of the analyte. Similarly, an anion exchange column (with positively charged stationary phase) will show no retention for cations but retain neutral polar molecules. This type of effect can be controlled by adjusting the pH of the mobile phase that influences the ionization of the analytes (for example, phosphorylated analytes can be present with one or two negative charges depending on the pH of mobile phase) or by the addition of IPA agents when ion pair type interactions may play a role in the separation. Some neutral stationary phase used in HILIC type separation may also induce electrostatic interactions if the aqueous mobile phase contains up to 20 mM salt that is the minimum concentration required for the formation of a complete electrical double layer on the surface of a charged stationary phase [20].

A useful example of utilization of this mechanism is the separation of peptides. Compared to HILIC type separation, the eHILIC is more sensitive to orientation effects on the interaction between analyte(s) and stationary phase. They have a role in separating species with same charge-to-mass ratio but with different spatial orientation of charged group [20].

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CHAPTER

13

Ion exchange, ion-moderated, and ligand exchange liquid chromatography

13.1 Retention and elution in ion exchange liquid chromatography

General comments

Ion exchange chromatography is dedicated to the separation of ionic species of the sample present in the mobile phase (water or water with a given proportion of a polar solvent). The ion species can be inorganic ions, but also organic ions such as organic acids, amines, amino acids, proteins, oligonucleotides, etc. The stationary phase is an ion exchange resin, i.e., a polymer that contains acidic groups (in case of a cation exchange) or basic groups (in case of an anion exchange) that are bonded to the resin. For example, a cation exchange material may contain covalently bonded sulfonic groups ($-SO_3^-$), carboxylic groups ($-COO^-$), etc., and an anion exchange may contain covalently bonded quaternary amine groups ($-N(CH_3)_3^+$), tertiary amines, etc. The counterion of the bonded acidic groups in the cationic resin can be H^+, Na^+, K^+, etc., and the counterion of the bonded basic groups in the anionic resin can be OH^-, Cl^-, CO_3^{2-}, etc. These ionic species are reversibly retained by ionic groups covalently bonded to the stationary phase.

Retention/elution in ion exchange chromatography

The retention and separation in ion chromatography for a cationic resin of the form $resX^-C^+$ consists of ionic exchange equilibria between the counterions C^+ of the bonded ionic groups on the resin and the analyte ions in the mobile phase. The equilibrium is based on the difference in the affinity for the resin of the ionic species that are separated. For the retention, the ionic analyte should have higher affinity for the resin than the counterions preexistent in the resin. The elution uses a mobile phase that contains competing ions (driving ions) that replace the analyte ions and "push" them out from the stationary phase. For an ion analyte M^{z+} in the mobile phase, involved in a cation exchange with the counterion C^+, the equilibrium is given by reaction 4.3.8 which is written once more below:

$$z\, resX^-C^+ + M^{z+} \rightleftarrows (resX^-)_z M^{z+} + zC^+ \tag{13.1.1}$$

The corresponding equilibrium constant given by Eq. 4.3.9 is written once more below:

$$K_{M,C} = \frac{[M^{z+}]_{res}}{[M^{z+}]_{mo}} \Big/ \left(\frac{[C^+]_{res}}{[C^+]_{mo}}\right)^z \tag{13.1.2}$$

https://doi.org/10.1016/B978-0-323-91177-1.00001-6

Eq. 13.1.2 indicates that the equilibrium 13.1.1 is displaced toward the right when $K_{M,C}$ is large and the concentration $[C^+]_{mo}$ is low. When the species C^+ is used as a driving ion, Eq. 13.1.2 shows that a higher concentration of C^+ (a lower pH in case of $C^+ = H^+$) leads to a smaller $[M^{z+}]_{res}$ which indicates that even an ion with lower affinity for the stationary phase can replace a strongly retained one if it is present at high enough concentration in the mobile phase. This process of modifying the concentration of the driving ions is used in IC for achieving retention and elution. Initially, the driving ion which has a lower affinity for the resin than the analyte is present at low concentration in the mobile phase, and the analyte ions with stronger affinity are retained. By increasing the concentration of the driving ions, the retained ions are eluted, the order of elution depending on their affinity for the resin.

The evaluation of equilibrium constant $K_{M,C}$ can be obtained using Gibbs–Donnan effect that explains the behavior of charged particles near a semipermeable membrane that are not distributed equally on the two sides of the membrane. In ion exchange, the surface of the resin can be equated with the semipermeable membrane, the ions being able to migrate freely outside and inside the resin, while the bonded ionic groups can be equated with large ions that cannot penetrate through the membrane generating in this way a potential difference. It can be considered that the ion exchange is a process combining features of heterogeneous chemical reaction and osmotic distribution [1].

When a semipermeable membrane separates two solution compartments and one side of the membrane contains water and the other side contains a solution of a dissociated salt consisting of small cations C^+ and anions A^- (e.g., Na^+ and Cl^-), the membrane is permeable to these ions, and the ions diffuse through the membrane. The concentration of the salt on both sides of the membrane tends to equalize and in time the final solutions on both sides of the membrane contain equal concentrations of both positive and negative ions, and the system is electrically neutral. No charges are accumulated on the two sides of the membrane.

There are, however, ions that have large volumes and are not able to diffuse freely through the membranes. If a semipermeable membrane separates two compartments and compartment No.1 contains a solution of a dissociated salt consisting of small ions C^+ and A^- and compartment No.2 contains the same small cation C^+ and a large anion B^- to which the membrane is impermeable, the diffusion of ions is not uniform. Since the compartment No.2 does not contain anions A^-, they will diffuse to the compartment No.2, but the large B^- will not diffuse to compartment No.1. This will result in a negative charge on compartment No.2 since $[A^-]_2 + [B^-]_2 > [A^-]_1$ (the index designates the compartment). Because of the excess of negative charge in compartment No.2, the concentration $[A^-]_2$ will remain smaller than $[A^-]_1$. The negative electrical gradient will attract additional C^+ from compartment No.1 to compartment No.2 that already had C^+ and establish electrical neutrality. However, there will be an unequal concentration of diffusible ions with $[C^+]_2 > [C^+]_1$ and $[A^-]_1 > [A^-]_2$. This uneven distribution of ions on the two sides of the semipermeable membrane is known as Gibbs–Donnan effect. The process is illustrated in Fig. 13.1.1.

At equilibrium, the electrochemical potentials $\overline{\mu}_X$ of the ionic species X (A^- and C^+) on both the two compartments No.1 and No.2 must be equal, which can be written as follows:

$$(\overline{\mu}_X)_1 = (\overline{\mu}_X)_2 \tag{13.1.3}$$

The expression of electrochemical potential can be written as follows:

$$\overline{\mu}_X = \mu_X + zF\Phi \tag{13.1.4}$$

In expression 13.1.4, μ_X is the chemical potential of ion X, z is the charge of the ion ($z = 1$ in Fig. 13.1.1), F is Faraday constant, and Φ is the

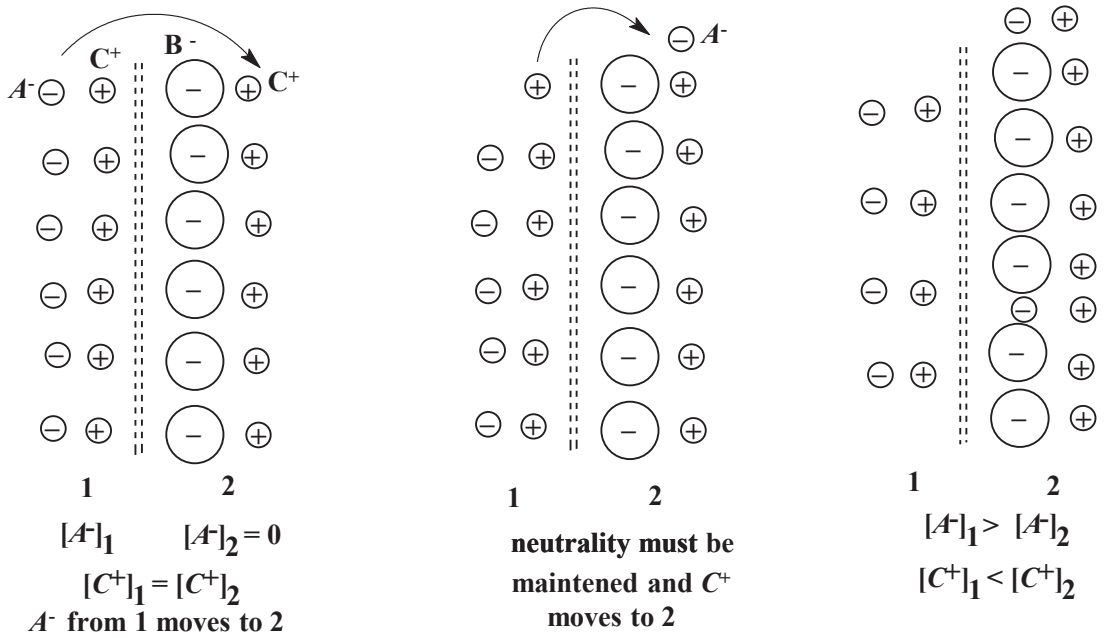


FIGURE 13.1.1 Illustration of Gibbs–Donnan effect. The tendency to have equal concentrations of diffusible ions on both sides of the membrane is compensated by the need for electrical neutrality.

local electrostatic potential. Based on Eq. 13.1.4, the equality 13.1.3 can be written in the following form:

$$\Phi_2 - \Phi_1 = \frac{1}{zF}[(\mu_X)_1 - (\mu_X)_2] \qquad (13.1.5)$$

The difference $\Phi_2 - \Phi_1$ is known as Donnan potential and is indicated as E_{Donnan}. On the other hand, the chemical potential for the species X in a solution with pressure p is given by the following formula:

$$\mu_X = \mu_X^0(p^0) + RT \ln a_X + (p - p^0)V_X \qquad (13.1.6)$$

where $\mu_X^0(p^0)$ is the chemical potential of species X in standard state, a_X is its activity, and V_X is its partial molar volume (partial molar volume of a substance X in a mixture is the change in volume per mole of X added to the mixture). From Eq. 13.1.5 and 13.1.6, the expression for Donnan potential for species X is given by the following expression [2]:

$$E_{Donnan} = \left(RT \ln \frac{(a_X)_1}{(a_X)_2} - \Pi V_X\right)/(zF) \qquad (13.1.7)$$

where Π is the osmotic pressure for the membrane (or the swelling pressure in the case of equilibrium between inside and outside of a resin at molar concentration of species X).

For very diluted solutions, the activities a_X can be replaced by molar concentrations, and in ion exchange, the surface of the resin can be equated with a semipermeable membrane with compartment No.2 being the inside of the resin (*res*) and compartment No.1 the mobile phase (*mo*). In this case, expression 13.1.7 gives the Donnan potential for an ion M^{z+} as follows:

$$E_{Donnan}(M) = \left(RT \ln \frac{[M^{z+}]_{mo}}{[M^{z+}]_{res}} - \Pi V_M\right)/zF \qquad (13.1.8)$$

For equilibrium 13.1.1, the Donnan potentials of the two species M^{z+} and C^{+} must be equal. This can be written as follows:

$$\left(RT\ln\frac{[M^{z+}]_{mo}}{[M^{z+}]_{res}}-\Pi V_M\right)/z=\left(RT\ln\frac{[C^{+}]_{mo}}{[C^{+}]_{res}}-\Pi V_C\right) \tag{13.1.9}$$

From this equality, formula 13.1.9 for $K_{M,C}$ will lead to the following expression:

$$K_{M,C}=\exp\left[\frac{\Pi}{RT}(zV_C-V_M)\right] \tag{13.1.10}$$

A similar expression can be obtained for an anion exchange process for a resin in the form $resY^{+}\ A^{-}$ involved in the equilibrium

$$zresY^{+}A^{-}+B^{z-}\rightleftarrows\left(resY^{+}\right)_zB^{z-}+zA^{-} \tag{13.1.11}$$

For equilibrium 13.1.11, the constant $K_{B,A}$ has the following expression:

$$K_{B,A}=\exp\left[\frac{\Pi}{RT}(zV_A-V)_B\right] \tag{13.1.12}$$

For the equilibrium 13.1.11 in which $z=1$, the equilibrium constant $K_{B,A}$ can be written as follows:

$$K_{B,A}=\frac{[B^{-}]_{res}}{[B^{-}]_{mo}}/\frac{[A^{-}]_{res}}{[A^{-}]_{mo}} \tag{13.1.13}$$

The evaluation of retention factor from a formula of the type $k'=K\Psi$ leads to the following expression:

$$k'(B^{-})=K_{B,A}\frac{[A^{-}]_{res}}{[A^{-}]_{mo}}\frac{V_{res}}{V_{mo}} \tag{13.1.14}$$

In Eq. 13.1.14 can be introduced the ion loading column capacity Q_{IC} obtained from the following expression:

$$Q_{IC}=[A^{-}]_{res}V_{res} \tag{13.1.15}$$

The use of Eq. 13.1.15 for Q_{IC} in expression 13.1.14 leads to the following formula:

$$k'(B^{-})=K_{B,A}\frac{Q_{IC}}{[A^{-}]_{mo}V_{mo}} \tag{13.1.16}$$

In ion exchange, the ion loading column capacity Q_{IC} can be obtained from the following expression:

$$Q_{IC}=\Omega w_{st} \tag{13.1.17}$$

where w_{st} is the weight of stationary phase and Ω is the loading capacity of the ion exchange resin expressed in miliequivalents ions/g sorbent (meq/g).

From Eq. 13.1.16 and Eq. 13.1.17, the retention factor k' for an anionic analyte B^{-} under isocratic conditions in the presence of a competing ion A^{-} can be written as follows [3]:

$$\log k'_B=\log K_{B,A}+\log\Omega+\log\frac{w_{st}}{V_{mo}}-\log[A^{-}]_{mo} \tag{13.1.18}$$

where $[A^{-}]_{mo}$ is the concentration of the anion A^{-} in the eluent, $K_{B,A}$ is the ion exchange selectivity coefficient between the anionic analyte B^{-} and the mobile phase competing anion A^{-}, Ω is the ion exchange capacity of the stationary phase, w_{st} is the weight of the stationary phase, and V_{mo} is the volume of mobile phase in the column. For a given anionic analyte and competing anion A^{-} from mobile phase, this equation can be formally reduced to the following logarithmic dependence:

$$\log k'_B=\alpha-\beta\log[A^{-}]_{mo} \tag{13.1.19}$$

where the regression parameters, α and β, can be determined from experimental measurements of the capacity factor and concentration of the competing ion A^{-}. Eq. 13.1.19 indicates that a decrease in the capacity factor is achieved when the concentration of the competing ions $[A^{-}]$ is increased. This shows that elution can

be achieved using gradient by increasing the concentration of the competing ions.

The use of complexing reagents in the mobile phase complicates the retention and elution process in ion exchange. As shown in Section 4.3, two equilibria are established in the presence of a ligand, as expressed by equilibrium 4.3.13. Eq. 4.3.15 shows that the concentration of the retained ions in the column ($[M^{+}]_{res}$) diminishes as the concentration of the ligand in the mobile phase and the complexation constant are higher. The separation can be modulated based on the retention of newly formed species, the ligand concentration, and the value of constant K_{LM} for the complexation equilibrium [4]. Negatively charged ligands L^{-} are sometimes used as complexing agents, and in the case of negatively charged complexes formed with metal ions, the separation of these complexes can be performed using anion exchange columns.

Besides the equilibrium aspects necessary to understand the ion exchange behavior, the kinetic factors are also very important in ion chromatography, since in the dynamic applications, in addition to the convective process of longitudinal flow of the fluid through the column and the movement of fluid in the void space between the resin particles, other factors are also important. Among these, the diffusion of the compound of interest through the solvent immobilized on the resin particles, the diffusion within the gel microchannels, and the kinetics of the exchange process in itself are factors determining the rate of exchange. Due to the complexity of the process, only the estimation of certain kinetic aspects is usually possible. An expression that estimates the time $t_{1/2}$ for half of the complete conversion of a resin from form C^{+} into form M^{z+}, when the limiting factor is the diffusion in the particle, is given by the following formula:

$$t_{1/2} = 0.0075 d_p^2 / D_{res} \qquad (13.1.20)$$

where d_p is the particle diameter and D_{res} is the diffusion coefficient in the resin. However, other expressions were developed to describe the kinetics of the ion exchange process [5].

Separation in ion chromatography

Since the constant $K_{M,C}$ is dependent on the nature of the ion M^{z+}, the separation of different ions can be achieved using ion exchange stationary phase. The selectivity α in this case for two ions $M_1^{z_1+}$ and $M_2^{z_2+}$ can be defined by the following formula:

$$\alpha = K_{M_1,C}/K_{M_2,C} = \frac{[M_1^{z_1+}]_{res}}{[M_1^{z_1+}]_{mo}} / \frac{[M_2^{z_2+}]_{res}}{[M_2^{z_2+}]_{mo}} \qquad (13.1.21)$$

Based on Eq. 13.1.21, the selectivity between the two ions $M_1^{z_1+}$ and $M_2^{z_2+}$ can be estimated the value of α as given by the following expression:

$$\alpha_{M_1,M_2} = \exp\left[\frac{\Pi}{RT}\left(z_1 V_{M_2} - z_2 V_{M_1}\right)\right] \qquad (13.1.22)$$

Eq. 13.1.22 indicates that in the separation of two ions by ion chromatography, the partial molar volume of these ions plays an important role and a larger difference in their partial molar volume leads to a better separation.

Depending on the affinity for the resin of species M_1^{z+} and M_2^{z+}, they can be separated, provided that α is large enough to assure separation. This implies that at equal concentrations in the mobile phase, the concentration in the resin of an ion with higher affinity for the resin will be higher and its retention will be stronger leading to longer retention times in the chromatographic process. The affinity for the resins of various inorganic ions is in the following order $M^{+} < M^{2+} < M^{3+} < M^{4+}$. This order is also verified experimentally. For the same valence cations, it was found that the affinity varies in general as follows: $Li^{+} < H^{+} < Na^{+} < NH_4^{+} < K^{+} < Rb^{+} < Ag^{+} < Tl^{+}$, and for divalent ions, $Be^{2+} < Mn^{2+} < Mg^{2+} < Zn^{2+} < Co^{2+} < Cu^{2+} < Cd^{2+} < Ni^{2+} < Ca^{2+} < Sr^{2+} < Pb^{2+} < Ba^{2+}$. For trivalent ions, the order is $Al^{3+} < Sc^{3+} > Y^{3+} > Eu^{3+} > Pr^{3+} > Ce^{3+} > La^{3+}$.

For the anions B^{-}, the order of affinity for the resin is in the order $B^{-} < B^{2-} < B^{3-}$. For the ions

with the same charge, the following order was established: $OH^- < F^- < CH_3COO^- < HCOO^- < H_2PO_4^- < HCO_3^- < Cl^- < NO_2^- < HSO_3^- < CN^- < Br^- < NO_3^- < HSO_4^- < I^-$. However, inversions in this order are possible due to the nature of the resin, formation of complexes, etc.

Retention of neutral molecules on ion exchange phases

In addition to the exchange of ions, ion exchange stationary phases are able to retain specific neutral molecules. There are two different ways in which this process can take place. The first is related to the retention based on the formation of complexes. Specific ions such as transition metals (Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, etc.) may be retained on a cation exchange resin and still have the capability to accept lone pair electrons from donor ligands such as amines. Using this mechanism, neutral ligand molecules can be retained on resins already treated with the transition metal ions.

The second process of retention of neutral molecules is based on adsorption on ion exchange matrix without involving an ion exchange process. Organic molecules can be adsorbed on the resin. For example, amines can be retained on a strong cation exchanger in K^+ form. The counterion is important in the adsorbing capability of the resin, and the elution is possible by using solutions of salts at different concentrations. A "salting out" effect is used to modify the adsorption, the variation of the distribution constant in the presence of the salt being described by the following formula:

$$\ln K_B(Salt) = \ln K_B(Salt = 0) + \kappa[Salt] \quad (13.1.23)$$

where $K_B(Salt)$ is the distribution coefficient for the analyte B in the presence of a salt, $K_B(Salt = 0)$ is the distribution constant in pure water, κ a constant specific for the system, and $[Salt]$ is the molar concentration of the salt. As shown in Eq. 13.1.23, the increase in salt concentration increases the adsorption, and in a chromatographic process using this effect, the elution is done by diluting the initial solution of the eluent [6].

Retention in ion-moderated chromatography

In ion-moderated chromatography, neutral molecules display selective partition between the liquid from the inside of the resin and the mobile phase. The retention inside the resin is based on weak polar interactions between the analyte and the stationary phase. Some ion-moderated phases may be considered as functioning based on ligand exchange mechanism.

Retention in ligand exchange and immobilized metal affinity chromatography

In ligand exchange chromatography, the stationary phase is a cation exchanger that carries ions of a transition metal such as Cu^{2+} or Ni^{2+}. The transition metal can form coordinative bonds with electron donor molecules, and the mobile phase must contain donor molecules such as a low concentration of ammonia or even water. These molecules are attached to the transition metal such that the stationary phase is loaded with the (weak) complexes of the transition metal. The analytes are also electron donor molecules such as amines, amino acids, or hydroxy compounds. The retention process consists of the replacement of the weak ligand (e.g., ammonia) with the analyte molecules that are stronger ligands. However, by increasing the concentration of ammonia in the mobile phase, or simply in isocratic mode by continuing to flush the column with the initial ammonia solution, the analyte ligand is eluted from the column (with the weaker ligand analytes eluting first) [7].

Ligand exchange chromatography has been further developed into a similar technique indicated as immobilized metal affinity chromatography. The difference from ligand exchange is that the stationary phase has covalently connected chelating groups able to form very strong coordinative complexes with transitional metals. These groups are typically derived from iminodiacetic acid or tricarboxymethylethylenediamine. The phase is pretrated with the metal ion solution, and during this process, weaker coordinative ligands such as water become part of the complex. The analytes are typically electron donor molecules such as amino acids, peptides, or proteins. These analyte molecules form relatively stable complexes with the inorganic metal. An immobilized complex involving the analyte, the metal, and the resin functionality is formed. The elution can be done with buffers that have a pH where the analyte to metal bond is weakened, or with an additive such as ammonia in the mobile phase, which competes with the analyte bonded on the column. The metal itself can also be eluted from the column using a strong complexing agent such as ethylene-diaminotetraacetic acid (EDTA). The metal (the same or different) can be later replaced on the column.

Ion exclusion

Ion exclusion is applied mainly for the separation of neutral molecules or weakly polar ones from ionic molecules. This type of separation can be practiced on ion exchange stationary phases having, for example, strong acid groups bonded to a solid support. Strong acids are not retained at all due to repulsive electrostatic forces (Donnan exclusion). Such compounds will elute in the void volume of the chromatographic column. Weak or very weak acids are not subject to Donnan exclusion, penetrate the pores of the stationary phase, and are separated based on differences in pK_a, size, and hydrophobicity. The mechanism in ion exclusion is more complex than in simple ion exchange chromatography.

13.2 Stationary phases and columns for ion exchange and related HPLC techniques

Types of ion exchange phases

The stationary phases used in ion exchange chromatography are basically similar to other stationary phases (see Section 8.1) having a solid porous support on which the active phase is bonded or coated. In IC, this phase contains ionic groups [8]. Most supports are either silica or organic polymers, but other materials are sometimes used for obtaining ion exchange phases (e.g., alumina, zeolites). Differently from RP-HPLC or HILIC, the organic polymers are more frequently employed as support materials in ion chromatography. These materials have a better stability toward extreme pH conditions than silica, and a wider pH range is sometimes necessary in ion chromatography. While silica-based LC columns are usually stable at pH between 2 and 8, polymeric ion exchangers (resins) can be stable in a wider range of pH of the mobile phase [9]. The main types of ion exchange stationary phases related to their functionality are indicated in Table 13.2.1.

Regarding the ion-moderated phases, they are usually made from a cation exchange material in H^+ or metal form (e.g., Na^+, K^+, Ca^{2+}, Pb^{2+}, Ag^+), which is used in specific conditions for the separation of neutral species. A different type of phase from the same category is based on crown ether moieties. The crown ether groups can be present in an organic based solid support or immobilized on silica. Crown ethers strongly bind certain cations, forming complexes. For example, 18-crown-6 ether has affinity for potassium ions, while 15-crown-5 has affinity for sodium. The crown ether can also display Donnan type exclusion and act as an ion exclusion phase. Other neutral materials, such as polyethylene glycol, exhibit retention abilities of various anions and used as the stationary phase for the separation of inorganic anions in ion chromatography [10].

TABLE 13.2.1 Types of functionalities in ion exchange phases.

Phase character	Strength	Examples of phase structure
Cation exchange	Weak	$-COO^-$, $-C_6H_4-O^-$, $-AsO_3H^-$
Cation exchange	medium	$-PO_2H^-$,
Cation exchange	Strong	$-PO_3H^-$, $-SO_3^-$
Anion exchange	Weak	$-NH_3^+$, $-[NH_2(CH_3)]^+$
Anion exchange	medium	$-[N(CH_3)_2(CH_2CH_2OH)]^+$
Anion exchange	Strong	$-[N(CH_3)_3]^+$, $-[N(C_2H_5)\ (CH_3)_2]^+$
Zwitterionic	–	$-N(CH_3)_2^+-(CH_2)_n-SO_3^-$ or $-CH(SO_3^-)-(CH_2)_n-N(CH_3)_3^+$
Amphoteric [a]	–	Various polymers

[a] *The presence in a polymeric structure of both acidic and basic groups such as $-N(CH_3)_2^+$- and $-SO_3^-$ is usually indicated as zwitterionic. An amphoteric group must be able to act as an acid in basic medium and as a base in acidic medium.*

Ion-moderated and ligand exchange phases

Ion-moderated stationary phases typically consist of a cation exchange stationary phase having covalently bonded groups such as $-SO_3^-$, with these groups having as counterion cations such as K^+, Na^+, Ag^+, Ca^{2+}, Pb^{2+} (in some cases H^+). Such columns are frequently utilized for the analysis of monosaccharides, oligosaccharides, hydroxy-organic acids, glycerol, sorbitol, etc., in an aqueous of almost aqueous mobile phase. The cation exchange material is usually an organic polymer based on styrene/divinylbenzene, and the specific metal form is maintained by having the ions at a low concentration in the mobile phase. Some ion-moderated polymeric columns that are commercially available are listed in Appendix 13.2.5. While most ion-moderated columns are used with an aqueous mobile phase, the "Lipid" columns are used with nonaqueous mobile phase.

A special type of ion-moderated stationary phase is utilized in the separation of cis/trans lipids and fatty acids. This stationary phase contains Ag^+ ions typically in a silica support type column such as ChromSpher 5 Lipids [11]. The separation of cis/trans isomers is based on differences in the interaction of π bonds in the lipid or fatty acid with the silver ions. Such columns are used with nonpolar organic mobile phases [12,13].

Ligand exchange phases are ion exchange phases loaded with a transition metal capable of forming coordinative bonds with the analyte. Special phases are used for immobilized metal affinity usually derived from iminodiacetic acid or tricarboxymethylethylenediamine. These phases are able to form very strong coordinative complexes with transitional metals that are further coordinating with the analytes possessing electron–donor functional groups ($-NH_2$, $-COOH$, $-OH$, $-SH$, and others). The order of elution is given by the order of stability of coordinating associations between analyte and transition metal ion from stationary phase [14]. This mechanism proved useful in separating chiral compounds [15].

Summary of procedures for the synthesis of ion exchange phases

The ion exchange stationary phases can be made using a variety of procedures. A classification of stationary phases used in IC based on their construction is indicated in Table 13.2.2.

Silica-based ion exchange phases include those with a bonded phase containing ionic groups, and also other types of phases

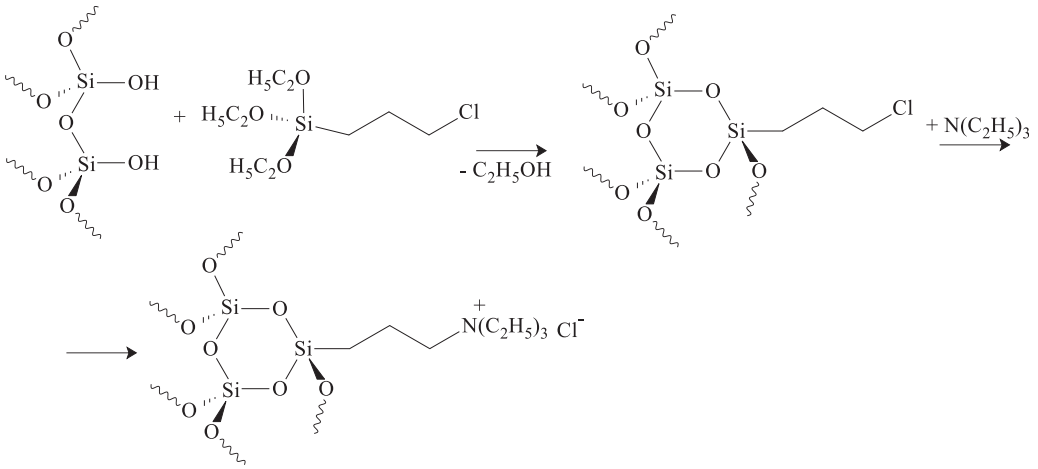
TABLE 13.2.2 Types of construction for ion exchange phases.

Phase support	Type of phase structure
Silica based	Bonded ionic groups
Silica based	Polymer coated
Organic polymer	Synthesized with ionic functional groups
Organic polymer	Surface functionalized
Organic polymer	Latex agglomerated
Inorganic silicates	Zeolites
Inorganic oxides	Alumina, silica

generated, for example, by coating a porous silica material with a polymer with specific ionic groups. Bonded ionic groups are more common in these types of ionic phases. The synthesis of such materials can be done using one step derivatization of porous (hydrated) silica with appropriate reagents. For example, acidic groups such as $-SO_3^-$ can be attached by using for silica derivatization, a silane containing ethylbenzenesulfonic groups. More commonly ionic phases are prepared in two-step reactions, one example of synthesis for an anion exchange phase being shown below (reaction 13.2.1):

Other procedures were described in the literature (e.g., Ref. [16]), such as reacting the silica surface with a silane containing an alkylthiol group followed by an oxidation of the thiol to a sulfonic group.

A large part of ion exchange stationary phases is based on organic polymers. These materials consist of an organic polymeric (resin) backbone containing covalently bound groups that are able to exist in ionic form. Among the common backbone polymers are the copolymers of styrene-divinylbenzene (PS-DVB), ethylvinylbenzene-divinylbenzene (EVB-DVB), polyvinyl



(13.2.1)

copolymers such as those obtained from polyvinyl alcohol (PVA), and various polymethacrylates [17]. Materials with ion exchange properties can also be obtained based on cellulose, dextrans, etc. The presence of the ionic groups on these resins makes these resins to act as polyelectrolytes. The main advantage of resin-based ion exchangers is their tolerance toward eluents with extreme pH values, between 0 and 14, in contrast to the silica-based stationary phases, whose pH limits are in many cases 2–7 and the pH range stability is extended only by special procedures (see Section 8.1). This wide range of pH values for organic polymers allows the use of selectivity effects on multicharged or weakly ionizable solutes. The use of polymeric resins has pressure limitations because they have lower mechanical resilience. Macroporous resins with high degree of cross-linking (in the interval from 4% to 12%, and for the commonly used ion exchangers 8% [18]) are relatively more rigid and stable, and although they have lower ion exchange capacity are used in HPLC and can be included in longer columns, at higher flow rates. Monoliths columns were also made from polymers such as polyacrylamide [19].

Several procedures are used for obtaining polymers with ion exchange properties. One such procedure consists of the synthesis of the polymer already containing the desired ionic groups in the monomer. Another procedure is the derivatization of an already polycondensed or polymerized resin. Many other synthetic paths have been either used or only explored for producing ion exchange resins. Some resins are prepared to have more than one type of functional group, others are made to contain unique structures, etc. Amphoteric ion exchangers with both basic and acidic groups, as well as ion exchangers with specific chelating properties, are also available for various applications.

Silver ion impregnated HPLC columns are typically made using a silica column or an ion exchange column (such as Nucleosil 5 SA containing phenylsulfonic acid groups bonded chemically to silica) and treating it with a solution of a silver salt, such as $AgNO_3$ [20].

Latex-agglomerated ion exchangers

The direct use of polymeric ion exchange materials as stationary phase for HPLC encounters several problems. One problem is the relative low mechanical stability even at moderate pressure, and the other is the swelling and shrinkage of the phase during the ion exchange process. These problems are significantly alleviated using latex-agglomerated ion exchange stationary phases. In addition to better mechanical stability and reduced swelling and shrinkage, these phases also offer high efficiencies and a better ion exchange capacity (between 0.03 and 0.1 mEquiv/g).

Latex-agglomerated exchangers contain an internal core particle (or support) that contains on its surface ionic functionalities. On this support is attached a monolayer of small diameter particles that carry functional groups consisting of bonded ions that are of the opposite charge with the functionality of the support. The groups of the outer particles have a double role, to attach the small particles to the core, and also to act as an ion exchanger for the ions in solution. The core particles are typically PS-DVB resin of moderate to high cross-linking, with a particle size in the range 5–25 μm. The outer microparticles consist of finally ground resin or monodisperse polymer (latex) with diameters up to 0.1 μm that have very high porosity, and are functionalized to contain an appropriate ion exchange group. This group determines the ion exchange properties of the composite particle. For example, an aminated latex produces agglomerated anion exchangers, although the core support contains groups such as SO_3^-. A sulfonated latex produces agglomerate cation exchangers although the core support has positive groups (see, e.g., Ref. [8]). The structure of such phase is schematically shown in Fig. 13.2.1.

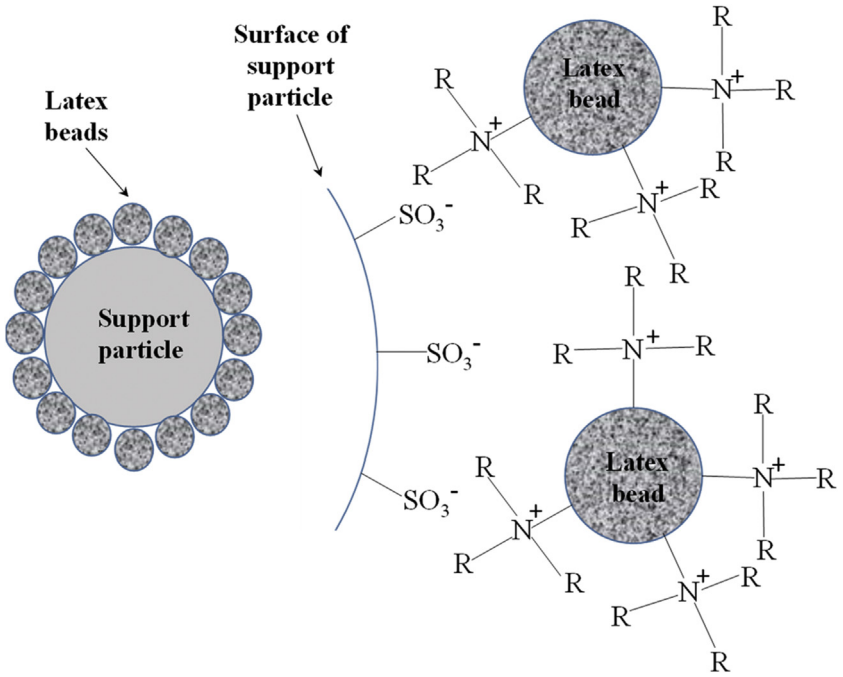


FIGURE 13.2.1 Schematic drawing of a latex-agglomerated type ion exchanger.

This kind of anion exchanger is chemically very stable, and even a high concentration of NaOH is unable to disrupt the ionic bonds between substrate particle and latex beads. The polymers used for latex formation include derivatized divynilbenzene − vinylbenzylchloride, polystyrene, and acrylate, but at lower degree of cross-linking [21]. Columns with latex-agglomerated technology can be used at column backpressures as high as 3000 to 4000 psi, which is close to the use of typical silica-based columns.

Other types of latex-agglomerated stationary phases can be produced. For example, Nafion (copolymer of tetrafluoroethylene with perfluorovinylether with terminal sulfonic groups) can be coated directly to an octadecyl-modified silica being attached by simple hydrophobic interactions. The final material can be used as cation exchanger, which has been proved to have a high selectivity. The schematic structure of Nafion is shown in Fig. 13.2.2

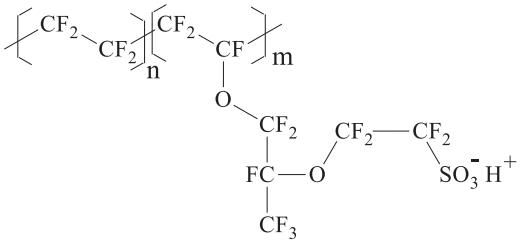


FIGURE 13.2.2 Idealized formula of Nafion polymer.

Cation exchange phases based on silica

Pure silica gel, synthetized by hydrolysis of tetraethoxysilane, is the first silica material used for separation of mono and divalent cations due to the dissociation of silanol groups at average pH values of mobile phase [22]. Silica-based ion exchange columns are preferable for some of their qualities, in particular their mechanical resilience and small variation in the volume in different mobile phases. However, silica-based columns can be typically used within a narrower pH range

compared to those based on organic polymers. In IC, it is frequently necessary to use basic or acidic mobile phases, and the acceptable pH range of use may be a limitation for some applications when using silica-based columns. Some commercially available silica-based cation exchange columns are listed in Appendix 13.2.1.

Attempts were made to use the advantages of silica support such as the large surface area and lack of volume changes in different mobile phases or when the counterion is changing, and at the same time to extend the pH range utilization. One path to reach this goal is to make polymer-coated silica phases [23]. The silica surface can be coated using "prepolymers" which are oligomers synthesized in a separate step and then applied to the support material and immobilized. For example, a prepolymer can be made using a mixture of butadiene and maleic acid (PBDMA) [9,24].

Organic polymeric cation exchange phases

Organic polymeric cation exchange columns are common. Styrene/divinylbenzene copolymers are the most widely used support materials for the obtaining this type of cation exchangers. Their resilience to a wide pH range is an advantage for IC applications. The ionic capacity is determined by the degree of sulfonation. Typical values for ionic capacity are 0.005−0.1 mEquiv/g, but some manufactured exchangers can reach higher values. Some cation exchange polymeric columns that are commercially available are listed in Appendix 13.2.2.

In this class of cation exchangers can be included the organic polymers containing carboxyl groups coated on silica. A special copolymer with cation exchanging property results from butadiene and maleic acid, using peroxides or γ-radiation as initiator [23]. Another possibility is to sulfonate the polymer coated or bound on the silica support, resulting a strong cation exchange capability [25].

Anion exchange phases based on silica

Silica-based IC stationary phases are characterized by higher chromatographic efficiencies and greater mechanical stabilities compared to those based on organic polymers. They have better mechanical resilience and do not show swelling and shrinkage in different solvents or when the counterion is different. Also silica-based columns can be used at temperatures up to 80 degrees. However, as indicated for cation exchange phases, silica-based columns typically have a narrower pH range of utilization (pH 2−8). The columns with up to $N/m \approx 20{,}000$ are common for phases with particles of 3 μm. The ion exchange capacities are in the range 0.1−0.3 mEquiv/g. A number of anion exchange columns with a silica backbone are commercially available. Some of these columns are listed in Appendix 13.2.3.

New silica-based stationary phases have been developed for anion exchange IC, by covalently bonding the ionic liquids incorporating imidazolium or pyridinium groups to the silica surface [26]. For example, a route of synthesizing a pyridinium moiety to the silica surface is based on a derivatization reaction of silica described in reaction 8.1.13, where R is $-(CH_3)_3-Cl$, followed by reaction with pyridine [16]. Besides anion exchanger character of these stationary phases, they can be involved in multimodal retention mechanisms due to the hydrophobic, ion−dipole, dipole−dipole, H-bond, and π-π interactions with the analytes.

Besides fully porous particles, pellicular phases consisting of much larger particles with a diameter of 25−40 μm and covered with 1−3 μm polymeric film are also available for HPLC use. The polymer can be, for example, lauryl methacrylate (Zipax SAX) with quaternary ammonium ion exchange functionality. The ion exchange capacity of pellicular materials is however lower, of about 0.01 mEquiv/g [9].

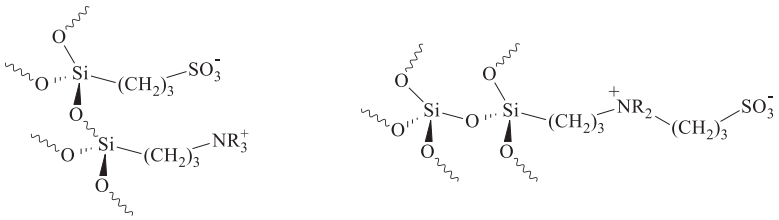


FIGURE 13.2.3 Examples of two structures silica-based zwitterionic IC stationary phases.

Organic polymeric anion exchange phases

Organic polymer anion exchange columns are very common. Similar to the case of cation exchange polymeric columns, the mechanical stability and the resilience of organic polymer-based phases to a wide pH range is an advantage for IC applications. Styrene/divinylbenzene copolymers, polymethacrylate, and other polyvinyl resins are common as support materials in the manufacturing process for polymer-based anion exchangers [9,27]. Some of the phases are made using latex technology. Chloromethylation and amination of various branches are the main procedures of introducing the functional groups on the polymeric surface [28]. Some of the phases are made using latex technology. Besides typical anions, some columns are designed to work at very high pH and capable of analyzing monosaccharides, disaccharides, specific oligosaccharides, and sialic acids [29]. Some anion exchange polymeric columns that are commercially available are listed in Appendix 13.2.4.

The use of conductivity detection following ion suppression is commonly used for anion detection. The columns can be designed specifically for use with aqueous NaOH (or KOH) eluent or with aqueous Na_2CO_3 eluent (e.g., Ref. [30]).

Zwitterionic stationary phases

Zwitterionic ion exchangers combine the positively and negatively charged sites in a single particle by attachment to the support of two different molecules with different functionalities or one type of molecule containing two functional groups of different nature (amino and sulfonic groups, for instance) [31]. These two possibilities are exemplified in Fig. 13.2.3.

Also, latex-agglomerated ion exchangers having an outer layer of anion exchange microbeads (as schematically exemplified in Fig. 13.2.1) can exhibit some cation exchange capacity. The analyte cations from mobile phase can have access to the sulfonic groups found on the core particle and interact with them, a process that can be reversible and leads to the elution of cations from the column. This property can be utilized in the separation of various inorganic anions and cations on the same HPLC column. Analytical applications are known for this type of zwitterionic stationary phases (Dionex HPIC-CAS1 column), using mobile phase containing phthalate—tetraborate and copper chloride [32].

Capillary IC columns

Besides columns with i.d. of 4.6 mm, 4.0 mm, or 2 mm, several capillary IC columns are commercially available (e.g., Ref. [33]). For example, Thermo Scientific/Dionex have commercially available columns with 0.4 mm i.d. (indicated as capillary). These columns are packed with the same material as the equivalent analytical scale version and produce separations similar to 4 mm i.d. columns (when appropriate flow is selected). The typical eluent flow rate in capillary columns must be selected only at about 1/100 from the flow rate of an analytical format.

Due to the smaller cross-sectional area, the flow rate on a capillary format can be scaled down to 10 μL/min. The first advantage of such flow is the reduction of the eluent usage. Also scaled down when using capillary columns must be the sample load. However, in IC, the most common detector is the conductivity detector, which is a concentration-sensitive detector (see Section 2.2). Because of the difference in the separation flow rates (e.g., 100 times lower than in conventional columns), the volume passing through the conductivity detector at a specific time is 100 times smaller with the capillary system than with the standard system and the concentrations of the separated analytes passing through the conductivity detector is 100 times higher. In this way, the capillary column provides increased mass sensitivity such that a much smaller sample can be injected with no loss or even with gain in sensitivity compared to standard columns and flows. One disadvantage of the use of capillary columns is the need for adequate instrumentation that may be different from the standard equipment. The dedicated equipment requires high-precision pumping at low flow rates, low volume injectors, and low volume conductivity cell for the IC detector (see Section 2.1) [34,35].

Other IC stationary phases

Columns with stationary phases containing a crown ether moiety attached to silica support are utilized in particular for chiral separations, when the crown ether has a specific chiral structure. They can be used also as stationary phases for the separation of alkali and alkaline-earth cations, ammonium, and amines by ion chromatography [36]. Crown ethers can also be used as additive in capillary ion chromatography when they act as a trapping selector of cations depending on the size ligand cavity and the size of cation. The free and cation-trapped crown ether molecules from the eluent are in reversible equilibrium of adsorption on a very hydrophobic stationary phase (e.g., triacontyl silica, C30) [37]. There are attempts to coat the silica with dendrimers in order to obtain anion exchanger for IC separations. An example is the dendrimer resulted from the polycondensation of methylamine with 1,4-butanedioldiglycidyl ether, which was coated on silica support and used for the separation of many anions (F^-, Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}, ClO_4^-) [38].

13.3 Characterization of ion exchange stationary phases

General comments

Besides common physical characteristics of the LC stationary phase (such as particle size, particle uniformity, porosity, pore size) and type of the active groups, ion exchange phases are also characterized by their ion capacity. The ion capacity of the ion exchanger is determined by the number of functional groups per unit weight of the stationary phase. The most commonly used units for ion capacity characterization are milliequivalents of charge per gram of dry packing and milliequivalents per mL of wet packing. Typical ion exchange capacity in IC is 10−100 mEquiv/g, but some commercial ion exchangers may reach higher values [39]). The counterion present in the stationary phase must be indicated together with the ion capacity since it affects the degree of swelling of the packing and hence its volume. The ion exchange capacity of a stationary phase plays a significant role in determining the concentrations of competing ions used in the mobile phase for elution. Higher capacity stationary phases generally require the use of more concentrated mobile phases. This is not a recommended feature in high-performance ion chromatography, since the use of the common conductometric detectors cannot function well with high salts concentrations. However, the lower ion exchange capacity

limits the sample loading of the phase, which must be low for low ion capacity phases. For strong ion exchange phases, the ionization of the stationary phase does not depend on the pH of the mobile phase, and regardless of the pH, the loading capacity is the same. For weak ion exchangers, the use of a pH of the mobile phase where the stationary phase is not ionized may considerably decrease the phase retention.

Another important characteristic of the IC columns is related to their hydrophobic character, which should be as low as possible. In many IC separations besides the inorganic ions, other molecules are present, and the hydrophobic character of the analyte should not interfere with the separation. However, the ionic groups of the stationary phase are typically bonded to an organic fragment that is either connected to silica or to an organic polymer. This part of the ionic stationary phase may influence the separation of the analytes by undesired hydrophobic interactions. Phases with low or ultralow hydrophobicity were manufactured, and this property is typically indicated for commercially available IC columns.

Besides ionic and hydrophobic interactions, some phases develop polar interactions with the analytes. The polarity of the stationary phase is not usually considered as detrimental. The polar character influences the retention of polar compounds, but the overall ion exchange character is dominant with both ionic and polar molecules. Polar character and ionic character do not act opposite to each other, and the polar interactions do not affect adversely the separations.

Other types of characterizations of ion exchange phases are related to their physical properties. Basically, the same physical characteristics are applicable to ion exchange columns as for other types of columns. Silica-based ion exchange phases usually have good mechanical resilience to the column backpressure, while for polymeric phases, the backpressure limit is usually lower. Changes in the phase volume in different mobile phases, in particular for polymeric type phases, are undesirable and phases with minor variation in volume are required for HPLC applications. Also, parameters such as thermodynamic exchange constant and the free energy of solute/phase interaction were used for phase characterization [40].

The van't Hoff plots are usually linear in IC, and can be used for the evaluation of the variation of standard enthalpy and entropy of retention for both anions and cations [41,42]. The retention of anions can be an exothermic or an endothermic process, and temperature can influence the selectivity of separation [35]. By classifying the type of ions in three groups: (a) weakly retained singly charged anions (iodate, bromate, nitrite, bromide, nitrate), (b) multiply charged anions (sulfate, oxalate, phosphate, thiosulfate), and (c) strongly retained singly charged anions (iodide, thiocyanate, and perchlorate), the change in temperature is ineffective at changing selectivity between anions of the same group, but affects selectivity for anions from different groups [43].

Ionic loading capacity measurement

Ion loading capacity of an ion exchange resin, Ω is defined as miliequivalents ions retained/g sorbent (meq/g) or per mL volume of swollen stationary phase. This value can be measured by titration with a strong acid or base solution, depending on the type of resin. However this approach is rather tedious [2]. Another procedure is based on elemental analysis for the identification of specific functional groups (e.g., measurement of sulfur for the evaluation of the number of SO_3H groups [44]). Ion exchange capacity can also be obtained using the measurements of transient pH change that occurs when a step is made in the ionic strength of the mobile phase with unmodified pH [45]. This method uses two solutions of exactly the same pH, but at different ion strengths (different buffer

concentrations) as mobile phase. The ion exchange column is first loaded with a specific ion in a high concentration of a buffer and, after equilibrated, the pH of the eluent is measured. By step decreasing, the buffer concentration part of the attached ions on the ion exchanger is released in the solution. For example, a column loaded with $H_2PO_4^-$ ions in a higher concentration of the buffer in the mobile phase will release $H_2PO_4^-$ ions when the buffer concentration is suddenly decreased. The released $H_2PO_4^-$ ions produce a pH drop (although the pH of the concentrated buffer solution and diluted buffer solution is the same). After a specific amount of ions are released, the equilibrium is reestablished and the pH is reset. The duration of the pH drop transient time is proportional with ionic capacity of the column allowing the evaluation of this capacity [46,47].

Solvent compatibility of ionic phases

Compatibility of the stationary phase with different organic solvents from the mobile phase is an important phase characteristic. The presence of an organic solvent in the mobile phase is common in IC, for assuring organic bases or acids solubility and affecting selectivity by changing the retention characteristics of the column [48]. Selectivity is affected by a complex process of modifying the solvation sphere of the ions and the strength of ionic analyte/solid phase interaction [49]. Silica-based ion exchange phases are less affected by the presence of an organic solvent in the predominantly aqueous mobile phase. However, ion exchanges based on organic polymers are prone to swelling even in the presence of a low level of organic solvent in an aqueous mobile phase. By increasing the polymer cross-linking, e.g., to as much as 50%, the swelling is significantly reduced in modern ion exchangers such that they are compatible with many solvents commonly used in IC. The swelling characteristics of the phase are important in the phase selection.

Phase affinity for specific ions

The affinity of the chromatographic column for certain ions is an important parameter for column selection. The columns typically come loaded with a counterion that has low affinity for the column. For example, the anion exchange columns typically come loaded with OH^-or with CO_3^{2-} ions. As indicated in Section 4.1, ion chromatography frequently uses conductivity detectors with ion suppression capability. The ion suppression eliminates the OH^- from the mobile phase by changing it into H_2O, while an ion X^- is changed into HX that is dissociated and produces high conductivity (similar mechanism works for CO_3^{2-}). Both OH^- and CO_3^{2-} ions have low affinity for anion exchange columns, and they can be easily replaced by ions such as F^-, Cl^-, Br^-, I^-, NO_3^-, ClO_3^-, SO_4^{2-}, PO_4^{3-}, etc. However, the ion exchange constant $K_{X,OH}$ (see expression 4.3.31 for a cation exchange) depends on the nature of the resin. For an anion X^- and the OH^-as a counterion, expression 4.3.11 can be written in the following form:

$$K_{X,OH} = \frac{[X^-]_{res}}{[X^-]_{mo}} / \frac{[OH^-]_{res}}{[OH^-]_{mo}} \tag{13.3.1}$$

The expression for retention factor of the column will be in this case given by Eq. 13.1.15 written once more below:

$$k'(X^-) = K_{X,OH} \frac{\Omega\, w_{st}}{[OH^-]_{mo} V_{mo}} \tag{13.3.2}$$

Expression 13.3.2 shows that at low hydroxide concentration in the mobile phase the retention factor k' for the ion X^- is high, and when the concentration of OH^- increases, k' decreases and the species X^- can be eluted from the column.

The values for $K_{X,OH}$ and for Ω for various columns are discussed in the literature [50,51] and reported by some column manufacturers [52]. For example, the values for $K_{X,OH}$, where X^- is F^-, Cl^-, Br^-, I^-, NO_3^-, ClO_3^-, SO_4^{2-}, and or PO_4^{3-}, are higher for stationary phases containing bonded trimethylamine or triethylamine in

comparison to alkanolamine. This can be explained by the more hydrophilic character of alkanolamine sites, which become more hydrated than trimethylamine and triethylamine moieties and display different affinity for the ions. For several Dionex columns (see Appendix 13.2.4), the values for $K_{Cl,OH}$ are in the following order: AS10 << AS15 << AS19 < AS24 < AS18 < AS11-HC ≈ AS20 < AS16. For the same types of columns, $K_{NO3,OH}$ is in the following order: AS10 << AS15 << AS24 ≈ AS19 ≈ AS18 < AS11-HC < AS20 < AS16. The column capacity Q_{IC} for OH^- of these columns is in the following order: AS24 < AS10 ≈ AS16 < AS15 < AS19 < AS18 < AS11-HC < AS20. Physical construction and chemical nature of the phase influence both $K_{X,OH}$ and for Q_{IC} values.

Hydrophobicity of IC columns

The hydrophobicity of the stationary phase for IC columns caused by various hydrophobic moieties in the phase construction (e.g., polymeric backbone) interferes in the ion exchange process and influences the retention and selectivity of IC columns. This characteristic can be detrimental in some separations, but also can be used to the advantage of certain separations. Hydrophobic contribution of a column can be evaluated by studying the retention of compounds that also have both ionic and hydrophobic moieties such as aliphatic carboxylic acids [53] or alkyl sulfonic acids [50]. For a series of homologues ions with a linear alkyl chain (for example, for a series of anions $H(CH_2)_n-X^-$) even methylene selectivity parameters $\alpha(CH_2)$ can be defined [39].

13.4 Selection of an ion exchange stationary phase

General comments

Strong ion exchange stationary phases are completely ionized over a wide range of pH. The degree of dissociation of weak ion exchangers depends on the mobile phase pH. For this reason, the ion exchange behavior of strong ion exchange phases, within typical range of pH operation, is not influenced by the pH of the mobile phase. The selection of a strong or weak ion exchange stationary phase is determined by the nature of the analytes and by the purpose of analysis. The use of weak ion exchangers allows a better modulation of the retention properties depending on the mobile phase.

Basic concept in the selection of ion exchange columns is simple: strong acids ($pK_a < 2$) are best separated on weak anion exchange columns, while the weak acids ($pK_a = 2-5$) are separated on a strong anion exchange column. Similarly, weak bases are well separated on a strong anion exchange column and strong bases ($pK_a > 10$) on a weak anion exchange column. However, the utilization of ion exchange separations is much more complex, and various vendors developed specific applications for their columns. As an example, the recommended applications for several Thermo/Dionex columns are indicated in Table 13.4.1 (see, e.g., Ref. [54]). Separation in IC depends besides the column on other separation parameters such as mobile phase composition, temperature, choice of gradient versus isocratic mode, etc. Since in IC the detection is frequently performed using conductivity detection, the ion suppression is an important step and a selection of the suppressor type and its parameters is required for a successful analysis. Specialized software guiding the column selections in IC is available in the literature [55].

Similar recommended applications can be found in the literature from other column manufacturers and for a variety of other columns. For example, for Rezex type columns working in ion-mediated type chromatography, the recommendations indicated in Table 13.4.2 can be found [56].

In addition to single column IC, more complex IC separations are sometimes practiced. The columns used in comprehensive multidimensional ion chromatography (IC x IC) are frequently selected to have orthogonal selectivity [57].

TABLE 13.4.1 Example of recommended applications for several Thermo/Dionex columns.

Column	Dimension mm	μEquiv/col[a]	Application with cation exchange
IonPac CS19-4 μm	250 × 4, etc.	2410	Common inorganic cations, amines
IonPac CS19	250 × 4, etc.	2410	Amines, morpholine, choline, etc.
IonPac CS18	250 × 2	290	Polar amines
IonPac CS17	250 × 4, etc.	1450	Amines
IonPac CS16	250 × 4, etc.	8400	Amines, ammonia, Li^+, Na^+
IonPac CS12A	250 × 4, etc.	2800	Common cations, Li^+, Na^+, Ca^{2+}, Mg^{2+}
IonPac CS5A	250 × 4, etc.	40	Hg^{2+}, transitional metals

Column	Dimension mm	μEquiv/col	Application with hydroxide mobile phase
IonPac AS27	250 × 4, etc.	220	Halogen anions
IonPac AS26	250 × 4, etc.	250	Haloacetic acids
IonPac AS25	250 × 4, etc.	350	Various inorganic anions
IonPac AS24A	250 × 4, etc.	560	Haloacetic acids
IonPac AS24	250 × 2	140	Halogen anions, small organic acids
IonPac AS20	250 × 4, etc.	310	Various anions
IonPac AS19	250 × 4, etc.	240	Various inorganic anions
IonPac AS18	250 × 4, etc.	285	Various anions
IonPac AS17-C	250 × 4, etc.	30	Fast analysis of various inorganic anions
IonPac AS16	250 × 4, etc.	170	Various inorganic anions
IonPac AS15	250 × 4, etc.	225	Various anions, carbohydrates, organic acids
IonPac AS11	250 × 4, etc.	45	Various inorganic anions, organic acids
IonPac AS7	250 × 4, etc.	100	Cr(VI)

Column	Dimension mm	μEquiv/col	Application with carbonate mobile phase
IonPac AS23	250 × 4, etc.	320	Inorganic anions
IonPac AS22	250 × 4, etc.	220	Inorganic anions, organic acids
IonPac AS14	250 × 4, etc.	65	Inorganic anions
IonPac AS9-HC	250 × 4, etc.	190	Inorganic anions
IonPac AS4A-SC	250 × 4, etc.	20	Fast analysis of inorganic anions

[a] *The mEquiv/col refers to the listed column dimension.*

TABLE 13.4.2 Example of applications for Rezex type columns.

Column	Part. size (μm)	Max. back pressure (PSI)	Applications
RAM-carbohydrate Ag^+ (8%)	8	1000	Monosaccharides
RCM-monosaccharide Ca^{+2} (8%)	8	1000	Monosaccharides and sugar alcohols
RHM-monosaccharide H^+ (8%)	8	1000	Monosaccharides, organic acids, fatty acids, alcohols, ketones, neutral compounds, and inorganic salts
RKP-potassium K^+ (8%)	8	1000	Glyphosate
RNM-carbohydrate Na^+ (8%)	8	1000	Sugars in matrices that contain a high level of sodium
ROA-organic acid H^+ (8%)	8	1000	Organic acids, carbohydrates, alcohols, fatty acids
RPM-monosaccharide Pb^{+2} (8%)	8	1000	Cellobiose, glucose, xylose, arabinose, mannose, etc.
RSO-oligosaccharides Ag^+ (4%)	8	1000	Oligosaccharides
RNO-oligosaccharides Na^+ (4%)	8	1000	Oligosaccharides
RFQ-fast acid H^+ (8%)	8	1000	Ethanol, acetic acid, glycerol, other alcohols
RCU-USP sugar alcohols Ca^{+2} (8%)	8	1000	Sugar alcohols such as sorbitol, mannitol

Separation of small ions by IC

Small inorganic ions are successfully separated using ion chromatography. For example, Na^+, NH_4^+, K^+, Mg^{2+}, and Ca^{2+} can be easily separated and determined using an IonPac CS16 column (carboxylic acid type) with the analytes eluted isocratically with methanesulfonic acid (MSA) that is electrochemically generated at a concentration of 30 mM. Similarly, anions such as F^-, Cl^-, NO_2^-, SO_4^{2-}, Br^-, NO_3^-, and PO_4^{3-} can be separated on strong anion exchange columns, such as IonPac AS18 using as eluent a 33 mM KOH solution in isocratic conditions at 1 mL/min. A similar analysis of small anions on an IC column is shown in Fig. 13.4.1. The separation was performed on an SI-90-4E column from Shodex, 250 × 4.0 mm that is a PVA gel-NR_4^+. The eluent used was a solution of 3.2 mM Na_2CO_3/$NaHCO_3$. The flow rate of the mobile phase was 0.7 mL/min. The separation was achieved in 25 min, and the detection was done using a conductivity detector (with suppression).

A considerable number of other separations of small inorganic ions are reported in the literature, with similar types of columns in general utilized [4].

Separation of ionic organic molecules

Many organic ionic molecules can be separated successfully using ion exchange chromatography. These molecules include among others organic acids, amines, and amino acids. One example of a separation for a standard mixture of 17 amino acids is shown in Fig. 13.4.2. The separation was achieved using

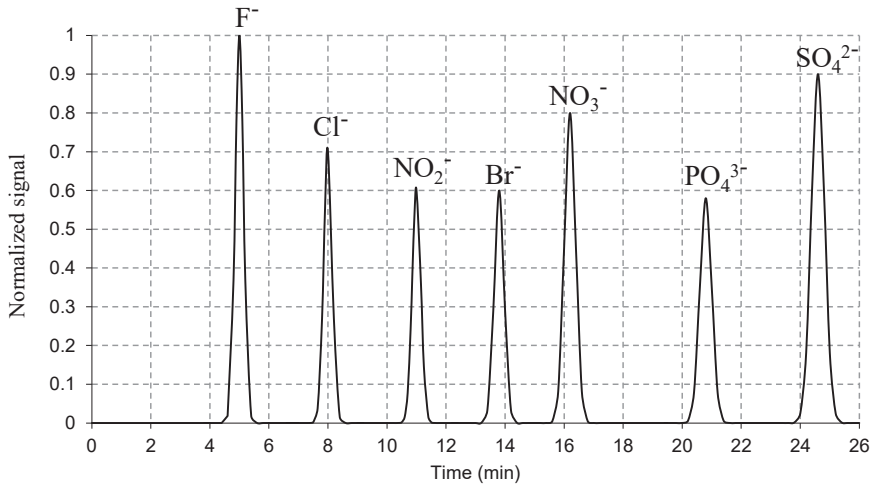


FIGURE 13.4.1 Chromatogram of a standard mixture of F^-, Cl^-, NO_2^- all 5 μg/mL and Br^-, NO_3^-, PO_4^{3-}, and SO_4^{2-} all 10 μg/mL, on an SI-90-4E from Shodex, 250 × 4.0 mm, elution with 3.2 mM $Na_2CO_3/NaHCO_3$ solution in water (conductivity detector).

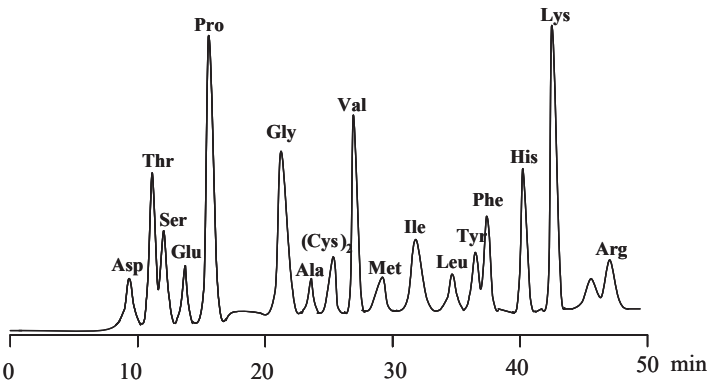


FIGURE 13.4.2 Chromatogram of a standard mixture of 17 amino acids using a Shim-pack Amino Na column and gradient elution, with fluorescence detection.

a Shim-pack Amino Na 100 × 6.0 mm analytical column and a Shim-pack ISC 38 × 4.6 precolumn using gradient elution (Solution A sodium citrate buffer at pH = 3.15, Solution B sodium acetate at pH 7.40, and Solution C, NaOH solution at pH = 12.4) [58,59]. The detection was done using postcolumn derivatization with NaOCl, *o*-phthalaldehyde, and N-acetyl-L-cysteine in carbonate buffer. The sample consisted of a mixture of amino acids, each at 0.1 μM/mL with injection volume of 10 μL, and the detection was done using an FLD, with Ex = 350 nm and Em = 450 nm.

Similar analyses were done using postcolumn derivatization with ninhydrin and UV detection (at 440 and 570 nm). Numerous methods using ion chromatography for a variety of ionic molecules are reported in the literature (e.g., Ref. [60]).

Separation of neutral organic molecules

Organic molecules that are considered neutral, such as carbohydrates and alcohols, can be successfully separated by IC. Carbohydrates have a very weak acidic character (with $pK_a > 12$), and using a basic mobile phase, it is possible to separate carbohydrates on an anion exchange column. For quantitation, pulsed amperometric detection is usually employed. An example of such separation on a CarboPac PA20 capillary column 150 × 0.4 mm is given in Fig. 13.4.3. The separation was done in isocratic conditions with eluent 10 mM KOH solution at 10 μL/min and PAD detection for a set of standards with 10 mM concentration and an injection of 0.4 μL.

A common procedure for the separation of carbohydrates and organic acids is the use of ion-moderated stationary phases. Specific columns are recommended for different types of analytes from this class. For example, the resins in Ca^{2+} form are recommended for monosaccharides and sugar alcohols, in H^+ form for organic acids, in Ag^+ form for oligosaccharides separation, etc. Temperatures at 60–80°C are typically used in these separations.

Separation of proteins and nucleic acids

Ion chromatography has been used successfully for protein and nucleic acids separations. For proteins, since they can be either positively or negatively charged depending on the pH of the mobile phase, both cation and anion exchange columns have been used for separation [61]. Polymer-based columns are in particular useful for such separations, and they were used for both analytical and preparative purposes. In principle, a protein will be positively charged at pH lower than isoelectric point (pI) and negatively charged at pH higher than pI. However, in practice the values of the pH of the mobile phase bracketing the pI value at 1.5–2 pH units are avoided. Some proteins show anomalous behavior around pI due to independent dissociation of different groups at different parts of the molecule. For this reason, it is common that cation exchangers are used in mobile phases with 1.5–2 pH units lower than pI, and anion exchangers in mobile phases more basic with 1.5–2 units than pI. Both strong and weak cation and anion exchange columns have been used for the separation. Similar to the case of RP columns, large pore materials are typically used in protein

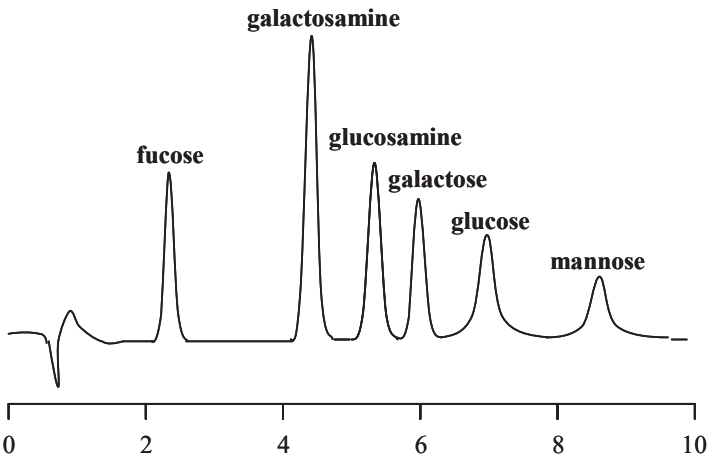


FIGURE 13.4.3 Example of separation of a set of carbohydrates standards on an anion exchange column (CarboPac PA20).

separations. Separation of proteins on ion exchange columns is also done using the chromatofocusing technique (see Section 13.5).

Nucleic acid, and in particular oligonucleotides, was successfully separated using ion exchange columns. Due to the phosphate groups in their molecules, these compounds have an ionic acidic character. Similar to the case of proteins, for the separation of larger nucleic acid molecules, stationary phases with large pores must be utilized.

13.5 Mobile phase in ion exchange and ion-moderated liquid chromatography

General comments

The main component of the mobile phase in IC is typically water in which a specific buffer, acid, or base is dissolved. The dissolved additives in the aqueous mobile phase are selected depending on whether anionic or cationic separation is practiced, for an analyte $B^{+/-}$ and must provide competing (driving) ions $A^{-/+}$ that replace reversibly the analyte retained on the stationary phase. The IC column is typically conditioned with the same mobile phase used for elution (except for cases when gradient is used). For a wide range of pH values for the mobile phase, the retention capacity for strong ion exchange stationary phases remains unchanged. However, the analytes are significantly influenced by pH values 2 pH units larger than the analyte pK_a. However, for weak ion exchangers, the mobile phase also influences the stationary phase ionization and therefore its retention capacity. The retention factor for an ion analyte B for the concentration $[A]_{mo}$ of the driving ion is given in IC by Eq. 13.1.19, written once more below:

$$\log k'_B = \alpha - \beta \log[A]_{mo} \qquad (13.5.1)$$

The values for the parameters α and β in Eq. 13.5.1 depend explicitly on the equilibrium constant $K_{B,A}$ for the analyte versus driving ions, the charges of the ions involved in the separation, the ion exchange capacity of the stationary phase, the weight of the stationary phase, and the volume of mobile phase in the column. The nature of the stationary phase, mobile phase, and of the analytes determines these parameters. The mobile phase properties can be modified using additives, selecting the pH, and even by adding an organic modifier such as acetonitrile or methanol [62]. It is common that the column in IC is preconditioned with the same mobile phase used initially for elution. In case of gradient, the composition of the mobile phase is modified, increasing the concentration of the driving ions $[A]$. Also, addition of ligands that can interact with the analytes may be used for facilitating the analyte elution.

A special type of solvent delivery system can be used in ion chromatography. This system is known as eluent generator (Dionex). When an eluent generator is used, the pump(s) deliver water, while the reagents necessary for the elution are generated electrochemically from special cartridges. For this purpose, DC current is applied to a special cartridge to produce either KOH for anion exchange eluents or MSA for cation exchange eluent. This type of eluent generator is typically offered as a whole assembly including other parts such as a degasser, and it is installed before the injector in the LC system (see Section 2.2).

The mobile phase in IC must be carefully selected in relation to the detection system. The most common detection technique in IC is based on conductivity, and the presence of acids, bases, or salts in the mobile phase may produce a large background for this type of detection. For this reason, two types of detection based on conductivity are practiced in IC: (1) with chemical suppression of the background signal and (2) without chemical suppression of the background signal. Chemical suppression can be achieved using a resin or a semipermeable membrane that eliminates mobile phase ions that cause

high conductivity background (see Section 1.4). The mobile phase must be selected in accordance with the type of conductivity detection [8].

Mobile phase in cation exchange chromatography

Various mobile phases are utilized in cation exchange chromatography, depending on the analytes, the selected column, and the detection system. Most mobile phases are aqueous, frequently containing diluted HCl, HNO_3, H_2SO_4, or CH_3SO_3H (concentration range between 2 and 50 mM/L). As discussed in Section 13.1, separation of different ions can be achieved based on their different retention constants $K_{M,C}$ (see Eq. 13.1.21). Since the retention follows the order $Li^+ < H^+ < Na^+ < NH_4^+ < K^+ < Rb^+ < Ag^+$, the elution can be done for monovalent ions using acids (H^+ driving ion) that generate a high enough concentration of H^+ to assure elution (see Eq. 13.5.1). However, the retention of divalent ions (or trivalent ions) is much stronger than that of monovalent ions, and the elution with acids requires a higher concentration of H^+ ions. This requirement is difficult to be applied because in detections without or even with chemical suppression, the background signal (conductance) caused by the mobile phase is very high. The use of a solution of $AgNO_3$ as mobile phase may elute divalent ions since Ag^+ does have a higher affinity than H^+ for the stationary phase. This procedure requires a suppressor column in Cl^- form for eliminating Ag^+ ions [63]. In case of strongly retained ions (e.g., transitional metallic ions), the elution is frequently achieved using a complexing agent, such as a solution of a weak organic acid (e.g., tartaric, or α-hydroxyisobutiric acid) that does not generate a large signal background (low H^+ concentration) and forms complexes with certain cations, such as of lantanides [64]. This procedure has been applied even for the elution of ions such as Ca^{2+}, Sr^{2+}, Pb^{2+}, and Ba^{2+} with 2,3-diaminopropionic acid and HCl in the mobile phase.

TABLE 13.5.1 Some buffers recommended for cation exchange LC.

Buffer/counterion	pK_a (at 25°C)	pH interval
Formic acid/Na^+ or Li^+	3.75	3.5–4.5
Acetic acid/Na^+ or Li^+	4.75	4.8–5.2
Maleic acid/Na^+	2.00	1.5–2.5
Malonic acid/Na^+ or Li^+	2.88	2.35–2.40
Citric acid/Na^+	3.13	2.60–3.60
Lactic acid/Na^+	3.80	3.60–4.30
Succinic acid/Na^+	4.20	4.30–4.80

Besides metallic cations, IC has been applied successfully for the analysis of organic cations, such as those from biogenic amines (e.g., putrescine, cadaverine, and histamine). In this case, a diluted solution of H_2SO_4 (5.0 mM/L) can be used as mobile phase [65]. The extension of ion chromatography to the analysis of various ionic molecules requires in many cases a specific pH of the mobile phase. This pH is specifically chosen to perform the separation and also to provide a mobile phase with electric conductivity that can be easily suppressed. Specific buffers are recommended to be used in IC, some of these being listed in Table 13.5.1.

Mobile phase in anion exchange chromatography

A variety of mobile phases are used in anion exchange chromatography. Electrochemical generation of OH^- ions (e.g., produced with a Dionex EG40 eluent generator) allows the use of water as a mobile phase. Buffers based on CO_3^{2-}/HCO_3^- are also widely used for separation of inorganic and organic anions. The major advantage of this buffer is related to the suppressor reaction, which leads to H_2CO_3, which is weakly dissociated and consequently its contribution to the background signal is very low. There are alternatives to carbonate/bicarbonate

buffer, such as solutions of amino acids. For pH > 7, only the carboxy group is dissociated and thus it plays its role in anion separation. Besides that, the suppressor reaction carried out at a pH corresponding to the isoelectric point of the amino acid converts it into a zwitterionic form with low contribution to the background signal. Another additive in anion exchange mobile phases is the tetraborate ion ($B_4O_7^{2-}$). The suppression of tetraborates is based on its change to H_3BO_3, which is weakly dissociated and thus it does not contribute to the background conductivity. However, $B_4O_7^{2-}$ has a low affinity for the stationary phases and for this reason it is only used for the elution of F^- and short-chain R-COO^- anions.

The mobile phases with low background conductivity can be used for nonsuppressed anion chromatography, and are usually based on diluted aqueous solutions of organic salts, such as benzoates, phthalates, or sulfobenzoates. These anions are also characterized by a significant affinity toward the stationary phase, and meanwhile they produce a relatively low conductivity of the mobile phase. The pH of mobile phase must be adjusted to 4–7 in order to favor the dissociation of the weak acid groups of the additive, which at their turn influence the retention process of the inorganic anionic species (e.g., F^-, Cl^-, Br^-, I^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, $S_2O_3^{2-}$, SCN^-). However, the background conductivities of these mobile phases are higher than the conductivity of carbonate/bicarbonate buffer after passing the suppressor column, which is of the 15–20 μS/cm level. Thus, at concentration of 0.5–1 mM/L of the mentioned organic salts, the background conductivity is situated between 60 and 160 μS/cm, which is high and affects the detection performances.

Besides inorganic ions, IC is also used for the separation of numerous other analytes that can be present in ionic form. For these compounds, the use of buffers that assure the formation of ionic form of the analytes is necessary. Similar to the case of cations, the easy elimination of the conductivity created by the mobile phase components using suppressors is one criterion for selecting such buffers. Table 13.5.2 lists several buffers used in anion exchange LC.

TABLE 13.5.2 Some buffers recommended for anion exchange LC.

Buffer/counterion	pK_a (at 25°C)	pH interval
Piperidine/Cl^-	11.12	10.6–11.6
Piperazine/Cl^-, $HCOO^-$	5.67	5.0–6.0
N-methylpiperazine/Cl^-	4.75	4.5–5.0
Ethanolamine/Cl^-	9.50	9.0–9.5
Diethanolamine/Cl^-	8.88	8.4–8.8
Triethanolamine/Cl^-	7.75	7.3–7.7
Histidine/Cl^-	5.95	5.5–6.0
1,3-Diaminopropane/Cl^-	10.46	9.8–10.3

Other mobile phases in anion exchange chromatography have been reported. Some such phases may have a multipart composition that can favor a complex separation process with ion exchange, ion exclusion, and ion-pairing principles for the separation [66].

When UV detection is used in ion chromatography, solutions of salts of phosphoric, sulfuric, or perchloric acid are suitable as mobile phase because these anions do not absorb strongly radiation in this spectral domain. When amperometric detection is chosen, the mobile phase acts as a support electrolyte and the electrolyte concentration must be about 50–100 higher than concentration of the anion analytes. In this case, hydroxide, chloride, chlorate, or perchlorate of alkali metals is used as supporting electrolyte for anion elution. EDTA can be used for the elution of very strongly retained polyvalent anions such as polyphosphates. Besides that, EDTA can form anionic complexes with many metallic ions by the control of pH of mobile phase, a property that can be used in separating metallic cations by anion exchange LC [21].

Gradient elution in ion chromatography

The use of gradient elution in IC is less common. The conductivity detection, which is usually employed in IC, is sensitive to changes in mobile phase composition, and isocratic separations are more convenient since they produce a constant background. In nonsuppressed IC, the use of a so-called isoconductive gradient, in which the conductances of the starting and finishing eluents used to obtain the gradient are equal, partially overcomes this difficulty, but the variation in eluotropic strength for isoconductive gradients is quite limited. In suppressed IC, the use of gradient elution is based on the availability of suppressors with sufficient capacity to ensure that the background conductance of the suppressed eluent remains essentially constant over the course of gradient. On the other hand, the eluotropic strength of an eluent with constant composition can be quite limited. For achieving separation, gradients are necessary in many cases, and they can be conveniently used in suppressed IC when good suppressors are available. Such suppressors can provide sufficient capacity to ensure that the background conductance of the suppressed eluent remains essentially constant over the course of gradient [67].

In gradient IC, similarly to other HPLC types of separation, an effective gradient retention factor k'_e can be used for describing retention. For a linear gradient, the expression for the effective retention factor can be approximated by an expression of the following form:

$$\log k'_{e,B} = \alpha' - \beta' \log \frac{\Delta[A]}{\Delta t} \qquad (13.5.2)$$

In Eq. 13.5.2, the intercept α' and slope β' are specific for the given separation system, the analyte B, and the competing (driving) ion A of the IC separation. The gradient ramp $\Delta[A]/\Delta t$ is expressed in mM/min, and it is assumed to start as the analyte reaches the head of the column. This dependence is illustrated in Fig. 13.5.1 for three anions (formate, acetate, and methansulfonate) eluted using OH^- driving ions under linear gradient mode for a separation on a Dionex AS11 IonPak column with mobile phase flow rate of 1.00 mL/min water, Dionex EG40 eluent generator that creates HO^- ions (KOH), an ASRS-II suppressor, and the detection based on conductivity [3,67].

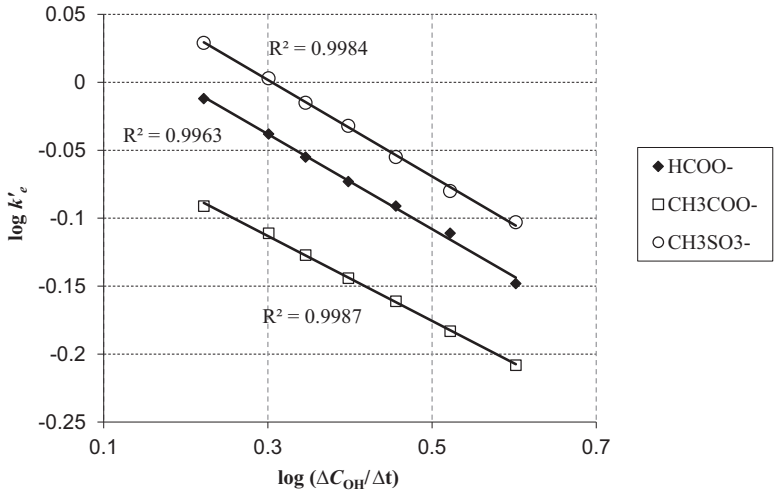


FIGURE 13.5.1 Linear dependence of log k'_e for three anions on linear gradient ramp.

Chromatofocusing

Cromatofocusing is a procedure typically used for separation of amphoteric compounds, according to their isoelectric point, most commonly proteins, on an ion exchange column that has a pH gradient within the column (not an externally applied solution gradient) [68,69]. A pH gradient can be produced within an ion exchanger column by combining the buffering capacity of the ion exchanger with that of a buffer in the mobile phase, in a similar manner as a pH gradient is formed if two buffers at different pH are gradually mixed [70,71]. For this purpose, the resin, which is, for example, an anion exchanger, is initially adjusted to a high pH value. The mobile phase has a different pH, e.g., slightly acidic, and as it flows, it changes the pH across the column [72]. The separation is based on the initial retention of the analyte (protein) at the high pH of the column. An eluent at a lower pH typically made from several buffer species (with a range of pK_a's) is passed through the column. This creates a physical gradient that migrates down the column and the protein moves with it. The mobile phase elutes the protein when the buffer exiting the column has a pH equal with pI of the protein. The range of pK_a's in the eluent must be selected to provide buffer capacity across the entire pH range of intended gradient [73].

Mobile phase in ion-moderated chromatography

The choice for mobile phase in ion-moderated chromatography is rather limited. The simplest mobile phase is pure deionized water, which has been proved useful, for example, in the analysis of carbonate ion, or for the separation of organic acids [74]. For some columns, a specific ion must be present in the mobile phase for maintaining column integrity. Examples are ions such as H^+, Ca^{2+}, Pb^{2+}, etc (e.g., diluted H_2SO_4 is used to generate H^+ ions). For the separation of organic acids, solutions of inorganic acids are usually used as mobile phase. This depends also on the type of the suppressor column employed in case of conductivity detection. In case of UV-detection, H_2SO_4 solution is also frequently used. The high retention of some aliphatic and aromatic carboxylic acids can be prevented by the addition in mobile phase of a small content of miscible solvents, such as methanol, ethanol, i-propanol, or acetonitrile.

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CHAPTER

14

Chiral HPLC separations

14.1 Separation process in chiral liquid chromatography

General comments

Stereoisomers that are mirror images to each other and are not superimposable although the atomic distances are the same in the molecules are called enantiomers. These compounds have the property called chirality. Chirality, which is needed for the existence of enantiomers, is commonly caused by the existence in the molecule of one or more tetrahedral carbon atom substituted with groups that are different and are bonded spatially different. The chirality in an enantiomer is characterized using the symbols R and S. For the assignment of a symbol R or S to a chiral carbon that has four different substituents, its substituents are at first ranked in a sequence a, b, c, d. The ranking of the substituents is based on specific rules. For example, for the four atoms directly attached to the asymmetric carbon, a higher atomic number outranks the lower one (e.g., O > N > C > H), and a higher atomic mass outranks the lower. For the same atoms directly attached to the asymmetric carbon, the priorities are assigned at the first point of difference. Double and triple bonds are treated by assuming that each such bonded atom is duplicated or triplicated (e.g., C*═O is treated as O─C*─O─C). More detailed rules are reported in the literature (e.g., Ref. [1]). Viewed in space and keeping the substituent d in the back of the page plan, when substituents a, b, and c are seen counterclockwise the carbon is labeled S, and when they are seen clockwise the carbon is labeled an R, as indicated in Fig. 14.1.1.

Besides having a chiral carbon, chiral molecules may be generated from other elements such as phosphorus or sulfur that are bonded to different substituents. Also, not only a chiral center (e.g., an asymmetric carbon) generates enantiomers. A chiral axis or a chiral plane also can lead to enantiomers (helicoidal chirality is also known). More than one asymmetric carbon can be present in a molecule. The stereoisomers generated by more than one asymmetric carbon can be mirror image one to the other (enantiomers) or may have different steric arrangements being diastereoisomers. For example, for a molecule with two chiral centers, these can be SS, SR, RS, and RR. The first couple of enantiomers is SS and RR and the second couple is SR and RS. The molecules SS and RS (or SR) are diastereoisomers, as well as RR and SR (or RS) which are also diastereoisomers. Diasteroisomers can be separated on common nonchiral stationary phases. The separation of enantiomers cannot be performed on nonchiral phases (separation on nonchiral phases is possible when using special chiral additives in the mobile phase). For enantiomer separation, a chiral stationary phase containing a chiral selector (center) must be used. Because of close similarity of the enantiomeric molecules, the separation can be rather

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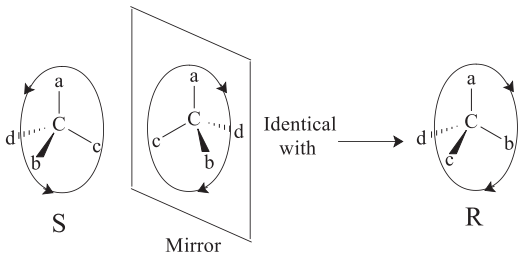

FIGURE 14.1.1 S and R molecules as mirror images, with one chiral carbon and four different substituents.

difficult. The quality of chiral separations can be evaluated similarly to that for other chromatographic separations using parameters such as selectivity α and resolution R. Several interaction mechanisms were suggested as responsible for the separation of chiral molecules (e.g., Ref. [2]).

Chiral recognition

For chiral stationary phases, the separation equilibrium can be considered mainly based on adsorption depending on differences in the intermolecular interactions between the enantiomers and the stationary phase [3]. Most chiral phases have been developed to be used in a mobile phase less polar than the stationary phase, similar to NPC and HILIC phases. However, the use of a mobile phase more polar than the stationary phase (e.g., containing methanol or even water) is also applicable with some chiral stationary phases. The separation in this case would be expected to be similar to that for RP-HPLC. However, the partition equilibrium is difficult to apply for explaining chiral separations. For this reason, even if some hydrophobic interaction may take place in the case of using a mobile phase having, for example, a high water content, probably the interaction between analytes and the stationary phase can be considered as dominated by the adsorption equilibrium. Typically, the interactions causing the separation are mainly of polar nature, including polar–polar, hydrogen bonding, π-donor–π-acceptor, and $\pi-\pi$ stacking [4]. Since the enantiomer molecules have very similar physical properties such as polarizability and dipole moments, the reason for differences in the interactions with the stationary phase is of geometric (steric) nature, such as the spatial access of the analyte to the chiral polar phase and access which is different for the two enantiomers (see Section 6.1). Different moieties with specific space orientation are present in both chiral analyte and chiral stationary phase. Each moiety is supposed to offer specific types of interactions. The particular example of enantioseparation of asymmetric polyhalogenated 4,4′-bipyridines on specific stationary phases (e.g., cellulose tris(3,5-dimethylphenylcarbamate)) revealed another interaction potentially involved in chiral recognition that is the halogen bond (see Section 5.1) [5].

Initial attempts to explain the difference in the retention between two enantiomers and a chiral stationary phase were based on the three points of interaction model between enantiomers and the chiral selector [6]. A schematic description of retention based on three-point interaction is illustrated in Fig. 14.1.2. The figure shows that a single point or two points of interaction (e.g., two hydrogen bonds formation) between the chiral stationary phase and the chiral analyte are not sufficient for providing difference in the enantiomer retention. For one point interaction (a↔A) as well as for two points interaction (a↔A and c↔C), both R and S enantiomers behave similarly. In the case of a three possible interaction points, solute S can establish all three points (a↔A, b↔B, and c↔C) of interactions, while solute R establishes only two points (a↔A and c↔C), such that the retention of the enantiomers is different (the third point of interaction for R may still be present but it is very weak) [2].

The three point of interaction model has a certain experimental base, and the type of stationary phase structure offering three different possible interactions is exemplified in Pirkle type stationary phases (e.g., Refs. [7,8]). In these phases, the three points of interaction are

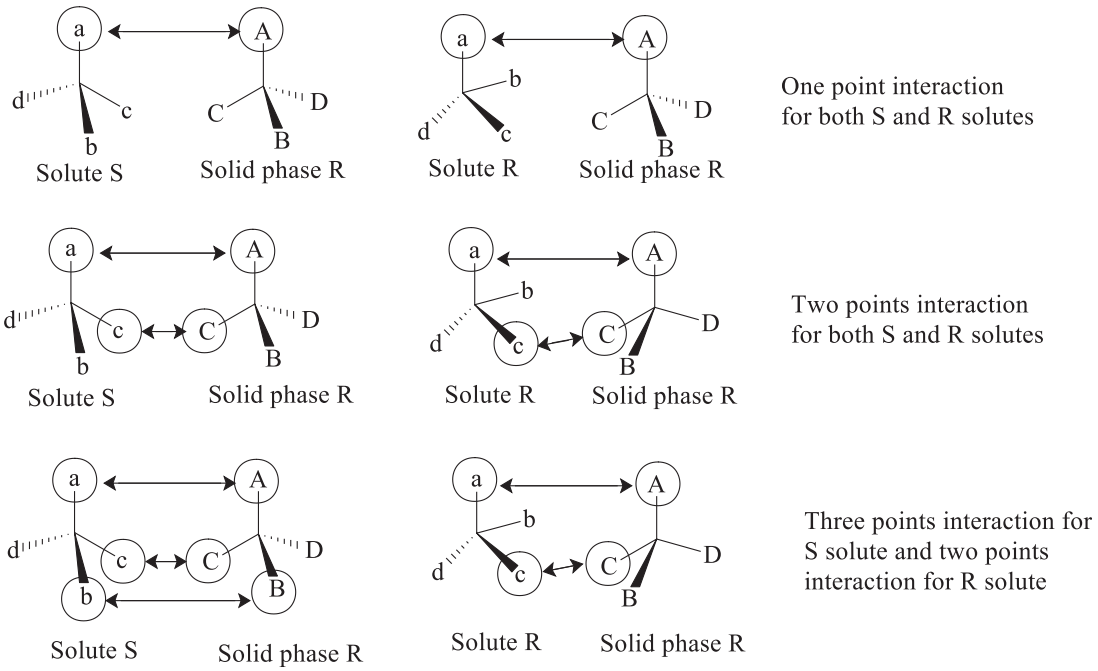

FIGURE 14.1.2 Illustration showing that one-point interaction (a↔A) and two-point interaction (a↔A and c↔C) are equal for the R and S enantiomers and do not lead to separation, while three points of interaction lead to differences.

different in nature (e.g., one donor type hydrogen bond, an acceptor type hydrogen bond, and π-donor−π-acceptor interactions). The three points of interaction model were however insufficient to explain some aspects of chiral separation, and multipoint interactions (some attractive and some repulsive) as well as the absence of certain constrains in the interaction of chiral molecules with the stationary phase were suggested to explain the separations [3,9–13].

Among the common types of chiral stationary phases are those based on cellulose and cellulose derivatives. Cellulose-based polymers have a linear structure, but the cellulosic polymeric chains form strong hydrogen bonds between them, which leads to an interstitial structure that allows molecular inclusions. The main types of interactions remain hydrogen bonding between stationary phase and the molecules of the analyte, but the inclusion leads to restrains in the movement of molecules. The restrained movement combined with the specific orientation of OH groups in cellulose leads to multipoint interactions that are different between the enantiomers.

A somewhat different type of mechanism in separation also involves differences in the inclusion properties in the stationary phase, but these inclusion properties are more important, while the hydrogen bonds are weaker compared to cellulose. The phases displaying this type of interaction are cyclodextrins, crown ethers, and amylose type phases. These phases have the ability to produce inclusion complexes with numerous molecules, depending on the guest molecule geometry. Cyclodextrins, for example, have a truncated conical cavity that forms an inclusion similar to a pseudorotaxane with one part of the chiral molecule. The inside of the cavity of a cyclodextrin is relatively hydrophobic, giving the cyclodextrins the ability to accept a

wide variety of molecular guests and limit the rotation of the included molecule. The still existing hydroxyl groups offer enough chiral centers that have different interactions with the remaining substituents of the chiral molecule. These interactions are of the types polar–polar and hydrogen bonding. Derivatized cyclodextrins (e.g., with acetyl groups) provide further differentiation in the types of interactions with the chiral molecule. Due to the chirality of the guest, different groups from the two enantiomers and from the cyclodextrin come in close proximity. The result is that the overall molecular interactions of the enantiomers R and S with the host molecule are different. The types of interactions are not specifically localized as in the case of Pirkle type phases but possibly still can be explained using the three-point interactions model. As an example, Fig. 14.1.3 shows a β-cyclodextrin partially derivatized with acetyl groups and anchored, e.g., to a silica surface, in interaction with two enantiomers. The figures shows one enantiomer having more interactions than the other, and therefore being stronger retained.

Other mechanisms for chiral separations

Other mechanisms can be used for chiral separations, such as the one based on ligand exchange chromatography using a chiral resin loaded with a transition metal (e.g., Cu^{2+}) capable of forming at the same time complexes with the solutes (enantiomeric analytes). The separation is based on the differences in the strength of the interactions (of coordinative and ionic type) of the solutes with the bonded metals ions in the asymmetric resin. Multiple mechanisms are involved in other materials that can be used as chiral stationary phases such as proteins or macrocyclic antibiotics that allow in certain cases the separation of enantiomers [14].

Special phases are used for immobilized metal affinity usually derived from iminodiacetic acid or tricarboxymethylethylenediamine. These phases are able to form very strong coordinative complexes with transitional metals that are further coordinating with the analytes possessing electron–donor functional groups ($-NH_2$, $-COOH$, $-OH$, $-SH$, and others) [15,16].

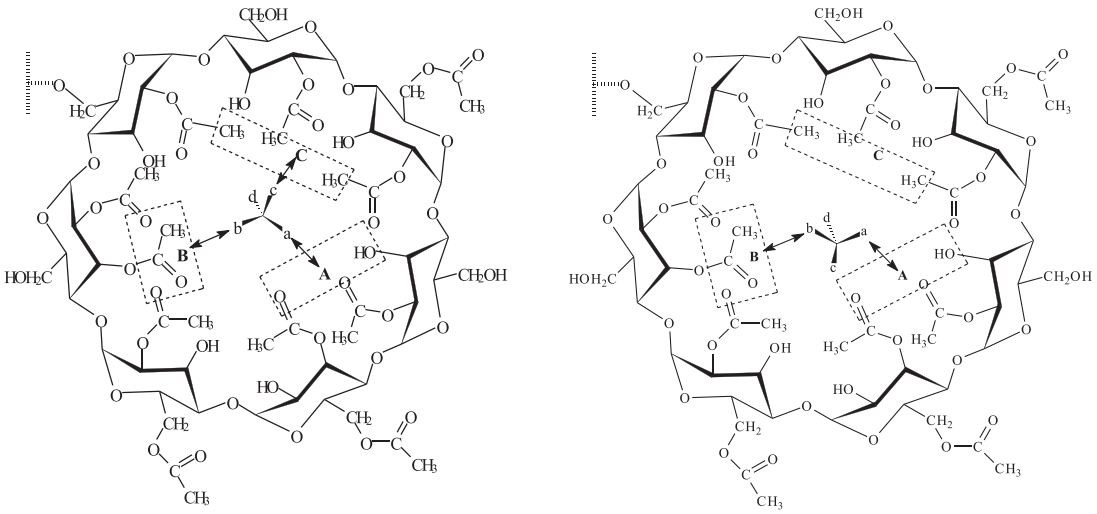

FIGURE 14.1.3 Types of interactions of a partially acetylated β-cyclodextrin and an S versus an R guest molecule (d substituent is along the cyclodextrin channel). Stationary phases are obtained by attaching the β-cyclodextrin on a silica gel surface.

Ligand exchange chiral stationary phases can be obtained by coating the C18 phase or by bonding covalently to silica gel chiral aminoalcohols [17]. For example, N-dodecyl-(1S,2R)-norephedrine or -carboxymethyl-N-dodecyl-(1S,2R)-norephedrine monosodium salt can be coated on a C18 stationary phase due to the strong interaction of dodecyl chain with C18 chain. The adjacent hydroxyl and amino groups from norephedrine moiety can coordinate Cu^{2+} ions the resulting sites having the capability to be involved in additional coordination with analyte molecules.

One special type of chiral separation involves the addition of a chiral additive in the mobile phase, with separation on a nonchiral stationary phase. Two different mechanisms are suggested for this type of separation: (1) the chiral additive forms diastereoisomeric complexes with the chiral analytes and these can be separated on achiral stationary phases and (2) the chiral additive is strongly retained on the stationary phase creating a chiral environment on which the diastereoisomers can be separated.

For the diastereoisomers formation in solution, the differences in the relative stability of these compounds and the differences between the partition between the mobile phase and the stationary phase allow the separation [18]. The formation of compounds in solution between the analyte and the chiral additive can be based on complexation, formation of various types of adducts, or ion pairing.

In the case of the retention of the chiral additive on the stationary phase, the retention and elution can be considered as caused by the same types of interactions as those previously discussed for chiral stationary phase. The chiral additive retained by the stationary phase should have differences in the interactions with different enantiomer analytes. Theoretical models for describing the separation of enantiomers based on the use of chiral additives in the mobile phase can be found in the literature [19].

14.2 Stationary phases and columns for chiral separations

Types of chiral phases

The separation of enantiomers typically requires a chiral stationary phase (CSP), although separations with a chiral agent in the mobile phase on achiral phases are also possible. Most chiral phases have the separation based on polar type interactions and are designed to be used in nonpolar solvents. Water being strongly polar may interfere with the polar interactions involved in chiral recognition. However, some chiral phases can use even a high level of water in the mobile phase. Such phases are useful in particular for the separation of biomolecules which may not be soluble in a mobile phase with very low polarity. Also, for MS detection, the absence of water or low level of a polar solvent such as methanol may produce no ionization of the analytes in ESI mode. For such cases MS-APCI can be used as an ionization mode. Also even if water is avoided, some chiral phases can be used in the presence of polar solvents such as methanol and an organic acid, base, or salt. The chiral phases are designed to work with analytes of different polarities. The type of utilization depends on both the nature of the stationary phase, and also on the mobile phase nature [20–22]. The types of utilization of chiral phases classify them in types similar to those used in nonchiral chromatography as follows [2]:

(1) Utilization similar to that practiced in normal phase chromatography (NPC) and applicable to the separation of many analytes, except some with high polarity. The phases are used with nonpolar or weakly polar solvents as mobile phases such as heptane with 20%–30% addition of ethanol or isopropanol. This is the most common type of utilization of chiral phases. A disadvantage of this type of chiral type of separation is related to the use of MS detection with an electrospray ion source (ESI). The

nonpolar solvents utilized for mobile phase are typically not compatible with ESI. A nonpolar organic solvent content prevents the ionization of dissolved solute molecules, as the solvent itself does not contain charge carriers like protons. Atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are still applicable although the use of polar additives in the mobile phase, like formic acid, acetic acid, or ammonium acetate, enhances sensitivity [23]. As an example, in Fig. 14.2.1 is shown the separation of (S)- and (R)-nicotine on a Chiracel OJ-3 column, 250 × 4.6 mm with 3 μm particles using isocratic mobile phase containing 85% hexane and 15% ethanol having as additives 3 mL/L trifluoroacetic acid (TFA), 3 mL/L triethylamine (TEA), and 2 mL/L formic acid. The sample contained 22.8 μg/mL racemic nicotine and the detection was performed using MS/MS with APCI ionization, monitoring 163.1 → 84.0 transition.

(2) Utilization similar to that practiced in reversed phase chromatography (RP) is applicable to many analytes having hydrophobic moieties, but less amenable for analysis of strongly polar compounds. The phases are used with (nonaqueous) more polar mobile phases than the stationary phase and some containing partially aqueous mobile phase. Among the mobile phase components can be methanol, acetonitrile, mixed or not with lower levels of nonpolar solvents and with or without additives such as ammonium acetate of ammonium formate, formic acid, etc. In some separations, water should not exceed 2%–3% in the mobile phase [24], but in other separations, water can be the major component [25]. As an example, in Fig. 14.2.2 is shown the separation of (S)- and (R)-nicotine on a Chiralpak AGP column, 150 × 4.0 mm with 5 μm particles using isocratic mobile phase with 82% water and 18% methanol having as additives 30 mmol $HCOONH_4$ and 0.3% NH_4OH. The sample

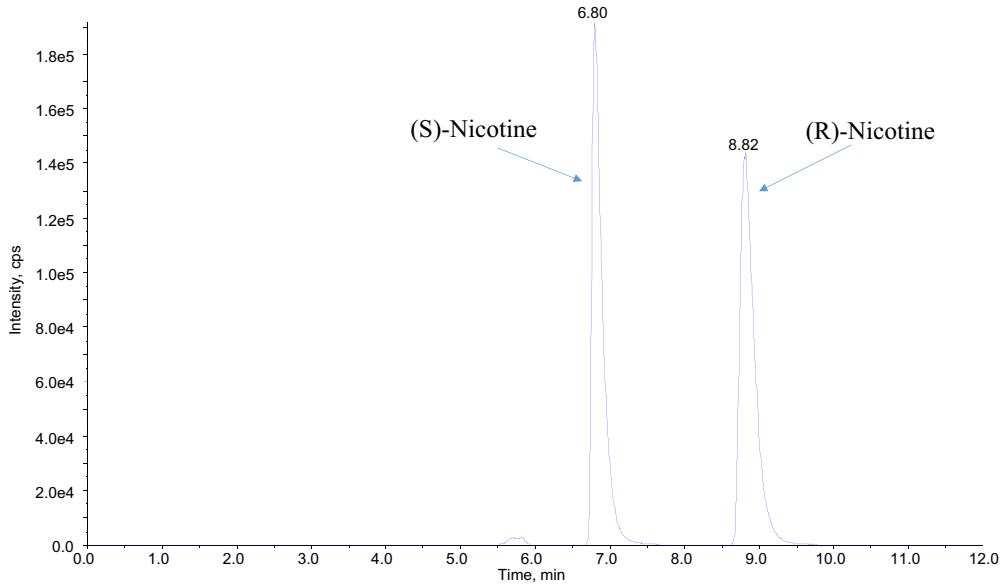

FIGURE 14.2.1 Chromatogram of a standard with 22.8 μg/mL nicotine racemic and separation on a Chiracel OJ-3 column with 85% hexane and 15% ethanol plus additives mobile phase.

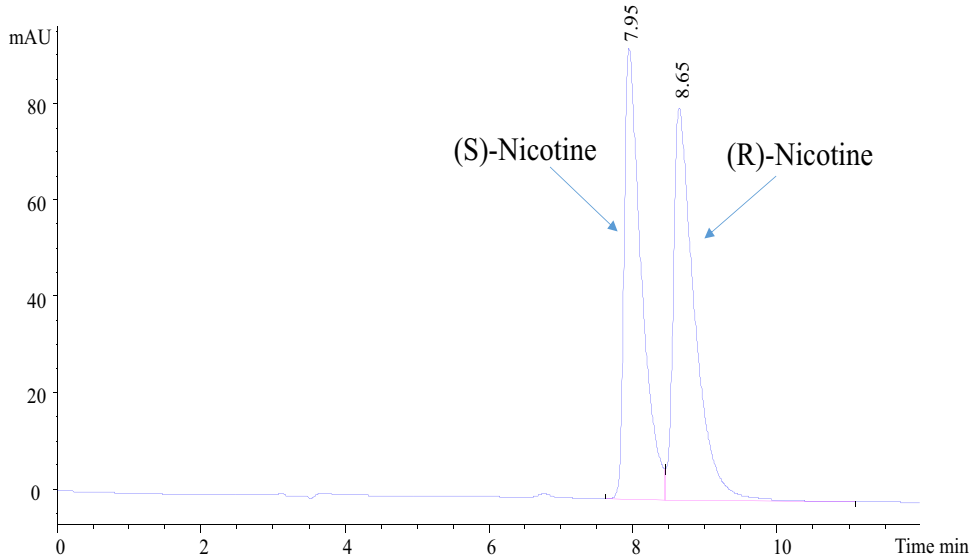

FIGURE 14.2.2 Chromatogram of a standard with 459.0 μg/mL nicotine racemic and separation on a Chiralpak AGP column with 82% water and 18% methanol plus additives mobile phase.

contained 459.0 μg/mL racemic nicotine and the detection was performed using UV absorption at 254 nm.

(3) Utilization applicable to polar molecules capable of forming strong hydrogen bonds (corresponding to HILIC nonchiral chromatography). In such cases, the phases are used with polar solvents such as methanol and acetonitrile with additives such as ammonium formate or low level of buffers such as acetic acid/trietanolamine or trifluoroacetic acid/trietanolamine, or specific chaotropic salts. Only special columns may be used with water in the mobile phase. Usually, if water is necessary in the mobile phase, it should not exceed a few %.

(4) Utilization applicable to ionizable compounds (corresponding to IC nonchiral chromatography). The phases are used with polar solvents such as methanol and acetonitrile with additives such as acetic acid/trietanolamine. Only low levels of water (2%–5%) are tolerable in some separations.

Regarding the construction/chemical composition, a variety of chiral phases were manufactured. All these phases have chiral centers. Based on their chemical composition, the chiral phases can be placed in specific classes that include (1) brush or "Pirkle" type, (2) cellulose based, (3) cyclodextrin or cyclofructan based, (4) amylose based, (5) crown ether based, (6) macrocyclic antibiotic type, (7) protein based, (8) ligand exchange type, and (9) chiral synthetic polymer type. Many chiral phases are placed on a silica gel surface in order to take advantage of the high porosity of this material. Some of the phases are just coated on silica, but some are connected with covalent bonds. Some organic polymers are also used to bind chiral phases. Numerous types of chiral phases are commercially available. In this section, some examples of chiral phases from each class are discussed [26,27].

Brush or "Pirkle" chiral phases

This type of stationary phase uses a silica support with the attached moieties capable of developing both donor and acceptor hydrogen bonds, and a group able to establish either π-donor, π-acceptor, or both π-donor and π-acceptor intermolecular bonds [22,28,29]. It is also required that the moiety has a stereospecific structure. Such structures are frequently obtained using an amino acid involved in an amide bond, and one or more aromatic moieties such as the "electron poor" dinitrobenzene which offers π-acceptor interactions or an "electron rich" moiety which offers π-donor interactions [30,31]. Pirkle chiral phases can be bonded to silica using either monofunctional or trifunctional reagents. An example of a Pirkle type phase and the path followed for its synthesis are shown in Fig. 14.2.3. This synthesis typically starts with aminopropyl silica that is derivatized with an amino acid such as (S)-leucine, followed by derivatization with 3,5-dinitrobenzoic acid (e.g., as acid chloride).

The difference in the interaction of the phase indicated in Fig. 14.2.3 with a chiral compound such as hexobarbital is schematically shown in Fig. 14.2.4 ((S)-hexobarbital is more effective hypnotic sedative than (R)-hexobarbital and analysis of the enantiomers ratio is necessary).

As schematically indicated in Fig. 14.2.4, the (S)-enantiomer is capable to form two different hydrogen bonds (one with the stationary phase acceptor C=O and one with the stationary phase donor NH) and a π-donor–π-acceptor interaction with the acceptor dinitrophenyl. At the same time, the (R)-hexobarbital forms only two bonds, the interaction of stationary phase acceptor C=O with the >N(CH_3) group being very week.

Other amino acids can be used in the synthesis such as (R)-leucine, (S)- or (R)-phenylglycine, (S)-proline, etc. [2,22,32–34]. Also, moieties different from π-acceptor 1,3-dinitrobenzene

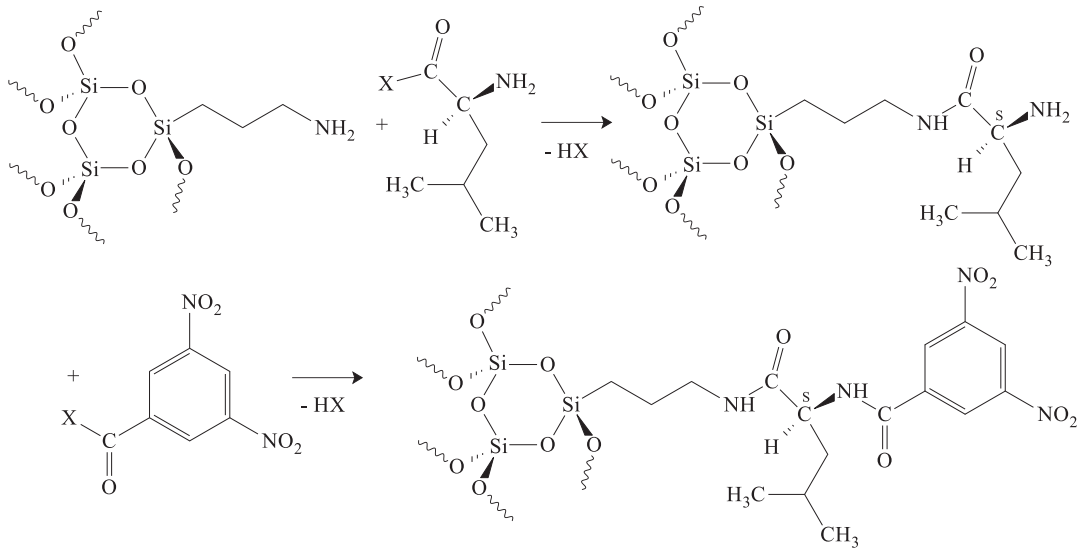

FIGURE 14.2.3 Schematic synthesis of a Pirkle chiral stationary phase containing a propyl handle to silica surface, an amide group connector, (S)-leucine as chiral group, and 1,3-dinitrobenzoyl moiety as π-acceptor group.

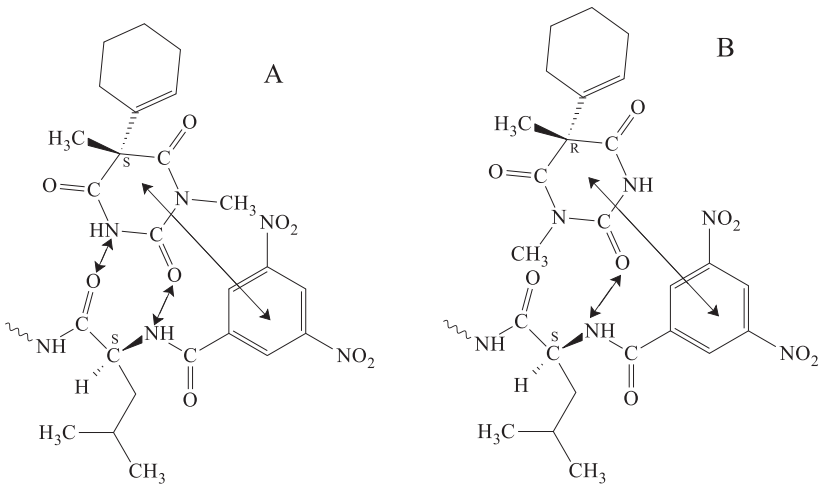

FIGURE 14.2.4 Schematic description of the interaction of (S)-hexobarbital (structure A) and of (R)-hexobarbital (structure B) with a Pirkle chiral stationary phase based on leucine.

and capable of offering π-donor interactions have been synthesized. Besides the use of a propylamino handle for the connection to the silica, the connection can be achieved using alkylglycidyl or alkylisocyanate handles.

Common utilized solvents for Pirkle phases are ethanol, isopropanol, hexane, CH_2Cl_2, etc. The phases can be used in water–alcohol mixtures, but the water content in the mobile phase must be very low. Water has the capability to form strong hydrogen bonds and interfere with the separation. Also, the (apparent) pH of the mobile phase must be in a relatively narrow range (pH = 2.5–7.5). Another limitation of these phases is that they are useable mainly with aromatic compounds (or compounds with a system of π electrons), since the π-donor or π-acceptor phases should interact with a π-acceptor or π-donor analyte, respectively. For the analysis of compounds not having such groups, derivatization before separation with reagents introducing, e.g., a π-donor group such as naphthylamine can be performed. Some examples of Pirkle type stationary phases are given in Appendix 14.2.1 [32,34]. Other Pirkle phases manufactured by Sumika Chem. Anal. Service are listed in Appendix 14.2.2. Various suppliers offer Pirkle type chiral chromatographic columns, including Advanced Separation Technologies (Astec) (3 types), IRIS Tecnol. (6 types), Machery-Nagel (2 types), Merck (2 types), Phenomenex (11 types), Regis Technologies (9 types), Sumika Chem. Anal. Service (11 types), YMC (2 types), etc.

Cellulose chiral phases

The chiral structure of cellulose and its capability to be derivatized at the active –OH groups allowed the synthesis of a number of chiral stationary phases. Some of these phases were obtained by derivatizing microcrystalline cellulose with groups such as triacetate (to form microcrystalline cellulose triacetate or MCTA), tribenzoate, trisphenylcarbamate, and tris(3,5-dimethylphenylcarbamate). In order to obtain a larger surface for the stationary phase, the derivatized cellulose material can be coated on a silica support [35,36]. The coating of silica

can be achieved either by simple adsorption on silica surface (physically coated) of the derivatized cellulose using a proper solvent or by simultaneous forming of bonds between cellulose and silica layer [37]. One possibility to anchor cellulose on silica is through glycidyl groups already attached to the silica surface by previous derivatization (see Section 8.1). Other procedures of binding cellulose to silica have been described in the literature [38]. The coating or binding must preserve the porous structure of silica. Typical derivatizations of the cellulose are performed as esters or as carbamates. Three examples of cellulose derivatization are given in Fig. 14.2.5.

Other substituents R can be used for various phases such as methoxy, acetyl, hydroxypropyl ether, naphthenylcarbamate, 3,5-dinitrophenyl carbamate, 3,5-dimethylphenylcarbamate, 2,6-dinitro-4-trifluoromethylphenyl ether, etc. [39]. Several commercially available chiral columns based on cellulose derivatives on silica support with different types of derivatization are listed in Appendix 14.2.3. Coating of macroporous polymers such as poly(2-aminoethyl methacrylate-co-ethylenedimethacrylate) with derivatized cellulose was also reported in the literature.

Besides silica generally used as support for coating with chiral materials, the surface of zirconia previously sintered but not rehydroxylated (see Section 8.1) can also be utilized as support, providing a stable surface for depositing the chiral polymers, such as derivatized cellulose with tris(3,5-dimethylphenyl-carbamate) in proportion of 3%–4% (w/w) [40,41].

The utilization of cellulose based columns requires a normal mobile phase, such as hexane-ethanol, hexane-isopropyl alcohol, etc. The use of these columns with a mobile phase containing some water can also be done, but the addition of a salt in the mobile phase, such as a perchlorate, is necessary to prevent the column degradation by the dissolution of the stationary active coating.

Amylose chiral phases

Amylose is one of the components of starch and the polymer can assume a helical shape and can form inclusion compounds, such as with iodine, fatty acids, and aromatic compounds. Similar with cellulose, it also offers the capability of hydrogen bonding and some hydrophobic interactions [42]. Amylose can also be derivatized to generate chiral stationary phases, typically in the form of carbamates in the form of tris(3,5-dimethylphenyl-carbamate), tris[(S)-α-methylbenzyl carbamate], or tris(chloro-2-methylphanylcarbamate). Amylose or derivatized amylose is placed on silica surface for achieving the high surface area of the phase [43]. Several commercially available chiral columns based on amylose derivatives on silica support with different types of derivatization are listed in Appendix 14.2.4.

Cyclodextrins and cyclofructans chiral phases

A number of chiral phases are cavity based on cyclodextrins or on derivatized cyclodextrins

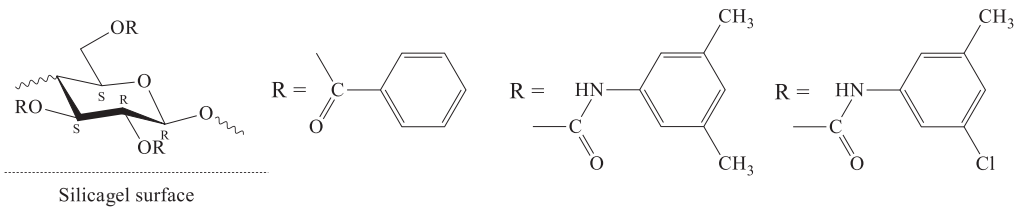

FIGURE 14.2.5 Examples of derivatized cellulose on a silica support.

[44]. These phases are placed on silica gel surfaces similarly to cellulose. Cyclodextrins are produced by the action of enzymes cyclodextrin glycosyltransferase and amylase on starch. The main types of cyclodextrins produced are α, β, and γ (with six, seven, and respectively, eight glucose residues), and they are generated by the enzymatic cleavage of starch polymeric chain and formation of cyclic oligomers. Larger cyclodextrins are also known. Cyclodextrins offer a series of advantages to be used as chiral stationary phases. They can be anchored to silica (e.g., through reactions with glycidyl groups bonded to silica, they offer a large number of chiral centers (30 for α, 35 for β, and 40 for γ-cyclodextrin), and also offer the possibility to be derivatized at their free OH groups with less polar substituents. The active stationary phase can be attached on silica support forming fully porous particles, or they can be in the form of core-shell particle (e.g., Ref. [45]). The separation on cyclodextrin type of phases is based on inclusion properties provided by their conical cavity of one part of the chiral molecule and differences in the interaction with the chiral OH or OR groups of cyclodextrin with the enantiomer remaining substituents. The difference in the size of enantiomers is important for the choice of α, β, or γ cyclodextrin, and the molecular fit determines the range of analytes that can be separated. For the inclusion, α-cyclodextrins will host single phenyl groups or napthyl end-groups, β-cyclodextrins will accept naphthyl groups and heavily substituted phenyl groups, and γ-cyclodextrins are useful for bulky steroid type molecules. Other interactions such as those involving the polar regions of an analyte and the surface hydroxyls of the stationary phase, and potential hydrophobic interactions in the cavity, provide the other two or more points of interaction required for chiral recognition.

Besides unmodified cyclodextrins, different modified cyclodextrins have been developed, and they expand the nature of compounds that can be separated. The derivatives are formed by bonding various groups onto the surface hydroxyls of the cyclodextrin cavity. The derivatives include acetyl (analogous to acetylated cellulose), (S)-hydroxypropyl ether, (S) or (R)-naphthylethylcarbamate (analogous to naphthyl Pirkle type columns), 3,5-dimethyl-phenylcarbamate, p-toluoylester, etc. These modified cyclodextrin phases have several advantages. They offer different polarities from simple cyclodextrins and are more stable to different mobile phases, allowing the use of a wider range of solvents. Cyclodextrins are used mainly in normal phase type separations, with no or little water/buffers. It is possible to operate cyclodextrin columns in an alternative polar organic mode with the mobile phase consisting of acetonitrile with up to 10% methanol plus up to 0.5% acetic acid and/or 0.5% triethylamine, and even in reversed phase conditions using acetonitrile/water mobile phase. Various suppliers offer chiral chromatographic columns based on derivatized cyclodextrins and resulting from α, β, or γ cyclodextrins. Among these suppliers are Advanced Separation Technologies (Astec) (Cyclobond 12 types), Machery-Nagel (4 types), Merck (2 types), Phenomenex (cyclodextrin with carboxymethyl functionalities bonded to methacrylate polymer), Showa Denko (4 types), Serva (3 types), Thermo Hypersil (2 types), YMC (3 types), etc. Besides cyclodextrins or modified cyclodextrins bonded to silica (typically with the help of a spacer), derivatized cyclodextrin (e.g., carboxymethyl cyclodextrin) can also be bonded to organic polymers. Stationary phases based on cyclofructans are also developed in the same manner as those based on cyclodextrin. Several commercially available chiral columns based on cyclodextrins or cyclofructans on silica support with different types of derivatization are listed in Appendix 14.2.5 [46,47]. New stationary phases for HILIC were synthesized by covalently attaching native cyclofructan 6 (CF6) to silica gel. The CF6 stationary phase has similar structural features to crown ether-based CSP, with enantioselectivity toward compounds

with amino groups [48]. The CF6 columns produced considerably different retention and selectivity patterns for various classes of polar analytes, including nucleic acid compounds, xanthines, β-blockers, salicylic acid and its derivatives, and maltooligosaccharides [49].

Crown ether chiral phases

Crown ethers–based chiral stationary phases are also cavity-based phases that can be used for enantiomer separations [50]. The crown ether can be bonded to the silica surface, for example, by the derivatization (as a second step) of aminopropyl silica (e.g., Chirosil RCA(+)). The crown macrocyclic groups consist of a polyether with the formula $[-(CH_2)_n-O-]_m$ ($n = 2$ or 3 and $m = 5-10$). The crown ether must achieve asymmetry by incorporating additional groups such as binaphthyl, biphenanthryl, tartaric acid, several carbohydrates, etc. A model chemical reaction with the formation of a bonded chiral phase starting with aminopropyl silica and tartaric acid modified crown ether is shown in Fig. 14.2.6 [51].

Among the commercially available crown ethers used for chiral separations are Daicel Chem. Ind., (Crownpack CR(+) and Crownpack CR(−)), Regis Technol. (Chirosil RCA(+) and SCA(−)), and Phenomenex (Sumichiral OA-8000). They are working mainly under reversed-phase type, and can be used for enantioseparation of racemates with amino groups, such as amino acids. In case of using stationary phases based on 1,1′-bi-2-naphthyl units into crown ether, the amino acids are kept into the crown ether cavity by $N-H^+ \ldots O$ hydrogen bonding, under the chiral barrier of naphthalene rings which are roughly perpendicular to the crown ether ring [52].

Macrocyclic antibiotics and glycopeptides

Another type of chiral stationary phase is based on macrocyclic type antibiotics immobilized on silica [53,54]. The macrocyclic antibiotics contain numerous chiral centers, functionalities that allow bonding to silica (using prederivatized silica with reactive groups) and capability

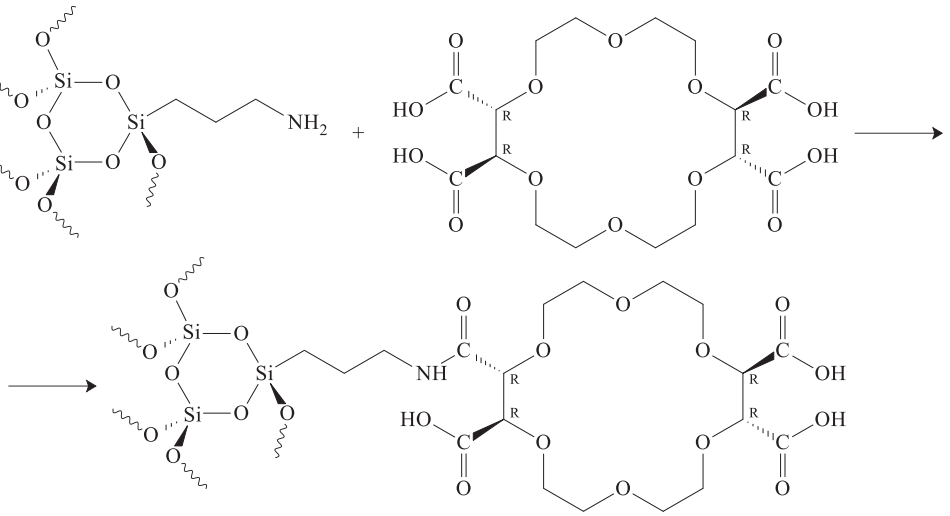

FIGURE 14.2.6 Schematic synthesis of a chiral stationary phase based on tartaric acid modified crown ether.

to offer π−π interactions, hydrogen bonding, inclusion/complexation, and ionic interactions. Several glycopeptide type antibiotics have been used for making stationary phases, including Rifamycin(s), Vancomycin (18 chiral centers), Avoparcin, Ristocetin, glycopeptide A-40,926 (MDL 62,476), and Teicoplanin (23 chiral centers). Macrocyclic antibiotics used as bonded stationary phase also include a family of thiopeptides with the parent compound being thiostrepton (17 chiral centers). The antibiotics that are glycopeptides have dissociable groups (−OH of phenolic type, $-NH_2$, −COOH, and −OH from carbohydrate). Some of the groups are capable to exist in zwitterionic form, and are likely to play a major role in the association with analytes during the chiral recognition. Similar to other chiral columns, the macrocyclic antibiotic type columns are used in normal phase but also in reversed phase mode. Glycopeptides immobilized on silica can be in the form of fully porous particles or as core-shell particles. Columns such as TeicoShell (silica base core-shell particle with teicoplanin active phase), VacoShell (silica base core-shell particle with vancomycin active phase), NicoShell (silica base core-shell particle with a modified macrocyclic glycopeptide active phase), Tag Shell (silica base core-shell particle with teicoplanin aglycone active phase) were successfully used for the separation of various tobacco alkaloids [55]. Docking simulations carried out for pantoprazole enantiomers, for example, have shown different number of interactions with teicoplanin aglycone CSP resulting in a calculated binding energy (ΔΔE) of about 0.3 kcal/mol [56].

Protein chiral phases

Proteins bonded to silica were also used as stationary phases for chiral separations. Proteins contain a large number of chiral centers and are known to interact with enantiomers to produce acceptable chiral selectivity, and can be bonded to silica, e.g., through one of their amino groups. Several types of proteins have been used so far for separation of enantiomers such as bovine serum albumin (BSA), human serum albumin, α1-acid glycoprotein, other glycoproteins, ovomucoid, ovoglycoprotein, avidin with cellobiohydrolase, and others. Some of these phases can be used in either normal phase or reversed phase mode with solvents such as isopropanol, ethanol, or acetonitrile in mixture with aqueous buffers [57,58]. Several commercially available chiral columns based on proteins on silica support are listed in Appendix 14.2.6.

Besides bonding directly to silica, proteins were also bonded to organic polymers such as acrylates and further coated on silica or silica functionalized with amino groups that provide a stronger bonding [59]. Another possibility to bound proteins to silica-based supports is to functionalize the silica surface to become an anion exchanger (e.g., quaternized polyvinylimidazole-coated silica) and then to bound electrostatically the proteins to its surface [60].

Ligand exchange chiral phases

Ligand exchange chromatographic columns contain chiral molecular units bonded on an organic polymer or on silica, the chiral units being capable of forming complexes with transition metal cations such as Cu^{2+}, Ni^{2+}, or Zn^{2+}. Some analytes with at least two electron-donating functional groups, such as amino acids, hydroxyacids, amino alcohols, or diols, are capable to form ternary complexes with the metal ions already bound to the chiral moiety from the stationary phase [4]. The enantiomer separation results from the difference between the stability constants of the ternary complexes. For example, (S)-proline attached to a solid phase forms a complex with Cu^{2+}. The ternary complex with

an (S) or (R) analyte such as leucine will have different stability constants [15]. Examples of ligand exchange phases are Astec CLC-L and CLC-D containing a chiral bidentate ligand and recommended to work in the presence of Cu^{2+} in the mobile phase, Davankov LEC (from Regis), Nucleosil Chiral1, Chiralpak WH, WM, and WE from (from Capital HPLC Lim.) [61], Chirex 3126 that contains D-penicillamine bonded on silica through a c18 handle in Cu^{2+} form, etc.

Chiral synthetic polymers

It is possible to synthetize chiral synthetic polymers that can be used as stationary phase for enantiomer separation. These polymers can be classified into three categories: addition polymers, condensation polymers, and cross-linked gels [62]. Such polymers can be bonded to silica in order to assure a high contact surface and mechanical rigidity. The polymer must contain chiral moieties to act as chiral selectors. One example is a polymer having diaminocyclohexylacrylamide chiral selectors with polymeric structure bonded to silica. Some other phases based on chiral polymers are commercially available (e.g., poly(diphenyl-ethylenediamine-bisacryloyl)) [63,64]. These stationary phases can be used in normal phase (NP) mode (e.g., hexane + isopropanol, or methylene chloride - + alcohol as a mobile phase), and also in NP with polar-organic mobile phase consisting of acetonitrile, methanol, and a polar additive (trifluoroacetic acid, triethanolamine, etc.). Several commercially available chiral columns based on synthetic polymers are listed in Appendix 14.2.7.

A number of chiral synthetic polymers were reported as synthesized, but they are not commercially available and were used only for research purposes. Examples include - trans-9,10-dihydro-9,10-ethanoanthracene-(11S,12S)-11,12-dicarboxylic acid bis-4 vinylphenylamide [65], - N, N′-[(1R,2R)-1,2-diphenyl-1,2-ethanediyl] bis-2- propenamide [66].

Among the polymers evaluated for potential use in chiral separations are also certain molecular imprinted polymers [67–69]. For example, 4-vinylpyridine can be used as a monomer with N-carbazolyl-L-aspartic acid as template for preparing a resin capable of separating N-carbobenzoxy-D,L-aspartic acids [70].

14.3 Characterization of chiral stationary phases

General comments

Several characteristics of chiral phases and columns are common to those used in other chromatographic techniques. Besides the obvious characteristics such as column dimensions, particle size of stationary phase, shape of particles, their mechanical resilience, range of pH stability, chemical stability in different solvents, and lack of bleeding, an important aspect related to chiral columns is related with their mode of utilization (NP, RP, HILIC, or IC like). Some columns are capable to be utilized in NP mode only, and other in more than one mode. For example, it is common for polysaccharide type chiral stationary phases (CSPs) to be effective under both normal-phase conditions and some under reversed-phase conditions (e.g., Lux Cellulose-2). Also some CSPs based on glycoproteins (e.g., Chiralpak AGP, a glycoprotein $alpha_1$ phase) can be used in RP mode. Water can be also present in the mobile phase of CSPs based on macrocyclic antibiotics such as VancoShell V. The capability of using a stationary phase in partially aqueous media is important related to the detection in MS-ESI mode where some water is usually necessary for obtaining ionization.

The main role of chiral phases is the efficient separation of enantiomers, which is characterized by *enantioselectivity*. Enantioselectivity follows the general definition of chromatographic

selectivity and can be characterized by the enantiomeric separation factor α given by expression 3.2.2 ($\alpha = k'_2/k'_1$, where k'_1 and k'_2 are the retention factors of the first and second enantiomer, respectively). It depends on which of the enantiomers forms the more stable association with the chiral selector from CSP and thus will be the more strongly retained species of the racemate. Enantioselectivity depends not only on the stationary phase, but also on the composition, pH, and temperature of the mobile phase [71].

Retention behavior of enantiomers on chiral stationary phases

The column efficiencies (N) of a typical 5 μm fully porous particle-based CSP can reach values situated in the domain of 25,000−50,000 plates/m, which are smaller than efficiency obtained for achiral columns (up to 250,000−350,000 plates/m) [72,73]. The efficiency can be improved by using sub-2 μm UHPLC chiral columns. Much higher efficiency (up to 400,000 plates/m) can be obtained for the capillary columns loaded with nonporous cyclodextrin-bonded silica-based particles [74].

With some exceptions (e.g., Ref. [52]), it is rather difficult to predict the efficiency of enantiomer separations and, when separated, the elution order of each enantiomer. For the identification of a specific enantiomer peak, isolated enantiomers should be injected on chiral column and their retention time measured. Except for polarimetric measurements, other physicochemical properties of enantiomers being identical, common detection techniques such as UV absorption of mass spectra do not differentiate the enantiomers. When isolated enantiomers are not available, information about their retention may be available in databases regarding enantiomer separation. For example, two known commercial databases, namely ChirBase [75] and Chirsource [76], contain information for over 200,000 chiral separations [77−79]. A theoretical possibility to predict elution order of enantiomers is offered by quantitative structure retention relationship (QSRR) techniques [80]. However, it is difficult to build accurate QSRR methods applied to chiral recognition (the use of QSRR to chiral compounds is usually indicated as quantitative structure enantioselective retention relationships, or QSERR), because it is difficult to find appropriate descriptors that give information about 3D structure of molecules. Other molecular modeling approaches were used to predict the results of chiral separations (e.g., AutoDock software [42]).

14.4 Selection of a chiral stationary phase

General comments

Chiral separations occupy an important sector of HPLC type analyses since many bioorganic molecules as well as many pharmaceutical drugs are chiral. The subject of chiral chromatography is covered in numerous publications including books (e.g., Refs. [26,81−85]), dedicated journals such as Chirality (Wiley) Tetrahedron: Asymmetry (Elsevier), numerous peer-reviewed publications, and companies application notes (e.g., Ref. [86]). However, the selection of a column in chiral separation and the development of a successful HPLC procedure remains a rather difficult subject. The differences in the interaction forces between enantiomers and the chiral selectors are typically small, and specific phases are more suitable than others for a specific separation. In addition, the restriction of using low level or no water in the mobile phase in many separations limits the type of polar compounds that can be separated using chiral phases. For this reason, trial and error in chiral separations is not uncommon. Theoretical considerations such as QSAR modeling are sometimes used for helping the chiral column selection [79].

Since chiral separations may be necessary only for a part of the compounds present in a

sample and the separation of all the components in a sample may be difficult using the chiral phase, bidimensional separations of samples containing chiral compounds are frequently utilized. In such separations, the components of the sample are initially separated on a nonchiral column with the enantiomer analytes not separated between themselves. A heart-cut can be taken for the region where the enantiomers elute, and the second separation is performed on a chiral column [87–91].

A number of chiral separations are reported to be performed on micro- or nano-HPLC columns [92–94]. This miniaturized technique includes chiral selectors, commonly used in HPLC, such as cyclodextrins, glycopeptide antibiotics, modified polysaccharides, and others [95]. Although conventional chromatography usually produces better peak symmetry and higher resolution, nanochromatography has the advantage of using very small samples [96].

Separation of the enantiomers of chiral compounds can also be achieved after derivatization with a chiral reagent, followed by separation on an achiral phase. For example, a mixture of (R)- and (S)-analytes can be derivatized with an (R)-reagent to generate (R)-,(R)- and (R)-,(S)- mixture of diastereoisomers that can be separated using achiral phases. The subject is presented in numerous publications but being part of sample preparation is beyond the purpose of present material (e.g., Ref. [97]).

The role of column selection in the development of a method for chiral separations

The selection of a chiral column for developing a separation is typically based on manufacturer recommendation. For the same task, it is possible to select several types of columns. Some recommendations for the use of Pirkle type columns on different classes of compounds are given in Table 14.4.1 (see also Appendix to Chapter 14).

A summary of other recommendations regarding the type of column to be used for a chiral separation is indicated in Table 14.4.2 [98]. The recommendations cover utilization mode (NP, RP, HILIC, or IC like), types of analyte, and detection mode.

The success in developing a good chiral separation is also highly dependent on the selection of the mobile phase, as also is in other types of

TABLE 14.4.1 Some recommendations for the use of Pirkle type columns.

Chiral selector	Compounds to be analyzed
(R)-phenylglycine and 3,5-dinitrobenzoic acid amide linkage	Carboxylic acids, alcohols, esters, sulfoxides
(R)-phenylglycine and 3,5-dinitroaniline urea linkage	Carboxylic acids, amino acids derivatives, compounds containing π-basic groups
(R)-1-naphthylglycine and 3,5-dinitrobenzoic acid amide linkage	Carboxylic acids, alcohols, esters, nonsteroidal antiinflammatory agents
(S)-valine and 3,5-dinitroaniline urea linkage	Carboxylic acids, amino acids derivatives, hydroxy acids
(S)-tert-leucine and 3,5-dinitroaniline urea linkage	Carboxylic acids, amino acids derivatives, benzodiazepines
(S)-valine and (R)-1-(α-naphthyl)ethylamine urea linkage	π-Acceptor derivatives of amines, carboxylic acids and amino acids, esters and amides of these acids, alcohols
(S)-proline and (R)-1-(α-naphthyl)ethylamine urea linkage	Amines, alcohols and amino acids, underivatized β-blockers, aromatic amines, pesticides

TABLE 14.4.1 Some recommendations for the use of Pirkle type columns.—cont'd

Chiral selector	Compounds to be analyzed
(S)-tert-leucine and (S)-1-(α-naphthyl) ethylamine urea linkage	Esters, amino alcohols, underivatized β-blockers, aromatic amines, cyano alcohols, pesticides
(S)-tert-leucine and (R)-1-(α-naphthyl) ethylamine urea linkage	Amines, amino alcohols, alcohols, underivatized β-blockers, aromatic amines, cyano alcohols, pesticides
(S)-indoline-2-carboxylic acid and (R)-1-(α-naphthyl) ethylamine, urea linkage	Amines, amino alcohols, alcohols
3,5-dinitrobenzoyl derivative of 1,2-diaminocyclohexyl bonded to silica by ether containing handle	Amides, alcohols, esters, ketones, acids, sulfoxides, phosphine oxides, heterocycles.
Dimethyl-N-3,5-dinitro-benzoyl-amino-2,2-dimethyl- 4-pentenyl phosphonate silica	β-blockers, π-acceptor derivatives of pharmaceuticals
N-(1-propylsilica-2-oxo-4-phenylazetidin-3-yl)-3,5-dinitrobenzamide	β-blockers
N-3, 5-dinitrobenzoyl-3-amino- 3-phenyl-2-(1,1-dimethylethyl)-propanoate bonded to silica by undecyl handle	Carboxylic acids, including nonsteroidal antiinflammatory agents.
3,5-dintrobenzoyl derivative of diphenylethylenediamine bonded to silica by tetradecanoic acid handle	Aryl carbinols, pharmaceuticals
1-N,1-N,3-N,3-N-Tetramethylidene-5-(1,2,3,4-tetrahydrophenanthren- 4-yl)carbamoyl]-benzene-1,3-diamine (propyl handle to silica)	Amides, epoxides, esters, ureas, carbamates, ethers, aziridines, phosphonates, aldehydes, ketones, carboxylic acids, alcohols, and nonsteroidal antiinflammatory drugs

TABLE 14.4.2 Suggestions for the utilization as type of chromatography of different types of chiral columns (not including Pirkle type).

Analysis type	Cellulose DMP	Cyclo-dextrin	Cyclo-fructan	Glyco-peptide	Immobilized protein	Ligand exchange	Synthetic polymer
Routine chiral column screening	3	3	3	3	2	1	2
Normal phase HPLC	3	2	3	2	1	1	3
Reversed-phase HPLC	1	3	2	3	3	2	1
Hydrophilic Interaction HPLC (HILIC)	1	2	2	3	1	1	1
Polar organic mode HPLC	3	2	3	3	1	1	3
Polar ionic mode HPLC	1	1	1	3	1	1	1
Polar/ionic analytes	2	2	2	3	3	1	1

(*Continued*)

TABLE 14.4.2 Suggestions for the utilization as type of chromatography of different types of chiral columns (not including Pirkle type).—cont'd

Analysis type	Cellulose DMP	Cyclo-dextrin	Cyclo-fructan	Glyco-peptide	Immobilized protein	Ligand exchange	Synthetic polymer
Amino acids, peptides	1	2	2	3	1	3	2
Primary amines, chiral	1	1	3	1	1	1	1
Nonaromatic organic acids	1	2	1	2	1	3	1
Bioanalysis (drugs in biological fluids)	2	3	1	3	2	1	1
Mass spec (LC/ESI)	2	3	3	3	3	1	1
Mass spec (LC/APCI)	3	3	3	3	1	1	2

Note: 3 = recommended, 2 = possible use, 1 = not recommended.

chromatographic separations [84,99]. In the development of chiral methods of separation, the mobile phase must be selected according to the type of utilization (NP, RP, HILIC, or IC like), and changes in the mobile phase composition are recommended for tuning a separation [100,101]. These changes include modification in the ratio nonpolar/polar solvent composition, addition of modifiers such as acid/base mixtures including the acids formic, acetic, trifluoroacetic, methanesulfonic, and the bases diethylamine, triethylamine, or salts such as ammonium acetate and ammonium formate. The content of water in the mobile phase can also be utilized for modifying the elution and separation.

14.5 Mobile phase in chiral HPLC

Mobile phase for chiral phases

Most chiral separations are performed on chiral stationary phases (CSPs). However, separations on achiral stationary phases with a chiral agent added to the mobile phase are also practiced. The mobile phases used in the two types of chiral separations are different.

Chiral phases can be utilized in normal phase mode (NP), reversed phase mode (RP), corresponding to HILIC (nonchiral chromatography mode), and applicable to ionizable compounds (corresponding to IC nonchiral chromatography) (see Section 14.2). For each of those modes of utilization, the mobile phase must have an appropriate composition [102].

Since hydrogen bonding is one common type of interaction utilized for differentiating the enantiomers, the presence of water in the mobile phase in many chiral separations may disturb such interactions. For this reason, the NP type utilization of chiral phases where no water or only very small levels are present in the mobile phase is the most common type of chiral separation. For normal phase utilization, the mobile phase is typically made from apolar solvents such as hexane or heptane with polar components such as methanol, ethanol, 2-propanol, chloroform, methylene chloride, lower ethers, or esthers [103]. Presence of water can be a problem not only for the separation but also for the miscibility with solvents such as hexane, heptane, or chloroform and methylene chloride.

In general, better separations are obtained at longer retention times, and therefore, lower levels of polar components in the mobile phase are typically used (in some separation as low as 2%–3%). The longer retention times are obtained using "weak" polar solvents such as

chloroform, methylene chloride, tetrahydrofuran, or ethyl acetate, which are less polar than methanol, ethanol, or acetonitrile [104]. One of the disadvantages of using apolar solvents and a total absence of water is the potential variability of the retention time in the chromatogram as traces of water are either absorbed by the mobile phase from the atmosphere or eliminated from the stationary phase by the flow of an anhydrous solvent. Also, in the case of MS used for detection, the ionization in ESI type is either absent or very low and APCI ionization type must be applied. The sensitivity of detection in APCI is usually lower than in ESI.

When the chiral phase is used in RP mode, the content of the polar solvent can be relatively low and water may be still absent in the mobile phase. Commonly used solvents are methanol, acetonitrile, tetrahydrofuran, 2-propanol, and ethanol. A high water content in the mobile phase is however not excluded for some chiral separations as illustrated in the example from Fig. 14.2.2. For cyclodextrin-based stationary phases, dimethylformamide and dimethylsulfoxide are also possible polar components of the mobile phase. The solvent selection may determine which interaction type is more important for the separation, e.g., π−π stacking, H-bonding, or hydrophobic interactions [105].

For HILIC and IC type utilization of chiral phases, the mobile phase must contain polar components such as methanol, acetonitrile, and water. For example, the chiral separations of basic compounds on polysaccharide phenylcarbamates using mobile phases with acetonitrile and 20%−30% water follow an HILIC-like separation [106]. Additives such as ammonium formate or low level of buffers such as acetic acid/trietanolamine or trifluoroacetic acid/trietanolamine or specific chaotropic salts can also be added to enhance the mobile phase polarity [107].

Enantioseparations can be strongly influenced by the pH of the mobile phase [103,108−112]. The control of pH can be achieved using a certain level of additives such as diethylamine (DEA), triethylamine, butylamine, or trifluoroacetic acid (TFA). The pH can also be controlled with buffers such as ammonium acetate, citrate buffers, triethylammonium phosphate, etc. [113]. The pH influences molecular structure as well as the formation/elimination of hydrogen bonds and plays an important role in the separation. Column stability is also affected by the mobile phase pH and temperature, many chiral phases being less resilient than RP columns to extreme pH values of the mobile phase. The typical pH range of utilization of many chiral phases is between 3 and 7 [114].

Besides the control of pH, the buffers may have additional roles in chiral separations. For example, the use of triethylamine/acetate buffer is preferred for separations on most cyclodextrin-based stationary phases, leading to better separations and better peak shapes. This can be explained by the special effect of the buffer on various types of interactions determining enantiomer separation. Some buffers such as triethylamine/acetate, formate, citrate, or trifluoroacetate are considered capable of forming inclusion complexes with the cyclodextrins and affect separation not only due to pH control. Buffer concentration is also important for achieving specific separations, and at high enough concentrations (e.g., higher than 1.5%), some buffers affect negatively enantioselectivity [108].

Gradient elution in chiral separations is less frequently utilized as compared to gradient utilization in RP-HPLC or HILIC. However, various published results on chiral separations use gradient. For example, gradient elution is useful for drug screening [115,116].

Mobile phase for chiral separations on achiral stationary phase

The separation of two enantiomers on achiral stationary phases requires a chiral additive in the

mobile phase to create a chiral environment. The use of a chiral additive to the mobile phase may involve two mechanisms of action. One mechanism involves the formation of diastereoisomeric complexes in solution between the analyte and the chiral additive and the other involves the retention of the chiral additive on the stationary phase, creating in this way a chiral environment for the separation. A simple theory can be developed to evaluate the factors influencing the separation in each case.

For the case of interaction in solution with diastereoisomers formation, the two enantiomers will be noted E and E^*, and the chiral additive will be noted Q. The equilibria for the enantiomer E are the following:

- Equilibrium between E in the mobile phase (mo) and in the stationary phase (st) described by the constant K_1 given by Eq. (14.5.1):

$$E_{mo} \rightleftarrows E_{st} \quad K_1 = \frac{[E]_{st}}{[E]_{mo}} \tag{14.5.1}$$

- Equilibrium between E and Q in the mobile phase to form the adduct EQ described by the constant K_2 given by Eq. (14.5.2):

$$E_{mo} + Q_{mo} \rightleftarrows EQ_{mo} \quad K_2 = \frac{[EQ]_{mo}}{[E]_{mo}[Q]_{mo}} \tag{14.5.2}$$

- Equilibrium between the complex E Q in the mobile phase and in the stationary phase described by the constant K_3 given by Eq. (14.5.3):

$$EQ_{mo} \rightleftarrows EQ_{st} \quad K_3 = \frac{[EQ]_{st}}{[EQ]_{mo}} \tag{14.5.3}$$

Since the enantiomer E is present both as free and as a complex with Q in both mobile phase and stationary phase, the distribution coefficient D_E for the enantiomer E between the mobile phase and the stationary phase is given by Eq. (14.5.4):

$$D_E = \frac{[E]_{st} + [EQ]_{st}}{[E]_{mo} + [EQ]_{mo}} \tag{14.5.4}$$

The value of $[E]_{st}$ can be obtained as a function of $[E]_{mo}$ from Eq. (14.5.1), and the value of $[EQ]_{st}$ can be obtained as a function of $[E]_{mo}$ and $[Q]_{mo}$ from Eqs. (14.5.3) and (14.5.2). Also, $[EQ]_{mo}$ can be expressed as a function of $[E]_{mo}$ and $[Q]_{mo}$ from Eq. (14.5.2). Including all these values in Eq. (14.5.4), the expression of D_E becomes

$$D_E = \frac{K_1 + K_2K_3[Q]_{mo}}{1 + K_2[Q]_{mo}} \tag{14.5.5}$$

Eq. (14.5.5) indicates that the distribution constant D_E for the enantiomer E is equal with K_1 if the chiral additive is absent ($[Q] = 0$) or if K_2 and K_3 are very small or zero. The increase in K_2, K_3, and $[Q]$ lead to the increase in D_E.

The corresponding distribution constant for the enantiomer E^* involves constants K_1^* (and $K_1 = K^*_1$), K^*_2, and K^*_3 (similarly defined as K_1, K_2, and K_3). Constants K^*_2 and K^*_3 can be assumed different from the corresponding ones for the enantiomer E because Q is assumed to interact differently with each enantiomer and because EQ and E^*Q can be viewed as two diastereoisomers, their retention on the achiral stationary phase being different. With these constants being different, the selectivity α given by $\alpha = D_E/D_{E^*}$ will be different from 1, and the separation is possible. The value of α depends on all equilibrium constants and the larger the differences between K and K^*, the better is the separation. Also, in general, an increase in $[Q]$ leads (up to a point) to an increase in α, indicating that more chiral additive favors the separation.t

For the case of retention of the chiral additive on the stationary phase, the additive Q is retained on the solid phase based on the equilibrium:

$$Q_{mo} \rightleftarrows Q_{st} \quad K_4 = \frac{[Q]_{st}}{[Q]_{mo}} \tag{14.5.6}$$

Since the enantiomer E is assumed to interact only with the adsorbed additive, the equilibrium

with the adsorbed additive can be written as follows:

$$E_{mo} + Q_{st} \rightleftarrows EQ_{st} \quad K_5 = \frac{[EQ]_{st}}{[E]_{mo}[Q]_{st}} \qquad (14.5.7)$$

The distribution coefficient D_E is given by the expression 14.5.4 where $[EQ]_{mo} = 0$. In this case, by substituting in D_E the values for $[E]_{st}$ (from Eq. 14.5.1), for $[EQ]_{st}$ (from Eq. 14.5.7) and for $[Q]_{st}$ (from Eq. 14.5.6), the following expression is obtained:

$$D_E = K_1 + K_4 K_5 [Q]_{mo} \qquad (14.5.8)$$

This expression shows that retention factor of the separation $k = D_E \Psi$ depends on the additive Q concentration and constants K_1, K_4, and K_5. Among these constants, only K_5 is different from K^*_5 (for the enantiomer E^*). Selectivity α is larger when the difference between K_5 and K^*_5 is larger ($K_1 = K^*_1$ and K_4 does not depend on E or E^*). The ratio D_E/D_{E^*} depends on all parameters and similar to the first model, the larger is the difference between K_5 and K^*_5, the better is the separation. The second model also shows that an increase in $[Q]$ leads (up to a point) to an increase in α, indicating that more chiral additive favors the separation. In every separation, one model or the other explains better the process, but it is likely that in most cases each type of interaction plays a certain role.

The solvents making the mobile phase in the case of chiral additives can still be similar to those from chiral NPC or RP-HPLC. Among the solvents utilized in such separations are acetonitrile, methanol, ethanol, mixture of such solvents with low levels of water, or nonpolar solvents such as hexane, heptane, etc.

A variety of additives can be used in the mobile phase for creating a chiral environment for the separation [18]. Among these are cyclodextrins, cationic β-cyclodextrins, BSA, alpha-1-acid glycoprotein (ORM), N-alkyl-L-hydroxyproline plus copper acetate added in the mobile phase, Cu^{2+}-L-phenylalanine in the mobile phase [117], etc.

One example of using this chiral additive is the separation of (±)-camphor and (±)-α-pinene using an RP column and α-cyclodextrin in the mobile phase [118]. In case of cyclodextrin used as a chiral modifier, solvents with higher hydrophobicity such as ethanol and iso-propanol are typically used for achieving faster elution. Among other examples of chiral additives are cationic β-cyclodextrins [119], BSA, and alpha-1-acid glycoprotein (ORM). In many cases, the influence of the additive on the separation is considered to be caused by the formation of complexes in the solution [120]. However, in the case of the additive N-alkyl-L-hydroxyproline plus copper acetate, used for the separation of certain enantiomers of amino acids on a C18 stationary phase, the chiral selector is strongly adsorbed onto the C18 surface, effectively forming a chiral stationary phase. For an aqueous mobile phase used for elution, there is virtually no column bleed when the additive is left out from the mobile phase after column conditioning. In this case, the equilibrium takes place between the chiral analyte and the chiral surface of the stationary phase, with the separation caused by differences in the interactions on the solid phase for each enantiomer.

Ion pairing mechanism for enantioseparation

One type of additives successfully utilized in chiral separation of the enantiomers of compounds that contain ionizable or strongly polar groups are chiral IPA followed by an ion pair chromatographic separation on achiral polar or hydrophobic stationary phases depending on the nature of chiral counterion and the composition of mobile phase. In principle, the chiral API will form with the analyte associates that act as diastereoisomers [121]. Among common chiral IP agents used for this purpose are, for example, (+)-10-camphor-sulphonic acid or (−)-10-camphor-sulphonic acid for the analysis of cationic compounds and quinine, quinidine,

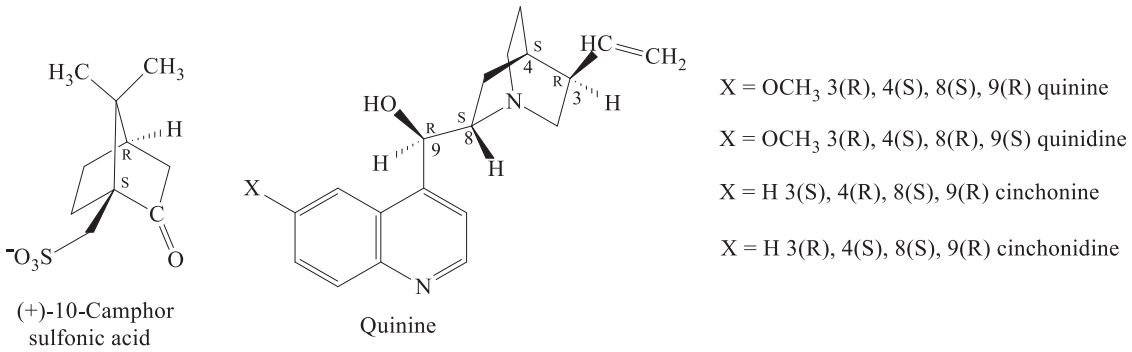

FIGURE 14.5.1 Formulas for (+)-10-camphor-sulphonic acid, and for quinine, quinidine, cinconine, and cinchonidine.

and cinchonidine, and cinchonine as counterion for the separation of acids [122,123] (Fig. 14.5.1).

The mobile phases typically used in such separations should be high in the organic phase component. The ion pair separation in NP mode can be performed on a silica stationary phase capable of separating the diastereoisomers.

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CHAPTER

15

Size exclusion HPLC

15.1 Separation process in size exclusion chromatography

General comments

Separation in size exclusion HPLC has a specific type of equilibrium, as presented in Section 4.4 and is not based on partition or adsorption. This type of chromatography separates the compounds according to their molecular size (hydrodynamic volume). When only size exclusion is taking place and no energetic interaction of the analyte and stationary phase is present, the separation is based on the difference in the conformational entropy outside of the pores of the stationary phase where they are in an "expanded" shape and inside the pore where their conformational entropy decreases. For a "pure" size exclusion process, the constant describing the retention equilibrium is given by Eq. 4.4.3 written once more below:

$$K_{\text{pure}} = \exp\left(\Delta S^0 / R\right) \tag{15.1.1}$$

As indicated in Section 4.4, size exclusion in reality is associated with additional energetic interactions between the analytes and the stationary phase involving some enthalpy changes such that the process is in fact described by an equilibrium constant K_{SEC} expressed by Eq. 4.4.6:

$$K_{SEC} = K_{\text{pure}} \exp\left(\frac{-\Delta \boldsymbol{H}^0 + T\Delta S^{*0}}{RT}\right) = K_{\text{pure}} K_{\text{interaction}} \tag{15.1.2}$$

In Eq. 15.1.2, $\Delta \boldsymbol{H}^0$ is the enthalpic term and ΔS^{*0} is the entropic term for these additional interactions.

Similar to any HPLC process, the retention in SEC of a given molecular species can be formally characterized by the reduced retention volume V'_R given by Eq. 3.1.24. From Eq. 3.1.24, the following expression can be written for the retention volume V_R:

$$V_R = V_0 + KV_{st} \tag{15.1.3}$$

In SEC, the equilibrium process takes place as a migration of the analytes between the interstitial volume of the column and the pores of the packing filling the column. In Eq. 15.1.3, the dead volume V_0 can be indicated for this process as the interstitial volume V_{inter}, and the volume V_{st} can be considered as equal to the volume of

https://doi.org/10.1016/B978-0-323-91177-1.00012-0

the pores of the packing V_{pores}. Therefore, the general Eq. 15.1.3 can be written for SEC separation in the following form:

$$V_R = V_{inter} + K_{SEC}V_{pores} \quad (15.1.4)$$

Using the same assumptions as for Eq. 15.1.4, the phase ratio that was described in general for HPLC in Section 3.1 as having Eq. 3.1.18, will be given in SEC by the following formula:

$$\Psi_{SEC} = \frac{V_{pores}}{V_{inter}} \quad (15.1.5)$$

Using Eq. 15.1.5, the expressions for retention factor k'_{SEC} will be given by the following formula:

$$k'_{SEC} = K_{SEC}\frac{V_{pores}}{V_{inter}} \quad (15.1.6)$$

The direct proportion between k'_{SEC} and Ψ_{SEC} expressed by Eq. 15.1.6 has, however, a problem. The value of Ψ_{SEC} may increase by an increase in the number of pores and not only by the increase in the (average) pore volume. If the pores are numerous but keep a small average volume, these pores cannot contribute to the retention of larger macromolecular analytes. In this case, Eq. 15.1.6 is not anymore valid, and the proportionality remains valid only for a specific range of molecular sizes. Also, due to the interactions with solvent molecules, the stationary phase is swollen resulting in a gel phase aspect, a process that may also affect the column pore volume [1]. The polymer separation in SEC mechanism on swollen stationary phases can be considered as a polymeric ternary system constituted of a solvent (A) polymeric solute (B) gel matrix (C) (or swollen gel) system. Under usual chromatographic conditions and from a thermodynamic point of view, this system can be considered as being formed by two phases coexisting in equilibrium: (a) a binary phase constituted by the polymer solution outside the pore (mobile phase) or inside the pore but far away from the pore walls and (b) a ternary phase (stationary phase) formed by the mixture of the polymer solution and the gel matrix [2].

The variation of the retention volume V_R as a function of entropy variation ΔS^0 for a hypothetical column where $V_{inter} = 5.9$ mL and $V_{pore} = 4.0$ mL is shown in Fig. 15.1.1 (interaction

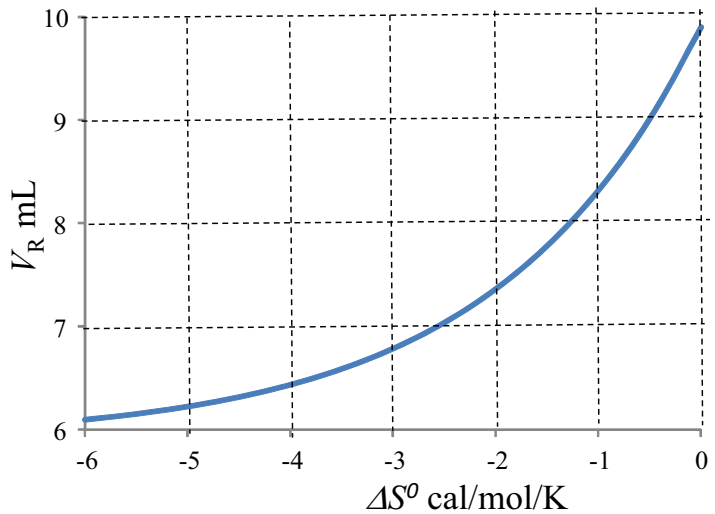

FIGURE 15.1.1 Variation of the retention volume V_R as a function of entropy variation ΔS^0 for a hypothetical system where $V_{inter} = 5.9$ mL and $V_{pore} = 4.0$ mL with no enthalpic contribution.

entropy corresponding for polystyrene in the range of *Mw* from 2500 to 3,000,000 [3]).

The graph from Fig. 15.1.1 shows that for molecular species with a negative (larger in absolute value) entropic term, the retention volume is smaller than for molecules with no variation in entropy between presence inside the pore or in the interstitial volume.

Eq. 15.1.2 allows the interpretation of separation in size exclusion based on the values of K_{SEC}. As shown in Section 4.4, ΔS^0 which is negative for a molecule entering the stationary phase pore is large in absolute value for macromolecules and this leads to a small K_{pure} and as a consequence a small K_{SEC}. Based on Eq. 15.1.6 (and shown in Fig. 15.1.1), this leads to a rapid elution of large molecules. Small molecules have a small ΔS^0 and K_{pure} is closer to 1. This leads to larger K_{SEC} and larger retention volumes.

In descriptive terms, an initial zero concentration of analyte in the porous stationary phase tends to become equal with the concentration of the analyte in the mobile phase, but for large molecules this tendency is opposed by the large negative entropy associated with the process. As a result, large molecules practically do not enter the pores and elute without retention (total exclusion). The macromolecules of medium size enter only some larger pores and are only partly retained, eluting faster than small molecules and slower than the very large ones, thus achieving separation. Small molecules will have only a small loss of entropy by penetrating the pores of stationary phase (K_{pure} close to 1) and entering the pores of stationary phase have a long path through the column bed and therefore large retention volumes (long retention times).

Depending on the molecule shape, its dimension that influences K_{pure} can be characterized in different ways. For example, for random coiled molecules, the shape can be described by a mean radius $\bar{r}$. The relation between K_{pure} and the radius $\bar{r}$ can be described by two formulas, depending if the radius $\bar{r}$ is smaller or larger than the mean pore radius $\bar{d}$ of the stationary phase [4]. These formulas are indicated below:

$$K_{\text{pure}} = 1 - \frac{2\bar{r}}{\sqrt{\pi}\,\bar{d}} \quad \text{for} \quad \bar{r} \ll \bar{d} \qquad (15.1.7)$$

$$K_{\text{pure}} = \frac{8}{\pi^2}\exp\left[-\left(\frac{\pi\bar{r}}{2\bar{d}}\right)^2\right] \quad \text{for} \quad \bar{r} \gg \bar{d}$$

The dimension of a molecule is also related to molecular weight, and as a result, it is expected that V_R will depend in some way on the molecular weight (*Mw*) of the polymeric material. This dependence is verified in practice, and the following formula has been proven as valid for a certain range of *Mw* for various polymers:

$$V_R(X) = A - B\log(Mw(X)) \qquad (15.1.8)$$

In Eq. 15.1.8, *A* and *B* are constants for polymers with different molecular weights but of the same type, and an ideal variation of V_R with the molecular weight is shown in Fig. 15.1.2.

In practice, Eq. 15.1.8 is frequently used for the evaluation of *Mw* for polymers [5]. However, Eq. 15.1.8 is valid only for a certain range of *Mw* values, and different molecular structures lead to different slopes for the dependence of V_R on log (*Mw*). The deviations from the ideal dependence

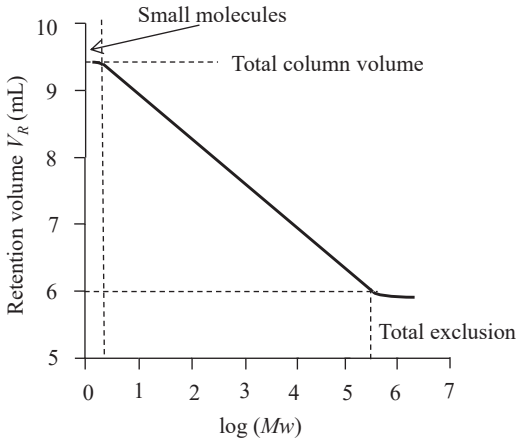

FIGURE 15.1.2 Ideal variation of elution volume V_R as a function of log (*Mw*) in SEC.

usually include linearity for only a narrow range of *Mw*, although a decrease in the retention time remains valid for larger molecules. For being used in *Mw* determination, the dependence between *Mw* and the elution volume must be calibrated in the molecular range of interest using polymer standards with known molecular weight and having similar structures (shape) to those that are analyzed. As indicated by Eq. 15.1.7, the K_{pure} depends in fact not on *Mw* but on the mean radius $\bar{r}$ of the molecule (or of the molecule hydrodynamic volume). Since molecules with different shape may have the same values for *Mw* but different molecular hydrodynamic volumes, only a calibration of Eq. 15.1.8 with polymer standards of the same shape as the analyte can provide correct results for the *Mw*.

In many cases, enthalpic interactions may also be encountered such that the more interacting polymers with the stationary phase appear as having lower *Mw* than their actual value. The presence of enthalpic contributions to the retention process can be detected based on chromatographic peak shape (sharp leading edge followed by tailing), unexpected retardation of the peak of polymers with known high *Mw*, poor reproducibility of retention time, or large changes in the retention time upon solvent changes.

Formulas for resolution *R* and peak capacity *P* have specific forms for SEC-HPLC separations. The specific expression for the resolution R_{SEC} between two adjacent peaks 1 and 2 assigned to two solute fractions with molecular weights denoted Mw_1 and Mw_2 (eluting in this order) can be calculated with the following formula:

$$R_{SEC} = \frac{2(t_{R,2} - t_{R,1})}{W_{b,1} + W_{b,2}} \cdot \frac{1}{\lg(Mw_2) - \lg(Mw_1)} \tag{15.1.9}$$

In Eq. 15.1.9, W_{b1} and W_{b2} are the peak widths at the peak base [6]. Other approaches are proposed, depending on the nature of the macromolecular species separated and the type of the porous stationary phase [7].

Peak capacity P_{SEC} parameter can be calculated from the following equation [8]:

$$P_{SEC} = 1 + \frac{\sqrt{L}}{2} \sum_{i=1}^{n} \frac{\Delta k'_{i-1,i}}{\sqrt{H_{i-1}} + \sqrt{H_i}} \tag{15.1.10}$$

In Eq. 15.1.10, *L* is the length column and *H* is the theoretical plate height of the corresponding peak. For SEC based on stationary phase particles of diameter (d_p), the value of P_{SEC} can be calculated by the following formula [9]:

$$P_{SEC} = 1 + \frac{1}{6}\sqrt{\frac{L}{d_p}} \tag{15.1.11}$$

15.2 Stationary phases and columns for size exclusion HPLC

General comments

Size exclusion chromatography (SEC) is a technique applied mainly for the separation of mixtures of organic polymers or for the separation between organic polymers and small molecules. SEC is also used for the estimation of molecular weight of polymers (see Eq. 4.3.62) [10]. For organic mobile phase, SEC is indicated as gel permeation chromatography (GPC) and for aqueous mobile phase SEC is indicated as gel filtration chromatography (GFC). Depending on the nature of the polymers, they may be soluble in an aqueous solvent with separation based on GFC or soluble in organic solvents with separation based on GPC. Solubilization of certain polymers can be a difficult task, and for this purpose, special solvents and sometimes elevated temperatures must be used. The organic polymers can be either synthetic or natural, many natural polymers being of biological origin. The average molecular weight of polymers separated by SEC varies in a large range starting with *Mw* of oligomers formed from several monomeric

units and being as high as $2 \cdot 10^7$ Da [11]. Size exclusion may be performed at higher pressures and be considered part of HPLC family, but also can be performed at low pressure. More recently, some success has been obtained in the attempts to perform SEC in UPLC mode, on particles below 2 μm diameter [12]. This technique is limited to the characterization of polymers with *Mw* up to about $2 \cdot 10^6$ Da, and beyond this limit, the deformation and/or mechanical shearing of large molecules at high flow rates may occur [13]. Stationary phases in SEC can be based on porous silica but also on other inorganic materials and very commonly on organic polymeric materials. Polymeric materials for making the stationary phase are more frequently used in SEC than in other HPLC procedures [11,14].

Silica-based SEC stationary phases and glass phases

Silica-based stationary phases are common for size exclusion separations, their advantages being mainly related to the good mechanical properties and the lack of swelling, such that their pore size is independent of the nature of mobile phase [15]. This typically is associated with good column efficiency. However, for SEC, polymer-based phases are more frequently utilized than silica-based ones, in particular due to the larger range of pore sizes offered by the polymeric materials (between 60 Å and 4000 Å). The progress made in obtaining good mechanical strength for newer organic polymers contributed to their utilization in HPLC. Silica-based phases have been used in gel permeation-GPC (with organic type mobile phase) but more often in gel filtration-GFC (with aqueous type mobile phase) modes. Pore size for SEC silica phases can be controlled similarly as for other silica-based phases (see Section 8.1), and usually pore sizes of 300 Å or larger are used in SEC.

Silica-based phases include bare silica as well as derivatized silica, similar to the construction of other bonded phases on silica. Bonded phase silica-based materials with large and controlled pore dimensions can be made to also have groups such as diol, diol on Zr-clad silica, glycol ether, polyether, and amide. Special polymer separation can be done on large pores C18 type columns [16]. These phases have frequently a propyl anchor group to the silica material. The derivatization of silica for SEC separations is performed for altering the interaction of pore walls with the analytes. The goal in SEC is to eliminate as much as possible the enthalpic interactions (see Section 4.4) between the stationary phase and the analytes. These interactions interfere with the "pure" entropic separation and affect the elution order of the analytes. The selection of the structure of the stationary phase allows recommendations for which types of analytes are expected to have less enthalpic interactions. For example, diol phases seem to be the most successful in separating proteins since they have minimal enthalpic interactions with them.

Other procedures are also used to limit the enthalpic interactions. For example, the purity of the silica material used for making the packing is very important in order to avoid enthalpic interactions with the analytes. Some parameters of the mobile phase may contribute to minimization of enthalpic interactions between stationary phase and analyte, such as added salts and other modifiers, or a specific pH [7]. The silica-based phases should be operated within the recommended range of pH, which is typically from 2.5 to 7.5. The silica-based phases are not stable outside this range. Several commercially available silica-based packings are listed in Appendix 15.2.1.

Synthesis of silica-based stationary phases for SEC columns uses similar procedures as those previously described for other silica type phases. Controlled hydrolysis of an alkoxysilane with the purpose of generating a silica gel with the appropriate pore size is the first step in the phase preparation (see Section 8.1). The aging of the

silica gel by controlling parameters such as temperature, pH, gelling time, addition of electrolytes for flocculation, addition of detergents, and specific solvents determines the growth of sol particles and of the reticulation to obtain the desired properties of the final material. Drying of the gel is also important for generating the desired silanol density. The silica gel can be used without modifications or can be derivatized, e.g., with diol groups.

Besides silica, porous glass has been used as packing material for SEC columns [17]. The surface of the porous glass can be derivatized with diol groups, similar to that of porous silica [18].

Polymer-based phases used in SEC

Polymeric-based SEC phases are more common than silica-based ones. Some phases are designed for GPC, a very common material used for this purpose being polystyrene-divinylbenzene (PS-DVB). This polymer is typically prepared by suspension polymerization (see Section 8.1), and depending on the proportion of cross-linking reagent (DVB), either soft gels are obtained (2%−12% DVB) or rigid polymers are generated (more than 20% DVB) [19]. Other phases are used for GFC (aqueous SEC), examples of these including several soft gels based on dextran or agarose, more rigid polymers such as hydroxylated poly(methyl methacrylate), and polyvinylalcohol copolymers.

The porosity of the polymeric materials is controlled by the conditions in which the polymerization is carried out. When more than 20% or more of a nonpolymerizable compound is present, this compound dissolving the monomer but not the polymer, a product with a macroporous structure is obtained. This macroporous structure remains in the dry state of material. Porosity of the stationary phase is critical for the separation of compounds within a specific range of *Mw*. However, the pore size of the polymer is difficult to measure when the polymer is swollen, and it is usually assessed with calibrants of known *Mw*. Examples of calibrants are sets of polystyrenes with different known *Mw* for GPC separations and sets of polyethylene oxide, dextrans, and proteins, also with different known *Mw*. Polymeric packings with a narrow range of pore size have a separation capacity concentrated in a limited molecular weight range. Although the resolution of such columns is high, their use for SEC analyses is possible only for a narrow molecular weight distribution of the sample. In practice, SEC columns of different pore sizes are connected in series to provide a wider molecular weight separation range. This procedure of multiple columns is not very convenient, and unique columns in which the pore size distribution of the stationary phase is broadened by blending together two or more phases are available. Mixed pore packings obtained by blending together several selected pore size materials can be made such that the column exhibits a linear calibration for analytes in a wide range of *Mw* [20,21].

The particle size of polymer-based packings is another parameter important for SEC column efficiency (height of theoretical plate). The packing materials must be as homogeneous as possible, and this is achieved by having particles of equal size and spherical shape, with uniform flow channels. Polymer particles with a highly monodisperse particle size distribution can be produced by a two-step microsuspension method. This process is based on using monosized polymer seed particles mixed with the low-molecular weight material during the polymerization. This process allows the preparation of monosized compact or macroporous particles of predetermined size in the range of 1−100 μm [22]. However, it is common that a separation by size of the synthesized polymer particles is still necessary for obtaining particles with a narrow size distribution. This is achieved by using sedimentation or centrifugation [23].

Information regarding the particle shape and size can be obtained by microscopic methods. Smaller particles offer improved resolution but result in higher operating pressures and can prove more difficult to pack.

Mechanical stability of the polymer is important for establishing the limit of flow rate operation. The maximum operating pressure of the packing should be below the compression point of the packing material. Since in both GPC and GFC it is possible that the mobile phase viscosity can be increased during a gradient separation, particular attention must be given to the potential changes in this parameter. SEC stationary phases may be sensitive to excessive backpressure that can collapse the phase and destroy the column.

Surface chemistry of the packing material is very important and must be selected such that any enthalpic contribution (interactions with the polymer surface) is minimal. For GFC, the packing materials are typically highly hydrophilic and should not possess charges. Supports for aqueous phase size exclusion chromatography may be produced by coating the particle surface with a hydrophilic cross-linked polymer. The enthalpic effects are difficult to completely eliminate, and salts (buffer) as well as organic modifiers are typically added to the mobile phase to diminish such interactions.

Chemical and temperature stability of the packing is related to its inertness to specific solvents, and typically, the cross-linked polymers are capable of being used with a wide range of solvents. Temperature stability of the packing is also necessary since some polymeric analytes are soluble in the mobile phase only at elevated temperature. Packing materials capable to be used at temperatures in the range 50–120 °C are common. Several phases used in GPC are listed in Appendix 15.2.2, and some used in GFC are listed in Appendix 15.2.3.

New developments

New development in SEC column technology included the use of core-shell phases and small particle size ones (sub 2-μm) [12]. For example, packed columns with small core-shell particles (1.3, 2.6, 2.9, and 3.6 μm d.p.) with silica and C18 silica displayed acceptable separation resolution in specific mass ranges of polystyrene standards, depending on the pore size [24]. A few newly commercially available SEC columns are listed in Appendix 15.2.4 [25]. The long separation times typical for SEC separation are significantly shortened on newer columns. Also, the decrease in particle size leads to an increase in column efficiency with better separations of biopolymers and more accurate results regarding evaluation of *Mw*.

15.3 Characterization of size exclusion phases and columns

General comments

The separation in SEC is influenced by specific characteristics of the packing materials. Several such characteristics of SEC columns/packing materials are described in Table 15.3.1 [26].

Column characterization should be made in association with the purpose of analysis and nature of the analytes [27]. The interaction between the SEC column and the analyte can be very different for different classes of macromolecules, and proteins, polymeric carbohydrates, and synthetic polymers may have very different behavior on the same type of column [28,29].

Porosity and particle size

Some of the characteristics indicated in Table 15.3.1 are relevant for any chromatographic column, such as resolution, column pressure, and chemical and thermal stability. Other

TABLE 15.3.1 Characteristics of SEC columns/packing materials.

Attribute	Variable	Importance	Common values	Range
Porosity	Pore size. Pore volume	Range of *Mw*	50–10^5 Å	50–10^5 Å
Pore size distribution	Range of pore size	Resolution		
Resolution	Column efficiency (*N*)	Separation efficiency	18,000	7000–25,000
Resolution	Particle size	Separation efficiency	5 μm, 7 μm	4–20 μm
Resolution	Particle shape	Separation efficiency	Spherical	Irregular sphere
Column pressure	Particle size	Flow rate, analysis time	500 psi	600–2000 psi
Inertness	Column activity	Utility for *Mw* evaluation	Inert	Some inertness
Recovery	Column activity	Recovery of the analyte	95%	Variable
Mechanical stability	Support type	Max working pressure	500, 2000 psi	Variable
Chemical stability	Support type. Phase type	Solvents, range of pH	Stable	Variable
Thermal stability	Support type.	Temperature of utilization	Up to 130°C	20–160°C
Load capacity	Amount of sample	Resolution	2–4 mg	2–4 mg

characteristics are more relevant for SEC columns. One of these is the stationary phase porosity, which is also important for other chromatography type phases, but in SEC it plays a crucial role in the separation mechanism. Porosity determines the range of molecular weights (*Mw*) that can be covered by a specific column. The resolution of the columns regarding the separation of two analytes of different molecular weights depends on pore size and also on pore size distribution. Columns with a wider range of pore diameters are able to separate a wider range of molecular weights for the analytes, but typically, the resolution of these columns is lower, as compared to columns with a narrow pore size distribution. However, columns with a narrow range of pore sizes (narrow pore size distribution) can be used only within a narrow range of molecular weights for the analytes.

Particle size diameter of the columns is also important for the resolution of the separation. For high column efficiency, packing materials in SEC should be fine, uniform, and spherical particles (3–20 μm), as in the common interactive HPLC. However, smaller particles (e.g., 3–5 μm), which lead to better resolution, also increase the column backpressure. In SEC, the columns have very frequently a limited value for the backpressure in order to avoid the collapse of the pores, which damages the column. For this reason, when columns with small particles are used, a decrease in the flow rate of the mobile phase is typically recommended (below 1 mL/min, e.g., 0.2 mL/min). The increase in the column length, which also enhances resolution, is associated with an increase in the column backpressure. The use of two or more columns in series for SEC, which is useful for covering the separations of a wider range of *Mw*, is also limited by the increase in the backpressure.

Inertness and recovery

Two other characteristics are the inertness of the phase and the recovery (which is related to inertness). The inertness of the SEC column is

important in particular when estimations of the *Mw* of polymers are done using SEC. When the column is not inert, other interactions besides size exclusion will influence the retention process, and the result for the *Mw* evaluation may be incorrect. From Eqs. 15.1.4 and 15.1.2, the following formula is obtained for the retention volume:

$$V_R(X) = V_{\text{inter}} + K_{\text{pure}}(X)K_{\text{interaction}}(X)V_{\text{pores}} \tag{15.3.1}$$

In Eq. 15.3.1, only $K_{\text{pure}}(X)$ depends on *Mw* of *X*. As shown by Eq. 15.1.1, $K_{\text{pure}}(X)$ depends only on ΔS^0 which depends only on *Mw*. On the other hand, $K_{\text{interaction}}(X)$ depends on specific (mainly enthalpic) interactions of compound *X* with the stationary phase. Different compounds having different $K_{\text{interaction}}(X)$, the retention volume V_R will not depend anymore only on *Mw*. For this reason, stationary phases as inert as possible having minimal interactions with the analyte are desirable for the use in the measurement of *Mw*.

For preparative reasons in particular, the recovery of the analytes is another important characteristic of the SEC columns. It is common that proteins or other biologically active materials are separated (e.g., for purification) on SEC columns. In some instances, it may be important that the material injected in the column is recovered and its biological activity is not altered. When the biological activity of the analyte must be preserved, the nature of the mobile phase also plays an important role in preserving biological activity. For such GFC separations, the mobile phase is either water or a dilute buffer [30].

15.4 Selection of a stationary phase in size exclusion HPLC

Selection factors for SEC columns

The main factors to be considered in the selection of the column (or of a set of columns) for SEC separations are (1) the purpose of separation, (2) the nature of the analytes to be separated, and (3) other analysis requirements. The purpose of separation in SEC may include the following: (1a) separation of small molecules from polymeric molecules, (1b) separation of different polymeric molecules, (1c) evaluation of molecular weight of polymers, and (1d) generation of a pure molecular fraction for further analysis of the separated macromolecules. Regarding the nature of the analytes to be separated, the following aspects should be considered: (2a) solubility of the analytes in aqueous/polar solvents (for GFC application), (2b) solubility of the analytes in organic nonpolar solvents (for GPC applications), and (2c) range of molecular weights expected for the analytes and matrix. Regarding other analysis requirements, these can be related to the following: (3a) general column properties, (3b) analysis time, and (3c) use of specific equipment such as specific detectors. Some details about these factors are further discussed. Various manufacturers offer information on column selection for various separation purposes (e.g., Ref. [31]).

(1a) Separation of small molecules from large molecules is easily achieved in SEC. For this reason, the selection of a column to separate large molecules from small molecules should not be a difficult task [32]. The column selection must consider other aspects such as the nature of the polymers, which indicates if GFC or GPC is going to be utilized. However, one important parameter to consider when separating large molecules from small molecules is the lack of enthalpic interactions of the macromolecular components with the stationary phase material (see Section 4.4). When strong interactions take place between the macromolecules and the stationary phase (interactions with enthalpic contribution are present), these macromolecules will be retained longer in the column, and they may elute in the region of small molecules (having larger V_R than expected) [33].

(1b) Separation of polymeric materials for analytical purposes is a common task for SEC analyses. In this type of separation, the selection of the SEC column also must consider the use of GFC or GPC depending on sample nature. For many natural polymers such as polymeric carbohydrates, proteins, and polyethylene glycols, GFC is usually utilized [34–36]. For many synthetic polymers, the solubility in organic nonpolar solvents makes GPC more appropriate for separation [4]. For successful separations, it is very useful to have some information about the *Mw* range of the components of the sample that must be separated. For the separation of mixtures of molecules within a wide range of *Mw* values, more than a single SEC column can be used in a series. In such cases, the column with the highest porosity is used first. For example, Phenogel columns cover a wide range of porosities (between 10^2 and 10^6) and can be used up to four columns in series for achieving some separations. Since high backpressure can damage gel porosities, the maximum acceptable backpressure (up to 1500 psi) of the column should be never exceeded. A reduced flow rate of the mobile phase is sometimes necessary for maintaining the correct backpressure, especially when more than one column in series is utilized for the separation. Recommendations from column manufacturers indicate the solvent compatibility and the *Mw* range where the column is effective [37]. The range also depends on the molecular structure of the polymeric molecule. For example, the recommended use of Toyopearl columns (Tosoh Bioscience) [38] for different types of analytes and for different ranges of *Mw* is illustrated in Fig. 15.4.1.

As indicated in Fig. 15.4.1, dextrans, globular proteins, and polyethylene glycols, having different molecular structures, are not covered in the same *Mw* range for a specific type of column. A variety of column formats are available for specific applications [39]. Particular

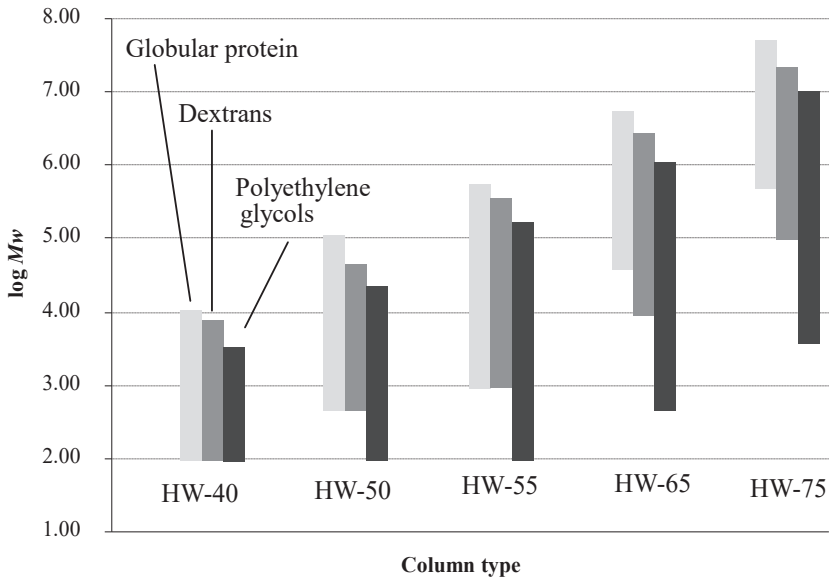

FIGURE 15.4.1 Types of Toyopearl columns recommended for use with polymers of different *Mw* and different chemical natures [38].

advantages and limitations as well as typical applications for each type of column are reported by the suppliers.

(1c) When the purpose of SEC separation is the *Mw* determination of a polymer or polymer mixture, the selection of the column type is very important. The linearity of the correlation between retention time t_R (retention volume V_R) and molecular weight (in fact molecular hydrodynamic volume) strongly depends on the nature of the polymer and the selected column. A particular problem in the use of SEC for the measurement of *Mw* is related to the dimensions of the pores and pore size distribution of the phase. As previously indicated (see Section 15.3), the columns with a narrow pore size distribution offer better resolution and allow a more precise measurement of *Mw* of analytes, but they can be applied only in a narrow range of *Mw* values. In order to achieve both higher resolution and separation for a wider range of *Mw*, SEC can be conducted using two to four columns of large dimensions (7.8 mm I.D. × 300 mm) connected in series. Similar to separation for polymer analysis, the range of utilization of SEC columns is also related to the nature of the analytes that are separated. The measurement of *Mw* is typically performed using a calibration with standards of known *Mw* (standards are available from different suppliers). For relevant results, the calibration must be performed using standards with a similar molecular structure as the analytes. For example, separation of a set of polystyrenes of different *Mw* on three Phenogel 10 μm GPC columns, 105, 104, and 103 Å, 300 × 7.8 mm, with tetrahydrofuran (THF) mobile phase, at a flow rate of 1 mL/min and refractive index detection is shown in Fig. 15.4.2. The injection volume was 100 μL of a solution 1% of seven standards of polystyrene. The plot of log *Mw* as a function of retention time is also shown in the figure.

As shown in Fig. 15.4.2, the plot of log *Mw* as a function of retention time is not perfectly linear, but after a calibration with standards, the measurement of MW can be done with good accuracy.

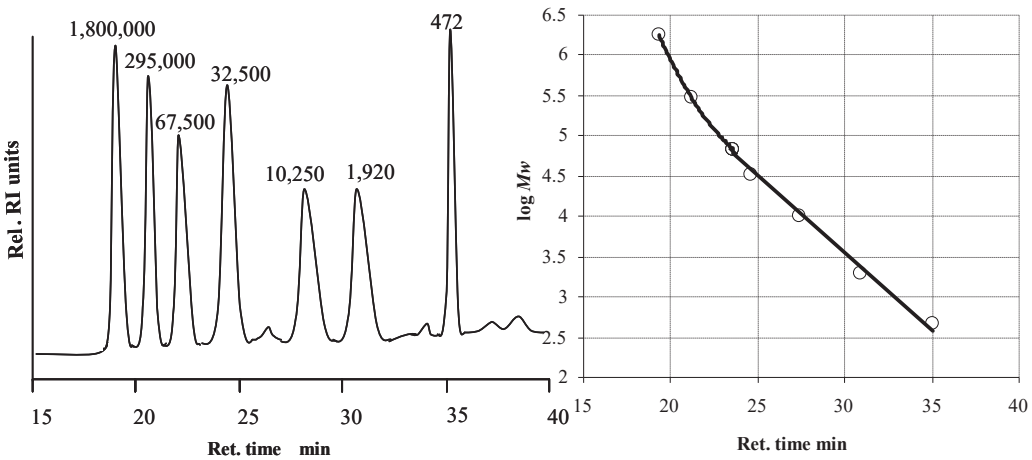

FIGURE 15.4.2 GPC separation of seven polystyrene standards (MW indicated above each peak) on three Phenogel 105, 104, and 103 Å, 300 × 7.8 mm columns. The plot of log *Mw* as a function of retention time is also shown.

(1d) Some SEC separations are performed with the purpose of further utilization of the separated macromolecules (such as proteins). In such cases, lack of denaturation and as complete as possible recovery from the HPLC column are very important [40]. Particular attention must be given to the nature of solvents, pH, and temperature of the mobile phase.

(2a) Solubility of the polymeric samples can be a challenging task, in particular because the sample concentration in SEC is typically higher than in other HPLC techniques (can be in the range of 1%−3%). Some polymers are soluble only in special solvents, e.g., dissolution of cellulose may require ionic solvents [41] or solvents such as water or diethylformamide but with additives such as LiOH and urea [42], and dissolution may affect the polymerization degree (and therefore the *Mw*) of the polymer. The selection of GFC or GPC is related to the polymer nature and the nature of the sample solvent.

(2b) The use of GPC is common for many synthetic polymers with hydrophobic molecules. For these polymers, solubilization may also be a problem and some polymers are soluble only at temperatures of 110−130 °C. For the analysis of such polymers, the columns (and the whole HPLC system) must be kept at elevated temperatures. Specific columns are recommended for GPC separations and maximum allowed temperatures are usually specified by the manufacturer (see Appendix for Chapter 15).

(3a) Some of the column general properties were already indicated, such as column efficiency, maximum column backpressure (limited by mechanical resilience of the phase), stability in specific solvents, and pH stability range (particularly for silica-based columns). Also, column loading capacity must be considered in SEC, since in SEC practice it is common to use rather concentrated samples for injection. The selection of the column for SEC separation must consider all those elements for a successful separation (e.g., https://www.sepax-tech.com/application_notes/SM1003.pdf).

(3b) The use of large column bunks (two to four columns in the order of decreasing pore size) achieves high resolution and accurate *Mw* measurement. However, it requires long analysis time and significant solvent consumption. Since there is increasing interest in developing high-speed SEC using a single column of small dimensions, special columns with higher phase resilience have been made commercially available (see Appendix for Chapter 15). Help in reducing the run time was also obtained from the use of mass spectrometers as detectors in SEC. The higher sensitivity of the mass spectrometers allowed the use of shorter columns with lower loading capacity and better resolution where the use of lower flow rates overcomes the backpressure problem [15].

Utilization of ultrahigh-pressure liquid chromatography (UPLC) in SEC is another attractive alternative to solve the problem of large solvent volumes and long analysis time. However, the implementation of UPLC for the analysis of macromolecules poses several challenges, including the following: a) development of packing materials with large pore diameters and pore volumes that are mechanically stable at ultrahigh-pressure, b) avoidance of high shear stress generated by the flow of polymer solutions, which may affect the conformation of the polymer chains, and c) assurance of proper diffusion of the polymers in the pore of the stationary phase during the separation. Effort is continually made in

solving such problems [16], and several phases recommended to be used in UPLC mode are commercially available (e.g., TSKgel UP-SW3000 from Tosoh Bioscience). Also, the possibility to perform fast and efficient two-dimensional analysis of polymers using ultrahigh-pressure liquid chromatography (UHPLC × UHPLC) was proved for the separations of some copolymers based on their chemical composition [43].

15.5 Mobile phase for size exclusion separations

General comments

In most cases, in size exclusion (in both known modes GFC and GPC), the mobile phase serves mainly to dissolve the sample and carry it through the column. Compared to RP-HPLC or HILIC, the mobile phase in SEC has a lower role in modulating the separation. However, polymer dissolution can be a challenging task, which is worsened by the fact that in SEC the samples are usually more concentrated that in other HPLC types (e.g., several mg/mL). Also, since the stationary phases in SEC are frequently polymers, the solvents used in SEC separations should not affect the structure of stationary phase. Column manufacturers typically indicate the range of solvents allowed on a specific column.

Dissolution of polymers is mainly determined by enthalpic interactions, since the gain in mixing entropy for polymers during dissolution is typically small. The intermolecular interactions between the polymer chain segments and solvent molecules have an associated energy which can be positive or negative. For a "thermodynamic good solvent" interactions between polymer segments and solvent molecules are energetically favorable and will cause polymer coils to expand. For a "poor solvent" polymer–polymer self-interactions are preferred, and the polymer coils will contract. The thermodynamic "quality" of the solvent depends on the chemical composition of both the polymer and solvent molecules, and also on the solution temperature. In solution, macromolecules can assume various shapes (conformations) such as globules, rods, coils, etc. In one special type of solvent (and at a specific temperature), the polymer behaves as in the bulk polymer without changing its conformation. Such solvent is called a theta solvent. The enthalpy of interactions with theta solvent must only compensate the effect of excluded volume expansion. In such solvent the measurement of *Mw* can be considered more accurate. Detailed discussion regarding polymer solutions and the influence of solvent on polymer conformation is reported in the literature [44,45].

The equilibrium constant K_{SEC} for the separation, and as a result the capacity factor k_{SEC}, depends on the effective mean radius $\overline{r}$ of the polymer. Since the shape of the macromolecule in solution and macromolecular hydrodynamic volume depends on the solvent choice, when SEC is used for the determination of molecular weight of the polymers, the resulting value for *Mw* may be influenced by the solvent choice [4]. Also, in the case of protein separation, the solvent may affect biological activity by changing the conformation of the protein. This can be a detrimental effect in cases when biological activity must be preserved. For other SEC utilizations, such as polymer identification, changes in molecular shape are less important.

In order to obtain reliable information regarding the macromolecular size (molecular weight) by SEC, besides the choice of a solvent that does not change conformation, it is essential that the separation is controlled exclusively by the entropy of the adsorption/desorption process (when the goal of the separation is, for example, only polymer purification, this is not an essential factor). Enthalpic interactions with the stationary phase lead to retention that is not a function of molecular size of the analyte. For such cases where enthalpic interactions must be diminished (or even eliminated), the solvent can be selected specifically for this purpose.

This solvent selection is typically done based on experimental trials and depends on the analyte and the stationary phase. Additives in the solution may sometimes affect the interaction between the analyte and the stationary phase, in particular for charged macromolecules. Due to the potential change of the polymer shape caused by the dissolution solvent, because sometimes stationary phase can be affected when the solvent is changed, and also because the solvent composition has in general little effect on SEC separation process, most SEC separations are performed in isocratic conditions.

The kinetics of partition is governed by the diffusion coefficient $D_{A,B}$, as defined by Eq. 7.2.4, which has different coefficients for different polymers and solvents. Also, the viscosity η of the solution involved in Eq. 7.2.4 can be calculated with empirical formulas for different polymeric solutions. For example, for NaOH/urea aqueous solutions of cellulose, η is calculated with the following formula [46]:

$$\eta = 3.85\ 10^{-2}(Mw)^{0.76} \qquad (15.5.1)$$

Data references for other polymeric solutions can be found in specialized databases (e.g., Ref. [47]).

Typical solvents for gel filtration

A variety of solvents are used for water-soluble (hydrophilic) polymers. For nonionic polymers, pure water can be used successfully as an eluent. For less hydrophilic polymers that may have some solubility problems in pure water, a certain percent of organic solvent in water can be used, and for ionic polymers, buffers and additives can be beneficial for the separation. Maintaining a constant ionic strength of the mobile phase is also sometimes necessary for reproducible results of molecular weight measurements. Some typical solvents used in water soluble polymers are given in Table 15.5.1.

The salts used for constant ionic strength may include NaCl, Na_2SO_4, $NaNO_3$, CH_3COONa, etc. The buffers that can be used include acetic acid/sodium acetate and other common buffers. The organic solvents may be methanol, acetonitrile, isopropanol, etc. The manufactures of the SEC columns usually indicate restrictions regarding the solvents that can be used for a separation.

Specific hydrophilic polymers may not be soluble in water, although they also do not dissolve in organic solvents. This is the case, for example, for cellulose and other polysaccharides, and

TABLE 15.5.1 Common solvent used in gel filtration.

Type of polymer	Typical eluents
Nonionic	Water, Water with 0.1–0.2 M salt for constant ionic strength Water with buffer at pH = 7
Nonionic with some hydrophobicity	Water with up to 20% organic modifier Water, organic modifier, 0.1–0.2 M salt, and/or buffer pH = 7
Anionic	Water with 0.1–0.2 M salt for constant ionic strength and with buffer at pH = 7–9
Anionic with some hydrophobicity	Water, organic modifier up to 20%, 0.1–0.2 M salt, and/or buffer pH = 7–9
Cationic	Water with 0.3–1.0 M salt for constant ionic strength and with buffer at pH = 2–7
Cationic with some hydrophobicity	Water with organic modifier, 0.3–1.0 M salt for constant ionic strength and with buffer at pH = 2–7

for specific proteins. In such cases, special solvents are necessary. For example, cellulose can be solubilized in dimethylacetamide + LiCl, urea or thiourea + NaOH [46,48], certain cationic polymers can be solubilized in formamide + LiCl or in dimethylformamide/triethylamine/pyridine, etc.

In case of proteins, specific restrictions are sometimes imposed on the mobile phase in order to promote solubilization, avoid protein adsorption on the stationary phase due to electrostatic and even hydrophobic interactions, and also to prevent the protein denaturation. For example, addition of salts such as NaCl, or Na_2HPO_4, of amino acids (glycine, alanine, or arginine), or to organic solvents (methanol, acetonitrile) may preclude protein adsorption, but higher contents than 5%−10% organic solvents or more than 0.1−0.4 mM/mL salts must be avoided in order to maintain protein biological activity [49]. Sometimes, for protein solubilization, a detergent such as sodium dodecylsulfate (SDS) is added. This compound leads to the dissociation of protein aggregates, but also increases protein hydrodynamic volume by formation of a protein−SDS complexes [50−52].

Typical solvents for gel permeation

For GPC, the solubilization of the polymer can also be a considerable challenge. Tetrahydrofuran is a common solvent for many polymers such as polystyrene, poly-(methylmetacrylate), epoxy resins, polycarbonates, polyvinylchloride, and polystyrene/acrylonitrile. Other solvents used in polymer analysis include toluene, chloroform, benzene, etc. [53]. Some common synthetic polymers such as polyethylene or polypropylene are not easily solubilized and they are soluble only in solvents like trichlorobenzene or methylcyclohexane at temperature above 90−100°C [54]. The solvents used in SEC separations should not affect the structure of stationary phase. Column manufacturers typically indicate the range of solvents allowed on a specific column.

15.6 Interaction polymer chromatography

General comments

Size exclusion chromatography is capable to separate molecules based on the steric interactions of the molecules and the stationary phase producing a separation by molecular size (hydrodynamic volume). This procedure is frequently utilized for the *Mw* characterization of polymers. However, a more detailed characterization is frequently required for the *Mw* distribution of polymers. The polymer molecules typically have a range of masses, and of topology of polymer chains or of other polymer properties such as type of end groups, and all these components lead to an average value for *Mw* (that is measured by conventional SEC). Because SEC is usually insufficient to characterize such details about polymer structure, a group of techniques indicated as interaction polymer chromatography (IPC) has been developed [55]. The IPCs are usually performed in combination with SEC and attempt to add different separation properties than steric by involving enthalpic separation interactions [56]. Among such techniques are liquid chromatography at the critical conditions, temperature−gradient interaction chromatography, interactive gradient polymer elution chromatography (GPEC), GPEC in precipitation-redissolution mode [57], barrier methods, etc. [56,58].

As an example, liquid chromatography at the critical conditions is a method of characterizing polymers that uses a solvent and a stationary phase involving both SEC and enthalpic interactions such that weak attractive enthalpic interaction effect is exactly compensated by entropic

SEC exclusion effect when the following relation is achieved (see Eqs. 15.1.1 and 15.1.2):

$$\Delta S^0 = \frac{-\Delta H^0 + T\Delta S^{*0}}{T} \qquad (15.6.1)$$

In such conditions, the polymer species satisfying the critical condition elutes near the elution time of unretained molecules, independent of its molecular mass (it has $k' = 0$). In this regime, usually only one of the components of the polymer undergoes chromatography under critical conditions and is indicated as "invisible," having no effect on separation, while other components take part in the chromatographic process according to an adsorption or a size exclusion mechanism and elute at a longer retention time. Establishing the proper conditions for such separations is typically difficult but allows for a very fine separation of a polymer components [55]. Most techniques practiced in IPC are also based on bringing the separation to critical conditions for one polymer component.

Gradient temperature in IPC

The equilibrium of various polymeric species between the mobile and the stationary phases is a complex process. The interaction enthalpy depends on the molar mass of polymeric species as well as on their tacticity, functionalization, topology, and chemical composition. The entropic component also depends on different characteristics of the molecular shape, in particular on hydrodynamic volume. However, only small differences are usually present between the macromolecules to be separated from a polymer with an average *Mw*. Nevertheless, two different polymeric species of the same *Mw*, one with a linear chain and other with branched structure, will interact differently with the stationary phase. By applying a temperature gradient to the separation process, these structural modifications can be better differentiated and used for the characterization of heterogeneity polymers. For example, temperature gradient interaction chromatography is based on variation of temperature kept during separation for achieving a very shallow gradient that affects retention conditions. Starting at a temperature below the one for critical conditions and increasing the temperature, the polymer interaction with the stationary phase takes initially place through enthalpic interactions, then reaches critical conditions, and follows to SEC separation.

Temperature gradient IPC can be performed with RP or NP conditions. Under RP mechanism, the polymeric species are separated according to enthalpic interactions instead of hydrodynamic volume. This is used for characterization of structural dispersity for various branched polymers (star-shaped, H-shaped, comb branched, dendritically branched, hyperbranched, miktoarm star block, or copolymers) [59]. The NP conditions utilize polar stationary phase, such as bare silica or diol bonded silica, and less polar mobile phase, and they are useful for the characterization of end-functionalized polymers of high molar mass, such as the class of homopolymers with different functional groups [60].

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CHAPTER

16

Affinity, immunoaffinity, and aptamer type HPLC

16.1 Separation process in immunoaffinity HPLC

General comments

Affinity chromatography is a liquid chromatographic technique typically used for protein and other bio-molecules separation. The separation in affinity chromatography is based on the interaction of a specific (biological) compound present in the mobile phase with compounds such as heparin, lectins, nucleotides, etc., bound to a support such as agarose (the neutral gelling fraction of the complex natural polysaccharide agar), cross-linked agarose, cross-linked dextrans (sepharose, sephacryl, etc.), or cellulose. In immunoaffinity (IAC), the biological compounds interact with its natural biological complement immobilized on the surface of a stationary phase [1]. Examples of such interactions are between an antigen and its antibody, between lectins and glycoproteins, between an immobilized metal ion and proteins containing amino acid residues that have affinity for the metal ion (e.g., histidine), between biotin and avidin, etc. The nature of the interaction is complex, and a variety of types of forces occur between the two participants (one participant being immobilized). In immunoaffinity, it is common that a sample passed through the column is separated into two bands. The first band elutes with a retention factor $k' = 0$ and contains all the compounds of the sample that do not bind to the affinity ligand. At this time, the analyte of interest should be strongly adsorbed to the ligand and should not elute ($k' = \infty$). A change in the mobile phase, such as a modification in pH, in the ionic strength, or in other parameters determines the analyte molecules to decouple from the complex with the immobilized complement and thus to elute and produce the second band [2,3].

Because on the capability of IAC to separate large molecules (e.g., proteins and nucleic acids) and small molecules, the technique is also known as high-performance affinity chromatography (HPAC) [4,5]. Essential to the method development for selecting a phase in HPAC is to consider the matrix support, the biological affinity pair, the immobilization coupling chemistry, and the elution conditions [6]. Detection in HPAC is made by known techniques, such as by fluorescence, chemiluminescence, and electrochemical detectors [7]. A large body of information regarding IAC is available (e.g., Refs. [8–10]).

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16.2 Types of phases and their preparation in affinity and immunoaffinity chromatography

General comments

Separation using immunoaffinity liquid chromatography (IAC) is a common technique for many analyses of biological samples. The technique can be applied in both low pressure [11] and high-performance chromatography (HPLC) modes [12]. Because of the importance of this technique in medical and biological applications, a significant number of publications are available on this subject. Details about immunoaffinity chromatography are given in a number of publications (e.g., Refs. [13,14]). Because the applications of IAC are very diverse and each application has its own particularities, it is not possible to provide general recommendations for the selection of a type of column to be used in IAC. Some of the main aspects on the preparation and characteristics of the stationary phases used in IAC are presented in this section.

Supports for stationary phases in immunoaffinity chromatography

Similar to other chromatographic stationary phases, the immunoaffinity phases have a support and an active component. For being used in HPLC, the support for IAC should be chemically and physically stable, with a good mechanical strength, and should have minimal nonspecific adsorption capability. Because IAC is used for large molecules separation, the particle size and pore size distribution of the support are very important parameters for assuring the access of the analyte to the active phase. The pore size for IAC is typical large, between 300 and 500 Å. The immobilization of ligands of interest should be such that high ligand accessibility is achieved, while the phase properties are not affected. A wide variety of materials, including organic and inorganic polymers, have been used for the design of these solid phase supports. One of the first materials used as support in IAC were natural polysaccharides such as agarose (agarose is a linear polymer made up of repeating units of agarobiose and it is one of the components of the natural polysaccharide agar), cross-linked agarose (sepharose), cellulose, cross-linked dextran (sephacryl), etc. Gels made from purified agarose have a relatively large pore size, making them useful for separation of large molecules. These materials are stable over a wide interval of pH (3−13) and are characterized by a high content of hydroxyl groups available for activation and derivatization. Their hydrophilic surface generally does not interact with proteins and exhibits only a low nonspecific adsorption. A major drawback of these materials is their poor mechanical strength and their swelling propensity in aqueous solvents. Other supports used in IAC for the stationary phase include silica [15] and synthetic organic polymers such as methacrylates, polyacrylamide, and copolymers of polyacrylamide with other polyvinyl polymer [14]. Although more resistant to pressure than polysaccharides, the organic supports still exhibit a lower pressure tolerance in comparison to inorganic materials such as silica. In addition, some of the synthetic polymers show swelling differences in the presence of organic solvents, a broader pore size distribution, decreased ligand binding efficiency, and nonspecific interaction due to their hydrophobic character compared to the phases based on natural polysaccharides. The stationary phases can also be porous, nonporous [16], or monolithic [17].

Monolithic supports based on glycidyl methacrylate-co-ethylene dimethacrylate (GMA-EDMA) have been shown to have some advantages in particular related to the lack of swelling and to the lower backpressure that eliminates the need of high mechanical resilience. Such columns are commercially available from BIA Separations (Slovenia) under the trade name of CIM disk monolithic columns. The main advantage of the GMA-EDMA support is the significant concentration of chemically reactive epoxy groups which can be used for immobilization of ligands, e.g., containing amino groups [18].

Silica is also used as support in IAC mainly because of its excellent stability under pressure and because it can be easily derivatized in order to introduce different functional groups. However, silica stability is not good in alkaline pH values and dissolves significantly above pH of 8. In addition, nonspecific interactions can occur between silanol groups and the basic parts of the biomolecules. Surface modification of silica can be performed either by chemical modification or physical adsorption of ligands in order to minimize this nonspecific adsorption and to introduce a high density of functional groups [19].

The active phase in immunoaffinity chromatography

The active phase in immunoaffinity can be very diverse, depending on the nature of the material immobilized on the stationary phase. One important group of active phases is that of antibodies or antibody-related moieties bonded to the solid support [20,21]. Stationary phases containing an immobilized antibody, enzyme, or other compounds such as heparin [22], lectins, nucleotides, etc., were successfully utilized in various chromatographic procedures, some as low pressure liquid chromatography and other in HPLC mode [23].

The two main types of antibodies that are used in IAC are polyclonal antibodies and monoclonal antibodies. Polyclonal antibodies are produced from multiple cell lines within a living body and they can bind a variety of antigens and with a range of binding strengths (the bonding takes place with the part of the antigen recognized by the immune system, known as epitope). Because monoclonal antibodies are generated from a single cell line, they bind to a single epitope with identical binding affinities. Recent advances have led to the production of artificial antibodies that have high binding affinities [24]. Before the antibodies can be used in IAC, some further purifications are often required. This purification may involve the use of ion exchange chromatography [25], precipitation with ammonium or dextran sulfate, or isolation on a low pressure LC immunoaffinity column [26].

Antibodies can be immobilized onto supports by using a variety of techniques that range from covalent attachment to adsorption-based methods. Of these techniques, those that make use of covalent attachment are the most common, but even these methods can range from the use of random attachment via amino or carboxyl groups to more site-selective immobilization approaches that make use of modified carbohydrate residues or thiol groups. The ideal situation in any of these immobilization methods is to have antibodies attached to the support in a way that does not affect the activity of the binding sites or the accessibility to these sites of the target compounds [27]. The immobilization can be done through free amine groups by using supports that have been activated with agents such as *N*,*N*′-carbonyldiimidazole, cyanogen bromide, *N*-hydroxysuccinimide, or tresyl chloride/tosyl chloride. Antibodies can also be immobilized through amine groups using a support that has been treated to produce reactive epoxy or aldehyde groups on its surface [28]. Antibodies can be covalently linked to IAC supports through more site-selective methods. This can be achieved by utilizing the free sulfhydryl groups of the protein. These groups can be used for immobilization by using techniques such as the divinylsulfone, epoxy, iodoacetyl/bromoacetyl, maleimide, or tresyl chloride/tosyl chloride methods. Another procedure for the immobilization of antibodies is using antibodies that have been reacted with biotin, and then are adsorbed to a support that contains immobilized avidin or streptavidin. The most popular biotinylation technique is to incubate antibodies with *N*-hydroxysuccinimide-*D*-biotin at pH 9. These linkages can resist many types of elution conditions without dissociation.

For silica-based stationary phases, a common material utilized as a starting material is silica pretreated with an aminosilane (e.g., γ-aminopropyltriethoxysilane, γ-aminopropylmethyl-diethoxysilane, γ-aminopropyldimethylethoxysilane).

The reaction of the propylamino silica surface with glutaraldehyde generates a reactive material on which an amino group of a protein (e.g., with lysine units in its structure) can be readily attached. The reactions are schematically shown in Fig. 16.2.1.

Other similar procedures use succinic anhydride instead of glutaraldehyde as an intermediate step of derivatization. Some proteins that contain phenolic groups (e.g., with tyrosine units) can be bound to the amino silica using azo coupling reactions.

For an organic support, the linking process is usually done using an activating reagent such as cyanogen bromide when the molecule to bind contains a free primary amine, sulfhydryl, or hydroxyl groups for attachment. On the activated support, proteins (or other molecules) with specific binding capability are further immobilized [29]. These can be selected by the user or can be general purpose immobilized compounds. Immobilized heparin, for example, acts with a specific binding site to retain certain proteins, lectin resins can be used for the separation of glycoproteins from other glycoconjugate molecules, and nucleotide resins are used for the separation of specific proteins [30]. Heparin resins, lectin resins, and others are commercially available. Specific immunoproteins also can be bound, for example, on agarose activated with cyanogen bromide or with other activation reagents such as 6-aminohexanoic acid, carbonyldiimidazole, thiol, etc. [28]. These types of materials have a very high specificity for the specific antigen that generates the immunoprotein. Affinity resins containing immobilized sugars and sugar derivatives and resins with immobilized biotin or avidin are also available. Several activation reagents for agarose and cross-linked dextrans are indicated in Table 16.2.1. Other resins are used having immobilized ligands such as *p*-amino benzamidine (ABA-5PW), *m*-aminophenyl boronic acid (Boronate-5PW), and iminodiacetic acid (CHelate 5 PW) all from Sigma. Numerous other immobilization procedures are reported in the literature [31,32].

The immunoaffinity type phases have excellent selectivity and work well in aqueous solutions, but each material must be developed for a specific analyte, the phases can be unstable toward organic solvents, and may be stable only in a narrow pH range. Some bioaffinity phases are used for HPLC separations [33–35], while others must be used at lower pressures (1–2 bar) since some materials impose considerable limitations regarding the maximum pressure that can be applied to the phase.

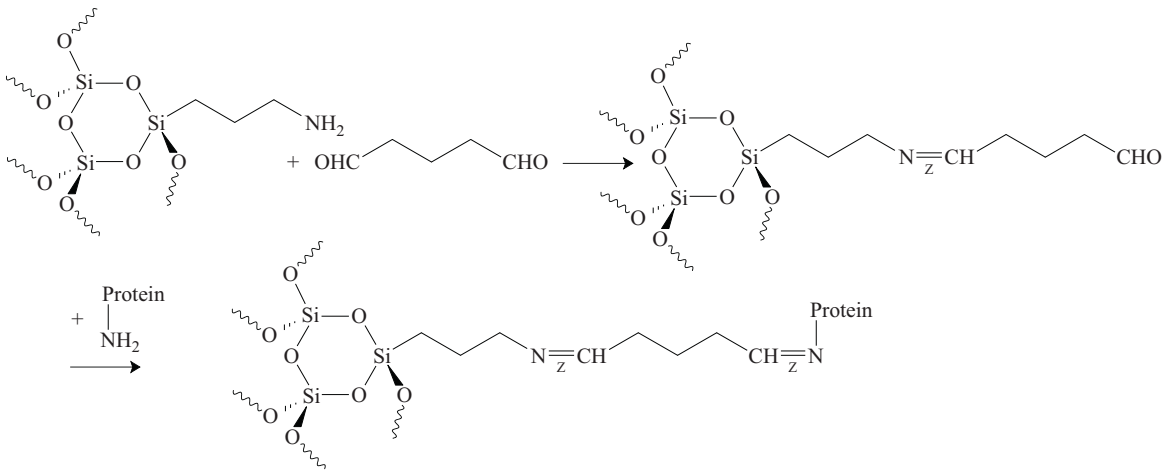

FIGURE 16.2.1 Schematic reaction showing the binding a protein to amino silica.

TABLE 16.2.1 Reagents used to make activated resins able to bind proteins.

Activating reagent	Linkage to resin by	Reactive group in the ligand	Specificity of the group to	Reaction conditions	Bond type to ligand	Stability
6-Aminohexanoic acid	Isourea	Carboxyl	Amine, with carbodiimide coupler	pH 4.5–6.0	Amide	Good
6-Aminohexanoic acid N-hydroxy-succinimide ester	Isourea	Succinimidyl ester	Amine	pH 6.0–8.0	Amide	Good
Carbonyldiimidazole	Carbamate	Imidazolyl carbamate	Amine	pH 8.0–10.0	Carbamate	Good below pH 10
Cyanogen bromide	Ester	Cyanate	Amine	pH 8.0–9.5	Isourea	Moderate
Epoxy	Ether	Epoxy	SH > NH	pH 7–8 SH pH 8–11 NH_2	SH: Thioether, NH_2 amino ether	Very good
N-hydroxy-succinimide ester	Isourea	Succinimidyl ester	Amine	pH 6.0–8.0	Amide	Good
Periodate	Oxidizes agarose, saccharides	Aldehyde	Amine	pH 4.0–10.0	Reductive amination with $NaBH_3(CN)$	Very good
Thiol	Isourea	Disulfide	Sulfhydryl	pH 6.0–8.0	Disulfide	Good in nonreducing conditions

Besides bioaffinity (immunoaffinity) phases, affinity chromatography can be also used for small molecule analysis, such as separation of amino acids on a column having ethylenediaminotetracetic acid moiety bound on a solid material, in the form of a transition metal chelate, capable of forming further chelates with the amino acids in solution. Such phases can be used, for example, in chiral separations (see Chapter 14).

Some stationary phases in immunoaffinity chromatography are based on aptamers [36]. Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule. Most aptamers are synthetic molecules but natural aptamers also exist. The oligonucleotides typically consist of a single strand of 20–100 bases and the peptides are typically small. Aptamers are made in vitro by combinatorial selection from a large sequence pool based on their capability to recognize a target (a selection methodology indicated as SELEX is typically utilized) [37]. Aptamers bind to this target through various noncovalent interactions such as electrostatic interactions, hydrophobic interactions, and induced fitting. Similar to antibodies, the aptamers can be grafted on a solid support.

The retention of the analytes on affinity, immunoaffinity, and aptamers grafted on solid supports is performed using specific conditions regarding pH, salinity, temperature variation, and mixtures of solvents specific for retention of the analyte. Recovery is further performed by changing those conditions, or in some cases

using denaturing agents such as urea or guanidine hydrochloride for antibodies, complexing agents such as EDTA for aptamers sensitive to cations, or even digestion using trypsin for target proteins. The applicability of affinity, immunoaffinity, and aptamer sorbents for numerous bioanalytical purposes is very common [38]. A detailed discussion on the subject of affinity materials for bioanalytical purposes is beyond the purpose of this book.

Other types of affinity chromatography

Affinity and immunoaffinity chromatography can be utilized in a variety of forms which led to new chromatography types such as pseudoaffinity or dye-ligand affinity chromatography [39]. Some synthetic dyes are capably to "stain" proteins by forming with them stable bonds. Such dyes can be attached to a solid support such as sepharose and form a stationary phase. This phase can be applied for protein purification. The procedure allows the separation of a range of proteins with similar active sites that bind to the dye which is designed to mimic substrates or cofactors binding to the active sites of proteins. Besides proteins, some other molecules of biological origin can be separated by this procedure [40].

16.3 Biomimetic liquid chromatography

General comments

Biomimetics, also known as biomimicry, is defined as the imitation of biological processes or models from nature aiming to solve various complex biological problems such as the drug delivery in biomedical applications, characterization of nanostructurated biohybrid materials, etc. [41]. Such processes and materials can be studied with the help of biomimetic liquid chromatography. This technique, *also known as* immobilized artificial membrane chromatography, is using *biomimicking* stationary *phases with multiple functionalities to imitate biological processes.* Biomimetic chromatography involves noncovalent interactions which are essential in many biological systems [42]. From various experimental designs of biomimetic LC process, a series of biological processes can be predicted. Examples of such biological processes studied indirectly by biomimetic LC are cell membrane distribution, protein–lipid interactions, blood–brain barrier penetration, skin permeability, and assessment of absorption, distribution, metabolism, and excretion [43–47].

Some HPLC techniques that rely on stationary phases containing biocomponents such as affinity HPLC, chiral HPLC based on glycopeptides or proteins, or size exclusion chromatography (SEC) using natural materials as stationary phase are not included in the category of biomimetic HPLC. Biomimetic HPLC is based mainly on stationary phases that mimic environment of a lipid membrane and they can be used in the assessment of interactions between molecules and biological membranes or for the estimation of permeability through cell membranes. The best model mimicking these natural processes is the liposomes. The distribution/permittivity from outside to inside the liposome could be an image of the same process taking place in cells.

Liposomes are artificially prepared vesicles consisting of natural and synthetic phospholipids that are widely used as a cell membrane mimicking platform in order to study various biochemical processes [48,49]. The property of liposomes as nanocarriers for drug delivery is based on encapsulating in their core the hydrophilic compounds and in their membrane the hydrophobic moiety containing molecules. Examples of compounds delivered by liposomes to the target tissue are antiviral, antifungal, antimicrobial, vaccines, antitubercular drugs, and gene therapeutics. Liposomes are characterized with respect to physical, chemical, and biological parameters [50] and their size as one of the main physical parameters is usually determined by

conventional SEC [51] or by sequential extrusion at relatively low pressure through a polycarbonate membrane [48].

Stationary phases used for biomimetic LC

There are two possibilities of covering an HPLC support with a phospholipid environment in order to obtain biomimetic stationary phases. A first possibility is to bind physically a proper ligand to the surface of an RP stationary phase (mainly C18), by its strong adsorption on its surface. Another possibility is to chemically modify a silica surface with the ligand resulting a bonded stationary phase containing a phospholipid moiety [52].

The stationary phases of octadecylsilica (ODS) used in RP-HPLC can be coated with ligands, such as phosphatidylcholine (lecithin) or acylcarnitine, in order to obtain half of the structure of a double-layer liposome based on these building blocks [53,54]. This is possible due to the many weak van de Waals interactions between the hydrophobic chains of phosphatidylcholine or acylcarnitine which result in a strong interaction between ligand and stationary phase. The result of this strong interaction is a stable structure of the resulted stationary phase surface. A schematic view of the resulted association between a C18 stationary phase and a zwitterionic phospholipid ligand (distearophosphatidylcholine) is shown in Fig. 16.3.1.

This surface imitates half of the layer of liposome, and the analyte molecule that is supposed to have both hydrophobic and hydrophilic moiety can interact with one or both of the zones from this coating. Similar surfaces can be obtained by derivatization of silica supports by introducing a phospholipid moiety resulting a biomimetic stationary phase, also known as immobilized artificial membranes (IAMs). Other types of IAMs contain human serum albumin (HAS) or α-acid glycoprotein (AGP), which are chemically bonded on silica skeleton. A general route for obtaining IAM surface based on silica includes three main steps: (1) the activation of terminal carboxyl group of phosholipide with carbonyldiimidazole; (2) the reaction of activated ligand with aminopropylsilicagel; and (3) end-capping of the surface with long and short anhydrides [55]. An example of IAM containing a phosphatidylcholine moiety bonded on aminopropylsilica support through a derivatization reaction is given in Fig. 16.3.2 [56]. Similarly, sphingomyelin biomimetic stationary phase for IAM chromatography can be obtained by derivatization of aminopropylsilica support [57] as shown in Fig. 16.3.2.

Various IAM columns manufactured by Regis Technologies Inc. (Morton Grove, IL, USA) are commercially available, such as IAM.PC, IAM.MG, and IAM.DD2 [58]. They can be used for measuring the retention time of analytes of interest, under isocratic or gradient elution,

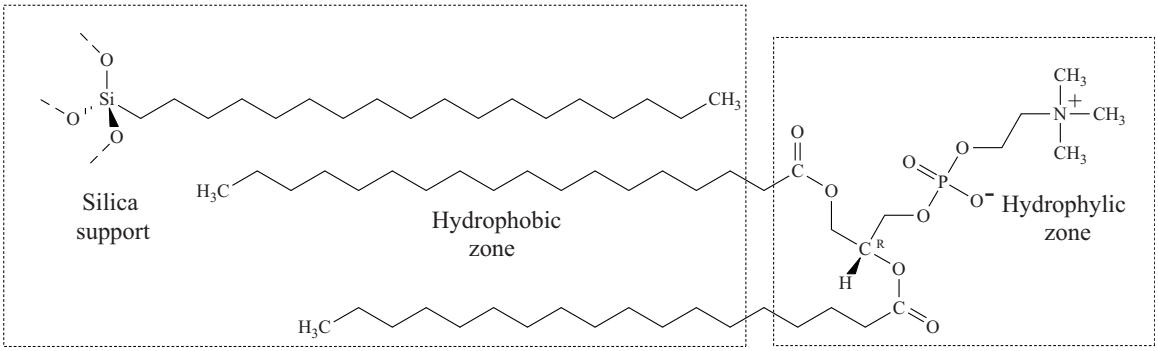

FIGURE 16.3.1 The principle of physical coating an RP stationary phase with phospholipid ligands.

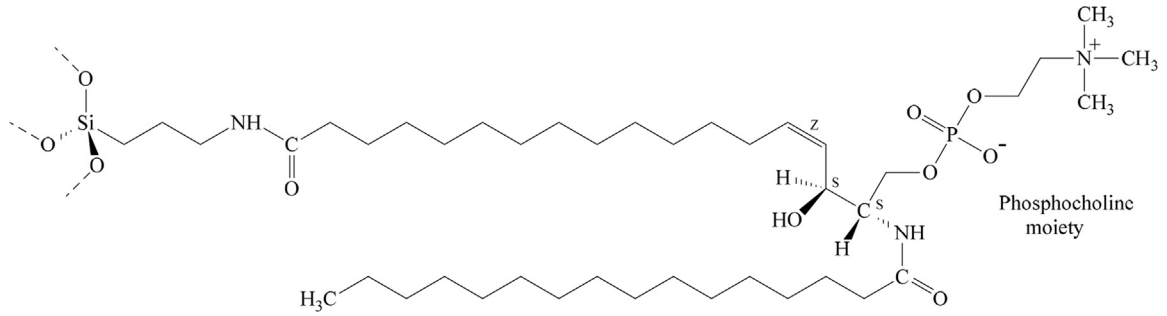

FIGURE 16.3.2 Chemical structure of immobilized artificial membrane containing a phosphatidylcholine region ((N-hexadecanoyl)-sphing-4-enine-1-phosphocholine).

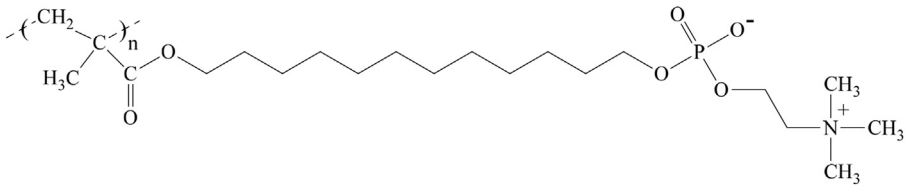

FIGURE 16.3.3 Chemical structure of monolith synthetized from dodecyl ester of methacrylic acid, with phosphocholine as terminal functionality.

using aqueous buffer at pH 7.4 or pH 6.5. For strongly retained compounds, the mobile phase can contain acetonitrile up to 70% (v/v), as organic component [59]. Other additives in the mobile phase can be salts or even detergents [60].

Biomimetic stationary phases containing phosphatidylcholine moiety can also be obtained by monolithic technology [61]. The monomers with phosphocoline moiety can be synthetized [62] and further they can be polymerized to obtained monolithic structures with a biomimetic surface. An example of monolithic structure with the methacrylate ester as starting monomer is presented in Fig. 16.3.3 [63].

The main problem with using biomimetic stationary phase is related to the low chemical and mechanical stability for their long-term use. These phases are easily affected by exposure to organic solvents or surfactants. A trend to alleviate these problems is the use of lipids containing polymerizable moieties incorporated into the hydrophilic or hydrophobic region of the lipid (acryloyl, methacryloyl, styryl, dienoyl, diacetylenyl, sorbyl, lipoyl) [64].

Retention in immobilized artificial membrane LC

The partition process of analytes between aqueous mobile phase and phospholipid based stationary phase is very similar to the partition to a liposome/water system. Liposome/water partition is characterized by the corresponding partition constant, denoted by $K_{L,w}$. In the liposome system, partitioning of the ionized species is influenced by electrostatic interaction with the membranes [65]. In many cases, the liposome–water partition constant is a more suitable molecular descriptor for the uptake of hydrophobic dissociable compounds into biological membranes than the corresponding octanol–water partition constant [66].

The partition equilibrium of the two processes, the one using a phospholipid-based stationary phase and the partition in liposome/water system, could be well characterized by $K_{L,w}$. This partition constant depends on pH, ionic strength, and compound concentration. Eq. 4.1.22 applied to the retention factor measured with IAM stationary phase $(k'_{biomim,w})$ can be related to the liposome/water partition by the following formula:

$$\log k'_{biomim,w} = \log K_{L,w} + \log \Psi_{IAM} \qquad (16.3.1)$$

The retention factor $k'_{biomim,w}$ can be measured experimentally for water as mobile phase or in case an organic solvent is used in the mobile phase from extrapolation to $\phi = 0$ of organic solvent content.

When experimental values for $\log k'_{biomim,w}$ are acquired, the value of $\log K_{L,w}$ can be obtained if Ψ_{IAM} is known. However, the values of Ψ_{IAM} are difficult to find [56,67]. The values of K_{Lw} are more realistic estimates of the membrane distribution of a compound than the values of octanol/water partition constant (K_{ow}), used in majority of models [68,69]. However, for many compounds, the two partition constants, K_{Lw} and K_{ow}, are well correlated [70].

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CHAPTER

17

Mixed-mode HPLC

17.1 Stationary phases with more than one type of active groups

General comments

Separation in most common types of HPLC is dominated by few main types of interactions, such as hydrophobic in RP-HPLC, polar in HILIC, ionic in IC, etc. However, even in these cases, the interactions between the analyte, stationary phase, and mobile phase involve a mixture of other interactions. For example, RP-HPLC is also associated with some polar contributions, HILIC is associated with some hydrophobic interactions, etc. A proof of mixed mechanisms is, for example, the nonlinear or even the U-shape dependence of retention factor on the mobile phase composition that can be interpreted as a switch of the retention mechanism involving different interactions between the analytes and stationary phase under the influence of the mobile phase composition [1]. A mixed mechanism is also encountered in case of SEC HPLC, which separates analytes by hydrodynamic volume but cannot eliminate some hydrophobic interactions with the stationary phase [2]. The so-called *mixed-mode* HPLC is related to special stationary phases having in their compositions at least two different functionalities which can be involved in different main interactions with the analytes.

The interactions additional to the main one play an important role in some separations. Therefore, it was an expected result that chromatographic column with intentional mixed functionalities in which the main interactions consist of two (or more) types of active groups was manufactured [3]. These columns are indicated as *mixed mode* [4,5]. Synthesis of such phases can be based on silica with specific two or three bonded phases or can be based on organic polymers (see Section 8.1). The stationary phase in mixed mode HPLC can be made by several procedures such as: 1) mixing two types of stationary phase particles into a single column, 2) bonding on a stationary phase support different types of ligands, 3) using embedded polar or ionic groups in a hydrophobic chain used for the derivatization of the solid support, 4) using for the derivatization of the solid support hydrophobic chains terminated (tipped) with polar or ionic groups. Homogeneity of the mixed mode stationary phase can be an issue for type 1 and 2 of these phases. Different fragment attachments to the silica surface to synthetize bonded phases with mixed-mode character were schematically presented in Fig. 8.1.10.

According to the types of interactions combined, the main mixed-mode stationary phases can be classified into three different groups: (1) reversed-phase/hydrophilic interaction (RP-HILIC), (2) reversed-phase/ion-exchange

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(RP-IC), and (3) hydrophilic interaction/ion-exchange (HILIC-IC). Besides these double mode mechanisms, there is the possibility of combining three different mechanisms into one HPLC column, the result of such combinations being, for example, an RP-HILIC-IC column [6]. However, the three-mode mechanism requires a much more complex procedure for synthesis of stationary phase [7,8]. Trimodal HPLC can also be the combination of RP with two IC mechanisms, one for anion exchange, and another for cation exchange [9,10].

Stationary phases with mixed mode including RP and HILIC capability

The mixed-mode stationary phases can be silica-based, polymer-based, hybrid between silica with polymers and monolithic [11]. They may have two distinct zones, one with hydrophobic character and the other with polar characteristic bound to a silica or polymeric support. An example of stationary phase was presented in Figure 11.2.3, based on a long hydrocarbon spacer and a tertiary amine linked to an amide groups as polar groups. Stationary phases with a silica backbone and mixed-mode capability containing hydrophobic groups (C8 or C18) and polar groups such as NH_2 or CN are usually synthesized directly by starting with appropriate reagents. For example, octyl and CN groups can be obtained by controlled hydrolysis of a mixture of a mixture of tetramethoxysilane (TMOS), cyanopropyltrimethoxysilane, and octyltrimethoxysilane [12]. Other possibilities of preparation of mixed-mode stationary phases have the starting step a derivatization of silicagel with 3-aminopropyltrimethoxysilane, 3-mercaptopropyl-trimethoxysilane, or 3-chloropropyltrimethoxysilane, followed by the reaction of terminal functionality with different organic reagents. Examples of such mixed-mode stationary phases are silica-based modified with peptide, poly-L-lactic acid, imidazolium-embedded C8, C18-diol, etc. [13].

Polymers based on poly(styrene-divinylbenzene), poly(glycidyl methacrylate-*co*-divinylbenzene), or poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) containing different attached groups can also be used, for example, for a phase providing hydrophobic and hydrophilic interactions [14–16]. Covalent organic frameworks can also be used as mixed-mode stationary phases [17].

Stationary phases with mixed mode including ion exchange capability

Some ionic exchange phases have ionizable groups and specific hydrophobic moieties that allow the phase to work in mixed mode, both reversed phase and ion exchange [18]. For example, Acclaim Mixed-Mode WCX-1 columns are packed with a silica-based stationary phase that incorporates both hydrophobic and weak cation exchange (WCX) properties. Unlike conventional RP material, the packing has a hydrophobic alkyl chain with a carboxylate terminus and offers the potential for a wide range of applications, depending on the pH of the mobile phase [19]. The Acclaim Mixed-Mode WCX-1 column offers multiple retention mechanisms, including RPLC, cation exchange, ion exclusion, and HILIC. These features make this column versatile in many applications that require different types of selectivity. For even further increase in selectivity necessary for the separation of oligonucleotides, mixed-mode columns were utilized in a multiple heart-cutting approach for bidimensional HPLC [20]. A mixed-mode type column is also Intrada organic acids (Imtakt USA), which is a proprietary multimode column optimized for the analysis of a variety of organic acids. In the same family of columns is Intrada amino acid that allows the separation of 55 amino acids within 10 min run using gradient mobile phase and MS/MS detection. The

column is indicated as normal phase ligand + ion exchange.

Columns containing a stationary phase that have both hydrophobic and weak anion exchange (WAX) properties are also known. For example, Acclaim Mixed-Mode WAX-1 columns are packed with a silica-based stationary phase that incorporates both hydrophobic and WAX groups. Retention of basic, neutral, and acidic molecules can be either independently or concurrently adjusted by changing ionic strength, pH, and organic solvents content in the mobile phase. In these columns, the presence of anion exchange functionality controls through electrostatic interactions the attraction of anions and repulsion of cations, while the alkyl chain controls the separations by hydrophobic interactions [21].

Some phases have HILIC and ion exchange capability. Such a phase, with HILIC and WAX capability, has been used for the analysis of surfactants [22].

In some phases, mixed mode refers to two or even three types of IC interactions. For example, a column providing strong cationic and zwitterionic type interactions has been reported to be used for the analysis of biogenic amines. The silica-based stationary phase contains a C5 linker an imbedded group used to create zwitterion ion exchange capability, a C4 spacer, and a strong terminal cation exchanger SO_3^- group [23]. A stationary phase containing zwitterionic, strong cation, and weak anion groups was also evaluated [24].

A special class of mixed-mode stationary phase contains ionic liquids bonded on silica surface as exemplified by the structures from Fig. 17.1.1.

The OmniPac PAX- and PCX-100 and -500 are latex-based columns having both hydrophobic and ion exchange character. The PAX anion exchange capacity is about 40 μeq per column and the PCX cation exchange capacity is approximately 120 μeq per column. The columns are 100% solvent compatible and allow the separation of inorganic and organic anions, having acid and base compatibility over 0−14 pH range. Latex coated on bare silica monolith confers its surface a dual mode mechanism (HILIC-IC) [25].

More complicated structures for mixed-mode stationary phases were also reported in the literature. For example, a reversed-phase/hydrophilic interaction/ion exchange (RPLC/HILIC/IEC) and chiral recognition mixed-mode stationary phase material was synthesized using 3-n-octadecyl-1-vinylimidazolium bromide and 6-(1-allylimidazolium)-cyclodextrin tosylate monomers used to functionalize β-cyclodextrin [26].

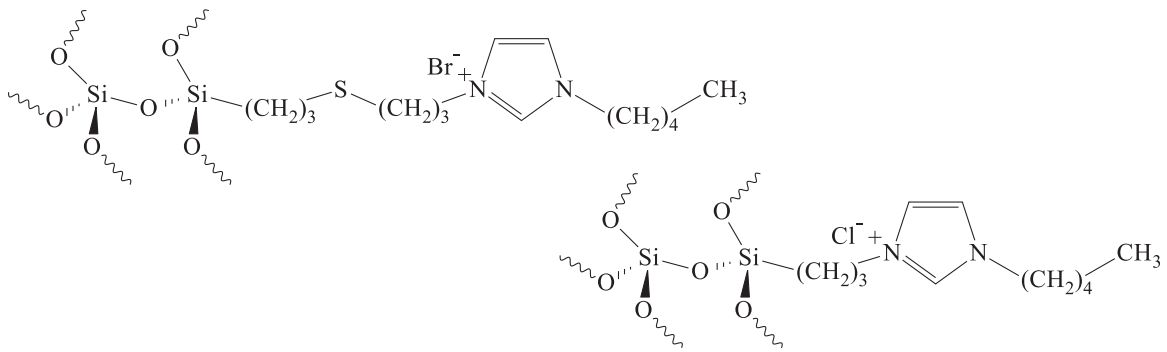

FIGURE 17.1.1 Examples of ionic liquid moiety bonded on silica.

17.2 Mobile phase in mixed-mode HPLC

General comments

Mixed-mode HPLC is applicable to samples containing a large variety of analytes, with different physico-chemical characteristics. For example, RP/HILIC mode can separate polar and hydrophobic analytes in a single analysis, while RP/IC can be applied to analyze samples with analytes having hydrophobic character and analytes with dissociable groups. The mobile phase in mixed-mode chromatography is selected based on the type of interactions offered by the stationary phase. For different types of mixed-mode HPLC, the separation selectivity can be modulated by the control of mobile phase composition (the nature and content of the organic component, pH, or ionic strength). The elimination of ion-pairing agents in the mobile phase as choice alternative for separating highly hydrophilic or charged analytes by IP-HPLC is an advantage of mixed-mode HPLC, due to the simplification of the composition of mobile phase and a better compatibility with MS detection [27].

For RP/HILIC mixed mode, the effect of acetonitrile is opposite to the two mixed mechanisms, as described by Eqs. 9.5.2 and 11.3.1. At low content of acetonitrile in the mobile phase composition, the hydrophobic effect is predominant and the hydrophobic analytes will have high retention due to their interactions with hydrophobic moiety of stationary phase. On the contrary, at high concentration of acetonitrile in mobile phase, the hydrophobic effect is diminished, while the hydrophilic interactions between polar groups of analytes and the polar moiety of stationary phase are favored, and thus the polar analytes have high retention. Therefore, the mobile phase composition influences the order of elution as well as the separation selectivity between analytes. This can also be observed for mixed-mode hydrophilic and ionic interactions with a strong cation exchange column, for example, used for the separation of peptides. For higher acetonitrile concentrations (70%–90%, v/v), the hydrophilic interactions are dominating and changed the column selectivity compared to the separations using 20%–50% acetonitrile, when the peptides elute in the order of their decreasing hydrophobicity [28].

The mobile phase pH can be used to control retention and selectivity, by influencing the dissociation degree of acid–base analytes. This parameter may also have influence on the surface charge in case of weak anion or cation exchanging stationary phases. The nature of buffer is typically controlled for MS/MS detection, when formic acid/ammonium formate is recommended. For some columns, a more complicated mixture of solvents is used as mobile phase. For example, for the amino acids separation on Intrada amino acid column, a mixture of acetonitrile, tetrahydrofuran, aqueous solution of ammonium formate, and formic acid are used as solvent A and acetonitrile with aqueous solution of ammonium formate as solvent B for a gradient elution program. For this separation, the sample solvent is also important and it is recommended to be 0.1 N HCl. Recommendation for using a specific mobile phase is given by manufacturers and can be found on the web (e.g., Ref. [29]).

The ionic strength can be used for modulating the retention in mixed-mode HPLC having HILIC or/and IC retention mechanisms. The effect of suppression and the electrostatic interactions or ion exchanging equilibria by ionic strength is opposite to the RP mechanism, where the increase of ionic strength enhances the hydrophobic effect on analytes. For example, the retention and order of elution for a test injected sample containing butylbenzene and 4-hydroxybenzoic acid on an Acclaim Mixed-mode WAX-1 HPLC column is significantly changed, when phosphate buffer (pH = 6) was changed from 20 to 100 mmol/L [29].

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CHAPTER

18

Utilization of HPLC in chemical analysis

18.1 Steps in development and implementation of an HPLC separation

General comments

Development and implementation of any chemical analysis should start with the collection of general information regarding the planned analysis, as shown in Figure 1.1.1. Staring with the collected information, the task of the analytical chemist is to select details about the analysis itself. Many aspects regarding the preliminary information about the sample can be missing. However, the collection of as much information as possible about a projected chemical analysis is very important. An iterative selection process may take place before achieving a successful analytical procedure. For stationary phase and mainly for mobile phase, several cycles of selection are common. This process of selection must be done within a specified time interval, which can be an important factor for the selection, development, and validation of an HPLC method [1].

Information for starting the development of an HPLC method

The general information regarding the analysis should contain the following: (1) the purpose of analysis, (2) general information about the material to be analyzed, (3) any knowledge about the chemical nature of the analytes and of the matrix of the sample, (4) the required type and quality of the results, (5) published data regarding already developed methods of analysis performed on the same or on similar samples, and (6) information regarding the necessary resources to perform the analysis and their availability in the laboratory.

(1) The purpose of analysis should be the first type of information obtained about an analysis. Sources of information about the purpose can be very diverse, the most common being the direct request from a customer and also should include self-imposed requests. Samples are frequently analyzed for health-related purposes (e.g., medical analyses, analysis of pharmaceuticals, analysis of metabolites), for evaluating environmental issues, for forensic purposes, for exploratory reasons, and for fundamental research. Information must be collected indicating the required type of analysis regarding qualitative, quantitative, semiquantitative, or both qualitative and quantitative.

(2) The general information about the material(s) to be analyzed should cover various aspects of the sample. Information about the sampling process is also very useful, in particular indicating the sample homogeneity, the age of the sample, and potential of contamination. If nonhomogeneous samples

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need to be analyzed, it should be known if the analysis must be performed in a specific way (e.g., after homogenization or selecting parts of the sample). Also, it must be known if the whole sample should be analyzed or only a specific part (surface, soluble component, selected points, etc.) Information about the sample physical state should be collected, indicating if the sample is gas, liquid, solid, solution, semisolid, or mixed phases. Also, knowledge should be collected weather the sample is inorganic, organic, composite, of biological origin, environmental, or of a special source. Other data about the sample should indicate the amount of sample available (large quantity, small quantity, readily available, uniqueness, etc.) and also the value of the sample. In some cases, the sample must be returned to the provider after a small amount has been used for analysis and this should also be known. Other information about the sample should indicate the sample thermal stability and perishability, as well as any safety concerns about the sample. The information about the sample should also indicate if the sample is of a new type, or if similar samples were previously analyzed. General information must specify whether a specific protocol must be followed during the analysis or that no regulations are imposed. Some analyses are required to be nondestructive, and in certain cases, the analysis is done in conjunction with preparative purposes, which also should be known. There are important issues related to analyses associated with preparatory purposes since some analyses are modifying the nature of the sample. If the biological activity of the sample must be preserved, this aspect should also be known.

(3) Preliminary knowledge about the chemical nature of the analytes and of the matrix of the sample includes data about chemical nature of the constituents, range of concentrations of the analytes, the strength of bonding of the analytes to the matrix, etc. General properties include physico-chemical data such as volatility of analytes and matrix, solubility in various solvents, acid–base character, hydrophilic–hydrophobic properties, reactivity, etc. The knowledge about the chemical nature of the analytes is essential. It is preferable to know the list of compounds (single or multiple component analysis) that must be analyzed or at least the class of the analytes (inorganic, organic, functional groups in organic compounds, ionic character, etc.). In case that very little information is available about the sample, preliminary analyses of the sample should be performed, not necessarily using HPLC. For example, a GC/MS analysis (with or without derivatization) may provide some valuable qualitative information.

(4) The required quality of the results and the future utilization of the results must satisfy specific requirement regarding accuracy, precision, and the number of samples characterized. Based on this request, important aspects of the planned analysis are selected such as the detection type, the sensitivity of the utilized detectors, etc. Also, it is important to know if the results are part of a larger study, if they can be compared with the results from other laboratories or with results on standard materials.

(5) The published data regarding already developed methods of analysis performed on the same or on similar samples are important for the selection and/or development of a method of analysis and contribute to the success of a chemical analysis. Such information may shorten significantly the time to have a method available for analysis and provides important guidance even in the development of a new method.

(6) The information regarding the necessary resources to perform the analysis and their availability in the laboratory determines which method should be selected or developed, and which materials and/or instruments must be purchased. This information indicates, for example, the need for preliminary purchases of

chromatographic columns, reagents, solvents, and standards. Related to the safety concerns, appropriate laboratory conditions must also be assured for the analysis of certain samples.

Selections to be made for an HPLC analysis

Several selections are required in the planning for an HPLC analysis. These include the following: (1) is HPLC an appropriate method for the analysis, (2) is there a need for sample preparation, (3) which is the appropriate type of HPLC, (4) which type of HPLC instrumentation should be used, (5) what is an appropriate analytical column for the analysis, (6) what is an appropriate mobile phase for the analysis, and (7) what detection type should be selected for the analysis.

Before deciding that HPLC is an appropriate technique for the sample analysis, all the available information must be evaluated. In some cases, better alternatives are possible, such as in the case of request for qualitative information when GC/MS may be applicable if the analytes are volatile enough or can be derivatized into volatile compounds. In some cases, simpler analyses such as a spectrophotometric measurement may provide sufficient information regarding the sample. Only after deciding that HPLC is the most appropriate method for analysis, the following steps should be followed.

Sample preparation is a common step in many analyses. This step is necessary for making a sample amenable for analysis, or to improve the performance of analysis, and it is always performed linked to a prospective core analytical procedure. Sample preparation is presented in many peer-reviewed papers, books, and on information from the web (e.g., Ref. [2]). Decision of the need of sample preparation is a topic frequently included in the analytical methods description.

After the decision of using an HPLC method as a core analytical procedure, and applying the necessary sample preparation to the sample, more details should be selected regarding the HPLC analysis itself. The choice of the HPLC type is a very important step for a successful analysis. The selection is made considering various sample properties, analysis requirements, instrumentation availability, etc. A preliminary scheme related to the choice of type of HPLC is shown in Fig. 18.1.1. One first parameter to consider is the expected range of molecular weight of the analytes. Small molecules (e.g., with $Mw < 5000$ Da) and large molecules (polymers with $Mw > 5000$) are treated differently. Another important parameter regarding the sample is its solubility. Samples soluble in water or water/organic–polar solvents are treated differently from samples soluble only in organic–nonpolar solvents. Another sample characteristic is the ionic or nonionic character. This type of evaluation of sample properties is also indicated in Fig. 18.1.1. Using as guidance the description of the sample given in Fig. 18.1.1, a preliminary selection of HPLC type appropriate for the analysis is also suggested in the figure.

Also, the scheme is very simplistic, since specific techniques can be used for other types of molecules than those suggested in Fig. 18.1.1. For example, RP-HPLC can be used successfully for protein analysis, and GPC can be used, if necessary for the separation of small molecules from large molecules. Several comments are further made regarding the choice of HPLC type.

(a) Organic nonpolar small molecules that are not soluble in water and possibly not soluble in organic polar solvents can be analyzed using normal phase chromatography (NPC) or NARP. NPC and nonaqueous reversed-phase chromatography (NARP) use solvent that can dissolve molecules such as carotenoids, triglycerides, sterols, etc., and molecular

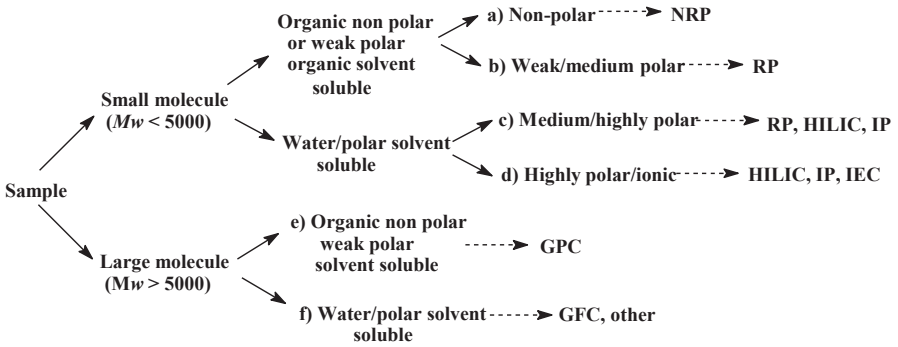

FIGURE 18.1.1 Diagram showing the sample properties useful for the preliminary selection of HPLC type of analysis.

solubility as well as types of interactions with the stationary phase are proper for the separation of this type of molecules.

(b) Small molecules having some polarity and soluble in organic solvents (polar or nonpolar) represent a very large class of compounds. This class includes pharmaceuticals, biological small molecules, compounds to be analyzed in food and beverages, in the environment, and in agricultural products. The main type of chromatography applied for the analysis of these compounds is RP-HPLC. This technique is extremely versatile and a large number of columns and combinations of mobile phases were developed for RP-HPLC applications. In case the separation of enantiomers is necessary for this group of analytes, chiral chromatography must be applied.

(c) Small molecules soluble in water or polar solvents that have polar groups but are not ionic also represent a very large class of compounds. This class also includes many pharmaceuticals, biological small molecules, compounds to be analyzed in food and beverages, in the environment, in agricultural products, etc. RP-HPLC is frequently used for the separation of these types of molecules, when some part of their structure contains hydrophobic moieties. For molecules with numerous polar groups such as amino acids or carbohydrates, HILIC chromatography can be used for the analysis. Also, in some cases, these types of molecules may be analyzed using ion pair (IP) chromatography by adding an ion pairing agent (IPA) in the mobile phase and using RP-HPLC type columns.

(d) Small ionic molecules that are water soluble can be analyzed by different types of HPLC. One type is ion chromatography, either cation exchange or anion exchange chromatography, depending on the particular type of ion. Highly polar or ionic molecules can also be analyzed using IP chromatography.

(e) Organic soluble large molecules are frequently separated for differentiation based on their molecular weight. This can be achieved using gel permeation chromatography. GPC is also used when the molecular weight of a polymeric material must be evaluated.

(f) In the type labeled as large molecules that are water soluble can be included a wide variety of compounds such as polymeric carbohydrates, proteins of different types, certain synthetic polymers, etc. Depending on the purpose of analysis and only if a separation based on molecular weight is necessary, the HPLC of choice will be gel filtration SEC. Other techniques can be used depending on sample properties, such as RP-HPLC, HIC (hydrophobic interaction chromatography), displacement, or bioaffinity chromatography.

One important criterion for selecting the type of HPLC is based on the polar or nonpolar (hydrophobic) character of the analyte. This character can be estimated using octanol/water partition constant log K_{ow} or coefficient log D_{ow} for the analyte. As described in Section 6.1, for neutral molecules, log $D_{ow} = \log K_{ow}$, but for molecules that can be present in ionic form, the molecules will have different structures depending on pH, and different log D_{ow}. In such cases, log D_{ow} at isoelectric point must be used instead of log K_{ow}. Fig. 18.1.2 suggests different chromatographic types to be selected depending on log K_{ow} of the analyte.

The simple scheme shown in Fig. 18.1.2 is able to capture only a few aspects of HPLC type selection. For example, the scheme shows that more than one technique can be applied for a specific class of molecules with a specific polarity [3]. Also, samples may contain complex mixtures of molecules, which may have a wide range of log K_{ow} values. In such cases, one alternative is to analyze one group of solutes by one technique, and another group by a different technique. The use of a more versatile column with a wide range of polarity of solvents is another alternative. Multimode separations also can be applied in cases of very different analytes.

Based on the selected HPLC type, further choices are made including column selection, mobile phase selection, detection type, quantitation method, etc. Detailed information regarding those choices has been described in Part 2 of this book.

Comments on the implementation of a method from the literature

Scientific literature, including various types of publications (articles in peer-reviewed journals, books, company catalogs and application notes, web information), contains the description of a large number of HPLC analytical methods for almost every possible analyte. For this reason, one convenient way for having a desired HPLC method of analysis is the use of one already described in the literature. The method from literature should describe the analysis of the same analytes and with a matrix close to the one of the samples of interest. Most analytical methods are developed with the capability to perform the analysis on samples where the matrix is not identical from sample to sample but not completely different. For this reason, the robustness of the analytical method to matrix variations is an important element to consider

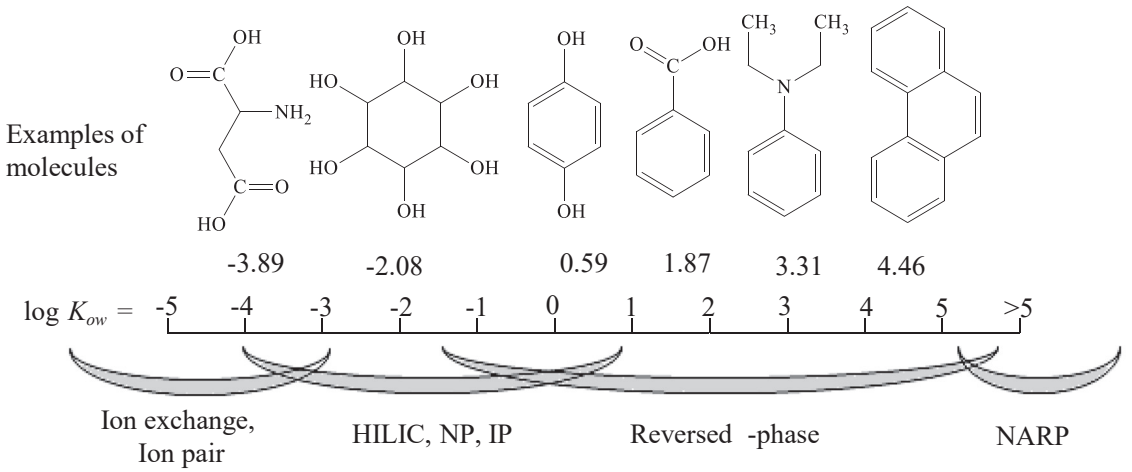

FIGURE 18.1.2 Different HPLC types preferentially utilized depending on log K_{ow} of the analyte molecule.

when attempting to implement a method from the literature. The implementation of such a method may encounter several problems: (1) differences in the matrix between the method from literature and the necessary method, (2) differences in the level of analytes, (3) differences in the required analysis time, and (4) differences between the recommended instrumentation and the available instrumentation.

(1) One potential problem in implementing a method from the literature is related to the differences between the matrix on which the method from the literature has been applied and the matrix of interest. The matrix differences may be not significant, or they can be critical for the method utilization. Sample preparation (different from the one from the literature) can be used in some cases for simplifying the matrix and possibly for increasing the content of the analyte in a processed sample. Potential matrix interference must be thoroughly verified and eliminated for the implemented method. In case that the utilization of the method from literature does not work with a particular sample matrix, modifications of the method are first recommended or addition of a sample preparation step, and only if these changes are not sufficient, a radically new method should be utilized.
(2) Another problem can be related to the content of the analyte in the samples of interest. When the content of the analytes in the samples of interest is too low for the recommended detection, the possibilities of changes to a more sensitive detector should be first evaluated. Also, the addition of a sample concentration step may be utilized.
(3) Some methods recommended in the literature may indicate a longer analysis time than acceptable, for example, for the analysis of a large number of samples. In such cases, modifications of the recommended method can be applied, such as using a shorter chromatographic column (if the separation is still acceptable), increase in the flow rate in the HPLC, or attempt to use UPLC instead of HPLC methodology (see Chapter 8).
(4) Other problems with the implementation of the method from the literature can be related to the availability of similar instrumentation, or the qualification of the similar HPLC system (which is assumed to be periodically checked). The replacement of pumping system from high pressure mixing to low pressure mixing (see Section 2.1) may generate changes in peak retention times or even in the resolution of certain peaks in the chromatograms. Changes of the chromatographic column may also be sometimes necessary. Columns of the recommended type but with different dimensions can be utilized in some instances, but the change in the retention time of the analytes, loading capacity of the column, and backpressure changes must be first evaluated. When a replacement with an equivalent but not identical column is intended, the selection of a new column should be made, for example, by using the PQRI approach (see Eq. 9.3.11) [4], which can indicate which column is expected to provide a similar separation.

The replacement of one type of detection with a different type, although some time possible, is not in general straightforward. For example, a change to RI detection requires the use of isocratic separations, which is not necessary for other detection types. The replacement of a more sensitive detector with a less sensitive detector may cause problems with the LOQ value for the analytes. The change from a less sensitive detector to a more sensitive detector also may pose problems. For example, the change from UV detection to MS detection (that may provide better sensitivity and selectivity) must assure that the mobile phase utilized with the UV detection is adequate for MS detection (all mobile phase components must be volatile for MS

use). Even when changing, for example, from MS detection to UV detection, it must be assured that the UV cut-off of the mobile phase is acceptable.

Improvement of a method from the literature

The need for better methods of analysis is common due to new demands in the quality of data and the requirements to analyze a diversity of new types of samples. Also, the HPLC instrumentation, the column quality, and detector performances are continuously improving. On the other hand, a large number of very good methods of analysis by HPLC type techniques are available in the literature. However, these methods may have not been developed using modern HPLC capabilities, or they may have aspects that can be modified for achieving better selectivity, sensitivity, or shorter run time. The need to apply a method from the literature to a different type of matrix or to add more analytes for quantitation is also common. Many older HPLC methods can be modified and improved to include new requirements and/or new capabilities. The main places where improvement can be made are the following: (1) the injection, (2) the HPLC column with the possibility to move the whole method from HPLC type to UPLC type, (3) the mobile phase, and (4) the detection.

(1) The changes regarding the injection may affect the injection volume, the injection solvent, or both. Smaller injection volumes may lead to better separation, and when moving from HPLC to UPLC, such changes are usually recommended. Lower amount of analyte in the HPLC system may lead to lower sensitivity of the method, which can be compensated, if necessary, by improving the detector sensitivity. An increase in sample volume (placing a larger amount of analyte in the system) may be useful in some analyses where a better sensitivity is required. Larger volumes of injected sample may reduce resolution, and in some cases, a modification of the initial mobile phase composition to a weaker eluent may help to achieve sample focusing at the head of the chromatographic column. Special injection techniques may also allow the injection of larger samples.

(2) The same types of columns that were previously available with 5 μm diameter particles (or larger) are now available in formats with smaller particles, in core-shell type particles, or as monolithic columns. The new columns offer better efficiency, and for monolithic columns lower backpressure. The new columns having smaller particles may produce higher backpressure and the shift from HPLC to UPLC must be considered if the instrumentation is available. Such change will offer better separation with more accurate capability for the integration of peaks (see Section 8.2) and much shorter retention times. The change of the column can be done not only regarding the column format, but also regarding the column type. Such change must consider an equivalent column that produces basically the same kind of separation, but with better performance (better resolution, better peak shape, longer column life). Column equivalence can be evaluated using PQRI approach, or can be recommended in vendor catalogs (see, e.g., Phenomenex, Chromatography Product guide 2016/2017 [5], Waters Chromatography Columns and Supplies Catalog [6], MAC-MOD resources [7], Thermo Scientific HPLC [8], Restek HPLC Column Selection [9]).

(3) Changes in the mobile phase are a useful procedure for improving a reported HPLC method. These changes may involve the following: (a) the modification of the percent composition of the mobile phase using the same

solvents as recommended in the initial method, (b) changes of gradient, and (c) changes in the chemical composition including different solvents and additives. The modification of percent composition of mobile phase may improve separation, may provide focusing at the column head of the analytes, and may reduce the run time for the analysis. The improvement of the separation and reduction of the run time can also be achieved by changing the gradient. The changes in the nature of the solvents used for making the mobile phase can bring more significant modifications of the chromatographic results. The effect of such change must be well evaluated before implementation, since it may affect not only the separation but also the detection. For example, the change of acetonitrile with methanol (or the other way around) can affect not only the separation, but also the sensitivity in the ionization of the analytes when MS is used for detection.

(4) Changes in the detection type are sometimes necessary for enhancing selectivity or sensitivity. For example, the change from RI to UV detection may improve sensitivity and selectivity. Although some analytes do not have strong chromophores, the detection in UV may still be possible at low wavelength. However, the UV cut-off of the solvent must be verified to be significantly below the setup detection UV wavelength, which is not a requirement for the RI detection. The increased availability of MS detectors and their excellent selectivity and sensitivity provides a good reason to change older methods with different types of detection to MS detection (MS or MS/MS). The change to MS detection may require a number of other changes, in particular to the composition of the mobile phase, which must be completely volatile. With MS detection, common buffers used in HPLC such as KH_2PO_4/K_2HPO_4 cannot be used. Buffers such as $HCOOH/HCOONH_4$ should replace nonvolatile buffers, and this may pose problems regarding the adjustment of the same pH of the mobile phase and the same ionic strength. Also, the use of MS detection may require a reduction in the mobile phase flow rate. This may cause the need to change the larger columns having high k' values for the analytes to smaller columns with lower k' values.

Development of a new HPLC method

The development of an entirely new HPLC analytical method is sometimes necessary. Besides the requirement to analyze new types of samples, the trend of reducing the sample preparation step requires the development of better HPLC analyses. This book provides detailed information regarding multiple choices available for developing an HPLC method. The use of the available literature for guidance is the first necessary step for the development of a new method. If possible, a set of initial experiments are recommended, to check the chromatographic response to some possible experimental parameters that will affect the results. Individual experience, trial and error experiments, and exchange of information on the web (e.g., using Chromacademy [10]) improve the results in developing a new HPLC method. The computer programs assisting in method development can also be useful (e.g., Drylab [11]).

Depending on the purpose of HPLC analysis, the method development is performed in different ways. For qualitative analysis, the process of identifying the analytes is less structured. HPLC has some capabilities for the identification of unknowns, but the identification of unknown compounds can be a challenging task. A separation of the analytes is necessary before detection, and this can be done by HPLC either with the intention of identifying the presence of certain expected compounds or just for screening and the detection of unknowns. In case of screening, the separation should provide differences in the retention times for compounds in a wide range of polarities. When searching for the presence

of specific compounds, standard mixtures of these compounds should be used first for assuring that they are well separated and their retention times (retention factors) can be used for identification. UV detection with the generation of a full spectrum can sometimes be useful for compound characterization, but mass spectrometric detection techniques (MS) and in particular tandem mass spectrometry (MS/MS) may offer more information about the compound nature.

HPLC is mainly used for quantitative analysis, and several steps can be described for a common method development geared toward quantitation: (1) preliminary experiments, (2) establishment of an acceptable separation of the analytes, (3) utilization of the unrefined method on a mixture of pure analytes, (4) first round of the evaluation of separation (good resolution, low peak asymmetry), (5) application of the method on a set of calibration standards, and (6) validation. The process can be iterative as a whole or only for particular steps.

Method optimization

Each newly implemented method, either adopted from the literature or entirely new, is subject to a process of various modifications with the goal of improvement. The extent of this improvement process can range from very limited modifications to a full optimization process. The need for a full optimization should always be critically evaluated, since the process may be time consuming and expensive and produce only minor improvements. On the other hand, the improvement process should continue until the method becomes entirely adequate for the goal of the analysis. The method optimization is related to the sample preparation and they influence each other, depending on how much the complexity of the sample is maintained.

The improvement process, frequently indicated as optimization (even without reaching a fully optimized method), consists of a number of trials with changes at various parts of the method or even of the whole method. This process may need to be repeated several times. The optimization of the chromatographic separation is an important part of improving a method, but other parts of the method may be subjected to changes with the goal of improvement. Such parts may include the injection volume, the detector settings, and the length of the chromatographic run.

The improvement process can be done by trial and error, or with multivariate statistical techniques. In a systematic optimization process, it is important to clearly establish the controlled variables (explanatory or independent) and the response variables. The relation between these two sets of variables is indicated in the "response surface" [12]. The statistical techniques utilize a minimum and a maximum value for independent variables, which defines the experimental domain to be investigated during the optimization. Among the designs used to determine response surfaces are the full and fractional factorial designs, central composite design, and Box–Behnken design [13].

The optimization process can be extended to the selection of a calibration type. For establishing a quantitation method, several procedures can be utilized besides simple calibrations with standards. These may include external calibration, internal normalization calibration, matrix-matched calibration, standard addition calibration, and signal to ratio calibration [14]. Most common calibration procedures use a simple calibration with a set of standards. There are different recommendations regarding the number of calibration standards and the number of replicates at each calibration level. IUPAC advises for method validation purposes the use of six or more calibration standards that should be run in triplicate in a randomized way [15]. ISO 8466 indicates for an initial assessment of the calibration performance to employ at least five calibration standards, although it

recommends 10, and 10 replicates of the lowest and highest standards. Method development performed on matrix reference materials is also preferred, but reference materials are not always available. These materials contain the analytes of interest plus the principal chemical compounds found into the matrix to be matched [16].

Method validation

For a new method, but also for a method adapted from literature with or without modifications but new for the laboratory where it will be practiced, a validation process is usually necessary [17]. Whereas the aim of optimization is to find out the set of experimental parameters that allow reaching a maximum or optimum information from the HPLC analysis, its validation is focused on proving that the analytical results are fit for their intended purpose. For the validation of an HPLC method, all of the variables of the method must be considered, including experimental parameters influencing the separation process (e.g., mobile phase composition, temperature, flow rate, elution program when gradient is used), as well as the HPLC detection. Also, the formal validation includes documented evidence indicating that the chromatographic process is performed consistently (column validation, number of injections on the same column, column batch, HPLC validation, etc.). Stability of the injected samples is also part of method validation.

Detailed information regarding validation can be found in a number of publications dedicated to this subject [18–24]. Validation typically requires the characterization of (1) specificity, (2) selectivity, (3) precision, (4) reproducibility, (5) accuracy, (6) linearity of calibrations, (7) linear range, (8) limit of detection (LOD), (9) limit of quantitation, (10) recovery of the analyte(s), (11) robustness, (12) ruggedness, and (13) stability of results. For each method, various levels of validation are necessary, depending on the purpose of analysis and requirements for the quality of results. Variability in the matrix components and verification of robustness of the method to these variations must always be performed for a new method. Because of the interferences that can be generated by the matrix, the quantitation of an analyte, although accurate in samples with one matrix, may generate inaccurate results with a different matrix. This problem may be encountered, for example, when the MS detection is used to compensate for poor separation in the chromatographic column. The MS detection being highly selective does not provide information about the coeluting compounds with the analytes if the detection is not set for that. The good separation in the chromatogram is for this reason always recommended, although the MS detector can differentiate one compound from another without the compounds being chromatographically separated.

The separation process is validated by measuring the precision of the retention time of target analytes (within a session and between sessions of analysis), the evolution of chromatographic resolution, peak efficiency, symmetry, and baseline stability. Detection is validated according to the precision and accuracy of peak area of target analytes, peak purity (when spectrometric detection is applied), linearity or other dependences in detector response, LOD, limit of quantitation, and baseline noise [25]. Injection step is also necessary to be validated, as it influences the detection parameters.

The validation of an analytical method depends on the acceptance criteria, before starting the process, and they should be specified in the protocol attached to this procedure according to the guidelines under whose recommendations the protocol is performed. In those cases, when the acceptance criteria are not fulfilled, an investigation should be performed in order to find out the cause of these differences. When the source of differences is not identified, it would be necessary to evaluate the potential impact the differences may have on the intended study and this

may necessitate further development. For example, in many recommendations, the precision of the retention time measurement as a relative standard deviation is 1% in isocratic elution and 2% for gradient program. The coefficient of determination R^2 for the detector response should be higher than 0.99, and the accuracy for each concentration level used in calibration should be situated between 85.0% and 115.0% of nominal concentration of a standard solution injected in the HPLC system.

18.2 Application of HPLC for quantitative analysis

General comments

The main utilization of HPLC is in quantitative analysis. As previously described in Section 2.2, many detectors utilized in HPLC have a linear response to the instantaneous concentration of the analyte. This is translated into a linear dependence of the area of the chromatographic peak on the mass of the analyte injected in the HPLC system, as it was shown in Section 3.4 (see Eq. 3.4.4.). For an ideal detector response, the following expression can be written:

$$A_{peak}(X) = \beta\, q_{inj}(X) \qquad (18.2.1)$$

In Eq. 18.2.1, $A_{peak}(X)$ is the peak area for a compound X and $q_{inj}(X)$ is the mass of the analyte X injected in the HPLC. The proportionality coefficient β (detection factor) depends on the detector sensitivity (and set electronic amplification). The analytes are injected in the HPLC in the form of a solution with volume V_{inj}, containing the analyte with the unknown concentration C_X. With the notation $q_{inj}(X) = q_X$ (where $q_X = C_X\ V_{inj}$) and $A_{peak}(X) = A_X$, Eq. 18.2.1 is equivalent with the following expression:

$$C_X = \frac{q_X}{V_{inj}} = b\, A_X \qquad (18.2.2)$$

In Eq. 18.2.2, $b = 1/(\beta \cdot V_{inj})$. The value of the proportionality coefficient b is derived from β, and depends on the detector sensitivity (and set electronic amplification), and on the volume V_{inj} of sample injected in the HPLC system.

Calibration procedures

In order to measure a concentration using expression 18.2.2, the value of coefficient b must be determined. This can be done by several procedures, the most common being by the use of calibration lines for a set of known concentrations $\{C_j\}$ of the pure compound to be analyzed (*calibration standard*) and the measured corresponding chromatographic peak areas indicated as $\{A_j\}$. After the areas are measured, the calibration lines are obtained by plotting the system of points $\{C_j, A_j\}$ and obtaining the equation of the line (trendline) using for example least-squares fitting. The calibrations can be done with pure solutions of the analyte, although it is preferable to make the calibrations by adding different levels of the calibration compound to a blank sample that does not contain the analyte. This procedure makes the analysis of the samples containing the calibration standards as close as possible to the analysis of a real sample and allows the subtraction of the overall influence of the matrix in the analysis. Some linear calibrations do not have a zero intercept as shown in Eq. 18.2.2 and the dependence can be written in the following form:

$$C_X = bA_X + a \qquad (18.2.3)$$

The parameters a (intercept) and b (slope) are also obtained from plotting the system of points $\{C_j, A_j\}$. This type of dependence may indicate some problems with the particular analytical method, such as sample decomposition, loss of sample in the chromatographic process due to selective adsorption, interfering signal from the blank sample, etc. A negative value for the parameter a indicates in general a loss of analyte,

while a positive value indicates background or interferences. In cases when the equation of the calibration curve is obtained from the equation of the trendline passing through the calibration points, it can be recommended to force the trendline through zero when zero response is known for the absence of the analyte in the sample.

Also, in some cases, for specific detectors of for specific analytes, the calibration is not linear. Nonlinearity may be due to overloading of detectors that otherwise are expected to have a linear response. Also, very low levels of analyte may lead to nonlinear response. For this reason, linearity must be verified for a whole range of concentrations, and particular attention must be paid to very low and very high concentrations. In some instances, a better fit between the analyte concentration and the detector response is obtained based on nonlinear fit (see, e.g., the response of light scattering detector by Eq. 2.2.37, which is logarithmic). Also, a quadratic dependence provides in some cases a much better fit than the linear one for the experimental data [26]. The equation for a quadratic dependence has the following form:

$$C_X = cA_X^2 + b\mathrm{A}_X + a \qquad (18.2.4)$$

The parameters a, b, and c can be obtained by the same procedure of fitting the system of points $\{C_j, \mathrm{A}_j\}$ using least-squares fitting. Although the linear calibration is preferred, quadratic calibrations are unavoidable in some analyses. In such cases, this type of calibration is more appropriate than forcing a linear calibration. For example, the linearity of fluorescence detection is valid only for low concentrations. A nonlinear calibration curve is sometimes necessary for fluorescence and chemiluminescence detectors. In LC-MS/MS, the nonlinear calibration is also necessary when a wide range of concentrations is present in samples. The nonlinear calibration may allow more accurate calculation of the concentration. However, a linear calibration curve is preferable when possible.

For compounds that have similar structures, the calibration curve for only one of the compounds to be analyzed in a mixture is utilized sometimes, and different compounds are quantitated based on the same calibration. This procedure should be used with caution and only when the calibration standards of a compound is not available.

Sample preparation, sometimes involving multiple operations, usually leads to changes in the analyte concentration compared to that from the initial sample. Also, in some analytical techniques, using, for example, MS detection, the variation of signal intensity is possible due to factors such as the variation in the composition of the matrix of the injected sample or due to the small variation in the stability of the spray of the MS instrument. Also, possible small variation in the injection volume or of the gradient elution program may influence the differences in the instrument response to the analyte concentration. For these reasons, in many quantitative techniques, an *internal standard* must be introduced with every chromatographic run. Internal standards are compounds that are absent in the real samples and are added in a constant amount at a chosen point during the analysis for verifying the reproducibility, accounting for sample losses, and in sample preparation for accounting for changes in the concentration from the raw sample to the processed sample due to the cleanup, concentration, etc. The most suitable internal standard is one that is chemically similar to the analyte and can be selectively separated against the analyte of interest and the pattern of the matrix sample. The internal standard must be chosen in such a way to behave in the analytical process as close as possible with the analytes, to not interfere with the analyte determination, and to give a chromatographic peak convenient to integrate.

The use of the internal standard for the correction for changes during sample preparation or to mimic instrument variability is done by replacing the use of peak area A_X in expressions 18.2.2, 18.2.3, or 18.2.4 with the normalized peak area of the analyte A_X/A_{IS}. For this purpose, an internal standard (*IS*) must be added in the same amount to the calibration standards and the raw sample that is analyzed. Using this procedure, a loss or a change in analyte concentration by x% is tracked by x% change in the concentration of the internal standard, such that the ratio A_X/A_{IS} remains the same regardless of the x% change. Since the internal standard is added in a constant known amount, the ratio A_X/A_{IS} remains proportional with A_X. In this way, the quantitation following the dependence expressed by Eq. 18.2.2 is based on the following expression:

$$C_X = b\frac{A_X}{A_{IS}} = b'A_X \qquad (18.2.5)$$

Expression 18.2.5 indicates that the peak area of the analyte X normalized by the constant peak area of the internal standard A_{IS} can be used for the true quantitation, regardless of some potential losses of the analyte, when the calibration is done for the set of known concentrations $\{C_j\}$ of the pure compound to be analyzed (*calibration standard*) and the corresponding normalized chromatographic peak areas $\{A_j/A_{IS}\}$. For the case of calibrations not having a zero intercept, the calibrations based on the ratio A_X/A_{IS} can be done following the expression:

$$C_X = b\frac{A_X}{A_{IS}} + a \qquad (18.2.6)$$

Besides the internal standards that are added in the sample such that they go through the sample preparation process, it is sometimes useful to add a *chromatographic standard* in the processed sample. This standard is a type of internal standard used only for verifying that the chromatographic process works properly, and it is introduced in the processed sample that is ready to be injected in the chromatograph.

A different quantitation technique besides that using a calibration curve is the standard addition. Standard addition method can be used to analyze an unknown sample of concentration C_X without the use of a calibration curve. It must be assumed, however, that the relation between the concentration and the peak area follows Eq. 18.2.2 and not Eq. 18.2.3 or Eq. 18.2.4. A (close to) perfect linearity between the response and sample concentration is necessary for successful use of standard addition technique. The unknown concentration C_X that should be analyzed is given by $C_X = q_X/V_X$ where V_X is a known volume of the sample containing the unknown quantity of X, q_X. For this sample, an initial peak area $A_X = A_0$ is measured. After the addition of the quantity $q_1 = C_1\ V_1$ of the compound to be analyzed, a second peak area A_1 is measured. Two equations can now be written: $q_X/V_X = b\ A_X$ and $(q_X + q_1)/(V_X + V_1) = b\ A_1$. The ratio of these two formulas leads to the following result:

$$\frac{(q_X + q_1)V_X}{(V_X + V_1)q_X} = \frac{A_1}{A_X} = \frac{A_1}{A_0} \qquad (18.2.7)$$

This relation can be easily rearranged to give the following:

$$C_X = \frac{q_1 A_X}{(V_X + V_1)A_1 - V_X A_X} \qquad (18.2.8)$$

The determination of C_X can also be obtained using a graph obtained using a line that passes through the points $(0, A_0)$ and (C_1, A_1) where $C_1 = q_1/(V_X + V_1)$. The equation of the line that passes through the two points has the following form (C and A variables):

$$\frac{C - C_1}{A - A_1} = \frac{C_0 - C_1}{A_0 - A_1} \qquad (18.2.9)$$

At the values $A = 0$ and since $C_0 = 0$, from Eq. 18.2.9 can be immediately obtained the value of

C_X (with negative sign) from the following expression ($A_X = A_0$):

$$C_X = C_1 - C_1\frac{A_1}{A_1 - A_0} \qquad (18.2.10)$$

This procedure is indicated in graphic form in Fig. 18.2.1.

When the addition of the standard does not dilute the sample ($V_1 \approx 0$ and $q_1/V_X \approx C_1$), Eq. 18.2.8 can be written in the form equivalent with Eq. 18.2.10:

$$C_X = \frac{C_1 A_X}{A_1 - A_X} \qquad (18.2.11)$$

For a more reliable result, the standard addition method can be used with multiple additions of known amounts of analyte $\{q_j\}$ $j = 1,2$ $\ldots.n$, leading to the concentrations $C_j = (q_X + q_j)/(V_X + V_j)$. The volume of the added solution with the j standard is V_j and the relation between the concentration C_j and the signal (peak areas A_j) is in this case given by the following relation:

$$C_j = bA_j \ (j = 1, 2, \ldots.) \qquad (18.2.12)$$

Eq. 18.2.12 is equivalent with the following set of equations:

$$q_j = -q_X + b\left(V_X + V_j\right)A_j \quad (j = 1, 2, \ldots\ldots..) \qquad (18.2.13)$$

The unknown values q_X and b (as parameters) can be obtained from the known values of V_X of the sample, V_j and q_j added, and the measured A_j using, for example, least-square fitting that generates the equation of the trendline for the points $\{q_j,(V_X + V_j)A_j\}$.

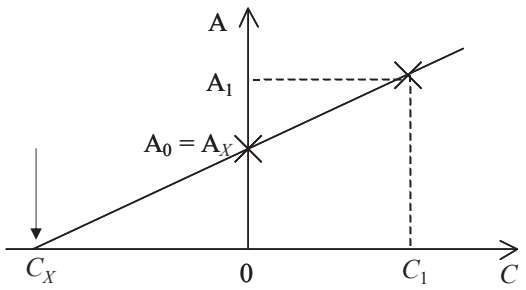

FIGURE 18.2.1 Graph showing the measurement of the unknown concentration C_X by standard addition technique.

Other procedures can also be used for quantitation. One of them is based solely on the peak area ratios for two compounds. For this procedure, a response factor $\boldsymbol{F}_X$ must be obtained initially. This response factor using an internal standard (*IS*) is calculated from the peak area A^*_{IS} of the internal standard and the peak area A^*_X of the compound X to be analyzed, both added to a blank sample or in pure solvent at equal or known amounts (concentration). When various sample preparation steps are part of the analysis, the samples with internal standard and with the compound to be analyzed must be both subjected to the same sample preparation steps. The ratio of the two chromatographic peak areas of the standard and analyte, usually obtained as an average of several measurements, gives the following response factor:

$$\boldsymbol{F}_X = \frac{A^*_{IS}}{A^*_X} \qquad (18.2.14)$$

Ideally, the value for $\boldsymbol{F}_X$ remains constant for an interval of values for the pair of concentrations of the standard and the sample. The concentration of the unknown is then obtained by measuring in the same run the peak area of the compound to be analyzed (at unknown concentration) and peak area of the standard using the following formula:

$$C_X = \boldsymbol{F}_X\left(\frac{A_X}{A_{IS}}\right)C_{IS} \qquad (18.2.15)$$

where A_X is the area of the compound X at unknown concentration, A_{IS} is the area of the standard at the concentration C_{IS}, and $\boldsymbol{F}_X$ is the response factor. In order to achieve a constant value for the response factor $\boldsymbol{F}_X$ in a range of concentrations, it is recommended that the two compounds, the internal standard and the analyte, be

chemically similar or even identical except for use of a labeled compound for the standard.

Selection of the internal standards in HPLC

In chromatographic analysis, the use of internal standards is very common and useful for obtaining more accurate [27]. Internal standards are mainly used in quantitative analysis as previously discussed, but they can also be useful in qualitative analysis. In order to behave similarly with the analyte in the sample preparation and in the chromatographic process, the internal standard must be a compound that is very similar but not identical to the chemical species of interest in the samples. In this way, the effects of sample preparation and of the core chromatographic process are very similar or even the same for the internal standard and for the analyte. The closest behavior during sample preparation or in chromatographic separation is typically seen between a compound and the same compound isotopically labeled. However, only the use of mass spectrometry as a detection procedure allows the differentiation between isotopically nonlabeled and labeled compounds. The isotopically labeled compounds (e.g., with deuterium H^2, C^{13} or N^{15}) are frequently used as internal standards, since mass spectrometers are frequently used as detectors [28,29]. Labeling with deuterium is often the most convenient choice, except the situation when deuterium/hydrogen exchange may occur [30] However, in cases when the detector does not differentiate between the labeled and nonlabeled compounds, or when the labeled compound is not available or it is too expensive, other compounds can be used as internal standards. These compounds are typically selected with properties similar to the analyte (same functional groups, not very different molecular weight, etc.). A common labeling isotope used in internal standards is deuterium. However, deuterium can be easily exchanged with hydrogen in ionizable compounds, and for this reason, compounds with ionizable deuterium must be avoided as internal standards.

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Appendix to Chapter 6

APPENDIX 6.1.1 Molar volumes (cm^3/mol) and solubility parameters δ $(cal/cm^3)^{1/2}$ for some common compounds used as solvents (at 25°C).[a]

Compound	*V*	δ	δ_d	δ_p	δ_a	δ_h
Acetic acid	71.3	12.4	7	N.R.L.[b]	N.R.L.	N.R.L.
Acetone	73.8	9.4	6.8	5	2.5	0
Acetonitrile	52.7	11.8	6.5	8	2.5	0
Anisole	108.7	9.7	9.1	2.5	2	0
Benzene	89.2	9.2	9.2	0	0.5	0
Benzonitrile	103.3	10.7	9.2	3.5	1.5	0
Bromobenzene	105.3	9.9	9.6	1.5	0.5	0
1-Butanol	91.5	9.6	N.R.L.	N.R.L.	N.R.L.	N.R.L.
CCI_3-CF_3	119.3	7.1	6.8	1.5	0.5	0
CCl_4	96.9	8.6	8.6	0	0	0
$CFCl_2$-CF_3	80.02	6.2	5.9	1.5	0	0
CH_2CI_2	64.4	9.6	6.4	5.5	0.5	0
$CHCl_3$	80.4	9.1	8.1	3	0.5	0
Chlorobenzene	102	9.6	9.2	2	0.5	0
CS_2	60.6	10	10	0	0.5	0
Cyclohexane	108.4	8.2	N.R.L.	0	0	0
Cyclohexanone	103.8	10.4	6.2	N.R.L.	N.R.L.	N.R.L.
Cyclopentane	93.21	8.1	N.R.L.	0	0	0
1,2-Dichloroethane	64.4	9.7	8.2	4	0	0
1,3-Dicyanopropane	94.6	13	8	8	3	0
Diethyl ether	104.4	7.4	6.7	2	2	0
Diethyl sulfide	109.98	8.6	8.2	2	0.5	0
Di-isopropyl ether	142	7	6.9	0.5	0.5	0

Continued

APPENDIX 6.1.1 Molar volumes (cm^3/mol) and solubility parameters δ $(cal/cm^3)^{1/2}$ for some common compounds used as solvents (at 25°C).[a] (*cont'd*)

Compound	*V*	δ	δ_d	δ_p	δ_a	δ_h
Dimethylformamide	77.3	11.5	7.9	N.R.L.	N.R.L.	N.R.L.
Dimethylsulfoxide	70.9	12.8	8.4	7.5	5	0
Dioxane	85.3	9.8	7.8	4	3	0
Ethanol	58.6	11.2	6.8	4	5	5
Ethanolamine	60.4	13.5	8.3	Large	Large	Large
Ethyl acetate	98.1	8.6	7	3	2	0
Ethyl bromide	75	8.8	7.8	3	0	0
Ethylene glycol	55.8	14.7	8	Large	Large	Large
Ethyl lactate	115	11	7.8	3.7		6.1
Formamide	39.7	17.9	8.3	Large	Large	Large
Isooctane	165.1	7	7	0	0	0
Methanol	40.6	12.9	6.2	5	7.5	7.5
Methyl acetate	79.5	9.2	6.8	4.5	2	0
Methyl benzoate	125.6	9.8	9.2	2.5	1	0
Methyl ethyl ketone	89.9	9.5	6.8	5	2.5	0
Methyl iodide	62.2	9.9	9.3	2	0.5	0
Methylene iodide	52.2	11.9	11.3	1	0.5	0
m-Xylene	123.4	8.8	8.8	0	0.5	0
n-Heptane	146.5	7.4	7.4	0	0	0
n-Hexane	131.1	7.3	7.3	0	0	0
Nitrobenzene	102.6	11.1	9.5	4	0.5	0
Nitromethane	53.7	11	7.3	8	1	0
n-Pentane	115.2	7.1	7.1	0	0	0
Octanol	158	10.3	5.2	N.R.L.	N.R.L.	N.R.L.
Perchloroethylene	102.2	9.3	9.3	0	0.5	0
Perfluoroalkanes	–	6	6	0	1	0
1-Propanol	75.1	10.2	7.2	2.5	4	4
2-Propanol	72.74	9.7	N.R.L.	N.R.L.	N.R.L.	N.R.L.
Propyl amine	81.7	8.7	7.3	4	6.5	0.5
Propyl chloride	88.2	8.3	7.3	3	0	0

APPENDIX 6.1.1 Molar volumes (cm^3/mol) and solubility parameters δ $(cal/cm^3)^{1/2}$ for some common compounds used as solvents (at 25°C).[a] (*cont'd*)

Compound	*V*	δ	δ_d	δ_p	δ_a	δ_h
Propylene carbonate	85.4	13.3	N.R.L.	N.R.L.	N.R.L.	N.R.L.
p-Xylene	123.4	8.8	8.8	0	0.5	0
Pyridine	80.6	10.4	9	4	5	0
Tetrahydrofuran	81.1	9.9	7.6	4	3	0
Toluene	106.6	8.9	8.9	0	0.5	0
Triethylamine	139.5	7.5	7.5	0	3.5	0
Water	18.02	21	6.3	Large	Large	Large

[a] *Only a limited number of compounds from the list are used as components of a mobile phase.*
[b] *N.R.L., Not reported in the referenced literature.*

Appendix to chapter 7

APPENDIX 7.1.1 Experimental solubility in water of various solvents used in HPLC.

Solvent	Solubility% in water	Solvent	Solubility% in water
Acetic acid	100	Formamide	100
Acetone	100	Heptane	0.0003
Acetonitrile	100	Hexane	0.001
Benzene	0.18	Iso-propanol	100
Butyl acetate	7.81	Methanol	100
Carbon tetrachloride	0.08	Methyl ethyl ketone	24
Chloroform	0.815	Methyl *tert*-butyl ether	4.8
Cyclohexane	0.01	Methylene chloride	1.6
1,2-Dichloroethane	0.81	n-Butanol	0.43
Diethyl ether	6.89	n-Propanol	100
Di-isopropyl ether	0.87	n-Octanol	0.06
Dimethylformamide	100	Pentane	0.004
Dimethylsulfoxide	100	Tetrahydrofuran	100
Dioxane	100	Toluene	0.051
Ethanol	100	Trichloroethylene	0.11
Ethyl acetate	8.7	Xylene	0.018

APPENDIX 7.1.2 Values of polarity (chromatographic strength) P' parameter and of separation parameters $\boldsymbol{x}_e$, $\boldsymbol{x}_d$, and $\boldsymbol{x}_n$ for several common solvents (*e*-ethanol, *d*-dioxane, *n*-nitromethane). The values for log K_{ow} are also listed.

No.	Compound	Group	P′	x_e	x_d	x_n	log K_{ow}
Non-polar, hydrocarbons							
1.	Carbon disulfide	0	0.3	–	–	–	1.95
2.	Carbon tetrachloride	0	1.6	–	–	–	3

(Continued)

APPENDIX 7.1.2 Values of polarity (chromatographic strength) P' parameter and of separation parameters x_e, x_d, and x_n for several common solvents (*e*-ethanol, *d*-dioxane, *n*-nitromethane). The values for log K_{ow} are also listed.—*cont'd*

No.	Compound	Group	P′	x_e	x_d	x_n	log K_{ow}
3.	Cyclohexane	0	0.2	–	–	–	2.67
4.	n-Decane	0	0.4	–	–	–	4.91
5.	n-Hexane	0	0.1	–	–	–	3.13
6.	Isooctane	0	0.1	–	–	–	3.71
7.	Squalane	0	1.2	–	–	–	12.86
Aliphatic ethers, other compounds							
8.	Dibutyl ether	1	2.1	0.44	0.18	0.38	2.77
9.	Diethyl ether	1	2.8	0.53	0.13	0.34	0.84
10.	Diisopropyl ether	1	2.4	0.48	0.14	0.38	1.67
11.	Hexamethylphosphoramide	1	7.4	0.47	0.17	0.37	−1.4
12.	Tetramethylguanidine	1	6.1	0.48	0.18	0.35	−0.16
13.	Triethylamine	1	1.9	0.56	0.12	0.32	1.26
Aliphatic alcohols							
14.	n-Butanol	2	3.9	0.58	0.18	0.24	0.81
15.	tert-Butanol	2	4.1	0.56	0.2	0.24	0.54
16.	Ethanol	2	4.3	0.52	0.19	0.29	−0.16
17.	Isopentanol	2	3.7	0.56	0.19	0.26	1.09
18.	Isopropanol	2	3.9	0.55	0.19	0.27	0.25
19.	Methanol	2	5.1	0.48	0.22	0.31	−0.52
20.	n-Octanol	2	3.4	0.57	0.19	0.25	2.58
21.	n-Propanol	2	4	0.54	0.19	0.27	0.36
Various, amides, nitrogenous, etc.							
22.	Diethylene glycol	3	5.2	0.44	0.23	0.33	−1.26
23.	2,6-Dimethylpyridine	3	4.5	0.45	0.2	0.36	1.02
24.	2-[2-(4-Nonylphenoxy) ethoxy]ethanol	3	–	0.38	0.22	0.4	5.15
25.	N,N-Dimethylacetamide	3	6.5	0.41	0.2	0.39	−0.58
26.	Dimethylformamide	3	6.4	0.39	0.21	0.4	−0.63
27.	Dimethylsulfoxide	3	7.2	0.39	0.23	0.39	−1.41
28.	Methoxyethanol	3	5.5	0.38	0.24	0.38	−0.57

APPENDIX 7.1.2 Values of polarity (chromatographic strength) P' parameter and of separation parameters x_e, x_d, and x_n for several common solvents (*e*-ethanol, *d*-dioxane, *n*-nitromethane). The values for log K_{ow} are also listed.—*cont'd*

No.	Compound	Group	P′	x_e	x_d	x_n	log K_{ow}
29.	Methyl formamide	3	6	0.41	0.23	0.36	−0.86
30.	2-Methylpyridine	3	4.9	0.44	0.21	0.36	0.89
31.	N-Methyl-2-pyrrolidone	3	6.7	0.4	0.21	0.39	−0.36
32.	Pyridine	3	5.3	0.42	0.23	0.36	0.76
33.	Quinoline	3	5	0.41	0.23	0.36	2.13
34.	Tetrahydrofuran	3	4	0.38	0.2	0.42	0.53
35.	Tetramethyl urea	3	6	0.42	0.19	0.39	−0.47
36.	Triethyleneglycol	3	5.6	0.42	0.24	0.34	−1.3
Various, acetic acid, formamide, etc.							
37.	Acetic acid	4	6	0.39	0.31	0.3	−0.22
38.	Benzyl alcohol	4	5.7	0.4	0.3	0.3	1.21
39.	Ethylene glycol (ethan-1,2-diol)	4	6.9	0.43	0.29	0.28	−1.21
40.	Formamide	4	9.6	0.37	0.34	0.3	−1.08
Chlorinated aliphatics							
41.	Ethylene chloride	5	3.5	0.3	0.21	0.49	1.5
42.	Methylene chloride	5	3.1	0.29	0.18	0.53	0.84
Nitriles, dioxanes, ketones, etc.							
43.	Acetone	6	5.1	0.35	0.23	0.42	0.11
44.	Acetonitrile	6	5.8	0.31	0.27	0.42	−0.17
45.	Acetophenone	6	4.8	0.33	0.26	0.41	1.53
46.	Aniline	6	6.3	0.32	0.32	0.36	1.14
47.	Benzonitrile	6	4.8	0.31	0.27	0.42	1.83
48.	Bis-(2-cyanoethyl) ether	6	6.8	0.31	0.29	0.4	−0.33
49.	Bis-(2-ethoxyethyl) ether	6	4.6	0.37	0.21	0.43	0.03
50.	γ-Butyrolactone	6	6.5	0.34	0.26	0.4	0.15
51.	Cyano morpholine	6	5.5	0.35	0.25	0.4	−0.05
52.	Cyclohexanone	6	4.7	0.37	0.22	0.42	1.49
53.	Dioxane	6	4.8	0.36	0.24	0.4	−0.09

(Continued)

APPENDIX 7.1.2 Values of polarity (chromatographic strength) P' parameter and of separation parameters x_e, x_d, and x_n for several common solvents (*e*-ethanol, *d*-dioxane, *n*-nitromethane). The values for log K_{ow} are also listed.—*cont'd*

No.	Compound	Group	P′	x_e	x_d	x_n	log K_{ow}
54.	Ethyl acetate	6	4.4	0.34	0.23	0.43	0.28
55.	Formyl morpholine	6	6.4	0.37	0.25	0.39	−0.85
56.	Methyl ethyl ketone	6	4.7	0.35	0.22	0.43	0.81
57.	Propylene carbonate	6	6.1	0.31	0.27	0.42	0.79
58.	Tetrahydrothiophene-1,1-dioxide	6	6.9	0.33	0.28	0.39	−0.59
59.	Tricresyl phosphate	6	4.6	0.36	0.23	0.41	6.63
60.	Tris-(2-cyanoethoxy) propane	6	6.6	0.32	0.27	0.41	−0.59
Aromatic hydrocarbons, aromatic ethers, nitro compounds							
61.	Benzene	7	2.7	0.23	0.32	0.45	1.97
62.	Bromobenzene	7	2.7	0.24	0.33	0.43	2.25
63.	Chlorobenzene	7	2.7	0.23	0.33	0.44	2.07
64.	Dibenzyl ether	7	4.1	0.3	0.28	0.42	3.57
65.	Dipentyl ether	7	3.4	0.27	0.32	0.41	3.66
66.	Ethoxybenzene	7	3.3	0.28	0.28	0.44	2.17
67.	Fluorobenzene	7	3.2	0.24	0.32	0.45	2.12
68.	Iodobenzene	7	2.8	0.24	0.35	0.41	2.9
69.	Methoxybenzene	7	3.8	0.28	0.3	0.43	1.82
70.	Nitrobenzene	7	4.4	0.26	0.3	0.44	1.91
71.	Nitroethane	7	5.2	0.28	0.29	0.43	0.38
72.	Nitromethane	7	6	0.29	0.32	0.4	0.02
73.	Toluene	7	2.4	0.25	0.28	0.47	2.49
74.	*p*-Xylene	7	2.5	0.27	0.28	0.45	3
Various and water							
75.	Chloroform	8	4.1	0.26	0.42	0.33	0.84
76.	*m*-Cresol	8	7.4	0.38	0.37	0.25	2.18
77.	1H,1H,7H-Dodecafluoroheptanol	8	8.8	0.33	0.4	0.27	3.45
78.	Tetrafluoropropanol	8	8.6	0.34	0.36	0.3	0.65
79.	Water	8	10.2	0.38	0.38	0.25	−0.65

APPENDIX 7.1.3 Values for $E_T(30)$ polarity for several common solvents.

Compound	$E_T(30)$	Compound	$E_T(30)$
Acetic acid	51.7	Formic acid	54.3
Acetonitrile	45.6	Glycerin	57
Benzene	34.3	*n*-Hexane	31
(*tert*-Butyl) methyl ether	34.7	*n*-Heptane	31.1
Carbon dioxide (40°C/150 bar)	28.5	Methoxybenzene	37.1
Carbon tetrachloride	32.4	1-Methylpyrrolidin-2-one	42.2
Chloroform	39.1	Nitrobenzene	41.2
Cyclohexane	30.9	Nitromethane	46.3
Deuterium oxide	62.8	*n*-Octane	31.1
1,2-Dichloroethane	41.3	1,2-Propanediol	54.1
Dibenzylether	36.3	Propionic acid	50.5
Dichloromethane	40.7	Propionitrile	43.6
Diethylether	34.5	Pyridine	40.5
Diglyme	38.6	Styrene	34.8
N,N-Dimethylformamide	43.2	Tetrahydrofuran	37.4
Dimethylsulfoxide	45.1	Tetrahydropyran	36.2
1,4-Dioxane	36	Thiophene	35.4
1,3-Dioxolan	43.1	Toluene	33.9
Diphenylether (30°C)	35.3	Trimethylphosphate	43.6
1,2-Ethanediol	56.3	p-Xylene	33.1
Formamide	55.8	Water	63.1

APPENDIX 7.1.4 Solvatochromic parameters **α**, **β**, and **π*** for several common solvents.

No.	Compound	α	β	π*	No.	Compound	α	β	π*
1.	Acetic acid	1.12	0.45	0.64	33.	Formamide	0.71	0.48	0.97
2.	Acetonitrile	0.19	0.4	0.75	34.	Formic acid	1.23	0.38	0.65
3.	Aniline	0.26	0.5	0.73	35.	Heptane	0	0	0
4.	Benzene	0	0.1	0.59	36.	Hexamethylphosphoramide	0	1.05	0.87
5.	Benzyl alcohol	0.6	0.52	0.98	37.	Hexane	0	0	0
6.	Butanoic acid	1.1	0.45	0.56	38.	Methanol	0.93	0.66	0.6
7.	1-Butanol	0.84	0.88	0.47	39.	Methyl acetate	0	0.42	0.6
8.	2-Butanol	0.69	0.8	0.4	40.	2-Methyl-2-propanol	0.68	1.01	0.41
9.	2-Butanone	0.06	0.48	0.67	41.	Morpholine	0.29	0.7	0.39
10.	Carbon disulfide	0	0.07	0.61	42.	Octane	0	0	0.01
11.	Carbon tetrachloride	0	0.1	0.28	43.	Octanol	0.77	0.81	0.4
12.	Chlorobenzene	0	0.07	0.71	44.	Pentane	0	0	−0.08
13.	1-Chlorobutane	0	0	0.39	45.	Pentanoic acid	1.19	0.45	0.54
14.	Chloroform	0.44	0	0.58	46.	Pentanol	0.84	0.86	0.4
15.	Cyclohexane	0	0	0	47.	Piperidine	0	1.04	0.3
16.	Cyclopentane	0	0	−0.087	48.	1,2,3-Propanetriol	1.21	0.51	0.62
17.	1,2-Dichlorobenzene	0	0.07	0.67	49.	1-Propanol	0.78	0.84	0.52
18.	1,1-Dichloroethane	0.1	0.1	0.48	50.	2-Propanol	0.76	0.95	0.48
19.	1,2-Dichloroethane	0	0.1	0.81	51.	Propionitrile	0.10	0.37	0.71
20.	Dichloromethane	0.13	0.1	0.82	52.	2-Propanone	0.08	0.43	0.71
21.	Diethyl ether	0	0.47	0.27	53.	Propanoic acid	1.12	0.45	0.58
22.	Diethyl sulfide	0	0.37	0.46	54.	Pyridine	0	0.64	0.87
23.	Diethylamine	0.3	0.7	0.24	55.	Pyrrolidine	0.16	0.7	0.39
24.	Diisopropyl ether	0	0.49	0.27	56.	Sulfolane	0	0.39	0.98
25.	N,N-Dimethylacetamide	0	0.76	0.88	57.	Tetrahydrofuran	0	0.55	0.58
26.	N,N-Dimethylformamide	0	0.69	0.88	58.	Tetramethylsilane	0	0.02	−0.09
27.	Dimethylsulfoxide	0	0.76	1	59.	Toluene	0	0.11	0.54
28.	Dioxane	0	0.37	0.55	60.	Triethylamine	0	0.71	0.14
29.	Ethanediol	0.9	0.52	0.92	61.	2,2,2-Trifluoroethanol	1.51	0	0.73
30.	1,3-Dioxolane	0	0.45	0.69	62.	m-Xylene	0	0.11	0.47
31.	Ethanol	0.86	0.75	0.54	63.	p-Xylene	0	0.12	0.43
32.	Ethyl acetate	0	0.45	0.55	64.	Water	1.17	0.47	1.09

The **α** scale describes hydrogen-bond donor interactions. The **β** scale describes hydrogen-bond acceptor interactions. The **π*** scale describes polarity and polarizability.

APPENDIX 7.1.5 Parameters *c*, *e*, *s*, *a*, *b*, and *v* for partition between water and a dry solvent.

Solvent	*C*	*e*	*S*	*a*	*b*	*v*
Methanol/dry	0.329	0.299	−0.671	0.08	−3.389	3.512
Ethanol/dry	0.208	0.409	−0.959	0.186	−3.645	3.928
Propan-1-ol/dry	0.148	0.436	−1.098	0.389	−3.893	4.036
Butan-1-ol/dry	0.152	0.438	−1.177	0.096	−3.919	4.122
Pentan-1-ol/dry	0.08	0.521	−1.294	0.208	−3.908	4.208
Hexan-1-ol/dry	0.044	0.47	−1.153	0.083	−4.057	4.249
Heptan-1-ol/dry	−0.026	0.491	−1.258	0.035	−4.155	4.415
Octan-1-ol/dry	−0.034	0.49	−1.048	−0.028	−4.229	4.219
Propanone/dry	0.335	0.349	−0.231	−0.411	−4.793	3.963
Acetonitrile/dry	0.413	0.077	0.326	−1.566	−4.391	3.364
Hexane	0.361	0.579	−1.723	−3.599	−4.764	4.344
Heptane	0.325	0.67	−2.061	−3.317	−4.733	4.543
Octane	0.223	0.642	−1.647	−3.480	−5.067	4.526
Cyclohexane	0.159	0.784	−1.678	−3.740	−4.929	4.577
Tetrachloromethane	0.26	0.573	−1.254	−3.558	−4.588	4.589
Toluene	0.143	0.527	−0.720	−3.010	−4.824	4.545
Benzene	0.142	0.464	−0.588	−3.099	−4.625	4.491
Carbon disulfide	0.047	0.686	−0.943	−3.603	−5.818	4.921

Parameter *e* is related to polarizability, *s* is related to dipolar effects, *a* is related to basicity, *b* is related to hydrogen bond acidity, and *v* is related molecular volume (*c* is log k' of a reference compound).

From M.H. Abraham, A. Ibrahim, A.M. Zissimos, Determination of sets of solute descriptors from chromatographic measurements, J. Chromatogr. A 1037 (2004) 29–47.

APPENDIX 7.2.1 Viscosity for several common solvents at 25°C.

Compound	Viscosity η (cP)	Compound	Viscosity η (cP)	Compound	Viscosity η (cP)
Acetone	0.32	N,N-Dimethylformamide	0.92	Methyl ethyl ketone	0.43
Acetonitrile	0.37	Dimethylsulfoxide	2.24	Methyl isobutyl ketone	0.58[a]
Benzene	0.607	Dioxane	1.54	Methyl *t*-butyl ether	0.36
n-Butanol	2.95	Ethanol	1.2	Methylene chloride	0.44
Carbon disulfide	0.36[a]	Ethyl acetate	0.45	Morpholine	7.42
Carbon tetrachloride	0.97	Ethylene glycol	19.9	n-Octanol	7.36

(Continued)

APPENDIX 7.2.1 Viscosity for several common solvents at 25°C.—*cont'd*

Compound	Viscosity η (cP)	Compound	Viscosity η (cP)	Compound	Viscosity η (cP)
Chloroform	0.57	n-Heptane	0.41	n-Pentane	0.23
Cyclohexane	1.0	n-Hexane	0.33	n-Propanol	2.27
Cyclohexanone	2.02	Isobutyl alcohol	4.7	Tetrahydrofuran	0.55
Cyclopentane	0.47	Isooctane	0.53	Toluene	0.59
Decane	0.92	Isopropanol	2.37	*p*-Xylene	0.648[a]
Diethyl ether	0.23	Methanol	0.6	Water	1.00
Diisopropyl ether	0.37	Methyl acetate	0.37		

[a] *Viscosity at 20°C.*

APPENDIX 7.2.2 Values of surface tension γ' in mN/m (dyne/cm) and the corresponding temperature coefficient in mN/(m·K) for several common solvents.

Compound	γ' 20°C mN/m	Temp. coeff. mN/(m·K)
Acetone (2-Propanone)	25.2	−0.112
Benzene	28.88	−0.1291
Carbon disulfide	32.3	−0.1484
Chlorobenzene	33.6	−0.1191
Chloroform	27.5	−0.1295
Cyclohexane	24.95	−0.1211
1,2-Dichloroethane	33.3	−0.1428
1,4-Dioxane	33	−0.1391
Ethanol	22.1	−0.0832
Isopropanol	23	−0.0789
Methanol	22.7	−0.0773
Methyl ethyl ketone (MEK)	24.6	−0.1199
N,N-Dimethylformamide (DMF)	37.1	−0.1400
n-Decane	23.83	−0.0920
n-Heptane	20.14	−0.0980
n-Hexane	18.43	−0.1022
Methylene chloride	26.5	−0.1284
Nitrobenzene	43.9	−0.1177
Nitromethane	36.8	−0.1678

APPENDIX 7.2.2 Values of surface tension γ' in mN/m (dyne/cm) and the corresponding temperature coefficient in mN/(m·K) for several common solvents.—*cont'd*

Compound	γ' 20°C mN/m	Temp. coeff. mN/(m·K)
1-Octanol	27.6	−0.0795
Propanol (25°C)	23.7	−0.0777
Pyridine	38	−0.1372
Toluene	28.4	−0.1189
Water	72.8	−0.1514

APPENDIX 7.2.3 Several properties of common solvents important for the separation process.

Compound	Formula	Dielectric constant ε	Dipole *m* (D) liq.	Dipole *m* (D) gas	Polariz. $4\pi\varepsilon_0$ (Å)^3
Acetic acid	$C_2H_4O_2$	6.2	1.92	1.75	5.33
Acetone	C_3H_6O	20.7	3.11	2.87	6.41
Acetonitrile	C_2H_3N	37.5	3.39	3.97	4.27
Benzene	C_6H_6	2.3	0.0	0.0	8.89
n-Butanol	$C_4H_{10}O$	17.8	2.96	1.6	9.21
Carbon disulfide	CS_2	2.6	0.0	0.0	6.79
Carbon tetrachloride	CCl_4	2.24	0.0	0.0	10.5
Chlorobenzene	C_6H_5Cl	5.5−6.3	1.39	1.72	11.06
Chloroform	$CHCl_3$	4.81	1.85	1.03	8.52
Cyclohexane	C_6H_{12}	18.5	0.2	0.61	11.07
Cyclohexanone	$C_6H_{10}O$	18.2	2.94	—	11.15
Cyclopentane	C_5H_{10}	2.0	0.0	0.0	9.2
Decane	$C_{10}H_{22}$	1.99	0.0	0.0	20.61
1,2-Dichloroethane	$C_2H_4Cl_2$	10.4	2.94	1.84	8.5
Diethyl ether	$C4H_{10}O$	4.34	1.27	1.13	9.33
Diisopropyl ether	$C_6H_{14}O$	3.88	1.26	1.13	12.94
N,N-Dimethylformamide	C_3H_7NO	37.6	3.85	—	7.69
Dimethylsulfoxide	C_2H_6SO	46.2	3.9	—	7.91
Dioxane	$C_4H_8O_2$	2.21	0.45	0.43	8.97
Ethanol	C_2H_6O	24.6	1.66	1.69	5.3
Ethyl acetate	$C_4H_8O_2$	6.02	2.05	1.78	9.28
Ethylene glycol	$C_2H_6O_2$	37.7	2.2	2.2	6.18
n-Heptane	C_7H_{16}	1.89	0.0	0.0	14.34
n-Hexane	C_6H_{14}	1.9	0.0	0.0	12.3

(Continued)

APPENDIX 7.2.3 Several properties of common solvents important for the separation process.—*cont'd*

Compound	Formula	Dielectric constant ε	Dipole *m* (D) liq.	Dipole *m* (D) gas	Polariz. $4\pi\varepsilon_0$ (Å)^3
Isobutyl alcohol	$C_4H_{10}O$	16.68	2.96	1.64	9.07
Isooctane	C_8H_{18}	1.94	0	0	16.18
Isopropanol	C_3H_8O	19.9	3.09	1.59	7.14
Methanol	CH_4O	32.7	2.97	1.69	3.38
Methyl acetate	$C_3H_6O_2$	6.68	1.74	1.68	7.36
Methyl ethyl ketone	C_4H_8O	18.5	3.41	—	8.35
Methyl isobutyl ketone	$C_6H_{12}O$	13.11	2.68	—	12.04
Methyl *tert*-butyl ether	$C_5H_{12}O$	4.0	1.25	—	10.88
Methylene chloride	CH_2Cl_2	9.08	1.9	1.54	4.54
Morpholine	C_4H_9NO	—	1.75	—	9.47
Nitrobenzene	$C_6H_5NO_2$	34.8	3.93	4.28	11.22
Nitromethane	CH_3NO_2	35.9	4.39	3.44	4.66
n-Octanol	$C_8H_{18}O$	3.4	1.61	—	17.42
n-Pentane	C_5H_{12}	2.1	0.0	0.0	10.27
n-Propanol	C_3H_8O	20.1	3.09	1.65	13.23
Pyridine	C_5H_5N	12.4	2.31	2.15	8.25
Tetrahydrofuran	C_4H_8O	7.58	1.63	1.63	8.14
Tetrahydrothiophene-1,1-dioxide	$C_4H_8O_2S$	43.3	4.69	—	11.46
Toluene	C_7H_8	2.37	0.38	0.37	10.97
Triethylamine	$C_6H_{15}N$	2.4	0.75	0.61	13.47
p-Xylene	C_8H_{10}	2.4	0.02	0.0	13.12
Water	H_2O	77.46	3.12	1.85	1.51

APPENDIX 7.2.4 Boiling point for several common solvents (at atmospheric pressure).

Solvent	Boiling point °C	Solvent	Boiling point °C
Acetone	56.3	n-Hexane	68.7
Acetonitrile	81.6	Isobutyl alcohol	98
Benzene	80.1	i-Octane	126
n-Butanol	117.7	Methanol	64.7
sec-Butanol	107.7	Methyl acetate	57.5
t-Butyl methyl ether	55.5	Methyl ethyl ketone	80
Carbon tetrachloride	76.5	Methyl i-butyl ketone	117.5
Chloroform	61.2	Methylene chloride	39.8
Cyclohexane	80.7	Morpholine	129
Cyclopentane	49.3	i-Pentane	27.7
Decalin	193	n-Pentane	36.1
n-Decane	174	i-Propanol	82.3
Dimethylformamide	153	n-Propanol	97.2
Dimethylsulfoxide	189	i-Propyl ether	69
Dioxane	101	Pyridine	115.3
Ethanol	78.3	Tetrahydrofuran	66
Ethyl acetate	77.1	Toluene	110.6
Ethyl ether	34.6	Triethylamine	88.8
Ethylene glycol	197.5	Trimethylpentane	115
n-Heptane	98.4	Water	100

APPENDIX 7.3.1 Physical properties of interest for detection for several common solvents.

Solvent	Refractive index	UV cut-off (nm)	Solvent	Refractive index	UV cut-off (nm)
Acetone	1.395	330	Isobutyl alcohol	1.384	220
Acetonitrile	1.344	190	i-Octane	1.404	210
Benzene	1.501	278	Methanol	1.329	205
n-Butanol	1.347	210	Methyl acetate	1.362	260
sec-Butanol	1.397	260	Methyl ethyl ketone	1.381	329
t-Butyl methyl ether	1.369	210	Methyl i-butyl ketone	1.394	334
Carbon tetrachloride	1.466	265	Methylene chloride	1.424	233
Chloroform	1.443	245	Morpholine	–	285
Cyclohexane	1.427	200	Pentane	–	190
Cyclopentane	1.406	198	i-Pentane	1.371	200
Decalin	1.476	200	n-Pentane	1.358	210
n-Decane	1.412	210	i-Propanol	1.375	205
Dimethylformamide	1.427	268	n-Propanol	1.383	210
Dimethylsulfoxide	1.476	268	i-Propyl ether	1.368	220
1,4-Dioxane	1.422	215	Pyridine	1.510	330
Ethanol	1.361	210	Tetrahydrofuran	1.408	212
Ethyl acetate	1.370	256	Toluene	1.496	285
Ethyl ether	1.353	215	Triethylamine	1.400	–
Ethylene glycol	1.427	210	Trimethylpentane	1.389	215
n-Heptane	1.385	200	o-Xylene	–	288
n-Hexane	1.375	195	Water	1.323	<190

APPENDIX 7.3.2 UV cut-off of several common additives and buffers.

Additive/buffer	UV cut-off (nm)	Additive/buffer	UV cut-off (nm)
Acetic acid	230	Formic acid	210
Acetate (Na^+, K^+, NH_4^+)	240	Phosphate (Na^+, K^+, NH_4^+)	210
Borate (Na^+, K^+, NH_4^+)	210	Triethylamine	235
Citrate (Na^+, K^+, NH_4^+)	250	Trifluoroacetic acid	210
Diethylamine	210	TRIS	225

APPENDIX 7.4.1 The pK_a values of the common weak acids and bases used for buffers preparation and their pH working range.[a]

Buffer	pK_a of acid or base	pH working range
CF_3COOH/CF_3COONH_4	0.3	1.5–2.5
H_3PO_4/KH_2PO_4	pK_1 2.15	1.1–3.1
KH_2PO_4/K_2HPO_4	pK_2 7.20	6.2–8.2
K_2HPO_4/K_3PO_4	pK_3 12.33	11.1–13.6
Citric acid K salt (tribasic)/HCl	pK_1 (citric) 3.13	2.1–4.1
Citric acid K salt (tribasic)/HCl	pK_2 (citric) 4.76	3.7–5.7
Citric acid K salt (tribasic)/HCl	pK_3 (citric) 6.40	4.4–6.4
$HCOOH/HCOONH_4$	pK (formic) 3.75	2.8–4.8
HCOOH/HCOOK	pK (formic) 3.75	2.5–5.0
$HCOONH_4$/HCOOH	pK (ammonia) 9.25	8.2–10.2
CH_3COOH/CH_3COOK	pK (acetic) 4.76	3.8–5.8
CH_3COOH/CH_3COONH_4	pK (acetic) 4.76	3.8–5.8
CH_3COONH_4/CH_3COOH	pK (ammonia) 9.25	8.2–10.2
$CH_3CH_2COOH/CH_3CH_2COOK$	4.86	3.6–6.1
$KHC_8H_4O_4$ (phthalate)/HCl	pK_1 2.89	2.2–3.8
$KHC_8H_4O_4$ (phthalate)/NaOH	pK_2 5.51	4–6.2
$H_2CO_3/KHCO_3$	pK_1 6.35	5.1–7.6
$KHCO_3/K_2CO_3$	pK_2 10.33	9.1–11.6
NH_4HCO_3/NH_4OH	pK_2 10.33	9.1–11.6
Tris (tris(hydroxymethyl) aminomethane)/HCl	8.06	7–9
1,3-Bis-tris propane/HCl	6.6, 8.3	6–9.5
H_3BO_4/NaOH	pK_1 9.24	9.2–11.0
NH_2CH_2COOH/NaOH	pK_2 9.78	8.5–12.9
Diethylamine/HCl	10.5	9.5–11.5
Triethylamine/HCl	pK_2 10.72	9.5–11.9
Pyrrolidine/HCl	11.27	10–12.5
1-Methylpiperidine/HCl	11.3	10.2–12.5
K_3PO_4/KOH	12.33	–

[a] *Note: In some potassium salts the cation can be replaced with sodium.*

APPENDIX 7.4.2 Solubility (mM/L) of potassium phosphate-based buffers[a] in different mobile phase compositions.

Organic content (%, vol.)	Acetonitrile	Methanol	Tetrahydrofuran
0–40	>50	>50	>50
50	>50	>50	25
60	45	>50	15
70	20	35	10
80	5	15	<5
90	0	5	0

[a] *Note: Solutions containing KH_2PO_4 and K_2HPO_4 are used as buffers in the range of pH = 4.8–8.0 (measured in water).*

APPENDIX 7.4.3 The values for pK_a and pK_b of several common acids or bases used as additives in the mobile phase.

Acid	pK_a	Base	pK_b
Trifluoroacetic	0.23	Pyrrolidine	2.73
Pentafluoropropionic acid	0.38	Triethylamine	3.25
Trichloroacetic	0.51	Dimethylamine	3.27
Phosphoric (1)	2.12	Ethylamine	3.35
Monofluoroacetic	2.60	n-Butylamine	3.4
Monochloroacetic	2.87	Trimethylamine	4.2
Citric (1)	3.08	Ethanolamine	4.50
Formic	3.75	Ammonia	4.75
Lactic	3.86	Hydroxylamine	8.06
Ascorbic (1)	4.10	Pyridine	8.77
Citric (2)	4.74	Aniline	9.13
Acetic	4.75		
Boric (1)	9.23		

APPENDIX 7.4.4 UV cut-off values of several common buffers and additives (for a specific concentration) and their suitability for LC/MS detection.

Buffer	UV cut-off	Concentration in UV	LC/MS detection suitability
Potassium formate/formic acid	210 nm	10 mM	No
Potassium acetate/acetic acid	210 nm	10 mM	No
Ammonium formate	210 nm	50 mM	Yes
Ammonium acetate	210 nm	50 mM	Yes
Trifluoroacetic acid	210 nm	10 mM	Yes
KH_2PO_4/H_3PO_4	<200 nm	0.1%	No
KH_2PO_4/K_2HPO_4	<200 nm	0.1%	No
Ammonium hydroxide/ammonia	200 nm	10 mM	Yes
Diethylamine/HCl	200 nm	10 mM	No
Triethylamine/HCl	200 nm	10 mM	No
Borate ($H_3BO_3/Na_2B_4O_7 \cdot 10H_2O$)	210 nm	10 mM	No
Glycine/HCl	230 nm	10 mM	No
Glycine/HCOOH	215 nm	10 mM	Yes
NH_4HCO_3/NH_4OH	210 nm	10 mM	Yes
Tri-K-Citrate/HCl	230 nm	10 mM	No
1,3- Bis-tris propane/HCl [bis(tris (hydroxymethyl)methylamino) propane]/HCl	225 nm	10 mM	No
1-Methylpiperidine/HCl	215 nm	10 mM	No
Pyrrolidine/HCl	210 nm	10 mM	No
Pyrrolidine/HCOOH	215 nm	10 mM	Yes

APPENDIX 7.4.5 Solubility of K_2HPO_4 in water/organic solvent and acceptable concentration in the mobile phase.

Organic content%	Methanol	Acetonitrile	Tetrahydrofuran
50% and lower	less than 50 mM	less than 50 mM	less than 25 mM
60%	less than 50 mM	45 mM	15 mM
70%	35 mM	20 mM	10 mM
80%	15 mM	5 mM	less than 5 mM
90%	5 mM	0 mM	0 mM

From J. Dolan, A Guide to HPLC and LC-MS Buffer Selection. www.ace-hplc.com.

Appendix to chapter 8

APPENDIX 8.2.1 USP classification of HPLC chromatographic columns (all stationary phases as spherical, unless indicated as (M) for monolith, and (I) for irregular shape).

Code	Description	Examples
L1	Octadecyl silane (C18) chemically bonded to porous silica or ceramic microparticles, 1.5–10 μm in diameter, or a monolithic rod.	Over 250 columns such as Luna C18(2), Luna C18(2)-HST, Gemini C18, Synergi Hydro-RP, Onyx C18 (M), Aquity UPLC BEH C18, Aquity UPLC Shield RP 18, Atlantis T3, μBondapack C18, Nova-Pak C18, Symmetry C18, ABridge C18, XTerra MS C18
L2	Octadecyl silane (C18) chemically bonded to silica gel of a controlled surface porosity that has been bonded to a solid spherical core, 30–50 μm in diameter.	Bondapak Prep C18
L3	Porous silica particles, 1.5–10 μm in diameter, or a monolithic silica rod.	Luna Silica(2), Aquity UPLC BEH HILIC, Atlantis HILIC Silica, Onyx Si (M), SunFire Silica, XBridge HILIC, Zorbax SIL
L4	Silica gel of controlled surface porosity bonded to a solid spherical core, 30–50 μm in diameter	Porasil Prep Silica
L5	Alumina of controlled surface porosity bonded to a solid spherical core, 30–50 μm in diameter.	–
L6	Strong cation exchange packing: sulfonated fluorocarbon polymer coated on a solid spherical core, 30–50 μm in diameter.	Adsorbosphere XL SCX, Partisil SCX, Zipax SCX, Zodiac Prep SCX
L7	Octyl silane (C8) chemically bonded to totally porous silica particles, 1.5–10 μm in diameter, or a monolithic silica rod.	Luna C8(2), Aquity UPLC BEH C8, Nova-Pak C8, Resolve C8, SunFire C8, Symmetry C8, XBridge C8, XTerra MS C8, XTerra RP8, Onyx C8 (M), Nucleosil C8, Zorbax C8, Zorbax SB-C8, Zorbax C8, Zorbax Eclipse XDB-C8, Hypersil-MOS, LiChrosorb-RP8/RP-SelectB, etc.
L8	An essentially monomolecular layer of aminopropylsilane (NH_2) chemically bonded to totally porous silica gel support, 3–10 μm in diameter.	Luna 10 μm NH_2, μBondapak NH_2, Waters Spherisorb NH_2, Zorbax NH_2, etc.
L9	Irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation exchange coating, 3–10 μm in diameter.	Partisil 10 μm SCX (I), Spherisorb SCX, Luna 10 μm SCX, Zorbax SCX
L10	Nitrile groups (CN) chemically bonded to porous silica particles, 3–10 μm in diameter.	Luna CN 100 Å, Capcell CN UG, μBondapak CN, Nova-Pak CN, Resolve CN, Waters Spherisorb CN

(Continued)

APPENDIX 8.2.1 USP classification of HPLC chromatographic columns (all stationary phases as spherical, unless indicated as (M) for monolith, and (I) for irregular shape).—*cont'd*

Code	Description	Examples
L11	Phenyl groups (C6H5) chemically bonded to porous silica particles, 1.5–10 μm in diameter.	Synergi Polar-RP, Luna Phenyl-Hexyl, Gemini C6-Phenyl, Prodigy PH-3, Aquity UPLC BEH Phenyl, μBondapak Phenyl, XBridge Phenyl, XTerra Phenyl
L12	Strong anion exchange packing made by chemically bonding a quaternary amine to a solid silica spherical core, 30–50 μm in diameter	AccelPlus QMA
L13	Trimethylsilane (C1) chemically bonded to porous silica particles, 3–10 μm in diameter.	Develosil TMS-UG (C1) 130 Å, TSKgel TMS-250, Waters Spherisorb C1
L14	Silica gel having a chemically bonded, strongly basic quaternary ammonium anion exchange coating, 5–10 μm in diameter.	Partisil 10 μm SAX (I), PartiSphere 5 μm SAX, Waters Spherisorb SAX
L15	Hexylsilane (C6) chemically bonded to totally porous silica particles, 3–10 μm in diameter.	PhenoSphere C6, Waters Spherisorb C6
L16	Dimethylsilane (C2) chemically bonded to totally porous silica particles, 5–10 μm in diameter.	Maxsil RP2 60 Å (I), Lichrosorb RP2
L17	Strong cation exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form, 7–11 μm in diameter.	Rezex RHM Monosaccharide, IC-Pak Ion Exclusion, IC-Pak Cation, Shodex RSpak DC-613, Rezex ROA
L18	Amino and cyano groups chemically bonded to porous silica particles, 3–10 μm in diameter.	Partisil PAC (I)
L19	Strong cation exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the calcium form, 9 μm in diameter.	Rezex RCM, Rezex RCU, Sugar-Pak 1, Shodex SC-1011
L20	Dihydroxypropyl groups chemically bonded to porous silica particles, 3–10 μm in diameter.	Luna HILIC Shodex PROTEIN KW-800 series, TSKgel QC-PAK 200 and 300, BioSuite 125, Insulin HMWP (I), Protein-Pak (I)
L21	A rigid, spherical styrene-divinylbenzene copolymer, 3–10 μm in diameter.	Polymerx RP-1, Phenogel 100 Å, IC-Pak Ion Exclusion, Shodex SP-0810
L22	A cation exchange resin made of porous polystyrene gel with sulfonic acid groups, about 10 μm in size.	Rezex ROA
L23	An anion exchange resin made of porous polymethacrylate or polyacrylate gel with quaternary ammonium groups, about 10 μm in size.	Shodex IEC QA-825, TSKgel BioAssist Q, TSKgel SuperQ-5PW, BioSuite Q AXC, BioSuite DEAE, Protein-Pak Q 8HR
L24	A semirigid hydrophilic gel consisting of vinyl polymers with numerous hydroxyl groups on the matrix surface, 32–63 μm in diameter.	YMC-Pac PVA-Sil, Toyopearl HW-type
L25	Packing having the capacity to separate compounds with a MW range from 100 to 5000 Daltons (as determined by polyethylene oxide), applied to neutral, anionic, and cationic water-soluble polymers. A polymethacrylate resin base, cross-linked with poly-hydroxylated ether (surface contained some residual carboxyl functional groups) was found suitable.	PolySep-GFC-P2000, Shodex OHpak SB-802.5HQ, Ultrahydrogel DP +120, TSK-gel G1000PW

APPENDIX 8.2.1 USP classification of HPLC chromatographic columns (all stationary phases as spherical, unless indicated as (M) for monolith, and (I) for irregular shape).—*cont'd*

Code	Description	Examples
L26	Butyl silane (C4) chemically bonded to totally porous silica particles, 1.5–10 μm in diameter.	Jupiter 300 C4, Aquity UPLC BEH300 C4, Delta-Pak C4, Symmetry300 C4, XBridge BEH300 C4
L27	Porous silica particles, 30–50 μm in diameter.	Sepra (I), Porasil (I), Nucleodur, YMS-Pack Silica
L28	A multifunctional support, which consists of a high purity, 100 Å, spherical silica substrate that has been bonded with anionic (amine) functionality in addition to a conventional reversed phase C8 functionality.	Altech mixed mode C8/anion, Generik C8/Amino, ProTec C8
L29	Gamma alumina, reversed phase, low carbon percentage by weight, alumina-based polybutadiene spherical particles, 5 μm diameter with a pore diameter of 80 Å.	Gamabond ARP-1, Gamabond Alumina Potency, Aluspher® RP-Select-B
L30	Ethyl silane (C2) chemically bonded to a totally porous silica particle, 3–10 μm in diameter.	Maxsil RP2 60 Å (I), Nucleosuil C2, APEX Prepsil C2
L31	A strong anion exchange resin quaternary amine bonded on latex particles attached to a core of 8.5 μm macroporous particles having a pore size of 2000 Å and consisting of ethylvinylbenzene cross-linked with 55 % divinyl benzene.	Ion Pac AS 10, Ion Pack AS 16
L32	A chiral ligand exchange packing- L-proline copper complex covalently bonded to irregularly shaped silica particles, 5–10 μm in diameter.	CHIRALCEL WH, Astec CLD-D (or –L), Nucleosil Chiral-1
L33	Packing having the capacity to separate proteins of 4000–400,000 daltons. It is spherical, silica based, and processed to provide pH stability.	BioSep-SEC-S2000, BioSep-SEC-S3000 BioBasic SEC 120, Nucleosil 125-5 GFC, Shodex KW-404
L34	Strong cation exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the lead form, about 9 μm in diameter.	Aminex Fast Carbohydrate, Rezex RPM Monosaccharide, Shodex Sugar SP0810, Nucleogel Sugar Pb
L35	A zirconium-stabilized spherical silica packing with a hydrophilic (diol-type) molecular monolayer bonded phase having a pore size of 150 Å.	Bio-Sep-SEC-S2000, Zorbax GF-250, Zorbax GF-450
L36	3,5-dinitrobenzoyl derivative of L-phenylglycine covalently bonded to 5 μm aminopropyl silica.	Nucleosil Chiral-3
L37	Polymethacrylate gel packing having the capacity to separate proteins by molecular size over a range of 2000–40,000D.	PolySep-GFC-P3000, Shodex OHpak SB-803HQ, Ultrahydrogel 250
L38	Methacrylate-based size exclusion packing for water-soluble samples.	PolySep-GFC-P1000, Shodex OHpak SB-802HQ, Ultrahydrogel
L39	Hydrophilic polyhydroxymethacrylate gel of totally porous spherical resin.	PolySep-GFC-P Series, Shodex OHpak SB-800HQ series, Shodex RSpak DM-614

(Continued)

APPENDIX 8.2.1 USP classification of HPLC chromatographic columns (all stationary phases as spherical, unless indicated as (M) for monolith, and (I) for irregular shape).—*cont'd*

Code	Description	Examples
L40	Cellulose tris-3,5-dimethylphenylcarbamate coated porous silica particles, 5–20 μm in diameter.	CHIRALCEL OD, Lux cellulose 1, Nucleocel Delta
L41	Immobilized α-acid glycoprotein on spherical silica particles, 5 μm in diameter.	Chiral-AGP
L42	Octylsilane and octadecylsilane groups chemically bonded to porous silica particles, 5 μm in diameter.	Chromegabond PSC, HiChrom RPB-250A
L43	Pentafluorophenyl groups chemically bonded to silica particles, 5–10 μm in diameter.	Curosil-PFP, Ultra-PFP, Pinnacle DB PFP (Restek) Allure PFP Propyl
L44	A multifunctional support, which consists of a high purity, 60 Å, spherical silica substrate that has been bonded with a cationic exchanger, sulfonic acid functionality in addition to a conventional reversed phase C8 functionality.	Chromegabond RP-SCX, Generik C8/SCX
L45	Beta cyclodextrin bonded to porous silica particles, 5–10 μm in diameter	Astec Cyclobond I, II, or II ser., Chiral CD-Ph, Nucleodex Beta-PM, ChiralDex
L46	Polystyrene/divinylbenzene substrate agglomerated with quaternary amine functionalized latex beads, 10 μm in diameter.	CarboPac PA1, Transgenomic AN1
L47	High capacity anion exchange microporous substrate, fully functionalized with a trimethylamine group, 8 μm in diameter.	CarboPac MA1, Hamilton PRP-X100, X110, Hamilton RCX-10
L48	Sulfonated, cross-linked polystyrene with an outer layer of submicron, porous, anion exchange microbeads, 15 μm in diameter.	IonPac AS5, IonPac AS7
L49	A reversed-phase packing made by coating a thin layer of polybutadiene onto spherical porous zirconia particles, 3–10 μm in diameter.	Zirchrom PBD, Discovery ZR-PBD
L50	Multifunction resin with reversed-phase retention and strong anion exchange functionalities. The resin consists of ethylvinylbenzene, 55 % cross-linked with divinylbenzene copolymer, 3–15 μm in diameter, and a surface area of not less than 350 m^2/g; substrate is coated with quaternary ammonium functionalized latex particles consisting of styrene cross-linked with divinylbenzene.	OmniPac PAX-500, Proteomix SAX-POR
L51	Amylose tris-3,5-dimethylphenylcarbamate-coated, porous, spherical, silica particles, 5–10 μm in diameter.	Chiralpak ADS, Nucleocel Alpha
L52	A strong cation exchange resin made of porous silica with sulfopropyl groups, 5–10 μm in diameter.	TSKgel SP-2SW, BioBasic SCX, Supelcosil LC-SCX

APPENDIX 8.2.1 USP classification of HPLC chromatographic columns (all stationary phases as spherical, unless indicated as (M) for monolith, and (I) for irregular shape).—*cont'd*

Code	Description	Examples
L53	Weak cation exchange resin consisting of ethylvinylbenzene, 55 % cross-linked with divinylbenzene copolymer, 3–15 μm diameter. Substrate is surface grafted with carboxylic acid and/or phosphoric acid functionalized monomers. Capacity not less than 500 μEq per column.	IonPac CS14
L54	A size exclusion medium made of covalent bonding of dextran to highly cross-linked porous agarose beads, about 13 μm in diameter.	Superdex peptide HR 10/30
L55	A strong cation exchange resin made of porous silica coated with polybutadiene-maleic acid copolymer, about 5 μm in diameter.	IC-Pak C M/D, Waters Spherisorb SCX, Universal Cation
L56	Isopropyl silane (C3) chemically bonded to totally porous silica particles, 3–10 μm in diameter.	Zorbax SB C3
L57	A chiral recognition protein, ovomucoid, chemically bonded to silica particles, about 5 μm in diameter, with a pore size of 120 Å.	Ultron ES-OVM
L58	Strong cation exchange resin consisting of sulfonated cross-linked styrene- divinylbenzene copolymer in the sodium form, about 7–11 μm diameter.	Rezex RNM-Carbohydrate, Aminex HPX-87N, Shodex SUGAR KS-801, -802
L59	Packing with the capacity to separate proteins by molecular weight over the range of 10–500 kDa. Spherical 10 μm, silica-based, and processed to provide hydrophilic characteristics and pH stability	BioSep-SEC-S3000, Biosuite 125, Nanofilm SEC-150, TSK-GEL G2000SW, G3000SW, etc.
L60	Spherical, porous silica gel, 3–10 μm in diameter, surface has been covalently modified with palmitamidopropyl groups and end-capped.	Acclaim polar Advantage, Ascentis RP-Amide, HALO RP-Amide, Prism RP, Supelcosil LC-ABZ
L61	Hydroxide-selective, strong anion exchange resin consisting of a highly cross-linked core of 13 μm microporous particles, pore size less than 10 Å, and consisting of ethylvinylbenzene cross-linked with 55 % divinylbenzene with a latex coating composed of 85 nm diameter microbeads bonded with alkanol quaternary ammonium ions (6 %).	Ion Pac AS-11, Ion Pac AG-11
L62	C30 silane bonded phase on a fully porous spherical silica, 3–15 μm in diameter.	Develosil Combi-RP, Develosil RP-Aqueous, Develosil RP-Aqueous-AR, ProntoSil c30, YMC-Pack Carotenoid, Zodiac 120 C30
L63	Glycopeptide teicoplanin linked to spherical silica (chiral phase)	CHIROBIOTIC V, T, TAG, R.
L64	Strongly basic anion exchange resin consisting of 8% cross-linked styrene-divinylbenzene copolymer with a quaternary ammonium group in the chloride form, 45–180 μm in diameter	AG 1-X8

(Continued)

APPENDIX 8.2.1 USP classification of HPLC chromatographic columns (all stationary phases as spherical, unless indicated as (M) for monolith, and (I) for irregular shape).—*cont'd*

Code	Description	Examples
L65	Strongly acidic cation exchange resin, consisting of 8% sulfonated cross-linked styrene-divinylbenzene copolymer with a sulfonic group in hydrogen form, 63−250 μm diameter	AG 50W-X2
L66	Crown ether coated on 5 μm silica gel substrate	CrownPak CR
L67	Porous vinyl alcohol copolymer with C18 alkyl group attached to the hydroxyl group of the polymer, 2−10 μm diameter	Supelcogel ODP-50, apHera C18, Asahipak ODP-40
L68	Spherical porous silica containing a polar group within or intrinsic to the hydrocarbon bonded phase (e.g., alkylamide)	Ultra II IDB
L69	Polymeric (ethylvinylbenzene/divinylbenzene) with strong anion exchange (quaternary amine) on latex beads, about 6.5 μm diameter	CarboPac PA20
L70	Cellulose tris(phenylcarbamate) coated on 5 μm silica	Chiralcel OC-H
L71	A rigid, spherical polymethacrylate, 4−6 μm diameter	RSpak DE-613
L72	(R)-Phenylglycine and 3,5-dinitroaniline urea linkage covalently to silica	Chirex 3012, Sumichiral OA-3300
L73	A rigid, spherical polydivinylbenzene particle 5−10 μm diameter	Jordi-gel DVB
L##	(Dalteparin sodium, anion exchange Dowex 1X8) − strongly basic (type I) anion exchange resin in chloride form	Dowex 1X8
L##	(Dalteparin sodium, cation exchange Dowex 50WX2) − strongly acidic cation exchange resin in H^+ form	Dowex 50WX2
L##	(Glucosamine, Shodex NH_2 P-50) Polyamine chemically bonded to cross-linked polyvinyl alcohol polymer, 5 μm diameter	apHera NH_2 Amino, Shodex NH_2 P50
L##	(Ethylhexyl triazone, FluoFix) Fluorocarbon chains chemically bonded to 5 μm spherical silica particles	Wakopak FluoFix-II 120E, 120E, 120N

From M. Marques, ed., "USP Chromatographic Columns," U. S. Pharmacopeia, Rockville, 2009−2010.

Appendix to chapter 9

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
1.	AccQ (C18)	Sph. 1.7	135	0.70	185	17	–	–	1	–	–
2.	ACE C18	5	100	–	300	15.5	111,000	–	1	–	3.56
3.	ACE C18-300	5	300	–	100	9	103,500	–	1	–	1.80
4.	ACE C18-HL	5	90	–	400	20	102,000	–	2	–	6.36
5.	ACE SUPERC18	1.7, 2, 3, 5	90	–	400	14.8	–	–	1	1.5–7.5	–
6.	ACE C18-AMIDE	1.7, 2, 3, 5	100	–	300	17.0	–	–	1	2–8	–
7.	AQUITY UPLC BEH C18	Sph. 1.7	130	0.70	185	17	–	–	1	–	–
8.	AQUITY UPLC CSH C18	Sph. 1.8	100	0.70	230	15	–	–	1	–	–
9.	AQUITY UPLC HSS C18	Sph. 1.8	100	0.70	230	15	–	–	1	1–8	–
10.	AQUITY UPLC HSS T3	Sph. 1.8	100	0.70	230	11	–	–	1	2–8	–
11.	AQUITY UPLC CSH Phenyl-Hexyl	Sph. 1.7	130	0.70	185	14	–	–	1	–	–
12.	AQUITY UPLC CSH Fluoro Phenyl	Sph. 1.7	130	0.70	185	10	–	–	1	–	–
13.	Atlantis T3 (C18)	Sph. 3, 5, 10	100	1.00	330	12	–	1.6	1	–	–
14.	Atlantis dC18	Sph. 3,5	100	–	330	12	–	–	1	3–7	–
15.	Aeris widepore XB-C18	C–S 3.6	–	–	25	–	–	–	3	1.5–9	–
16.	Aeris widepore XB-C8	C–S 3.6	–	–	25	–	–	–	4	1.5–9	–

(Continued)

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
17.	Aeris widepore XB-C4	C−S 3.6	–	–	25	–	–	–	2	1.5–9	–
18.	Aeris PEPTIDE XB-C18	C−S 1.7, 2.6, 3.6, 5	100	–	200	10	–	–	1	1.5–9	–
19.	Aqua C18	Sph. 3, 5	125	1.05	320	15	–	–	2	2.5–7.5	–
20.	Aqua C18	Sph. 5	200	1.15	215	11	–	–	4	2.5–7.5	–
21.	Ascentis C18	Sph. 5	100		450	25	–	3.7	2	2–8	–
22.	Ascentis Express C18 C−S	Sph. 5	90	–	–	–	–	–	2	2–9	–
23.	Boltimate C18	C−S 2.7	90		120	9	200,000	–	1	2–8.5	–
24.	Boltimate Phenyl-Hexyl	C−S 2.7	90		120	7	200,000	–	1	2–8.5	–
25.	Boltimate EXT-C18	C−S 2.7	90		120	8	200,000	–	1	1.5–12	–
26.	Boltimate PFP	C−S 2.7	90		120	5	200,000	–	1	1.5–10	–
27.	Bondaclone C18	Irreg. 10	148	1.10	300	10 m.	–	1.61	4	2.5–7.5	–
28.	µBondapak C18	Irreg. 10	125	–	330	10	36,000	–	3	2.5–7.5	1.68
29.	Capcell Pak AG C18	5	120	–	300	15	51,000	–	4	–	3.25
30.	Clarity Oligo-RP	Sph. 3, 5, 10	110	–	375	14	–	–	2	1–12	–
31.	Clarity Oligo-WAX	Sph. 10	360	–	–	–	–	0.80	2	1–11	–
32.	Clarity Oligo-MS	C−S 1.3, 1.7, 2.6, 5	100	–	200	12	–	–	3	1.5–10	–
33.	Columbus C8	Sph. 5	110	–	375	13	–	–	1	2.5–7.5	–
34.	Columbus C18	Sph. 5	110	–	375	19	–	–	1	2.5–7.5	–
35.	CORTEX C18+ (trifunct.)	Sph. 1.6, 2.7	90	0.26	100	5.7	–	–	1	–	–
36.	CORTEX C18 (trifunct.)	Sph. 1.6, 2.7	90	0.26	100	6.6	–	–	1	–	–
37.	Delta-Pak C4	Sph. 5, 15	100	1.00	300	7.3	–	–	1	–	–
38.	Delta-Pak C18	Sph. 5, 15	100	1.00	300	17	–	–	1	–	–

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
39.	Develosil ODS-HG	5	140	–	300	18	85,500	–	3	–	2.97
40.	Develosil ODS-MG	5	100	–	450	15	66,000	–	1	–	6.17
41.	Develosil ODS-UG	5	140	–	300	18	92,000	–	2	–	3.74
42.	Discovery HS C18	Sph. 5	180	–	200	12	–	3.0	2	2–8	–
43.	Exsil ODS	5	100	–	200	11	93,000	–	2	–	2.97
44.	Exsil ODS1	5	100	–	200	11	114,000	–	4	–	1.92
45.	Exsil ODSB	5	100	–	200	12	82,000	–	4	–	1.68
46.	Gemini C18	Sph. 3, 5,10	110	1.10	375	14	75,500	–	1	1–12	4.10
47.	Gemini C6-Phenyl	Sph. 3, 5	110	–	375	12	–	–	3	1–12	–
48.	Gemini NX-C18	Sph. 3, 5,10	110	–	375	14	–	–	4	1–12	–
49.	HyperClone BDS C8	Sph. 3, 5	130	0.60	155	7	–	–	3	2.0–7.5	–
50.	HyperClone BDS C18	Sph. 3, 5	130	0.60	155	11	–	–	4	2.0–7.5	–
51.	HyperClone MOS (C8)	Sph. 3, 5	120	0.60	155	6.6	–	–	4	2.0–7.5	–
52.	HyperClone ODS (C18)	Sph. 3, 5	120	0.60	155	10	–	–	3	2.0–7.5	–
53.	HyperClone CN (CPS)	Sph. 3, 5	120	0.60	155	4	–	–	1	2.0–7.5	–
54.	Hichrom RPB	5	110	–	340	14	97,500	–	2	–	3.00
55.	Hypersil BDS C18	5	130	–	170	11	76,500	–	2	–	2.66
56.	Hypersil GOLD	5	180	–	200	10	91,000	–	4	–	1.99
57.	Hypersil HyPurity C18	5	180	–	200	13	73,000	–	1	–	2.34
58.	Hypersil ODS	5	120	–	170	10	94,500	–	2	–	2.66
59.	Hypersil SAS C1	Sph. 5	120	–	170	3		–	2	2–8	–
60.	IB-Sil C18	Sph. 3, 5	125	0.75	165	11 m.	–	3.27	3	2.5–7.5	–
61.	IB-Sil C18	Sph. 5	125	0.75	165	7.5 m.	–	4.29	4	2.5–7.5	–
62.	Inertsil ODS	5	100	–	350	14	73,500	–	4	–	3.63
63.	Inertsil ODS-P	5	100	1.15	450	29	–	–	4	1	–
64.	InertSustain C18	2,3,5	100	–	350	–	–	–	1	1–10	–

(Continued)

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
65.	InertSustain AQ-C18	1.9,3,5	100	–	350	–	120,000	–	1	1–10	–
66.	InertSustain C8	2,3,5	100	–	350	–	–	–	1	1–10	–
67.	InertSustain AX-C18[f]	Sph. 3, 5	200	1.0	200	8			1	1–9	
68.	Inertsil ODS-HL	3,5	100	–	450	–	–	–	1	1–8	–
69.	Inertsil ODS3	5	100	–	450	15	60,500	–	3	–	8.94
70.	Inertsil ODS2	5	150	–	320	18.5	32,000	–	3	–	4.35
71.	Inertsil C4	5	150	–	320	7.5	–	–	1	1–10	–
72.	Jupiter C4	Sph. 5,10, 15	300	–	170	5	–	6.30	3	1.5–10	–
73.	Jupiter C5	Sph. 5,10	300	–	170	5.5	–	5.30	2	1.5–10	–
74.	Jupiter C18	Sph. 5,10, 15	300	–	170	13.34	–	5.50	2	1.5–10	–
75.	Jupiter Proteo	Sph. 4, 10	90	–	475	15	–	–	1	1.5–10	–
76.	Kinetex EVO C18	C–S 1.7, 2.6, 5	100	–	200	11	–	–	2	1–12	–
77.	Kinetex C18	C–S 1.3,1.7, 2.6, 5	100	–	200	12	–	–	4	1.5–8.5	–
78.	Kinetex XB-C18	C–S 1.7, 2.6, 5	100	–	200	10	–	–	3	1.5–8.5	–
79.	Kinetex C8	C–S 1.7, 2.6, 5	100	–	200	8	–	–	3	1.5–8.5	–
80.	Kinetex Biphenyl	C–S 1.7, 2.6, 5	100	–	200	11	–	–	2	1.5–8.5	–
81.	Kinetex Phenyl-Hexyl	C–S 1.7, 2.6, 5	100	–	200	11	–	–	2	1.5–8.5	–
82.	Kinetex F5	C–S 1.7, 2.6	100	–	200	9	–	–	2	1.5–8.5	–
83.	Kromasil C18	5	100	–	340	19	99,000	–	4	–	7.32
84.	LiChrosorb RP-18	10	100	–	300	17	74,000	–	1	–	2.99
85.	LiChroSph. RP-18	5	100	–	350	21.6	80,000	–	2	–	4.90
86.	Luna PFP(2)	Sph. 3, 5	100	1.00	400	11.5	–	2.20	3	1.5–9.0	–
87.	Luna Phenyl-Hexyl	Sph. 3, 5,10, 15	100	1.00	400	17.5	–	4.00	4	1.5–9.0	–
88.	Luna C5	Sph. 5,10	100	1.00	440	12.5	–	7.85	4	1.5–9.0	–
89.	Luna C8	Sph. 5,10	100	1.00	440	14.75	–	5.50	3	1.5–9.0	–
90.	Luna C8(2)	Sph. 3, 5,10,15	100	1.00	440	13.5	–	5.50	3	1.5–9.0	–

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
91.	Luna C18	Sph. 5,10	100	1.00	440	19	–	3.00	3	1.5–9.0	–
92.	Luna C18(2)-HST	Sph. 2.5	100	1.00	400	17.5	–	3.00	2	1.5–9.0	–
93.	Luna C18(2)	Sph. 3, 5,10,15	100	1.00	400	17.5	88,000	3.00	2	1.5–9.0	4.85
94.	Luna Omega C18	Sph. 1.6, 3, 5	100	–	260	–	350,000	–	1	1.5–8.5	–
95.	Luna Omega Polar C18	Sph. 1.6, 3, 5	100	–	260	11.0	–	–	1	–	–
96.	Luna C5	Sph. 5, 10	100	–	440	12.5	–	–	1	–	–
97.	Luna CN	Sph. 3, 5,10	100	1.00	400	7	–	3.80	1	1.5–7.0	–
98.	Luna Phenyl-Hexyl	Sph. 3, 5,10,15	100	–	400	17.5	–	–	–	–	–
99.	Luna PFP	Sph. 3.5	100	–	400	–	–	–	–	–	–
100.	Novapak C18	Sph. 4	60	–	120	7.3	60,000	–	2	–	2.01
101.	Novapak C8	Sph. 4	60	–	120	4	–	–	1	2–8	–
102.	Novapak CN	Sph. 4	60	–	120	3	–	–	1	2–8	–
103.	Novapak Phenyl Hexyl	Sph. 4	60	–	120	5	–	–	2	2–8	–
104.	Nucleosil C18	5	100	–	350	15	101,000	–	4	–	4.14
105.	Nucleosil C18AB	5	100	–	350	24	87,000	–	3	–	2.77
106.	Nucleoshell RP 18	C–S 2.7	90	–	–	7.5	250,000	–	1	1–11	–
107.	Nucleodur C18 Gravity	1.8, 3, 5	110	–	–	18	80,000	–	1	1–11	–
108.	Onix C18	Monolith	130	1.00	300	18	–	3.60	3	2.0–7.5	–
109.	Partisil ODS	10	85	–	350	5	47,500	–	2	–	1.35
110.	Partisil ODS2	10	85	–	350	15	41,000	–	2	–	3.60
111.	Partisil ODS3	10	85	–	350	10.5	52,000	–	2	–	2.51
112.	PhenoSph.e C1	Sph. 3, 5,10	80	0.50	220	4 m.	–	1.80	4	2.5–7.5	–
113.	PhenoSph.e C6	Sph. 3, 5,10	80	0.50	220	6 m.	–	2.27	1	2.5–7.5	–
114.	PhenoSph.e C8	Sph. 3, 5,10	80	0.50	220	6 m.	–	3.54	2	2.5–7.5	–

(Continued)

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
115.	PhenoSph.e ODS(1)	Sph. 3, 5,10	80	0.50	220	7 m.	–	1.74	3	2.5–7.5	–
116.	PhenoSph.e ODS(2)	Sph. 3, 5,10	80	0.50	220	12 m.	–	2.50	4	2.5–7.5	–
117.	PhenoSph.e CN	Sph. 3, 5,10	80	0.50	220	4 m.	–	2.50	4	2.5–7.5	–
118.	PhenoSph.e NEXT C8	Sph. 3, 5	120	–	380	10	–	–	3	2.5–7.5	–
119.	PhenoSph.e NEXT C8	Sph. 3, 5	120	–	380	14	–	–	3	2.5–7.5	–
120.	PhenoSph.e NEXT Phenyl	Sph. 5	120	–	380	11	–	–	3	2.5–7.5	–
121.	PolymerX RP-1	Sph. 3, 5,10,15	100	–	410	–	–	–	2	0–14	–
122.	Poroshell EC-C18	C–S Sph 1.9, 2.7, 4	120	–	130	10	–	–	1	2–8	–
123.	Poroshell SB-C18	C–S Sph 1.9, 2.7, 4	120	–	130	8	–	–	4	1–8	–
124.	Poroshell HPH-C18	C–S Sph 1.9, 2.7, 4	100	–	95	–	–	–	1	3–11	–
125.	Poroshell CS-C18	C–S Sph 1.9, 2.7, 4	100	–	95	–	–	–	2	1–11	–
126.	Poroshell SB-Aq	C–S Sph 1.9, 2.7, 4	120	–	130	–	–	–	4	1–8	–
127.	Prodigy ODS(2)	Sph. 5	150	1.10	310	18.4 m.	48,000	3.50	2	2.0–9.0	4.01
128.	Prodigy C8	Sph. 5	150	1.10	310	12.5 m.	48,000	5.00	1	2.0–9.0	–
129.	Prodigy ODS(3)	Sph. 3, 5,10	100	1.00	450	15.5 m.	62,000	–	2	2.0–9.0	6.05
130.	Prodigy Phenyl (PH-3)	Sph. 5	100	–	450	10.0 poly.	62,000	–	4	2.0–9.0	–
131.	Resolve C18	5	90	0.5	200	10.2	45,500	–	4	–	3.16
132.	Resolve C8	5	90	0.5	200	5.1	45,500	–	4	–	3.16
133.	SiliaChrom AQ C18	3, 5, 10	100	–	380	18	–	–	1	1.5–9	–
134.	SiliaChrom AQ C8	3, 5,10	100	–	380	14	–	–	1	1.5–9	–
135.	SiliaChrom dt C18	2.5, 3, 5,10	100	–	410 –440	18	–	–	1	1.5–9	–
136.	SiliaChrom XT C18	3, 5, 10	150	–	200	15	–	–	1	1.5–12	–

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover $\mu M/m^2$	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
137.	SiliaChrom XT Fidelity C18	3, 5, 10	100	–	380	21	–	–	1	1.5–12	–
138.	SiliaChrom SB C18	3, 5, 10	150	–	200	12	–	–	1	0.5–7.5	–
139.	SiliaChrom SB C18-300	5	300	–	80	5	–	–	1	0.5–7.5	–
140.	SiliaChrom SB C8	5	150	–	200	7	–	–	1	1.0–7.5	–
141.	SiliaChrom SB C8-300	5	300	–	80	3	–	–	1	1.0–7.5	–
142.	SiliaChrom XDB C18	5	150	–	200	15	–	–	1	1.5–9.0	–
143.	SiliaChrom XDB C8	3, 5	150	–	200	8	–	–	1	1.5–9.0	–
144.	SiliaChrom XDB1 C18	3, 5	100	–	380 –400	22	45,000	–	1	1.5–10	2.14
145.	SiliaChrom XDB1 C18-300	5, 10	300	–	80	8	–	–	1	1.5–9.0	–
146.	SiliaChrom XDB2 C18	3, 5,10	100	–	380 –400	18	28,000	–	1	1.5–9.0	0.61
147.	SiliaChrom XDB1 C8	5, 10	100	–	380 –400	14	–	–	1	1.5–8.5	–
148.	SiliaChrom XDB1 C8-300	5	300	–	80	4	–	–	1	1.5–8.5	–
149.	SiliaChrom XDB1 C4	5	100	–	380 –400	7	–	–	1	1.5–8.5	–
150.	SiliaChrom XDB1 C4-300	3, 5, 10	300	–	80	3	–	–	1	2.0–8.0	–
151.	SiliaChrom XDB1 C1	5	100	–	380 –400	3	–	–	1	1.5–8.5	–
152.	SiliaChrom XDB1 C1-300	5	300	–	80	1	–	–	1	2.0–8.0	–
153.	SiliaChrom XDB1 CN	5, 10	100	–	380 –400	5	–	–	1	2.0–8.5	–
154.	SiliaChrom XDB1 CN-300	5	300	–	80	3.5	–	–	1	2.0–8.0	–
155.	SiliaChrom XDB1 Phenyl	3, 5	100	–	380 –400	12	–	–	1	1.5–9.0	–

(Continued)

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
156.	SiliaChrom XDB1 Phenyl–300	3, 5	300	–	80	4.5	–	–	1	2.0–8.0	–
157.	SphereClone C6	Sph. 5	80	–	200	6	–	–	2	2.5–7.5	–
158.	SphereClone C8	Sph. 3, 5	80	–	200	6	–	–	4	2.5–7.5	–
159.	SphereClone ODS (1)	Sph. 3, 5	80	–	200	7	–	–	3	2.5–7.5	–
160.	SphereClone ODS (2)	Sph. 3, 5	80	–	200	12	–	–	3	2.5–7.5	–
161.	Spherisorb	Sph. 3, 5	80	0.50	220	6.2	–	–	4	–	–
162.	SunFire C18	Sph. 2.5, 5	100	0.90	340	16	91,500	–	1	–	6.05
163.	SunFire C8	Sph. 2.5, 5	100	0.90	340	12	–	–	1	–	–
164.	Synergy Fusion-RP	Sph. 2.5	100	–	400	12	–	–	2	1.5–9.0	–
165.	Synergy MAX-RP	Sph. 2.5	100	–	400	17	–	–	2	1.5–9.0	–
166.	Synergy Hydro-RP	Sph. 2.5	100	–	400	19	–	–	2	1.5–7.5	–
167.	Synergy Polar-RP	Sph. 2.5	100	–	400	11	–	–	4	1.5–7.5	–
168.	Synergy Fusion-RP	Sph. 4, 10	80	1.05	475	12	–	–	1	1.5–9.0	–
169.	Synergy MAX-RP	Sph. 4, 10	80	1.05	475	17	–	3.21	2	1.5–9.0	–
170.	Synergy Hydro-RP	Sph. 4, 10	80	1.05	475	19	–	2.45	3	1.5–7.5	–
171.	Synergy Polar-RP	Sph. 4, 10	80	1.05	475	11	–	3.15	4	1.5–7.5	–
172.	Symmetry C18	Sph. 3.5, 5	100	0.90	335	19.1	92,000	–	1	–	6.11
173.	Symmetry C8	Sph. 3.5, 5	100	0.90	335	11.7	–	–	1	–	–
174.	Symmetry C4	Sph 5	300	–	110	2.8	–	–	2	2–8	–
175.	Symmetry300 C18	Sph. 3.5, 5	300	0.80	110	8.5	–	–	1	–	–
176.	SymmetryShield RP18	Sph. 5	100	0.90	335	17	–	–	1	–	–
177.	SymmetryShield RP8	Sph. 3.5, 5	100	0.90	335	15	–	–	1	–	–
178.	TSKgel Protein C4-300	3	300	–	–	3	–	–	1	–	–
179.	TSKgel ODS-140HTP	2.3	140	–	–	6	–	–	1	–	–
180.	TSKgel ODS-100V	3, 5	100	–	–	15 m.	–	–	1	–	–

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
181.	TSKgel ODS-120A	5,10	150	–	–	22	–	–	4	–	–
182.	Ultracarb C8	Sph. 5	60	0.80	550	14 m.	–	2.71	3	2.5–7.5	–
183.	Ultracarb ODS (20)	Sph. 3, 5	90	0.75	370	22 m.	–	3.53	3	2.5–7.5	–
184.	Ultracarb ODS (30)	Sph. 5	60	0.80	550	31 m.	–	4.06	3	2.5–7.5	–
185.	Vydac 218TP	5	300	–	70	8	63,000	–	2	–	1.49
186.	Waters Sph.isorb ODS1	5	80	–	220	6.2	100,500	–	2	–	2.23
187.	Waters Sph.isorb ODS2	5	80	–	220	11.5	91,500	–	1	–	3.63
188.	Waters Sph.isorb ODSB	5	80	–	220	11.5	92,000	–	2	–	3.03
189.	XBridge C18	Sph. 2.5, 3.5, 5	130	0.70	185	18	–	–	1	1–12	–
190.	XBridge C8	Sph. 2.5, 3.5, 5	130	0.70	185	13	–	–	1	1–12	–
191.	XBridge Shield RP18	Sph. 2.5, 3.5, 5	130	0.70	185	17	–	3.3	1	1–12	–
192.	XBridge BEH C18	Sph. 3.5, 5	130	0.70	185	18	–	–	1	1–12	–
193.	XBridge Phenyl (trifunct.)	Sph. 2.5, 3.5,5	135	0.70	185	15	–	3.0	1	1–12	–
194.	XSelect CSH C18	Sph. 2.5, 3.5, 5	130	0.70	185	15	–	–	1	1–11	–
195.	XSelect CSH Phenyl-Hexyl	Sph. 2.5, 3.5, 5	130	0.70	185	14	–	–	1	1–11	–
196.	XSelect CSH Fluoro-Phenyl	Sph. 2.5, 3.5, 5	130	0.70	185	10	–	–	1	1–8	–
197.	XTerra RP18	Sph. 3.5, 5	125	0.70	175	15	–	–	1	2–12	–
198.	XTerra Shield RP18	Sph. 3.5	125	0.70	175	15	–	–	1	2–12	
199.	XTerra RP8	Sph. 3.5, 5	125	0.70	175	13.5	–	–	1	2–12	–
200.	XTerra MS C18	Sph. 2.5, 3.5, 5	125	0.70	175	15.5	–	–	1	2–12	–
201.	XTerra MS C8	Sph. 2.5, 3.5, 5	125	0.70	175	12	–	–	1	2–12	–
202.	XTerra Phenyl	Sph. 3.5, 5	125	0.70	175	12	–	–	1	2–12	–
203.	YMC J'Sph.e ODS H80	4	80	–	510	22	64,500	–	4	–	7.03

(Continued)

APPENDIX 9.2.1 Common physical and chemical properties of various hydrophobic columns. Retention factor k' is given for toluene in a mobile phase consisting of 80% CH_3OH and 20% aqueous buffer 0.25 mM KH_2PO_4 at pH = 6 and at 24°C (in isocratic conditions).—*cont'd*

No.	Column name	Particle diam. d_p µm[a]	Pore size Å	Pore vol. mL/g	Surface area m^2/g	C%[b]	Theor. plate N/m[c]	Cover µM/m^2	End-cap[d]	pH stabil.[e]	Ret. factor k' toluene
204.	YMC J'Sph.e ODS M80	4	80	–	510	14	58,000	–	3	–	2.66
205.	YMC ODS A	5	120	–	300	17	99,500	–	3	–	3.53
206.	YMC ODS AM	5	120	–	300	17	83,500	–	2	–	3.49
207.	YMC Pro C18	5	120	–	335	16	105,000	–	4	–	3.53
208.	Zorbax Extend C18	5	80	–	180	12.5	80,500	–	3	–	4.57
209.	Zorbax ODS	5	70	–	330	20	85,500	–	3	–	5.47
210.	Zorbax Rx-C18	5	80	–	180	12	90,500	–	2	–	3.71
211.	Zorbax SB-C18	3.5, 5	80	–	180	10	103,000	–	2	–	3.16
212.	Zorbax Eclipse XDB-C18	1.8, 3.5, 5	80	–	180	10	96,000	–	2	2–9	4.06

[a] *C–S indicates core-shell, Sph. indicates spherical particles, Irreg. indicates irregular particles, no specification for unknown particle shape.*
[b] *m. after the value of C% indicates monomeric type derivatization for the bonded phase.*
[c] *Plate number N/m refers to 5 µm particles if more than one particle dimension is indicated. However, many columns are available in more than one particle size formats. For the estimation of* N *for columns with fully porous particles, the following expression can be used:* $N \approx 1000L/Ct.\ d_p$ *with* Ct. ≈ *2, 2.5, or 3 and* d_p *the diameter of the particle.*
[d] *1 indicates very low silanol activity to 4 high silanol activity.*
[e] *pH range 2–12 indicates silica-organic support.*
[f] *C18 and tertiary amino groups.*

APPENDIX 9.2.2 Examples of polymeric columns and their main characteristics.

Name	Polymer	Size μm	Pore Å	*N*/m	pH range	Funct. group	Max press(psi)	Equivalent
Asahipak ODP	Polyvinyl alcohol	5	250	56,000	2–13	C18	2250	C18
Asahipak ODP40	Polyvinyl alcohol	4	250	68,000	2–13	C18	1950	C18
Asahipak C8P	Polyvinyl alcohol	5	250	45,000	2–13	C8	2250	C8
Asahipak C4P	Polyvinyl alcohol	5	250	40,000	2–13	C4	2250	C4
PolymerX	Styrene divinyl benzene	3, 5, 7	100	–	0–14	None	–	C18
Shodex ODP2 HP	Polyhydroxymethacrylate	5	40	80,000	2–12	None	2250	C18
Shodex DE	Polymethacrylate	4	100	70,000	2–12	None	2250	C18
Shodex DS	Polymethacrylate	3.5	100	70,000	2–13	None	3000	Mixed mode
Shodex RP18-413	Styrene divinyl benzene	3.5	100	80,000	1–13	None	3300	Mixed mode
Shodex RP18-613	Styrene divinyl benzene	3.5	100	80,000	2–13	None	3300	Mixed mode
Shodex RP18-415	Styrene divinyl benzene	6	430	36,000	2–13	None	3300	Mixed mode
Shodex NN	Polyhydroxymethylacrylate	10	100	40,000	2–12	None	2250	Mixed mode
Shodex JJ	Polyvinyl alcohol	5	100	32,000	2–11	None	1400	Mixed mode
TSKgel Octadecyl-2PW	Styrene divinyl benzene	5	125	–	0–14	C18	–	C18
TSKgel Octadecyl-4PW	Styrene divinyl benzene	7, 13	500	–	0–14	C18	–	C18
TSKgel Phenyl-5PW	Styrene divinyl benzene	10, 13	1000	–	0–14	Phenyl	–	Phenyl
TSKgel Octadecyl-NPR	Styrene divinyl benzene	2.5	None	–	0–14	C18	–	C18

APPENDIX 9.2.3 Examples of columns resilient to 100% aqueous mobile phase.

Column name	Manuf.[a]	Particle size µm	Pore size Å	Description
Acclaim PolarAdvantage	1	2.2, 3, 5	120	Embedded sulphonamide
Acclaim PolarAdvantage ll	1	2.2, 3, 5	120	Embedded amide
Accucore AQ	1	2.6	80	C18 with polar end-capping
Accucore Polar Premium	1	2.6	150	Amide embedded C18
ACE AQ	2	2, 3, 5, 10	100	C18 with integral polar functionality
ACE C18-AR	2	3, 5, 10	100	C18 with integral phenyl group
AquaSep	3	3, 5	100	C8 with embedded ether group
Chromegabond ODS-PI	3	3, 5	120	Ureide embedded
CAPCELL PAK C18 AQ	4	3, 5	80	C18
COSMOSIL C18-PAQ	5	5	120	C18 phase with polymeric linkage
Develosil RPAQUEOUS	6	3, 5	140	C30, monofunctional
Develosil RPAQUEOUS-AR	6	3, 5	140	C30, trifunctional
Epic Polar	3	1.8, 3, 5, 10	120	Embedded ether group
HALO RP-Amide	2	2.7	90	Polar embedded amide
Hydrosphere C18	7	2, 3, 5	120	Hydrophilic C18 surface
Hypersil GOLD AQ	1	1.9, 3, 5, 8	175	Alkyl chain with polar end-capping
Inertsil ODS-EP	8	5	100	C18 phase with polar embedded group
NUCLEODUR C18 Pyramid	9	1.8, 3, 5	110	C18 with hydrophilic end-capping
NUCLEODUR PolarTec	9	1.8, 3, 5	110	Polar embedded group
NUCLEOSIL Nautilus	9	3, 5	110	C18 with polar embedded group
NUCLEOSIL Protect 1	9	5	100	Protective polar group
ProntoSil C18 (or C8) ace-EPS	10	3, 5	120	C18 or C8 with embedded amide group
ProTec-RP	3	3, 5	100	C8, C18, or Phenyl with embedded amide
Synergy 4u Hydro-RP	11	2.5, 4, 10		C18 with polar end-capping
Spursil C18 and C18-EP	12	3, 5, 10	100	C18 with proprietary polar modification
SymmetryShield	13	3.5, 5	100	C18 or C8 with polar embedded group
Syncronis aQ	1	1.7, 3.5	100	C18 with polar end-capping
YMC ODS-AQ	7	3, 5	120	C18 with hydrophilic end-capping
ZORBAX Bonus-RP	14	1.8, 3.5, 5	80	C14 chain with embedded amide
ZORBAX SB-Aq	14	1.8, 3.5, 5	80	Proprietary

[a] *Manufacturer; 1-Thermo scientific, 2-Advanced Chromatography Technology (ACT), 3-ES Industries, 4-Shiseido, 5-Nacalai Tesque, 6-Nomura, 7-YMC, 8-GL Sciences, 9-Macherey-Nagel, 10- Bischoff, 11-Phenomenx, 12-Dikma Technologies, 13-Waters, 14-Agilent Technologies.*

APPENDIX 9.2.4 Several more recent hydrophobic columns.[a]

Column name	Manufacturer	Phase	Particle size	Type
Acucore Biphenyl	Thermo Fisher Scientific	Biphenyl	2.6	Core-shell
Acquity Premier C18	Waters Corp.	C18	1.8	Hybrid
Acquity Premier Phenyl-Hexyl	Waters Corp.	Phenyl-hexyl	1.8	Hybrid
Acquity Premier C18-Carbamate	Waters Corp.	C18 carbamate (embedded)	1.8	Hybrid
AdvancedBio Oligonucleotide[b]	Agilent Technologies	C18	2.7	Core-shell
AdvanceBio Peptide Plus	Agilent Technologies	Charged C18	2.7	Core-shell
AdvanceBio EC-C18	Agilent Technologies	C18	2.7	Core-shell
Ascentis Express Biphenyl	Milipore Sigma	Biphenyl	2.7	Core-shell
Ascentis Express RP-Amide	Millipore Sigma	C18 amide embedded	2.7	Core-shell
Atlantis Premier BEH C18 AX	Waters Corp.	C18 and alkylamine	1.7, 2.5	Hybrid
Aurashell Amide 18	Horizon	Embedded amide C18	2.7, 5	Core-shell
Aurashell C18 Ultra	Horizon	C18	2.7, 5	Core-shell
Aurashell C18/AR	Horizon	C18 and phenyl	2,7, 5	Core-shell
Boltimate Core-Shell Series	Welch	C18, phenyl-hexyl, pentafluorophenyl (PFP)	2.7	Hybrid
Cortecs	Waters Corp.	C8, phenyl	1.6, 2.7	Core-shell
C8-30HT[b]	ChromaNik Technologies Inc.	C8	3.4	Core-shell
DeltaSil 100 C18	Watrex Praha	C18	3, 5	Porous
HALO 2 Peptide ES-C18[b]	Advanced Materials Technol.	C18	2	Core-shell
Halo PFAS	Advanced Materials Technol.	C18	2.7	Core-shell
Halo PFAS Delay Column	Advanced Materials Technol.	Highly retentive alkyl	2.7	Core-shell
Halo PAH	Advanced Materials Technol.	Trifunctional C8	2.7	Core-shell
Horizon Amide 18	Horizon	Embedded amide C18	1.6, 3.5	Porous
Horizon C18/AR	Horizon	C18 and phenyl	1.6, 3.5	Porous
InfinityLab Poroshell 120 CS-C18	Agilent	Charged C18	2.5	Core-shell
Kinetex EVO C18	Phenomenex	C18	1.7, 2.6	Core-shell
Kinetex PS C18	Phenomenex	C18 (positive charge surf.)	2.6	Core-shell

(Continued)

APPENDIX 9.2.4 Several more recent hydrophobic columns.[a]—*cont'd*

Column name	Manufacturer	Phase	Particle size	Type
Luna Omega	Phenomenex	C18	1.6	Porous
MAbPac RP[b]	Thermo Fisher Scientific	Phenyl	4	Polymeric
Poroshell HPH C18 and C8	Agilent Technologies	C18, C8	4	Core-shell
Poroshell 120 SB-C18	Agilent Technologies	C18	1.9, 4	Core-shell
Poroshell 120 SB-Aq	Agilent Technologies	?	1.9, 4	Core-shell
Raptor FluoroPhenyl	Restek Corp.	PFP	2.7, 5	Core-shell
Roc	Restek Corp.	C18, C8, phenyl-hexyl, cyano, silica	3, 5	Porous
Selectra Aqueous C18	UCT Inc.	Polar-enhanced C18	1.8, 3, 5	Porous
Selectra EtG	UCT Inc.	Proprietary	3	Porous
SiliaChrom Plus PFP	SiliCycle Inc.	PFP	5, 10	Porous
SMT-C30	Separation Methods Technologies. Inc.	C30	5	Porous
SpeedCore Aqua	Fortis	Polar end-capped	2.6	Core-shell
Speed Core Bio Peptide	Fortis	C18, C8, and C4	2.6	Core-shell
Speed Core C8	Fortis	C8	2.6	Core-shell
Sun Shell	ChromaNik Technologies Inc.	S18, C30	2.6	Core-shell
Sun Shell PFP&C18	ChromaNik Technologies Inc.	C18 and PFP	2.6	Core-shell
Sun Shell HFC18-30, C8-30, C4-30[b]	ChromaNik Technologies Inc.	C18, C8, C8	2.6	Core-shell
SunShell Biphenyl	ChromaNik Technologies Inc.	Biphenyl	2.6	Core-shell
SunShell Biphenyl	ChromaNik Technologies Inc.	Biphenyl	5	Porous
SunShell C18	ChromaNik Technologies Inc.	C18	3.5	Core-shell
SunArmor	ChromaNik Technologies Inc.	C18 and RP-AQUA	3, 5	Porous
Suniest PFP&C18	ChromaNik Technologies Inc.	C18 and PFP	5	Porous
Ultisil Series	Welch	C18, C8, phenyl, C4, C3, CN, PFP, C30, Polar-RP	1.8, 3.5	Porous
Xtimate Hybrid Series	Welch	C18, C8, phenyl-hexyl, C4, CN, Polar-RP	1.8, 3,5, 10	Hybrid
YMC-triart Prep Bio200 C8	YMC Co. Ltd.	C8	10	Hybrid

[a] *These columns are available in different formats such as 150 × 4.6 mm, 100 × 2.1 mm.*
[b] *Columns used for large molecules separations.*

APPENDIX 9.3.1 List of various columns used in RP-HPLC with the values for log k' for acenaphthene and for log α for the separation of amitriptyline/acenaphthene in a mobile phase 65% CH_3OH/35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH =7.0 (v/v).

No.	Column description	log k'	log α
1.	GL Sciences Intersil ODS-EP (Embedded)	−0.26	1.43
2.	Waters Xterra RP8 (Embedded)	−0.12	0.65
3.	Shodex Silica C18P	−0.11	1.39
4.	Waters Xterra RP18 (Embedded)	−0.10	0.88
5.	Supelco Supelcosil LC-ABZ (Embedded)	−0.09	0.99
6.	Supelco Supelcosil LC-ABZ+ (Embedded)	−0.08	0.96
7.	Supelco Discovery HS PEG	−0.07	0.14
8.	Waters XBridge Shield RP18 (Embedded)	−0.06	1.04
9.	Waters SymmetryShield RP18 (Embedded)	−0.05	1.19
10.	Supelco Discovery RP Amide C16 (Embedded)	−0.04	0.79
11.	Waters SymmetryShield RP8 (Embedded)	−0.02	0.91
12.	AMT RP-Amide	−0.02	1.08
13.	Supelco Ascentis Expres RP-Amide (Embedded)	−0.02	1.10
14.	Dionex Acclaim PA (Embedded)	−0.01	1.27
15.	ES Industries EPIC C18	−0.01	1.34
16.	YMC Carotenoid C30	0.00	0.98
17.	Supelco Ascentis RP-Amide (Embedded)	0.01	1.23
18.	Agilent Zorbax Bonus RP (Embedded)	0.02	0.88
19.	Thermo Hypersil Prism	0.02	0.96
20.	Waters XSelect HSS C18	0.02	1.26
21.	Phenomenex Gemini NX C18	0.03	0.91
22.	Metachem Polaris C18-A	0.05	1.06
23.	Waters XSelect CSH C18	0.05	1.08
24.	Keystone Prism (Embedded)	0.06	0.94
25.	Thermo Hypersil Elite C18	0.06	1.18
26.	Supelco Ascentis Express C18	0.06	1.27
27.	Shiseido Capcell Pack C18	0.07	0.78
28.	Waters XSelect CSH Phenyl-Hexyl	0.07	0.82
29.	AMT Halo C18	0.07	1.24

(Continued)

APPENDIX 9.3.1 List of various columns used in RP-HPLC with the values for log k' for acenaphthene and for log α for the separation of amitriptyline/acenaphthene in a mobile phase 65% CH_3OH/35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH =7.0 (v/v).—*cont'd*

No.	Column description	log k'	log α
30	Phenomenex Prodigy C18	0.08	1.20
31.	Supelco Discovery HS C18	0.08	1.31
32.	Waters Acquity UPLC CSH Phenyl-Hexyl	0.09	0.81
33.	Thermo Hypersil HyPurity C18	0.09	1.00
34.	Waters Symmetry C18	0.09	1.39
35.	Agilent Zorbax Eclipse XDB C18	0.10	1.21
36.	Supelco Ascentis C18	0.10	1.41
37.	YMC Pack Pro C18 RS	0.10	1.55
38.	Supelco Discovery C18	0.11	1.01
39.	Thermo Hypersil BDS C18	0.11	1.08
40.	Waters Aquity UPLC CSH C18	0.11	1.08
41.	Azko Nobel Kromasil C18	0.11	1.39
42.	GL Sciences Intersil ODS-2	0.12	1.26
43.	Imtakt Cadenza CD-C18	0.12	1.33
44.	Waters Xbridge C18 (Acquity UPLC BEH C18) (Embedded)	0.13	1.11
45.	GL Sciences Intersil ODS-3	0.13	1.36
46.	ACT Ace C18	0.14	1.17
47.	Nomura Devosil C30 UG 5	0.14	1.23
48.	Varian Pursuit C18	0.15	1.01
49.	Agilent Zorbax Extend C18	0.15	1.28
50.	Waters SunFire C18	0.15	1.32
51.	Mackery Nagel Nucleodur Gravity C18	0.15	1.34
52.	GL Sciences Intersil ODS 3V	0.15	1.42
53.	Waters Xterra MS C8	0.16	0.65
54.	Phenomenex Luna C8 (2)	0.16	0.91
55.	CITI L-Column ODS	0.16	1.39
56.	Agilent Zorbax Rx C18	0.17	1.19
57.	Phenomenex Luna C18	0.17	1.23
58.	Dionex Acclaim C18	0.17	1.34

APPENDIX 9.3.1 List of various columns used in RP-HPLC with the values for log k' for acenaphthene and for log α for the separation of amitriptyline/acenaphthene in a mobile phase 65% CH_3OH/35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH =7.0 (v/v).—*cont'd*

No.	Column description	log k'	log α
59.	Waters XTerra MS C18	0.18	1.09
60.	Waters Xbridge C8 (Acquity UPLC BEH C8)	0.19	0.63
61.	Phenomenex Prodigy C8	0.19	0.67
62.	Agilent HC-C18	0.19	1.21
63.	YMC Pack Pro C18	0.19	1.25
64.	Agilent Zorbax Eclipse Plus	0.19	1.27
65.	Shiseido Capcell Pak MGIII	0.19	1.30
66.	Thermo Hypersil GOLD (C12)	0.20	0.83
67.	Keystone Spectrum (Embedded)	0.20	0.85
68.	Waters SunFire C8	0.20	0.96
69.	Phenomenex Kinetex XB-C18	0.20	1.03
70.	Phenomenex Kinetex C18	0.21	1.06
71.	YMC ODS AQ	0.21	1.20
72.	Shiseido Capcell Pak MGII	0.21	1.33
73.	Waters Xterra Phenyl	0.22	0.71
74.	YMC Pro C8	0.22	0.97
75.	Phenomenex Synergy Max RP	0.22	1.18
76.	Phenomenex Luna C18 (2)	0.22	1.21
77.	Waters Acquity UPLC HSS C18	0.22	1.26
78.	Phenomenex Gemini C6-Phenyl	0.23	0.94
79.	Waters Symmetry C8	0.23	0.99
80.	Shiseido Capcell Pack SG120 C18	0.23	1.26
81.	Thermo Hypersil BDS C8	0.24	0.77
82.	YMC Hydrosphere C18	0.25	1.12
83.	YMC Basic	0.26	0.76
84.	Phenomenex Gemini C18	0.26	1.16
85.	Waters XSelect HSS T3	0.27	1.18
86.	Merck Purospher RP 18e	0.27	1.34
87.	Nomura Develosil ODS SR 5	0.27	1.50

(Continued)

APPENDIX 9.3.1 List of various columns used in RP-HPLC with the values for log k' for acenaphthene and for log α for the separation of amitriptyline/acenaphthene in a mobile phase 65% CH_3OH/35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH =7.0 (v/v).—*cont'd*

No.	Column description	log k'	log α
88.	Waters Xbridge Phenyl (AcquityUPLC BEH Phenyl)	0.28	0.77
89.	Waters Atlantis dC18	0.28	1.14
90.	ACT Ace C8	0.29	0.81
91.	Agilent Zorbax Eclipse XDB C8	0.29	0.93
92.	Phenomenex Aqua C18	0.29	1.09
93.	Waters Acquity UPLC HSS T3	0.31	1.16
94.	Waters Nova-Pak C8	0.32	0.77
95.	GL Science Intersil C8	0.32	0.89
96.	Azko Nobel Kromasil C8	0.33	0.94
97.	Machery Nagel Nucleodur Sphinx RP	0.33	1.06
98.	Waters Atlantis T3	0.33	1.18
99.	Shiseido Capcell Pack C18 AQ	0.33	1.21
100.	Varian Pursuit Diphenyl	0.34	0.23
101.	Agilent TC-C18	0.34	0.96
102.	Phenomenex Synergy Fusion RP (Embedded)	0.34	1.08
103.	Waters Nova-Pak C18	0.34	1.14
104.	GL Science Inertsil ODS-SP	0.35	0.86
105.	Supelco Supelcosil LC DB-C18	0.36	1.04
106.	Supelco Discovery Cyano	0.37	−0.3
107.	Phenomenex Luna Phenyl Hexyl	0.37	1.05
108.	Agilent Zorbax Stable-Bond C18	0.37	1.18
109.	Keystone Fluophase PFP	0.38	1.14
110.	Restek Ultra Phenyl	0.41	0.51
111.	TSK-Gel 80Ts	0.41	1.13
112.	ZirChrom PBD	0.43	0.71
113.	YMC J'Sphere H80	0.43	1.48
114.	Thermo Hypersil ODS	0.44	1.02
115.	Restek Ultra PFP	0.45	0.04
116.	Thermo Hypersil BDS Phenyl	0.46	0.21

APPENDIX 9.3.1 List of various columns used in RP-HPLC with the values for log k' for acenaphthene and for log α for the separation of amitriptyline/acenaphthene in a mobile phase 65% CH_3OH/35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH =7.0 (v/v).—*cont'd*

No.	Column description	log k'	log α
117.	Waters XSelect CSH Fluoro-Phenyl	0.46	0.51
118.	Supelco Supelcosil LC DB-C8	0.46	0.7
119.	GL Sciences Inertsil CN-3	0.47	0.28
120.	Waters Acquity UPLC CSH Fluoro-Phenyl	0.47	0.51
121.	Supelco Discovery HS F5	0.47	1.10
122.	Merck Purospher RP18	0.48	1.19
123.	YMC Pack CN	0.49	0.02
124.	W. R. Grace Altima C18	0.52	1.39
125.	Restek Allure Ultra IBD	0.54	0.76
126.	Phenomenex Synergy Polar-RP	0.54	0.94
127.	YMC J'Sphere M80	0.54	1.12
128.	Alltech Alltima C18	0.54	1.36
129.	Waters XSelect HSS Cyano	0.55	0.17
130.	Agilent Zorbax Eclipse XBD Phenyl	0.55	0.69
131.	Waters Acquity UPLC HSS Cyano	0.56	0.17
132.	Waters Nova-Pak Phenyl	0.57	0.59
133.	Waters Spherisorb ODSB	0.57	1.10
134.	Merck Lichrosphere RP Select B	0.58	0.76
135.	Restek Allure PFP Propyl	0.58	1.28
136.	YMC Pack Ph	0.59	0.46
137.	Thermo Hypersil CPS CN	0.60	−0.27
138.	GL Science Inertsil 3 CN	0.61	0.26
139.	Merck Lichrosorb RP Select B	0.61	0.92
140.	Mac-Mod HydroBond AQ C8	0.62	0.75
141.	W. R. Grace Altima C8	0.64	0.77
142.	YMC J'Sphere L80	0.65	0.73
143.	Machery Nagel Nucleosil C18	0.66	1.16
144.	Alltech Alltima C8	0.67	0.78
145.	Phenomenex Kinetex PFP	0.69	1.08

(Continued)

APPENDIX 9.3.1 List of various columns used in RP-HPLC with the values for log k' for acenaphthene and for log α for the separation of amitriptyline/acenaphthene in a mobile phase 65% CH_3OH/35% aqueous buffer 20 mM K_2HPO_4/KH_2PO_4 at pH =7.0 (v/v).—*cont'd*

No.	Column description	log k'	log α
146.	Agilent Zorbax SB-CN	0.72	0.16
147.	Agilent Zorbax Stable-Bond C8	0.74	0.78
148.	Agilent Zorbax Rx C8	0.80	0.69
149.	Waters mBondapack C18	0.80	0.75
150.	Waters Bondapak C18	0.81	0.75
151.	Keystone Fluophase RP	0.84	0.22
152.	Waters Spherisorb ODS-2	0.85	1.24
153.	Waters XSelect HSS PFP	0.91	0.86
154.	Waters Acquity UPLC HSS PFP	0.91	0.86
155.	W. R. Grace Platinum C18	0.93	0.77
156.	Alltech Platinum C18	0.94	0.77
157.	Phenomenex Luna CN	0.95	−0.02
158.	Thermo Hypersil Phenyl	0.96	0.23
159.	Agilent Zorbax SB-Aq	0.96	0.38
160.	Keystone Fluofix 120N	1.00	−0.02
161.	GL Sciences Inertsil Ph-3	1.03	0.46
162.	Agilent Zorbax SB-Phenyl	1.05	0.55
163.	Waters Nova-Pak HP CN	1.06	−0.56
164.	Waters Spherisorb S5 CN RP	1.17	−0.46
165.	Alltech Platinum EPS C18	1.20	0.49
166.	W.R. Grace Platinum EPS C18	1.20	0.52
167.	Waters XSelect HSS C18 SB	1.24	0.78
168.	Waters Acquity UPLC HSS C18 SB	1.24	0.82
169.	Waters Resolve C18	1.32	1.29
170.	Waters Spherisorb S5 Ph	1.62	−0.12
171.	Waters Spherisorb ODS-1	1.63	0.86

APPENDIX 9.3.2 List of C18 columns with USP parameters (mobile phase 80/20 methanol/aqueous buffer 60 mM phosphate v/v, pH = 7.0).

Column	H_y	CTF	CFA	TFA	BD	Manuf.[a]
218 TP 300 C18	–	1.4	7	1.3	5	6
238 MS 300 C18	0.4	–	0.7	1.6	2.5	6
Acclaim 120 C18	2.3	1.2	5.6	1.6	3.2	4
Acclaim 120 C8 (for comparison)	1.1	1.5	2.5	1.5	3.4	4
Acclaim 300 C18	0.7	1.2	1.7	1.3	3.3	4
Acclaim Polar Advantage	1.5	1.5	3.2	1.6	2.6	4
Acquity UPLC BEH C18	1.6	1.1	3.8	1.1	3.1	18
Acquity UPLC BEH Shield RP18	1.3	1.4	2.4	1.1	3.3	18
Alltima C18	3.4	1.1	13.5	2.5	2.8	5
Alltima C18 WP	1.4	1.3	4.3	5.3	5.2	5
Alltima HP C18	1.4	1.3	3.5	1.2	2.6	5
Alltima HP C18 Amide	1.1	1.5	2.2	1.1	2.8	5
Alltima HP C18 AQ	3.1	1	26.1	1.5	2.1	5
Alltima HP C18 EPS	0.4	1	8.4	1.2	1.3	5
Alltima HP C18 High load	3.6	1.2	6.9	1.2	2.4	5
Allure	1.4	1.9	12.7	4.4	4.1	11
Aquasil C18	1.5	1.9	23	2.8	2.2	16
Ascentis C18	2.7	1	6.2	1.3	3.6	15
Atlantis dC18	1.7	1.1	5.1	2.4	1.6	18
BetaBasic 18	1.6	6.6	4.1	2.7	3.2	16
BetaMax Neutral	4.2	1.6	11	3.9	3.6	16
BETASIL C18	3.2	1.6	7.6	2	3.6	16
Bio Basic 18	0.9	1.3	2.2	2.1	4.2	2
Capcell C18 ACR	2.9	1.2	6.3	3.1	3.5	14
Capcell C18 AG 120	2.4	1.7	7.8	5.4	2.3	14
Capcell C18 AQ	1.8	1.8	5.5	2.1	1.6	14
Capcell C18 MG	2.5	1.2	6.2	1.5	2.8	14
Capcell C18 MGII	2.5	1.3	6.1	1.1	2.8	14
Capcell C18 SG 120	1.9	1.3	4.7	2.3	2.1	14
Capcell C18 UG 120	1.9	1.1	4	2	2.4	14

(Continued)

APPENDIX 9.3.2 List of C18 columns with USP parameters (mobile phase 80/20 methanol/aqueous buffer 60 mM phosphate v/v, pH = 7.0).—*cont'd*

Column	H_y	CTF	CFA	TFA	BD	Manuf.[a]
Capcell C18 UG 80	3	1.7	6.4	5.3	2.6	14
Chromolith Perf RP-18e	0.8	1.5	3.1	2.9	3.3	7
Cosmosil 5C18-AR-II	2.4	1.2	14.4	2.3	3.4	8
Cosmosil 5C18-MS-II	2	1.1	4.7	1.7	2.8	8
Cosmosil 5C18-PAQ	1.2	2.2	2.5	1.5	2	8
Delta-Pak C18 100A	2	2	6.3	1.9	3.5	18
Denali C18	1.7	1.5	3.9	1.2	3.1	5
Discovery C18	1.1	1.1	2.8	1.6	3	15
Discovery C18 WP	0.8	1.1	1.8	1.4	3.6	15
Discovery HS C18	2.4	1	5.4	1.3	3.8	15
Everest C18 300A	0.8	1.5	2.3	3	4.3	5
Genesis C18	1.8	1.2	4.7	2	3.8	5
Genesis C18 AQ	1.8	1.1	5.4	1.9	4	5
Hydrosphere C18	1.5	1.3	4.4	1.9	1.9	19
Hypersil BDS 18	1.5	1.5	3.5	2	3.1	16
Hypersil ODS	1.5	3.4	4.3	3.6	3.2	16
Hypersil ODS-2	1.5	2	5.6	4.1	2.4	16
Hypersil PAH	1.2	2.2	12	2.6	4.6	16
HyPurity C18	1.3	1.4	3.5	2.1	3.3	16
Jsphere H80	3.3	1.3	8.8	2.9	3.2	19
Jsphere L80	0.9	1.4	3	2.8	0.9	19
Jsphere M80	1.6	1.4	5	2.7	1.4	19
Luna 5 µ C18(2)	2.2	1.2	5.3	1.1	3.4	10
Matrix C18	2.65	1.557	6.78	1.445	3.144	9
MicroBondapak C18	1	6	7.5	4	1.1	18
Monitor C18	2.1	1.438	5.71	1.966	3.34	9
Nova-Pak C18	1.5	7.5	4.6	3	2.7	18
Orosil C18	2.82	1.072	7.54	1.34	2.542	9
Pinnacle DB	1.4	1.8	4.3	2.8	3.7	11
Pinnacle II	1.4	3.4	5.1	3.4	3.7	11
Platinum EPS C18	0.4	1.4	14.5	3.5	1.2	5

APPENDIX 9.3.2 List of C18 columns with USP parameters (mobile phase 80/20 methanol/aqueous buffer 60 mM phosphate v/v, pH = 7.0).—*cont'd*

Column	H_y	CTF	CFA	TFA	BD	Manuf.[a]
Prestige C18	2.21	1.194	6.61	2.176	3.084	9
Prevail C18	2	1	23	2	2.6	5
Prevail Select C18	2	1.1	4	1.1	3.2	5
ProntoSil 120-5-C18-ace-EPS	2	1.2	3.8	1.6	2.8	3
ProntoSil 120-5-C18-AQ	2	1	6.7	2.6	2.1	3
ProntoSil 120-5-C18-AQ Plus	2.2	1.4	14.2	3.5	3.2	3
ProntoSil 120-5-C18-H	2.3	1	6.1	1.8	2.9	3
ProntoSil 120-5-C18-SH	2.2	1.8	10.2	2.2	3	3
ProntoSil 200-5-C18-ace-EPS	1.1	1.2	2.2	1.2	3.2	3
ProntoSil 200-5-C18-AQ	1	2.2	3.1	2.4	2.1	3
ProntoSil 200-5-C18-H	1.1	1.7	2.9	2.2	2.9	3
ProntoSil 300-5-C18-ace-EPS	0.7	1.3	1.4	1.8	3.2	3
ProntoSil 300-5-C18-H	0.7	1.6	2.2	2.7	2.9	3
ProntoSil 60-5-C18-H	3.9	1.7	12.5	4	2.9	3
Purospher RP-18	1.5	1.2	7.5	1.8	1.6	7
Purospher RP-18 end-capped	2.4	1.1	7.1	1	1.7	7
PurospherSTAR RP-18e 3 μm	2.1	1.2	5.7	1.5	3.3	7
PurospherSTAR RP-18e 5 μm	2.6	1.4	7.8	1.9	3.3	7
Reliachrom C18	1.64	3.047	2.65	2.43	3.751	9
Reliasil C18	2.24	6.906	10.92	7.123	2.637	9
SepaxGP-C18	2.5	1.2	7.7	2.54	3.3	12
SepaxHP-C18	2	1.2	6.8	1.81	3.3	12
Shim-pack VP-ODS	2.2	1.4	5.7	1.5	2.4	13
Shim-pack XR-ODS	2.1	1.2	5.1	1.4	2.6	13
Shim-pack XR-ODS II	3.4	2.5	8.5	4.9	2.4	13
Supelcosil LC18	1.3	2	4.5	13	3.1	15
Superiorex	2.8	1.7	7.5	3	2.8	14
Superspher 100 RP-18 end-capped	2.3	7.9	6	4.1	2.6	7
Symmetry C18	2.2	1.7	5.1	1.7	3.2	18
SymmetryShield RP18	1.6	1.5	3.1	1.2	3.3	18
Synergy 4μ Hydro-RP	2.6	1.2	–	3.3	4.0	1
TSKgel Octyl-80Ts	1.2	1.7	2.9	2.8	2.8	17

(Continued)

APPENDIX 9.3.2 List of C18 columns with USP parameters (mobile phase 80/20 methanol/aqueous buffer 60 mM phosphate v/v, pH = 7.0).—*cont'd*

Column	H_y	CTF	CFA	TFA	BD	Manuf.[a]
TSKgel ODS-100S	2.4	1.2	5.2	2	3	17
TSKgel ODS-100V 3 µm	1.9	1	6	1.4	1.8	17
TSKgel ODS-100V 5 µm	2.1	1.1	5.9	1.3	1.8	17
TSKgel ODS-100Z	3	1.1	7.2	1.3	2.6	17
TSKgel ODS-120A	1.6	1.7	16.4	6.2	2.7	17
TSKgel ODS-120T	2	2.8	9.8	8.3	2.8	17
TSKgel ODS-80TM	2.1	2.9	11.1	4.7	2.1	17
TSKgel ODS-80Ts	2.2	1.2	6.4	2.8	2.1	17
TSKgel Super-Octyl	0.5	1.5	0.9	1.7	1.9	17
TSKgel Super-ODS	1.1	2	3.7	4.4	2.7	17
TSKgel Super-Phenyl	0.2	1.9	1.9	3.6	3.9	17
Ultra	1.4	1.2	7.7	2.7	3.7	11
Viva 300	1.4	1.1	2.7	2.1	3.5	11
Xbridge C18	1.6	1.1	3.8	1.1	3.1	18
Xbridge Shield RP18	1.3	1.4	2.4	1.1	3.3	18
Xterra MS C18	1.5	1.1	3.3	1.3	2.2	18
Xterra RP18	1	1.2	1.7	1.1	2.3	18
YMC ODS-A	1.9	1.5	5	2.4	2.6	19
YMC ODS-AL	1.8	1.5	13.6	2.8	2.6	19
YMC ODS-AM	1.9	1.5	5.1	2.4	2.7	19
YMC ODS-AQ	1.9	1.5	6	2.9	2.2	19
YMC Pro C18	1.9	1.3	5	1.5	2.5	19
YMC Pro C18 RS	3.3	1.2	7.5	1.3	3	19
Zorbax Eclipse XDB C18	2.4	1	6.1	1.8	4	1
Zorbax Extend C18	2.4	1.1	5.9	3.4	3.8	1
Zorbax Rx C18	2.1	1.4	8.2	6.7	3.5	1
Zorbax StableBond C18	2	1.1	7.3	2.3	2	1

BD, the bonding density in µmol/m^2; *CFA*, retention factor k' for amitriptyline; *CTF*, chelating tailing factor for quinizarin; H_y, retention factor k' for ethylbenzene; *TFA*, tailing factor for amitriptyline.

[a] Manufacturer*: 1-Agilent Technologies, 2-Bio-Rad, 3-Bischoff, 4-Dionex, 5-Grace/Davison, 6-Grace/Vydac, 7-Merck KGaA (EDM Millipore), 8-Nacalai Tesque, 9-Orochem Technologies, 10-Phenomenex, 11-Restek, 12-Sepax Technologies, 13-Shimadzu, 14-Shiseido, 15-Supelco, 16-Thermo Scientific, 17-Tosoh Bioscience, 18-Waters, 19-YMC.*

Source: http://apps.usp.org/app/USPNF/columnsDB.html.

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Acclaim 120 C18	1.032	0.018	−0.143	−0.027	0.086	−0.002	B	L1	1
Acclaim 120 C8	0.857	0.004	−0.274	0.011	0.086	0.016	B	L7	1
Acclaim C30	0.973	−0.022	−0.126	0.004	0.353	0.320	B	L62	2
Acclaim Mixed-Mode HILIC-1	0.517	−0.086	0.000	0.126	0.067	0.893	B	L60	2
Acclaim Mixed-Mode WCX-1	0.432	−0.099	−0.606	0.168	−0.156	0.990	B	L60	2
Acclaim Organic Acid	0.833	−0.063	−0.385	−0.001	−0.316	0.349	Other		1
Acclaim Phenyl-1	0.689	−0.150	−0.548	0.068	0.013	0.150	Phenyl	L11	2
Acclaim Polar Advantage	0.855	−0.068	−0.116	0.023	−0.270	0.357	EP	L60	1
Acclaim PolarAdvantage II	0.740	0.015	−0.554	0.214	−0.225	0.670	EP	L60	1
Acclaim 300 C18	0.957	−0.018	−0.170	0.019	0.261	0.222	B	L1	1
Accucore 150-C18	1.027	0.046	−0.037	−0.009	0.200	0.171	B	L1	2
Accucore 150-C4	0.738	−0.008	−0.309	0.023	0.130	0.224	B	L26	2
Accucore aQ	1.071	0.050	0.002	−0.059	0.060	0.508	EP	L60	2
Accucore Biphenyl	0.706	−0.180	−0.317	0.025	0.077	0.727	B	L11	2
Accucore C18	1.092	0.054	0.055	−0.040	0.072	0.095	B	L1	2
Accucore C30	0.978	−0.020	−0.143	−0.002	0.321	0.462	B	L62	2
Accucore C8	0.924	0.015	−0.084	−0.003	0.002	0.186	B	L7	2
Accucore PFP	0.729	−0.098	−0.249	−0.036	0.873	1.655	Fluoro	L43	2
Accucore Phenyl-Hexyl	0.786	−0.047	−0.223	0.006	0.055	0.574	Phenyl	L11	2
Accucore Phenyl-X	0.781	−0.103	−0.554	0.044	−0.294	−0.082	Phenyl	L11	2
Accucore Polar Premium	0.871	0.103	−0.567	0.217	−0.207	0.787	EP	L60	2
Accucore RP-MS	1.000	0.024	−0.015	−0.006	0.027	0.057	B		2
Accucore XL C18	1.089	0.059	0.051	−0.038	0.086	0.384	B	L1	2
Accucore XL C8	0.926	0.020	−0.161	−0.001	0.020	−0.432	B	L7	2
ACE 5 AQ	0.804	−0.051	−0.129	0.034	0.009	0.167	EP		3
ACE 5 C18	1.000	0.027	−0.096	−0.007	0.143	0.096	B	L1	3
ACE 5 C18-300	0.983	0.025	0.046	0.012	0.262	0.237	B	L1	3
ACE 5 C18-AR	0.881	−0.059	−0.208	−0.016	0.079	0.153	Phenyl	L1	3
ACE 5 C18-HL	1.045	0.052	−0.088	−0.031	−0.038	−0.110	B	L1	3
Ace 5 C18-PFP	0.899	−0.021	−0.246	−0.080	−0.001	−0.995	B	L1	3
ACE 5 C4	0.674	−0.018	−0.178	0.026	0.090	0.316	B	L26	3
ACE 5 C4-300	0.710	−0.014	−0.183	0.039	0.166	0.356	B	L26	3
ACE 5 C8	0.827	0.005	−0.180	0.026	0.110	0.160	B	L7	3

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
ACE 5 C8-300	0.786	−0.003	−0.112	0.032	0.145	0.387	B	L7	3
ACE 5 CN	0.409	−0.107	−0.729	−0.008	−0.086	0.441	CN	L10	3
ACE 5 CN-300	0.460	−0.074	−0.165	0.030	0.151	0.856	CN	L10	3
ACE 5 Phenyl	0.647	−0.138	−0.296	0.027	0.132	0.466	Phenyl	L11	3
ACE Phenyl-300	0.599	−0.105	−0.234	0.032	0.164	0.548	Phenyl	L11	3
Acquity UPLC BEH C18	1.000	0.028	−0.366	0.007	0.142	0.088	B	L1	4
Acquity UPLC BEH C8	0.855	−0.008	0.095	0.056	0.220	0.777	B	L7	4
Acquity UPLC BEH Phenyl	0.764	0.077	−0.051	0.062	0.292	0.586	Phenyl	L11	4
Acquity UPLC BEH Shield RP-18	0.907	0.016	−0.031	0.133	−0.055	0.416	EP		4
Acquity UPLC CSH C18	0.968	0.000	−0.355	0.199	0.065	0.157	EP	L60	4
Adamas C18-AQ	0.847	−0.069	−0.162	0.011	−0.067	0.664	B	L1	5
Adamas C18-Classic	1.006	−0.024	−0.101	−0.001	0.036	0.083	B	L1	5
Adamas C18-Extreme	1.051	0.022	−0.017	−0.030	0.037	0.167	B	L1	5
Adamas C18-Select	0.948	−0.066	0.422	0.006	0.131	1.201	B	L1	5
Adamas C18-X-Bond	1.038	0.013	0.095	−0.034	−0.053	0.639	B	L1	5
Adsorbosphere C18	0.989	−0.073	0.070	−0.044	1.496	1.683	A	L1	6
Adsorpbosphere UHS C18	1.103	0.004	0.402	−0.046	−0.125	−0.125	B	L1	6
Advantage 300	0.867	−0.001	0.123	0.020	0.597	1.110	A	L1	7
Advantage Armor C18 120A	0.962	−0.014	−0.076	−0.004	0.077	0.261	B	L1	7
Aeris PEPTIDE XB-C18	0.992	−0.021	−0.095	0.016	0.071	0.314	B	L1	8
Aeris WIDEPORE XB-C18	0.934	−0.032	−0.134	0.068	0.073	0.360	B	L1	8
Aeris WIDEPORE XB-C4	0.699	−0.021	−0.310	0.017	0.084	0.195	B	L26	8
Aeris WIDEPORE XB-C8	0.788	−0.038	−0.169	0.073	−0.042	0.518	B	L7	8
Allsphere ODS1	0.733	−0.160	0.387	0.002	0.846	1.142	A	L1	6
Allsphere ODS2	1.004	−0.040	0.243	−0.028	0.960	1.281	A	L1	6
Alltima AQ	0.882	−0.070	0.301	0.016	0.158	1.157	B		6
Alltima C18	0.993	−0.014	0.035	−0.013	0.092	0.391	B	L1	6
Alltima C18-LL	0.780	0.085	−0.165	0.041	−0.056	0.367	B	L1	6
Alltima C18-WP	0.938	−0.062	0.027	0.002	−0.079	−0.081	B	L1	6
Alltima C8	0.756	0.015	−0.279	0.009	−0.062	0.288	B	L7	6
Alltima HP C18	0.985	−0.020	−0.040	0.006	0.177	0.199	B	L1	6
Alltima HP C18 Amide	0.497	−0.026	0.357	0.124	−0.019	0.926	EP	L60	6
Alltima HP C18 EPS	0.655	−0.104	0.401	0.036	0.459	0.955	EP	L1	6

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Alltima HP C18 High Load	1.080	−0.066	0.066	−0.040	−0.322	−0.244	B	L1	6
Alltima HP C8	0.834	0.010	−0.116	0.035	0.122	−0.418	B	L7	6
Alltima HP CN	0.469	−0.012	−0.556	0.009	−0.187	0.440	CN	L10	6
Allure Aqueous C18	0.968	−0.105	0.395	−0.044	0.006	1.389	B	L1	9
Allure Basix	0.438	−0.077	−0.423	−0.003	0.016	0.865	CN	Other	9
Allure Biphenyl	0.716	−0.192	−0.298	0.029	−0.010	0.694	Phenyl		9
Allure C18	1.131	0.052	0.046	−0.049	−0.037	0.020	B	L1	9
Allure Organic Acids	0.910	−0.125	0.530	−0.022	0.191	1.502	B	L1	9
Allure PFP Propyl	0.833	−0.265	0.051	0.348	1.109	1.659	Fluoro	L43	9
Alphabond (C18)	0.845	−0.094	0.061	0.001	0.579	1.760	A	L1	6
Apex C18	0.985	−0.035	0.013	0.042	1.246	2.311	A	L1	10
Apex C8	0.869	−0.071	0.235	0.177	1.364	1.373	A	L1	10
Apex II C18	1.008	−0.074	0.235	0.123	2.039	2.690	A	L1	10
ApHera C18 Polymer	0.838	−0.010	−1.106	0.001	−0.554	7.511	B	L67	11
Aqua C18	0.966	−0.030	0.033	0.009	0.068	0.276	B	L1	8
Aquasil C18	0.805	0.114	0.265	0.011	0.230	1.196	EP		2
Armor C18 3um	0.964	−0.016	−0.079	−0.002	0.122	0.296	B	L1	7
Ascentis C18	1.077	0.058	0.030	−0.042	−0.088	−0.084	B	L1	11
Ascentis C8	0.899	0.024	−0.180	−0.002	−0.124	−0.035	B	L7	11
Ascentis ES Cyano	0.541	−0.101	−0.495	0.013	0.133	−0.449	CN	L10	11
Ascentis Express 5 C18	1.125	0.053	0.053	−0.050	0.084	0.197	B	L1	11
Ascentis Express 5 F5	0.711	−0.118	−0.097	−0.067	1.056	1.705	Fluoro	L43	11
Ascentis Express Biphenyl	0.702	−0.177	−0.230	0.031	0.208	0.923	Phenyl	L11	11
Ascentis Express C18	1.136	0.053	0.023	−0.052	0.067	0.107	B	L1	11
Ascentis Express C8	0.915	0.015	−0.117	−0.005	−0.002	0.172	B	L7	11
Ascentis Express ES-CN	0.565	−0.098	−0.415	0.014	0.031	1.043	CN	L10	11
Ascentis Express F5	0.721	−0.124	0.067	−0.074	1.059	1.839	Fluoro	L43	11
Ascentis Express Peptide ES-C18	0.918	−0.042	0.263	0.019	0.192	0.813	B		11
Ascentis Express Phenyl-Hexyl	0.797	−0.088	−0.263	−0.010	0.094	0.483	Phenyl	L11	11
Ascentis Express RP-Amide	0.848	0.063	−0.416	0.198	−0.471	0.266	EP	L60	11
Ascentis Phenyl	0.721	−0.108	−0.288	−0.008	0.001	0.579	Phenyl	L11	11
Ascentis RP-Amide	0.843	0.078	−0.496	0.183	−0.781	0.087	EP	L60	11
Athena C18	0.997	0.002	−0.030	−0.013	0.094	0.265	B	L1	12

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Athena C18-WP	0.953	−0.030	−0.203	−0.003	−0.052	0.066	B	L1	12
Athena C8	0.831	−0.021	−0.166	0.009	0.026	0.274	B	L7	12
Athena Phenyl	0.587	−0.114	−0.396	0.033	0.067	0.345	Phenyl	L11	12
Atlantis dC18	0.917	−0.031	−0.193	0.001	0.036	0.087	B	L1	4
Atlantis T3	0.941	−0.035	−0.181	0.006	0.029	0.713	B	L1	4
Balancil C18	0.918	−0.037	−0.170	0.025	0.109	0.734	B	L1	13
BAS MF-8954	0.979	−0.069	0.181	0.022	1.081	1.397	A	L1	7
BetaBasic CN	0.426	−0.043	−0.453	0.014	0.014	0.904	CN	L10	2
BetaBasic Phenyl	0.582	−0.159	−0.411	0.049	0.104	0.758	Phenyl	L11	2
BetaMax Acid	0.635	0.057	−0.597	0.376	−2.064	−0.510	EP		2
BetaMax Base	0.470	−0.060	−0.391	0.010	0.014	1.146	EP		2
Betasil C18	1.063	0.033	0.034	−0.014	0.095	0.099	B	L1	2
Betasil C8	0.875	−0.003	−0.164	0.031	0.084	0.076	B	L7	2
Betasil Phenyl-Hexyl	0.707	−0.053	−0.294	0.028	0.054	0.357	Phenyl	L11	2
BioBasic 4	0.691	−0.009	−0.191	0.032	0.188	0.390	B	L26	2
BioBasic CN	0.390	−0.033	−0.823	−0.005	−0.070	0.537	CN	L10	2
BioBasic Phenyl	0.493	−0.233	−0.671	0.217	0.014	0.390	Phenyl	L11	2
Biobond C18	0.968	−0.021	−0.154	0.004	0.224	0.189	B	L1	14
Biobond C4	0.706	−0.001	−0.315	0.024	0.140	0.181	B	L26	14
Biobond C8	0.823	−0.008	−0.264	0.028	0.172	0.155	B	L7	14
BioResolve RP mAb	0.586	−0.140	−0.490	0.058	0.180	0.250	Phenyl	L11	4
BIOshell A160 Peptide C18	0.890	−0.059	0.250	0.024	0.177	0.819	B	L1	11
BIOshell A160 Peptide CN	0.481	−0.098	−0.353	0.028	0.076	0.838	CN	L10	11
BIOshell A400 Protein C18	0.940	−0.052	−0.202	0.063	0.177	0.261	B	L1	11
BIOshell A400 Protein C4	0.704	0.005	−0.847	0.013	0.109	0.155	B	L26	11
Bondclone C18	0.824	−0.056	−0.125	0.044	0.078	0.347	B	L1	8
Brava BDS C18	0.935	−0.033	0.033	0.012	0.281	0.768	B	L1	6
Cadenza 5CL-C18	1.016	0.004	0.093	−0.046	0.148	0.307	B	L1	15
Cadenza CD-C18	1.057	0.031	0.083	−0.028	0.113	0.042	B	L1	15
CAPCELL C18 ACR	1.025	0.045	0.073	−0.015	0.037	0.111	B	L1	16
CAPCELL C18 AG120	1.030	0.060	0.122	−0.065	0.543	0.628	A	L1	16
CAPCELL C18 AQ	0.867	−0.046	−0.068	0.014	−0.093	0.402	EP	L1	16
CAPCELL C18 MG	1.005	0.010	0.042	−0.007	0.079	0.007	B	L1	16

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
CAPCELL C18 SG120	0.987	0.031	0.093	−0.023	0.121	0.197	B	L1	16
CAPCELL C18 UG120	1.007	0.036	0.037	−0.012	0.016	0.001	B	L1	16
Capcell Pak C18 IF	0.957	0.025	−0.201	−0.001	−0.206	−0.010	B	L1	16
Capcell Pak C18 MG III	0.957	0.002	−0.116	−0.014	−0.115	0.046	B	L1	16
Capcell Pak C18 MGII	1.011	0.011	0.047	−0.006	0.007	−0.009	B	L1	16
CAPCELL PAK C8 DD	0.836	0.020	−0.154	0.015	−0.111	−0.075	B	L7	16
CAPCELL PAK C8 UG120	0.854	0.037	−0.097	−0.013	−0.046	−0.010	B	L7	16
Chromegabond WR C18	0.979	0.026	−0.159	−0.003	0.320	0.283	B	L1	17
Chromegabond WR C8	0.855	0.025	−0.279	0.024	0.200	0.144	B	L7	17
Chromolith HighResolution RP-18e	0.999	0.019	0.017	−0.001	0.206	0.285	B	L1	18
Chromolith Performance RP-8e	0.833	−0.014	−0.010	0.014	0.071	0.572	B	L1	18
Chromolith RP18e	1.003	0.029	0.008	−0.014	0.103	0.187	B	L1	18
Clipeus C18	1.002	0.003	−0.043	−0.010	0.079	0.341	B	L1	19
Clipeus C8	0.822	−0.014	−0.180	0.023	0.095	0.241	B	L7	19
Clipeus Cyano	0.423	−0.065	−0.424	0.010	0.055	0.786	CN	L10	19
Clipeus Phenyl	0.586	−0.113	−0.303	0.031	0.073	0.488	Phenyl	L11	19
Cogent Bidentate C18	0.950	0.059	−0.130	0.004	0.785	2.266	A	L1	20
Cogent Bidentate C8	0.681	−0.113	0.369	0.000	0.195	1.351	B	L7	20
Cogent HPS C18	1.021	−0.011	−0.071	−0.014	0.106	0.089	B	L1	20
Cogent hQ C18	0.908	−0.066	0.377	0.005	0.190	2.180	B	L1	20
Cogent UDC Cholesterol	0.625	0.227	0.528	0.069	0.745	1.212	Other		20
Cortecs C18	1.075	0.043	−0.108	−0.037	0.063	0.035	B	L1	4
Cortecs C18+	1.037	−0.005	0.175	0.026	−0.033	0.537	B	L1	4
Cortecs C8	0.882	0.020	−0.210	−0.007	0.028	0.047	B	L7	4
Cortecs Phenyl	0.764	−0.088	−0.310	0.009	0.042	0.590	Phenyl	L11	4
Cortecs T3	0.954	−0.036	−0.160	0.016	0.150	0.190	B	L1	4
Cortecs UPLC Shield RP18	0.869	−0.023	−0.280	0.100	−0.190	0.096	EP	L60	4
Cosmicsil Abra C18	0.810	0.031	−0.482	0.214	−0.178	0.139	C18	L1	21
Cosmicsil Adore 100 CN	0.339	−0.067	−0.488	0.008	0.099	0.901	B	L10	21
Cosmicsil Adze C18	0.997	−0.019	−0.138	−0.013	−0.082	0.069	B	L1	21
Cosmicsil Agate 100 C18	1.024	−0.026	−0.070	0.009	0.048	0.263	B	L1	21
Cosmicsil Agate RP C18	1.021	0.017	−0.007	−0.007	0.185	0.230	C18	L1	21

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Cosmicsil Agate RP C8	0.846	−0.008	−0.178	0.022	0.034	0.262	C8	L7	21
Cosmicsil APT C18	1.023	0.004	−0.064	−0.007	0.135	0.196	C18	L1	21
Cosmicsil AQ C18 120	0.958	−0.039	−0.072	0.013	0.133	0.295	B	L1	21
Cosmicsil Aster C18 XD	1.076	0.038	0.042	−0.024	−0.086	0.084	B	L1	21
Cosmicsil Aura ODS	0.948	−0.040	−0.185	0.009	−0.047	0.089	B	L1	21
Cosmicsil BDS C18	0.854	−0.035	0.195	−0.012	0.476	0.929	B	L1	21
Cosmicsil Glory C18	1.001	0.007	−0.170	0.003	0.172	0.222	C18	L1	21
COSMOSIL 5-C18-PAQ	0.822	−0.027	−0.342	0.053	−0.353	0.047	EP		22
Cosmosil 5PYE	0.671	−0.271	−0.283	0.092	0.521	1.318	Other		22
COSMOSIL C18-AR-II	1.017	0.011	0.126	−0.029	0.116	0.494	B	L1	22
COSMOSIL C18-MS-II	1.031	0.042	−0.132	−0.014	−0.118	−0.027	B	L1	22
Cosmosil piNap	0.665	−0.176	−0.258	0.034	0.188	0.789	Phenyl		22
Curosil-PFP	0.695	−0.079	−0.267	−0.004	0.119	0.379	Fluoro	L43	8
DeltaPak C18 100A	1.028	0.019	−0.018	−0.011	−0.051	0.024	B	L1	4
DeltaPak C18 300A	0.955	−0.013	−0.105	0.016	0.235	0.286	B	L1	4
Denali 120A C18	1.052	0.042	0.125	−0.014	0.143	0.222	B	L1	23
Develosil C30-UG-5	0.976	−0.036	−0.196	0.011	0.158	0.176	B	L62	24
Develosil ODS-HG-5	0.980	0.015	−0.172	−0.008	0.187	0.221	B	L1	24
Develosil ODS-MG-5	0.963	−0.036	−0.165	−0.003	−0.012	0.051	B	L1	24
Develosil ODS-UG-5	0.996	0.025	−0.146	−0.004	0.150	0.155	B	L1	24
Discovery Amide C16	0.720	0.013	−0.625	0.218	−0.092	−0.025	EP	L60	11
Discovery BIO Wide pore C18	0.836	0.014	−0.254	0.028	0.121	0.119	B	L1	11
Discovery BIO Wide pore C5	0.654	−0.019	−0.305	0.029	0.091	0.219	B		11
Discovery BIO Wide pore C8	0.839	0.018	−0.224	0.034	0.206	0.194	B	L7	11
Discovery C18	0.984	0.027	−0.128	0.004	0.176	0.153	B	L1	11
Discovery C8	0.832	0.011	−0.238	0.029	0.119	0.143	B	L7	11
Discovery CN	0.397	−0.110	−0.615	−0.002	−0.035	0.513	CN	L10	11
Discovery HS F5	0.673	0.084	−0.284	0.008	0.912	1.429	Fluoro	L43	11
Discovery HS PEG	0.318	0.027	−0.713	0.128	−0.531	0.387	EP		11
Durashell C18	1.042	0.041	−0.066	−0.038	−0.191	−0.174	B	L1	13
EC Nucleosil 100-5 Protect 1	0.544	0.048	−0.411	0.309	−3.213	−0.573	EP		25
Econosil C18	0.966	−0.066	0.376	−0.032	1.026	1.339	A	L1	6
Econosphere C18	0.818	−0.128	0.036	−0.017	1.046	1.522	A	L1	6

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Endeavorsil C18	1.021	0.043	−0.090	−0.020	−0.078	−0.009	B	L1	14
Epic C18	0.950	−0.027	−0.203	−0.007	−0.131	−0.041	B	L1	17
Epic C4	0.779	0.019	−0.315	−0.004	−0.200	0.061	B	L26	17
Epic C8	0.893	0.022	−0.194	−0.001	−0.102	0.038	B	L7	17
Epic Phenyl Hexyl	0.670	−0.088	−0.379	0.004	−0.148	0.305	Phenyl	L11	17
EU Reference Column	1.004	0.001	0.264	0.006	0.178	0.449	B	L1	26
Excel C18-Amide	0.791	0.023	−0.489	0.199	−0.060	0.176	EP		3
Excel CN-ES	0.811	−0.005	−0.429	−0.010	−0.052	0.153	CN	L10	3
Excel SuperC18	0.997	−0.003	−0.201	−0.013	0.030	0.009	C18	L1	3
Exsil C8	0.756	−0.076	−0.044	−0.014	0.472	0.974	A	L7	27
Exsil ODS	0.992	−0.036	0.292	−0.040	0.836	1.229	A	L1	27
Flare C18	0.806	−0.073	−0.470	0.841	−1.480	2.410	Other		28
Flare C18+	1.137	−0.308	0.730	0.966	−0.507	1.178	Other		28
Flowrosil C18 AQ	0.894	−0.040	−0.182	0.024	0.112	0.171	B	L1	29
Flowrosil C8	0.857	0.001	−0.173	0.024	0.110	0.166	B	L7	29
Flowrosil ODS	1.010	0.004	−0.121	0.000	0.162	0.142	B	L1	29
Fluophase PFP	0.675	−0.129	−0.311	0.065	0.817	1.375	Fluoro	L43	2
Fluophase RP	0.698	0.028	0.103	0.039	1.034	1.417	Fluoro	L43	2
Fortis C18	0.960	−0.023	−0.180	−0.009	−0.167	0.111	B	L1	30
Fortis C8	0.876	0.020	−0.223	0.001	−0.148	−0.041	B	L7	30
Fortis Cyano	0.383	−0.051	−0.399	0.016	0.011	0.872	CN	L10	30
Fortis H2O	1.038	0.032	0.141	−0.025	0.006	0.101	EP		30
Fortis Phenyl	0.615	−0.157	−0.402	0.037	0.084	0.772	Phenyl	L11	30
Fortis UniverSil C18	0.925	−0.027	−0.124	0.048	0.233	0.254	B	L1	30
Fortis UniverSil HS C18	0.992	−0.012	−0.117	−0.012	−0.065	0.082	B	L1	30
Gazelle C18	1.010	0.018	0.049	−0.010	0.164	0.209	B	L1	31
Gemini C18 110A	0.967	−0.008	0.027	0.013	−0.091	0.195	B	L1	8
Gemini C6-Phenyl 110A	0.803	0.032	−0.348	−0.005	−0.406	−0.266	Phenyl	L11	8
Gemini-NX C18	0.969	0.010	−0.204	−0.018	−0.184	−0.135	B	L1	8
Genesis AQ 120A	0.960	−0.036	−0.157	0.007	0.060	0.233	B	L1	10
Genesis C18 120A	1.005	0.004	−0.069	−0.007	0.139	0.124	B	L1	10
Genesis C18 300A	0.974	0.005	−0.086	0.013	0.266	0.270	B	L1	10
Genesis C4 300A	0.615	−0.057	−0.397	0.036	0.143	0.249	B	L26	10

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APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Genesis C4 EC 120A	0.646	−0.058	−0.331	0.027	0.063	0.400	B	L26	10
Genesis C8 120A	0.829	−0.016	−0.082	0.018	0.055	0.300	B	L7	10
Genesis CN 120A	0.424	−0.114	−0.681	−0.013	−0.001	0.573	CN	L10	10
Genesis CN 300A	0.397	−0.108	−0.645	−0.009	0.025	0.397	CN	L10	10
Genesis EC C8 120A	0.863	0.005	−0.174	0.023	0.064	0.141	B	L7	10
Genesis Phenyl	0.609	−0.140	−0.368	0.031	0.133	0.588	Phenyl	L11	10
GraceSmart RP 18	0.832	−0.035	0.070	0.002	0.071	0.740	B	L1	6
GraceSmart RP 18 5u	0.832	−0.035	0.070	0.002	0.071	0.740	B	L1	6
GROM Saphire 110 C18	1.055	−0.002	0.085	0.000	−0.030	0.115	B	L1	32
GROM Saphire 110 C8	0.835	−0.032	−0.103	0.031	−0.093	0.255	B	L7	32
GROM-SIL 120 Octyl-6 MB	0.872	0.001	−0.007	0.029	−0.017	0.135	B	L7	32
GROM-SIL 120 ODS-3 CP	1.029	0.019	0.093	−0.005	0.099	0.123	B	L1	32
Grom-Sil 120 ODS-4 HE	0.970	−0.037	−0.089	0.010	−0.037	0.263	B	L1	32
GROM-SIL 120 ODS-5 ST	1.035	−0.001	0.134	−0.005	0.135	0.121	B	L1	32
Haisil 300 C18	0.946	−0.003	−0.035	−0.001	0.428	0.683	A	L1	19
Haisil HL C18	1.045	−0.039	−0.078	−0.029	0.041	−0.057	B	L1	19
Halo 5 C18	1.148	0.055	0.025	−0.055	0.073	0.100	B	L1	33
Halo 5 C8	0.920	0.017	−0.118	−0.002	−0.002	0.205	B	L7	33
Halo 5 ES-CN	0.558	−0.102	−0.492	0.012	0.081	0.967	CN	L10	33
Halo 5 PFP	0.711	−0.122	−0.070	−0.052	1.159	1.847	Fluoro	L43	33
Halo 5 Phenyl-Hexyl	0.799	−0.091	−0.295	−0.004	0.115	0.429	Phenyl	L11	33
Halo AQ-C18	1.002	−0.036	0.099	−0.048	0.156	0.864	B	L1	33
Halo Biphenyl	0.708	−0.183	−0.279	0.028	0.047	0.990	Phenyl	L11	33
Halo C18	1.107	0.048	0.006	−0.050	0.056	0.040	B	L1	33
Halo C30	0.938	−0.046	−0.140	0.023	0.170	0.350	B	L62	33
Halo C8	0.913	0.028	−0.132	−0.008	−0.011	0.188	B	L7	33
Halo ES-CN	0.566	−0.110	−0.344	0.021	0.126	1.152	CN	L10	33
HALO Peptide ES-C18	0.890	−0.059	0.250	0.024	0.177	0.819	B	L1	33
HALO PFP	0.702	−0.117	−0.073	−0.062	1.168	0.972	Fluoro	L43	33
Halo Phenyl-Hexyl	0.789	−0.094	−0.233	−0.006	0.101	0.456	Phenyl	L11	33
Halo RP-Amide	0.859	0.080	−0.384	0.190	−0.417	0.312	EP	L60	33
Heavy C18	1.040	0.036	−0.137	−0.035	−0.083	−0.128	B	L1	19
Hichrom 300 5 RPB	0.944	0.028	0.044	0.015	0.226	0.216	B	L1	34

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Hichrom RPB	0.964	0.027	0.106	0.003	0.153	0.143	B	L1	34
Hitachi LaChrom C18-PM	1.127	0.069	−0.019	−0.068	−0.267	−0.144	B	L1	35
HSS C18	1.022	0.039	−0.136	−0.020	0.059	−0.009	B	L1	4
HSS C18 SB	0.730	−0.120	0.400	0.020	0.400	1.410	B	L1	4
HSS T3	0.949	−0.021	−0.173	−0.002	0.031	0.180	B	L1	4
HxSil C18	0.848	−0.077	0.303	0.017	0.230	1.054	B	L1	36
HxSil C8	0.684	−0.075	0.089	0.030	0.066	0.856	B	L7	36
HyperClone BDS C18 130A	0.988	0.032	0.019	−0.016	0.194	0.425	B	L1	8
HyperClone BDS C8 130A	0.847	0.008	−0.146	0.016	0.231	0.261	B	L7	8
HyperClone CN (CPS)	0.408	−0.086	−0.550	−0.003	0.920	1.045	CN	L10	8
HyperClone MOS C8 120A	0.847	−0.057	0.043	0.130	1.142	1.120	EP	L60	8
HyperClone ODS C18 120A	1.030	−0.020	0.060	0.090	1.030	0.960	A	L1	8
HyperClone PAH	0.980	−0.008	0.187	−0.024	1.169	1.116	Other		8
Hypersil 100 C18	1.048	0.022	0.118	0.031	0.405	0.348	A	L1	2
Hypersil BDS C18	0.993	0.016	−0.095	−0.009	0.337	0.281	A	L1	2
Hypersil Beta Basic-18	0.993	0.033	−0.099	0.001	0.163	0.126	B	L1	2
Hypersil Beta Basic-8	0.834	0.016	−0.248	0.029	0.110	0.115	B	L7	2
Hypersil Betamax Neutral	1.098	0.036	0.067	−0.031	−0.038	0.012	B	L1	2
Hypersil Bio Basic-18	0.974	0.025	−0.100	0.007	0.253	0.217	B	L1	2
Hypersil Bio Basic-8	0.821	0.012	−0.233	0.029	0.231	0.210	B	L7	2
Hypersil Elite	0.958	0.031	0.151	−0.010	0.314	0.739	A	L1	27
Hypersil GOLD	0.881	0.002	−0.017	0.036	0.162	0.479	B	L1	2
Hypersil GOLD aQ	0.915	−0.010	−0.065	−0.019	0.371	0.638	A	L1	2
Hypersil GOLD C4	0.705	−0.003	−0.285	0.026	0.110	0.235	B	L26	2
Hypersil GOLD C8	0.820	0.010	−0.150	0.030	0.090	0.210	B	L7	2
Hypersil GOLD CN	0.397	−0.035	−0.886	−0.019	−0.069	0.660	CN	L10	2
Hypersil GOLD PFP	0.624	−0.081	−0.116	−0.022	0.379	0.991	Fluoro	L43	2
Hypersil GOLD Phenyl	0.650	−0.091	−0.362	0.030	0.161	0.416	Phenyl	L11	2
Hypersil ODS	0.974	−0.026	−0.122	0.020	0.913	0.974	A	L1	2
Hypersil ODS	0.973	−0.051	−0.094	0.019	1.100	0.895	B	L1	2
Hypersil ODS-2	0.985	0.016	0.139	−0.011	0.254	0.370	B	L1	2
Hypersil PAH	0.949	−0.057	0.234	−0.017	1.439	1.724	A	L1	2
Hypersil Prism C18 RP	0.645	0.089	−0.459	0.301	−2.817	−0.716	EP		2

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APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Hypersil Prism C18 RPN	0.678	−0.001	−0.068	0.230	−0.544	0.625	EP		2
Hypurity Advance	0.412	−0.056	−0.095	0.249	−1.332	0.785	EP	L60	2
Hypurity C18	0.980	0.025	−0.091	0.003	0.192	0.167	B	L1	2
HyPurity C4	0.713	0.000	−0.291	0.028	0.121	0.252	B	L26	2
Hypurity C8	0.833	0.011	−0.201	0.035	0.157	0.161	B	L7	2
Hypurity Cyano	0.451	−0.049	−0.492	0.021	−0.016	0.839	CN	L10	2
Inertsil C8-3	0.830	−0.004	−0.268	−0.017	−0.334	−0.362	B	L7	37
Inertsil C8-4	0.678	−0.039	−0.386	−0.014	−0.094	−0.154	B	L7	37
Inertsil CN-3	0.369	0.049	−0.808	0.083	−2.607	−1.297	CN	L10	37
Inertsil ODS-2	1.007	0.045	−0.079	−0.014	−0.139	0.446	B	L1	37
Inertsil ODS-3	0.990	0.022	−0.146	−0.023	−0.474	−0.334	B	L1	37
Inertsil ODS-4	0.911	−0.026	−0.226	−0.030	−0.029	−0.143	B	L1	37
Inertsil ODS-EP	0.800	0.060	−1.520	0.050	−0.620	−0.070	EP	L60	37
Inertsil ODS-HL	1.070	0.010	−0.037	−0.066	0.016	−0.013	B	L1	37
Inertsil ODS-P	0.978	−0.028	0.611	−0.039	0.234	1.479	B	L1	37
Inertsil ODS-SP	0.858	−0.027	−0.221	−0.023	−0.048	−0.073	B	L1	37
Inertsil Ph-3	0.526	−0.179	−0.133	0.040	0.121	0.735	Phenyl	L11	37
Inertsil WP300 C18	0.938	−0.015	−0.117	0.001	0.202	0.163	B	L1	37
Inertsil WP300 C8	0.793	−0.015	−0.212	0.013	0.122	0.069	B	L7	37
InertSustain AQ-C18	0.939	−0.050	−0.230	0.001	−0.003	−0.095	B	L1	37
InertSustain C18	1.010	0.055	−0.152	−0.087	−0.081	−0.195	B	L1	37
InertSustain C8	0.868	−0.005	−0.270	−0.021	−0.035	−0.122	B	L7	37
InertSustain Cyano	0.445	−0.040	−0.370	−0.015	−0.077	0.830	CN	L10	37
InertSustain Phenyl	0.569	−0.135	−0.375	0.035	−0.044	0.320	Phenyl	L11	37
InertSustain Phenylhexyl	0.743	−0.043	−0.370	−0.015	−0.109	−0.204	Phenyl	L11	37
InertSustainSwift C18	0.882	−0.013	−0.310	−0.001	0.130	0.074	B	L1	37
InertSustainSwift C8	0.785	−0.014	−0.320	0.000	0.070	0.000	B	L7	37
Innoval C18	1.046	0.049	−0.024	−0.034	−0.066	0.027	B	L1	13
Inspire C18	1.063	−0.052	−0.078	−0.040	−0.081	−0.106	B	L1	14
Inspire C8	0.889	−0.025	−0.212	−0.004	−0.193	−0.014	B	L7	14
J'Sphere H80	1.130	0.050	−0.020	−0.060	−0.240	−0.160	B	L1	38
J'Sphere L80	0.760	−0.030	−0.210	0.000	−0.400	0.340	B	L1	38
J'Sphere M80	0.920	−0.020	−0.120	0.000	−0.290	0.130	B	L1	38

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Jupiter 300 C18	0.945	0.031	−0.225	0.008	0.234	0.218	B	L1	8
Jupiter 300 C4	0.698	0.008	−0.426	0.019	0.152	0.141	B	L26	8
Jupiter 300 C5	0.729	0.021	−0.382	0.016	0.129	0.330	B		8
Kanak C18	1.055	0.041	−0.054	−0.028	0.047	−0.031	B	L1	39
Kanak C4	0.730	−0.002	−0.289	0.017	0.042	0.304	B	L26	39
Kanak PheHex	0.763	−0.090	−0.304	0.011	0.152	0.417	Phenyl	L11	39
Kinetex Biphenyl 100A	0.697	−0.173	−0.583	0.034	0.122	0.817	Phenyl	L11	8
Kinetex C18 100A	0.963	0.009	−0.137	−0.011	0.007	0.125	B	L1	8
Kinetex C8	0.864	0.013	−0.208	−0.009	−0.089	0.002	B	L7	8
Kinetex EVO C18	1.010	−0.006	−0.174	−0.024	−0.108	−0.010	C18	L1	8
Kinetex F5	0.725	−0.064	−0.320	−0.046	0.110	2.660	Fluoro	L43	8
Kinetex PFP 100A	0.688	0.089	−0.273	−0.038	0.943	1.538	F	L43	8
Kinetex Phenyl-Hexyl	0.795	−0.091	−0.258	0.016	0.062	0.236	Phenyl	L11	8
Kinetex XB-C18	0.975	−0.013	−0.083	0.023	−0.046	0.305	B	L1	8
Kromasil 100 5 C18	1.050	0.030	−0.070	−0.020	0.030	−0.050	B	L1	40
Kromasil 100 5 C4	0.730	0.000	−0.330	0.010	0.000	0.000	B	L26	40
Kromasil 100 5 C8	0.860	0.010	−0.210	0.010	0.050	0.000	B	L7	40
Kromasil KR60 5 CN	0.440	−0.130	−0.570	−0.010	0.210	1.030	CN	L10	40
LaChrom C18	0.993	0.013	−0.151	−0.006	−0.278	−0.120	B	L1	35
LaChrom C18-AQ	0.907	−0.023	−0.137	0.011	−0.327	0.192	B	L1	35
LaChrom C18-NE	0.962	−0.024	0.360	−0.008	0.138	0.772	B	L1	35
LaChrom C8	0.856	0.022	−0.199	0.013	−0.414	−0.022	B	L7	35
LaChrom CN	0.425	−0.064	−0.372	0.006	−0.020	0.795	CN	L10	35
LaChrom Ph	0.594	−0.105	−0.324	0.024	−0.003	0.450	Phenyl	L11	35
Leapsil C18	1.052	0.044	−0.072	−0.035	−0.029	−0.055	B	L1	14
LiChrosorb RP-18	0.909	−0.070	0.151	−0.080	0.714	0.976	A	L1	18
LiChrospher 100 RP-18	1.006	−0.021	0.183	−0.036	0.646	0.896	A	L1	18
LiChrospher 60 RP-Select B	0.747	−0.060	−0.042	0.006	0.108	0.864	B	L7	18
Luna C18	1.018	0.025	0.072	0.008	−0.361	−0.036	B	L1	8
Luna C18(2)	1.002	0.024	−0.124	−0.007	−0.269	−0.174	B	L1	8
Luna C5	0.800	0.035	−0.252	0.003	−0.278	0.114	B		8
Luna C8	0.875	0.037	−0.015	0.024	−0.400	0.133	B	L7	8
Luna C8(2)	0.889	0.041	−0.222	−0.001	−0.300	−0.170	B	L7	8

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Luna CN	0.452	−0.112	−0.323	−0.024	0.439	1.321	CN	L10	8
Luna Omega C18	0.976	−0.003	−0.187	−0.007	−0.018	0.005	B	L1	8
Luna Omega Polar C18	0.860	−0.070	−0.158	0.020	0.003	0.558	EP	L60	8
Luna PFP(2)	0.753	−0.076	−0.382	−0.051	0.088	0.548	Fluoro	L43	8
Luna Phenyl-Hexyl	0.782	−0.118	−0.277	−0.004	0.004	0.387	Phenyl	L11	8
Matrix C18	0.934	−0.046	0.068	−0.003	0.071	0.765	B	L1	31
MicroBondapak C18	0.790	−0.070	−0.030	0.010	0.280	0.850	A	L1	4
MicroBondapak Phenyl	0.585	−0.152	−0.247	0.021	0.359	0.976	Phenyl	L11	4
Microsorb 100-5 C8	0.875	0.061	0.091	0.192	0.613	0.823	A	L7	41
Microsorb 100-5 Phenyl	0.711	−0.140	−0.195	0.163	0.604	0.787	Phenyl	L11	41
Microsorb 300-5 C4	0.666	−0.028	−0.315	0.036	0.207	0.419	B	L26	41
Microsorb-MV 100 CN	0.357	−0.241	−0.852	−0.029	0.148	0.785	CN	L10	41
Monitor C18	0.981	−0.004	−0.131	0.001	0.021	0.063	B	L1	31
Nova-Pak C18	1.049	0.004	0.098	−0.027	0.546	0.563	A	L1	4
Nova-Pak C8	0.899	−0.028	−0.094	0.006	0.611	0.621	A	L7	4
Nova-Pak CN HP 60A	0.362	−0.165	0.100	0.000	0.691	1.175	CN	L10	4
Nova-Pak Phenyl	0.704	−0.159	−0.300	0.015	0.767	0.812	Phenyl	L11	4
Nucelosil 100-5-C8 HD	0.865	−0.008	−0.174	0.029	0.045	0.188	A	L7	25
Nucleodur 100-5 C18	0.977	−0.024	0.009	−0.030	0.143	0.590	B	L1	25
Nucleodur 100-5 C8	0.779	−0.042	−0.152	−0.007	0.048	0.825	B	L1	25
Nucleodur C18 Gravity	1.056	0.041	−0.097	−0.025	−0.080	0.316	B	L1	25
Nucleodur C8 Gravity 5 micron	0.868	0.032	−0.240	0.000	−0.158	0.631	B	L1	25
Nucleodur HTEC C18	1.045	0.038	−0.022	−0.015	−0.482	0.136	B	L1	25
Nucleodur Isis	1.023	0.055	−0.078	−0.029	−0.019	0.153	B	L1	25
Nucleodur PAH C18	1.006	0.008	0.574	−0.022	0.343	1.216	B	L1	25
Nucleodur PFP	0.712	−0.059	−0.265	−0.036	0.023	0.810	Fluoro	L43	25
Nucleodur POLARTEC C18	0.858	0.168	−0.259	0.351	−3.398	−0.787	B	L1	25
Nucleodur Pyramid	0.958	0.003	−0.130	−0.016	−0.289	0.210	B	L1	25
Nucleodur Sphinx RP	0.805	−0.071	−0.274	0.000	0.022	0.722	Phenyl	L1	25
Nucleoshell RP 18	1.109	0.036	0.077	−0.040	0.082	0.266	B	L1	25
Nucleosil 100 5 C18 HD	0.961	−0.021	−0.126	0.009	0.089	0.150	A	L1	25
Nucleosil 100 5 C18 Nautilus	0.702	0.003	−0.483	0.268	−0.441	0.486	EP	L60	25
Nucleosil 300 5 C18	0.860	−0.081	−0.008	0.014	0.453	0.984	A	L1	25

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Nucleosil C18	0.906	−0.052	0.012	−0.030	0.321	0.730	A	L1	25
Nucleosil C8	0.575	−0.134	0.038	0.017	0.282	1.122	A	L7	25
OmniSpher 5 C18	1.055	0.051	−0.033	−0.029	0.122	0.058	B	L1	41
Onyx Monolithic C18	1.012	0.021	0.227	−0.018	0.120	0.430	B	L1	8
Onyx Monolithic C8	0.824	0.003	−0.006	0.004	−0.020	0.441	B	L7	8
Orosil C18	0.981	−0.032	−0.137	0.002	−0.048	0.155	B	L1	31
Orosil C18-ER	0.907	−0.054	0.356	0.026	0.157	1.054	B	L1	31
Orosil C18-HC	1.041	0.013	0.128	−0.020	−0.014	0.361	B	L1	31
Orosil Polar C18	1.021	−0.041	0.603	−0.017	0.291	1.369	B	L1	31
Partisil C8	0.749	−0.071	−0.099	0.074	0.035	0.546	B	L7	8
Partisil ODS(3)	0.810	−0.079	−0.007	0.002	0.317	0.902	A	L1	8
Peerless Basic C-18	0.988	−0.015	−0.156	−0.015	−0.164	−0.096	B	L1	42
Peerless C-18	0.999	−0.025	0.033	−0.013	−0.003	0.454	B	L1	42
Phalanx C18	0.953	−0.030	−0.209	−0.006	−0.075	−0.016	B	L1	19
Pinnacle DB Aqueous C18	0.698	−0.148	0.228	0.036	0.379	1.138	A	L1	9
Pinnacle DB Biphenyl	0.641	−0.175	−0.343	0.051	0.193	0.516	Phenyl	L11	9
Pinnacle DB C18	1.014	0.025	−0.033	−0.005	0.364	0.280	A	L1	9
Pinnacle DB C8	0.835	0.002	−0.198	0.037	0.269	0.231	A	L7	9
Pinnacle DB Cyano	0.376	−0.076	−0.380	0.007	0.221	0.722	CN	L10	9
Pinnacle DB PFP Propyl	0.648	−0.069	−0.253	0.054	0.598	1.106	F	L43	9
Pinnacle DB Phenyl	0.577	−0.124	−0.465	0.046	0.169	0.289	Phenyl	L11	9
Pinnacle II Biphenyl	0.642	−0.195	−0.247	0.054	0.465	0.732	Phenyl	L11	9
Pinnacle II C18	1.038	0.019	−0.030	0.003	0.445	0.349	A	L1	9
Pinnacle II C8	0.852	−0.006	−0.117	0.035	0.394	0.435	A	L7	9
Pinnacle II Cyano	0.370	−0.083	−0.213	−0.010	0.200	0.788	CN	L10	9
Pinnacle II PAH	0.977	−0.023	0.353	−0.020	0.746	1.188	A	L1	9
Pinnacle II Phenyl	0.594	−0.134	−0.405	0.061	0.370	0.492	Phenyl	L11	9
Platinum C18	0.786	−0.076	−0.098	0.005	0.400	0.694	A	L1	6
Platinum C8	0.584	−0.056	−0.225	0.005	0.251	0.391	B	L7	6
Platinum EPS C18	0.619	−0.170	0.306	0.026	0.688	1.701	A	L1	6
Platinum EPS C18 300	0.450	−0.058	0.379	0.016	0.247	1.291	EP		6
Platinum EPS C8	0.420	−0.152	0.151	0.026	0.509	1.369	A	L7	6
Platinum EPS C8 300	0.584	−0.113	−0.136	0.089	0.481	0.961	EP		6

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APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Polar C18	1.043	−0.086	0.645	−0.035	1.071	1.557	B	L1	31
Polaris Amide-C18	0.840	0.116	−0.336	0.345	−1.659	−0.556	EP	L60	41
Polaris C18-A	0.928	0.007	−0.227	0.061	0.149	0.160	EP	L1	41
Polaris C18-Ether	0.943	−0.013	−0.122	0.027	0.164	0.553	EP	L1	41
Polaris C8-A	0.601	−0.007	−0.609	0.104	−0.074	0.208	EP	L7	41
Polaris C8-Ether	0.705	−0.023	−0.312	0.040	0.095	0.269	EP	L7	41
Poroshell 120 Bonus-RP	0.686	−0.030	−0.573	0.180	−0.670	−0.017	EP	L60	43
Poroshell 120 EC-C18	1.023	0.008	−0.130	−0.004	0.161	0.123	B	L1	43
Poroshell 120 EC-C8	0.877	0.011	−0.232	0.023	0.127	0.090	B	L7	43
Poroshell 120 EC-CN	0.421	−0.057	−0.476	0.002	0.045	0.870	CN	L10	43
Poroshell 120 HPH C18	1.029	0.005	−0.140	−0.014	0.073	−0.004	B	L1	43
Poroshell 120 HPH C8	0.887	0.010	−0.250	0.008	−0.019	−0.073	B	L7	43
Poroshell 120 PFP	0.630	−0.059	−0.460	0.015	−0.038	0.741	Fluoro	L43	43
Poroshell 120 Phenyl-Hexyl	0.752	−0.083	−0.394	0.018	0.136	0.140	Phenyl	L11	43
Poroshell 120 SB-AQ	0.581	−0.120	−0.133	0.051	−0.014	0.670	EP	L96	43
Poroshell 120 SB-C18	0.956	−0.041	0.168	0.025	0.210	0.763	B	L1	43
Poroshell 120 SB-C8	0.726	−0.087	0.068	0.044	0.087	0.807	B	L7	43
Precision C18	1.002	0.003	−0.042	−0.010	0.079	0.341	B	L1	44
Precision C18-PE	0.976	−0.018	−0.085	−0.001	0.005	0.168	EP	L1	44
Precision C8	0.821	−0.014	−0.180	0.022	0.095	0.241	B	L7	44
Precision C8-PE	0.814	−0.021	−0.159	0.017	0.051	0.279	EP	L7	44
Precision CN	0.431	−0.114	−0.485	0.019	−0.041	0.606	CN	L10	44
Precision Phenyl	0.595	−0.136	−0.296	0.027	0.099	0.508	Phenyl	L11	44
Prevail Amide	0.862	−0.063	0.251	0.033	0.058	1.209	EP	L60	6
Prevail C18	0.888	−0.070	0.315	0.022	0.107	1.206	B	L1	6
Prevail C8	0.617	−0.089	0.039	0.041	0.081	1.072	B	L7	6
Prevail Select C18	0.822	0.029	−0.368	0.141	−1.057	0.455	B	L1	6
Primesep A	0.570	−0.199	0.057	0.032	2.732	2.600	Other		45
Primesep B	0.497	−0.004	0.034	0.584	−1.869	−1.357	Other		45
Primesep C	0.513	−0.146	−0.265	0.124	1.038	1.547	Other		45
Primesil C18 3 micron	1.020	0.025	−0.060	−0.001	0.014	0.120	C18	L1	29
Primesil C18 5 micron	1.040	−0.002	−0.043	−0.013	0.015	0.150	C18	L1	29

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Primesil C18(2)	1.000	−0.004	−0.057	−0.006	0.109	0.180	C18	L1	29
Primesil C8	0.858	0.009	−0.186	0.026	0.100	0.125	B	L7	29
Primesil ODS-P	0.752	0.090	−0.670	0.042	−0.240	−0.015	EP	L60	29
Prodigy ODS(2)	0.995	0.030	−0.114	−0.001	−0.091	0.237	B	L1	8
Prodigy ODS(3)	1.023	0.025	−0.131	−0.012	−0.195	−0.134	B	L1	8
Prodigy Phenyl-3	0.529	−0.195	0.055	0.022	0.230	1.467	Phenyl	L11	8
Promosil C18	1.064	0.036	−0.068	−0.012	−0.002	−0.090	B	L1	13
ProntoSIL 120 C1	0.413	−0.079	−0.085	0.020	0.042	0.656	B	C13	26
ProntoSIL 120 C18 ace-EPS	0.772	0.042	−0.590	0.228	−0.304	0.041	EP		26
ProntoSIL 120 C18 AQplus	0.947	−0.017	0.214	0.041	−0.133	0.605	EP		26
ProntoSIL 120 C18 H	1.005	0.008	−0.106	−0.004	0.125	0.156	B	L1	26
ProntoSIL 120 C18 SH	1.031	0.018	−0.109	−0.024	0.113	0.402	B	L1	26
ProntoSIL 120 C18-AQ	0.973	−0.007	−0.082	0.004	0.137	0.224	B	L1	26
ProntoSIL 120 C8 ace-EPS	0.532	−0.007	−0.852	0.213	−0.282	0.094	EP		26
ProntoSIL 120 C8 SH	0.739	−0.062	−0.081	0.013	0.076	0.526	B	L7	26
ProntoSIL 120 CN EC	0.427	−0.053	−0.320	0.015	0.019	0.768	CN	L10	26
ProntoSIL 120 Phenyl	0.568	−0.158	−0.201	0.022	0.176	0.712	Phenyl	L11	26
ProntoSIL 120-3-C30	0.919	−0.130	0.571	−0.003	0.507	1.788	A	L62	26
ProntoSIL 200 C18 ace-EPS	0.765	0.021	−0.566	0.214	0.026	0.143	EP		26
ProntoSIL 200 C18 AQ	0.973	−0.011	−0.057	0.006	0.125	0.288	B	L1	26
ProntoSIL 200 C18 H	0.955	−0.001	−0.121	0.016	0.163	0.218	B	L1	26
ProntoSIL 200 C30	0.909	−0.099	0.347	0.007	0.305	1.171	A	L62	26
ProntoSIL 200 C4	0.549	−0.063	−0.221	0.038	0.086	0.511	B	L26	26
ProntoSIL 200 C8 SH	0.761	−0.026	−0.195	0.024	0.125	0.238	B	L7	26
ProntoSIL 300 C18 ace-EPS	0.762	0.025	−0.579	0.211	−0.054	0.136	EP		26
ProntoSIL 300 C30	0.893	−0.107	0.322	0.030	0.401	1.547	A	L62	26
ProntoSIL 300 C30 EC	0.925	−0.047	−0.018	0.012	0.303	0.458	B	L62	26
ProntoSIL 300 C4	0.471	−0.093	−0.074	0.055	0.115	0.786	B	L26	26
ProntoSIL 300 C8 SH	0.739	−0.041	−0.131	0.028	0.156	0.405	B	L7	26
ProntoSIL 300-5-C18 H	0.956	−0.012	−0.090	0.015	0.238	0.249	B	L1	26
ProntoSIL 60 C18 H	1.158	0.041	0.066	−0.078	0.102	0.263	B	L1	26
ProntoSIL 60 C4	0.686	−0.072	0.108	0.001	−0.056	1.201	B	L26	26

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
ProntoSIL 60 C8 SH	0.929	−0.015	0.161	−0.017	−0.313	1.005	B	L7	26
ProntoSIL 60 Phenyl	0.705	−0.194	−0.003	−0.010	0.411	1.510	Phenyl	L11	26
ProntoSIL CN	0.370	−0.114	−0.414	−0.028	0.168	0.668	CN	L10	26
ProntoSIL HyperSORB 120 ODS	0.951	−0.065	0.039	−0.021	0.795	1.315	A	L1	26
ProntoSIL SpheriBOND 80 ODS1	0.700	−0.190	0.367	0.010	1.453	2.400	A	L1	26
ProntoSIL SpheriBOND 80 ODS2	1.010	−0.026	0.153	−0.037	0.731	1.008	A	L1	26
Prosphere 100 C18	0.883	−0.073	0.305	0.017	0.181	1.517	B	L1	6
Prosphere 300 C4	0.689	−0.015	−0.059	0.027	0.312	0.684	B	L26	6
Prosphere C18 300A	0.903	−0.012	0.176	0.013	0.577	1.266	A	L1	6
Proto 300 C18	0.962	0.016	−0.132	0.005	0.224	0.147	B	L1	19
Purospher RP-18	0.841	0.235	0.155	0.300	−0.964	0.901	B	L1	18
Purospher STAR RP18e	1.003	0.013	−0.071	−0.037	0.018	0.044	B	L1	18
Pursuit C18	1.001	0.004	−0.166	0.012	0.245	0.226	B	L1	41
Pursuit DP	0.574	−0.133	−0.538	0.047	0.153	0.261	Phenyl	L11	41
Pursuit PFP	0.658	−0.089	−0.253	−0.021	0.050	0.531	F	L43	41
Pursuit UPS C18	1.064	0.063	−0.050	−0.008	−0.076	0.207	B	L1	41
Pursuit XRs C-18	1.046	0.039	−0.121	−0.034	−0.014	−0.102	B	L1	41
Pursuit XRs C-8	0.882	0.020	−0.226	0.001	−0.152	−0.067	B	L7	41
Pursuit XRs DP	0.630	−0.136	−0.433	0.033	0.045	0.393	Phenyl	L11	41
Reliasil C18	0.879	−0.004	0.326	0.048	0.592	0.822	B	L1	31
Resolve C18	0.968	−0.127	0.335	−0.046	1.921	2.144	A	L1	4
Robusta C18	1.056	−0.002	0.025	−0.025	−0.019	0.203	B	L1	5
Selectosil C18	0.911	−0.054	0.034	−0.009	0.296	0.743	B	L1	8
Sepax Bio-C18	0.919	−0.026	−0.133	0.018	0.219	0.228	B	L1	46
Sepax HP-C18(2)	0.959	−0.024	−0.187	−0.007	−0.134	0.055	B	L1	46
SepaxBio-C4	0.663	−0.014	−0.291	0.022	0.109	0.228	B	L26	46
SepaxBio-C8	0.774	−0.025	−0.272	0.025	0.164	0.219	B	L7	46
SepaxBR-C18	1.019	0.037	−0.078	−0.017	−0.019	0.060	B	L1	46
SepaxGP-C18	1.014	0.014	−0.112	−0.019	0.103	0.096	B	L1	46
SepaxGP-C4	0.699	−0.014	−0.261	0.009	−0.026	0.204	B	L26	46
SepaxGP-C8	0.847	0.009	−0.137	0.015	−0.077	0.265	B	L7	46
SepaxGP-Phenyl	0.571	−0.118	−0.274	0.029	0.055	0.564	Phenyl	L11	46

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
SepaxHP-C18	0.951	−0.026	−0.102	0.001	0.070	0.221	B	L1	46
SepaxHP-Cyano	0.426	−0.056	−0.471	0.005	−0.001	0.756	CN	L10	46
Shim- pack XR-ODS II	1.086	0.039	0.076	−0.026	−0.006	0.204	B	L1	47
Shim-pack Arata C18	0.782	0.032	−0.503	0.236	−0.520	0.079	B	L1	47
Shim-pack Velox Biphenyl	0.707	−0.190	−0.244	0.046	0.063	1.070	B	L11	47
Shim-pack Velox C18	1.100	0.051	0.101	−0.034	0.111	0.196	B	L1	47
Shim-pack Velox PFPP	0.688	−0.130	0.116	−0.043	0.443	1.590	B	L43	47
Shim-pack Velox SP-C18	0.983	−0.045	0.385	0.010	0.106	1.060	B	L1	47
Shim-pack XR-C8	0.862	0.010	−0.160	0.017	−0.044	0.217	B	L7	47
Shim-pack XR-ODS	1.009	0.032	−0.106	−0.018	−0.061	0.170	B	L1	47
Shim-pack XR-Phenyl	0.705	−0.080	−0.264	0.010	−0.021	0.486	Phenyl	L11	47
Shodex C18-4D	1.007	0.027	0.021	−0.010	−0.110	0.107	B	L1	48
Sphereclone ODS(2)	0.975	−0.045	0.278	−0.051	0.866	1.326	A	L1	8
Spherisorb C8	0.763	−0.091	−0.032	0.053	0.737	1.142	A	L7	4
Spherisorb ODS-1	0.682	−0.186	0.323	0.018	0.843	1.297	A	L1	4
Spherisorb ODS-2	0.962	−0.076	0.070	0.034	0.908	1.263	A	L1	4
Spherisorb S5 ODSB	0.975	0.027	0.240	0.384	−0.642	1.680	B	L1	4
Spursil C18	0.961	0.009	−0.185	−0.049	0.429	0.765	A	L1	14
Spursil C18-EP	0.832	0.107	−0.509	0.226	−1.123	0.898	EP	L60	14
Sunfire C18	1.031	0.034	0.044	−0.014	−0.186	−0.099	B	L1	4
Sunfire C8	0.856	0.036	−0.122	0.006	−0.278	0.006	B	L7	4
Sunniest C18	1.021	0.010	−0.169	−0.023	0.089	0.803	B	L1	49
Sunniest C8	0.845	0.005	−0.304	−0.002	0.036	−0.018	B	L7	49
Sunniest PFP	0.615	−0.153	−0.049	−0.040	1.133	1.827	Fluoro	L43	49
Sunniest PhE	0.656	−0.085	−0.469	0.008	0.044	−0.022	Phenyl	L11	49
Sunniest RP-AQUA	0.958	−0.024	−0.210	−0.008	0.142	0.098	EP	L60	49
Sunshell C18	1.086	0.028	−0.124	−0.056	−0.043	−0.084	B	L1	49
Sunshell C8	0.907	0.007	−0.235	−0.028	−0.065	−0.126	B	L7	49
Sunshell PFP	0.663	−0.176	0.140	−0.076	1.106	2.143	Fluoro	L43	49
Sunshell Phenyl	0.813	−0.076	−0.323	0.017	−0.061	−0.090	Phenyl	L11	49
Sunshell RP-AQUA	0.898	−0.040	−0.272	0.013	0.127	0.095	EP	L60	49
Supelcosil LC-18	1.018	−0.047	0.181	0.162	1.595	1.752	A	L1	11

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Supelcosil LC-18-DB	0.979	−0.026	0.047	0.114	0.481	0.531	A	L1	11
Supelcosil LC-8	0.834	−0.048	−0.027	0.086	1.117	1.094	A	L7	11
Supelcosil LC-8-DB	0.819	−0.036	−0.072	0.143	0.446	0.554	A	L7	11
Supelcosil LC-PAH	0.851	−0.025	0.104	−0.030	0.642	0.830	A	L1	11
Supersphere 100 RP-18e	1.030	0.025	−0.028	−0.011	0.352	0.266	B	L1	18
Svea C18	1.055	0.041	−0.054	−0.028	0.047	−0.031	B	L1	50
Svea C4	0.730	−0.002	−0.289	0.017	0.042	0.304	B	L26	50
Svea C8	0.873	0.014	−0.170	0.012	0.029	0.150	B	L7	50
Svea CN	0.440	−0.046	−0.650	−0.010	0.010	0.820	CN	L10	50
Svea PFP	0.633	−0.085	−0.260	0.024	0.400	1.280	Fluoro	L43	50
Svea PheHex	0.763	−0.090	−0.304	0.011	0.152	0.417	Phenyl	L11	50
Symmetry 300 C18	0.984	0.031	−0.051	0.003	0.228	0.202	B	L1	4
Symmetry 300 C4	0.659	−0.016	−0.428	0.014	0.101	0.184	B	L26	4
Symmetry C18	1.052	0.063	0.018	−0.021	−0.302	0.123	B	L1	4
Symmetry C4	0.687	−0.012	−0.290	0.022	0.217	0.305	B	L26	4
Symmetry C8	0.893	0.049	−0.205	0.021	−0.509	0.283	B	L7	4
SymmetryShield C18	0.850	0.027	−0.411	0.093	−0.728	0.136	EP		4
SymmetryShield C8	0.730	−0.006	−0.550	0.103	−0.623	0.138	EP		4
SynChropak RP8	0.639	−0.099	0.109	0.029	0.223	0.940	A	L7	51
SynChropak RPP	0.746	−0.115	0.230	0.033	0.259	1.286	A	L1	27
SynChropak RPP 100	0.918	−0.059	−0.072	0.123	0.225	0.317	A	L1	27
Syncronis aQ	0.984	−0.004	−0.100	−0.038	0.066	1.077	EP	L60	2
Syncronis C18	1.043	−0.003	−0.086	−0.019	−0.026	0.038	B	L1	2
Syncronis C8	0.840	−0.024	−0.227	0.010	−0.058	0.116	B	L7	2
Syncronis Phenyl	0.750	−0.082	−0.320	0.008	−0.031	0.176	Phenyl	L11	2
Synergi Fusion-RP	0.879	−0.030	−0.014	0.008	−0.238	0.362	EP		8
Synergi Hydro-RP	1.022	−0.006	0.169	−0.042	−0.077	0.260	EP	L1	8
Synergi Max-RP	0.989	0.028	−0.008	−0.013	−0.133	−0.034	B	L1	8
Synergi Polar-RP	0.654	−0.148	−0.257	−0.007	0.057	0.778	EP	L1	8
Targa C18	0.977	−0.019	−0.070	0.000	0.013	0.175	B	L1	19
Targa C8	0.821	−0.023	−0.221	0.004	−0.027	0.174	B	L7	19
Targetsil C18 HPH	0.973	−0.014	−0.084	0.010	0.148	0.208	B	L1	29

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Targetsil C18 Polar	0.778	0.017	−0.463	0.185	−0.301	0.018	B	L1	29
Thermo CN	0.404	−0.111	−0.709	−0.009	−0.029	0.491	CN	L10	2
Titan C18	1.011	0.004	−0.084	−0.047	−0.052	0.088	C18	L1	11
Topsil C18	0.972	−0.011	−0.019	0.009	0.124	0.312	B	L1	52
Triart Bio C4	0.673	−0.015	−0.350	0.020	0.130	0.064	B	L26	38
TSKgel CN-80Ts	0.373	−0.025	−0.617	0.011	−0.369	0.426	CN	L10	53
TSKgel Octyl-80Ts	0.814	−0.005	−0.253	0.017	0.089	0.456	B	L7	53
TSKgel ODS-100S	1.032	0.065	−0.092	−0.034	−0.003	−0.032	B	L1	53
TSKgel ODS-100V	0.901	−0.043	−0.226	−0.009	−0.060	−0.020	B	L1	53
TSKgel ODS-100Z	1.032	0.018	−0.135	−0.031	−0.064	−0.161	B	L1	53
TSKgel ODS-120A	0.896	−0.039	0.280	0.001	0.265	0.963	B	L1	53
TSKgel ODS-120T	0.977	0.030	0.114	−0.011	0.195	0.227	B	L1	53
TSK-gel ODS-140HTP	1.002	0.051	−0.251	−0.032	0.134	0.142	B	L1	53
TSKgel ODS-80T	0.960	−0.037	0.145	−0.010	0.164	0.589	B	L1	53
TSKgel ODS-80Ts	0.971	−0.015	−0.132	−0.004	0.010	0.292	B	L1	53
TSKgel ODS-80Ts QA	0.940	−0.030	−0.118	0.005	0.004	0.361	B	L1	53
TSKgel OligoDNA RP	0.864	0.028	0.143	0.010	0.315	0.652	A	L1	53
TSKgel Super-Octyl	0.824	0.034	−0.155	0.010	0.126	0.230	B	L7	53
TSKgel Super-ODS	0.998	0.030	−0.048	−0.019	0.154	0.237	B	L1	53
TSKgel Super-Phenyl	0.580	−0.107	−0.146	0.016	0.085	0.672	Phenyl	L11	53
Ultimate AQ-C18	0.868	−0.033	−0.055	0.029	0.058	0.363	B	L1	52
Ultimate LP-C18	0.913	−0.044	0.160	0.026	0.150	0.770	B	L1	52
Ultimate PFP	0.636	−0.100	−0.170	−0.023	0.450	1.100	Fluoro	L43	52
Ultimate Plus C18	0.979	−0.003	−0.120	0.015	0.160	0.170	B	L1	52
Ultimate Polar-RP	0.773	0.026	−0.430	0.190	−0.270	0.100	B	L60	52
Ultimate XB-C18	1.005	0.011	−0.046	0.001	0.118	0.133	B	L1	52
Ultimate XB-C8	0.835	0.001	−0.074	0.028	0.069	0.281	B	L7	52
Ultimate XB-CN	0.394	−0.074	−0.300	0.007	0.092	0.920	CN	L10	52
Ultimate XB-Phenyl	0.651	−0.108	−0.250	0.032	0.150	0.476	Phenyl	L11	52
Ultimate XS-C18	0.982	−0.009	0.450	−0.016	0.220	1.200	B	L1	52
Ultisil AQ-C18	0.872	−0.037	−0.072	0.031	0.054	0.323	B	L1	52
Ultisil LP-C18	0.916	−0.042	0.150	0.026	0.140	0.760	B	L1	52

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APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Ultisil Polar-RP	0.773	0.028	−0.440	0.190	−0.250	0.037	B	L60	52
Ultisil XB-C18	1.009	0.012	−0.016	0.005	0.118	0.142	B	L1	52
Ultisil XB-C8	0.841	0.001	−0.108	0.027	0.063	0.244	B	L7	52
Ultisil XB-Phenyl	0.651	−0.108	−0.273	0.032	0.144	0.466	Phenyl	L11	52
Ultra Aqueous C18	0.808	−0.128	0.378	0.013	0.229	1.255	B	L1	9
Ultra Aromax	0.741	−0.179	−0.375	0.021	0.195	0.375	Phenyl	L11	9
Ultra Biphenyl	0.661	−0.189	−0.283	0.042	0.204	0.721	Fhenyl	L11	9
Ultra C1	0.613	−0.054	−0.408	0.016	−0.032	0.055	B	L13	9
Ultra C18	1.051	0.033	−0.032	−0.023	0.057	−0.003	B	L1	9
Ultra C4	0.738	−0.010	−0.276	0.019	0.032	0.318	B	L26	9
Ultra C8	0.871	0.013	−0.199	0.019	−0.032	−0.078	B	L7	9
Ultra Cyano	0.409	−0.041	−0.801	−0.011	−0.110	0.628	CN	L10	9
Ultra IBD	0.672	−0.035	−0.052	0.233	−0.564	0.860	EP	L68	9
Ultra II Aqueous C18	0.784	−0.154	0.321	0.015	0.468	1.163	B	L1	9
Ultra II Aromax	0.739	−0.193	−0.344	0.022	0.478	0.640	Phenyl	L11	9
Ultra II Biphenyl	0.652	−0.198	−0.275	0.047	0.434	0.764	Phenyl	L11	9
Ultra II C18	1.041	0.021	−0.037	−0.015	0.264	0.181	B	L1	9
Ultra II IBD	0.674	−0.043	−0.022	0.225	−0.257	0.864	EP	L68	9
Ultra II PFP Propyl	0.674	−0.084	−0.303	−0.006	0.769	1.213	Fluoro	L43	9
Ultra PFP	0.446	−0.055	−0.245	0.016	−0.221	0.152	Fluoro	L43	9
Ultra PFP Propyl	0.622	−0.102	−0.314	−0.035	0.456	1.047	Fluoro	L43	9
Ultra Phenyl	0.580	−0.127	−0.338	0.039	0.041	0.438	Phenyl	L11	9
Ultra Quat	0.858	0.016	−0.170	0.017	0.026	0.134	Other		9
Ultracarb ODS (30)	1.114	0.016	0.377	−0.050	−0.311	0.731	B	L62	8
Ultracore SuperC18	1.093	0.030	−0.111	−0.050	−0.029	0.028	C18	L1	3
UltraCore SuperPhenylHexyl	0.809	−0.074	−0.347	−0.014	−0.014	−0.007	Phenyl	L11	3
UltraSep ES AMID H RP18P	0.751	−0.013	−0.101	0.259	−0.527	0.855	EP		54
UltraSep ES PHARM RP18	0.953	−0.061	0.435	−0.057	0.593	1.674	A	L1	54
Ultrasphere Octyl	0.896	0.016	0.003	0.086	0.157	0.547	B	L7	34
Ultrasphere ODS	1.085	−0.014	0.173	0.068	0.279	0.382	B	L1	34
Unisol C18	0.950	−0.017	−0.108	−0.009	−0.108	0.092	B	L1	13
Unison UK-C18	0.981	−0.019	0.015	−0.011	0.110	0.070	B	L1	15

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
VDSpher PUR 100 C18-E	1.030	−0.003	−0.003	−0.016	0.010	0.150	B	L1	55
VDSpher PUR 100 C18-H	0.809	−0.073	−0.140	0.015	0.033	0.970	B	L1	55
VDSpher PUR 100 C18-M	0.903	−0.077	0.510	0.000	0.120	1.510	B	L1	55
VDSpher PUR 100 C18-M-SE	1.070	0.037	−0.044	−0.037	−0.060	0.022	B	L1	55
Venusil ABS C18	0.824	−0.066	0.166	0.050	0.047	0.644	B	L1	13
Venusil ABS C8	0.677	−0.078	−0.001	0.044	0.119	0.569	B	L7	13
Venusil HLP	0.547	−0.015	−0.649	0.254	−0.926	1.636	EP	L60	13
Venusil PFP	0.519	−0.158	0.120	0.011	0.350	1.144	Fluoro	L43	13
Venusil XBP Phenyl	0.626	−0.118	−0.234	0.020	0.024	0.776	Phenyl	L11	13
Venusil XBP Phenyl-Hexyl	0.678	−0.170	0.128	0.034	0.156	1.203	Phenyl	L11	13
Venusil XBP Polar-Phenyl	0.552	−0.214	0.016	0.052	−0.034	1.066	Phenyl	L11	13
Venusil XPB C18(2)	1.010	0.007	−0.081	−0.022	−0.116	0.072	B	L1	13
Venusil XPB C18(L)	0.945	0.025	−0.135	−0.002	0.152	0.318	B	L1	13
Venusil XPB CN	0.450	−0.053	−0.631	−0.013	−0.018	0.737	CN	L10	13
Vision C18 B	0.689	−0.111	0.350	0.031	0.390	1.410	A	L1	6
Vision C18 HL	0.992	0.056	0.057	−0.013	0.133	0.143	B	L1	6
VisionHT C18	0.786	−0.076	−0.098	0.005	0.400	0.694	B	L1	6
VisionHT C18-P	0.636	−0.165	0.287	0.033	0.698	1.618	B	L1	6
Viva Biphenyl	0.605	−0.183	−0.304	0.047	0.337	0.544	Phenyl	L11	9
Viva C18	0.980	0.016	−0.076	0.013	0.359	0.305	A	L1	9
Viva C4	0.708	−0.011	−0.285	0.031	0.223	0.311	A	L26	9
Viva C8	0.830	0.006	−0.222	0.036	0.262	0.222	A	L7	9
Viva PFP Propyl	0.626	−0.065	−0.252	0.029	0.283	0.762	Fluoro	L43	9
Vydac 201TP	0.901	−0.022	0.409	−0.004	0.394	1.026	A	L1	23
Vydac 218MS	0.770	0.182	0.111	−0.373	0.659	1.234	A	L1	23
Vydac 218MSC18	0.881	−0.013	0.295	0.005	0.171	0.823	B	L1	23
Vydac 218TP	0.909	0.009	0.345	−0.005	0.279	0.670	B	L1	23
Vydac C18 Monomeric	0.977	0.044	0.031	−0.002	0.139	0.275	B	L1	23
Vydac Everest	0.993	0.049	0.121	0.004	0.065	0.341	B	L1	23
Wakosil 5 C8 RS	0.802	−0.008	−0.272	0.001	−0.117	0.097	B	L7	27
Wakosil II 5 C18 AR	0.998	0.075	−0.055	−0.034	0.070	0.010	B	L1	27
Wakosil II 5 C18 HG	1.039	0.036	0.015	−0.023	0.009	0.210	B	L1	27

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Wakosil II 5 C18 RS	0.964	−0.008	−0.160	−0.009	−0.070	0.046	B	L1	27
XBridge C18	1.007	0.028	−0.097	0.009	0.178	0.138	B	L1	4
XBridge C8	0.805	0.018	−0.296	0.018	0.129	0.063	B	L7	4
XBridge Phenyl	0.732	−0.078	−0.358	0.037	0.190	0.206	Phenyl	L11	4
Xbridge Protein BEH C4	0.546	−0.073	−0.210	0.043	0.007	0.120	B	L26	4
XBridge Shield RP18	0.835	−0.026	−0.372	0.095	−0.122	−0.051	EP		4
XSelect CSH C18	0.954	−0.002	−0.179	0.118	0.082	0.171	B	L1	4
XSelect CSH Fluoro-Phenyl	0.498	−0.138	−0.052	0.055	−0.321	0.793	Fluoro	L43	4
XSelect CSH Phenyl-Hexyl	0.708	−0.059	−0.435	0.129	−0.068	0.223	Phenyl	L11	4
Xselect HSS Cyano	0.479	−0.116	−0.224	0.043	0.082	1.180	CN	L10	4
Xselect HSS PFP	0.592	−0.164	0.146	−0.020	0.740	1.774	Fluoro	L43	4
XTerra C18 RP	0.757	−0.043	−0.483	0.097	−0.170	−0.173	EP		4
XTerra C8 RP	0.657	−0.049	−0.604	0.099	−0.187	−0.198	EP		4
XTerra MS C18	0.984	0.012	−0.143	−0.015	0.133	0.051	B	L1	4
XTerra MS C8	0.803	0.005	−0.293	−0.005	0.058	−0.009	B	L7	4
XTerra Phenyl	0.683	−0.079	−0.363	−0.003	0.119	0.029	Phenyl	L11	4
Xtimate C18	1.000	−0.001	−0.083	−0.011	0.155	0.283	B	1	52
Xtimate C8	0.855	−0.014	−0.185	0.008	0.013	0.173	B	L7	52
YMC Basic	0.820	0.000	−0.230	0.020	0.070	0.090	B	L7	38
YMC Hydrosphere C18	0.937	−0.022	−0.129	0.006	−0.139	0.157	B	L1	38
YMC-Pack ODS-AQ	0.960	−0.030	−0.130	0.000	−0.060	0.100	B	L1	38
YMC-Pack Pro C18 RS	1.110	0.050	−0.060	−0.050	−0.170	−0.220	B	L1	38
YMC-Pro C18	1.010	0.010	−0.120	0.000	−0.150	0.000	B	L1	38
YMC-Pro C8	0.890	0.010	−0.210	0.000	−0.320	0.010	B	L7	38
YMC-Triart Bio C18	0.954	−0.008	−0.139	0.011	0.261	0.210	B	L1	38
YMC-Triart C18	0.929	−0.020	−0.190	−0.033	−0.023	−0.139	B	L1	38
YMC-Triart C18 ExRS	1.170	0.063	−0.004	−0.096	−0.139	−0.254	B	L1	38
YMC-Triart C8	0.830	−0.007	−0.317	−0.007	−0.023	−0.102	B	L7	38
YMC-Triart PFP	0.586	−0.182	−0.014	−0.045	0.363	1.360	Fluoro	L43	38
YMC-Triart Phenyl	0.723	−0.072	−0.376	−0.008	−0.021	−0.084	Phenyl	L11	38
ZirChrom-EZ	1.040	0.117	−0.999	−0.001	2.089	2.089	Other		56
ZirChrom-MS	0.948	−0.170	−0.451	0.326	0.483	0.550	Other		56

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
ZirChrom-PBD	1.284	0.158	−0.384	−0.072	2.188	2.188	Other	L49	56
ZirChrom-PS	0.589	−0.232	−0.477	0.062	1.750	1.750	Other		56
Zodiac C18	1.066	0.052	−0.069	−0.039	−0.184	−0.152	B	L1	57
Zodiac C18(1)	0.973	0.008	−0.124	0.019	0.204	0.156	B	L1	57
ZodiacSil 120-5 C18 AQ	0.974	−0.007	−0.082	0.003	0.139	0.224	B	L1	57
ZodiacSil 120-5-C18 ace EPS	0.773	0.042	−0.590	0.228	−0.302	0.041	EP		57
ZodiacSil 120-5-C18 AQ Plus	0.948	−0.017	0.214	0.041	−0.131	0.605	EP		57
ZodiacSil 120-5-C18H	1.006	0.008	−0.106	−0.004	0.127	0.156	B	L1	57
ZodiacSil 120-5-C18SH	1.032	0.018	−0.109	−0.024	0.115	0.402	B	L1	57
ZodiacSil 200-5-C18 ace EPS	0.766	0.021	−0.566	0.214	0.028	0.143	EP		57
ZodiacSil 200-5-C18AQ	0.974	−0.011	−0.057	0.006	0.127	0.029	B	L1	57
ZodiacSil 200-5-C18H	0.956	−0.001	−0.121	0.016	0.165	0.218	B	L1	57
ZodiacSil 300-5-C18 ace EPS	0.763	0.025	−0.579	0.211	−0.052	0.136	EP		57
ZodiacSil 300-5-C18H	0.957	−0.012	−0.090	0.015	0.240	0.249	B	L1	57
ZodiacSil 60-5-C18H	1.159	0.041	0.066	−0.078	0.104	0.263	B	L1	57
Zorbax Bonus RP	0.654	0.107	−1.046	0.373	−2.971	−1.103	EP	L60	43
Zorbax C18	1.089	0.055	0.474	0.060	1.489	1.566	A	L1	43
Zorbax C8	0.974	−0.041	0.216	0.176	0.974	1.051	A	L7	43
Zorbax Eclipse PAH	1.031	−0.015	0.688	−0.051	0.072	1.409	B	L1	43
Zorbax Eclipse Plus C18	1.030	0.007	−0.072	−0.020	−0.004	0.020	B	L1	43
Zorbax Eclipse Plus C8	0.889	0.017	−0.172	−0.005	−0.042	0.051	B	L7	43
Zorbax Eclipse XDB-C18	1.077	0.024	−0.063	−0.033	0.055	0.088	B	L1	43
Zorbax Eclipse XDB-C8	0.919	0.025	−0.219	−0.008	0.003	0.012	B	L7	43
Zorbax Eclipse XDB-CN	0.456	−0.068	−0.312	0.003	0.074	0.994	CN	L10	43
Zorbax Extend C18	1.098	0.050	0.012	−0.041	0.030	0.016	B	L1	43
Zorbax Rx-18	1.077	0.037	0.309	−0.038	0.096	0.415	B	L1	43
Zorbax Rx-C8	0.792	−0.076	0.116	0.018	0.012	0.948	B	L7	43
Zorbax SB-AQ	0.593	−0.120	−0.083	0.038	−0.136	0.736	EP	L96	43
Zorbax SB-CN	0.502	−0.108	−0.224	0.042	−0.146	1.047	CN	L10	43
Zorbax SB-Phenyl	0.623	−0.161	0.065	0.038	0.033	1.089	Phenyl	L11	43
Zorbax StableBond 300A C18	0.905	−0.050	0.045	0.043	0.254	0.701	B	L1	43
Zorbax StableBond 300A C3	0.526	−0.122	−0.195	0.047	0.057	0.357	B	L56	43
Zorbax StableBond 300A C8	0.701	−0.085	0.002	0.047	0.146	0.820	B	L7	43

(Continued)

APPENDIX 9.3.3 List of available columns with PQRI parameters (mobile phase 50/50 acetonitrile/aqueous buffer 60 mM phosphate v/v).—*cont'd*

Column	H*	S*	A*	B*	C(2.8)*	C(7.0)*	Silica[a]	USP	Manuf.[b]
Zorbax StableBond 80A C18	0.996	−0.032	0.264	−0.001	0.136	1.041	B	L1	43
Zorbax StableBond 80A C3	0.601	−0.124	−0.081	0.038	−0.084	0.810	B	L56	43
Zorbax StableBond 80A C8	0.795	−0.079	0.137	0.018	0.014	1.020	B	L7	43
Zorbax XDB-Phenyl	0.665	−0.127	−0.242	0.019	0.063	0.584	Phenyl	L11	43

A*, hydrogen bonding basic solute; *B**, hydrogen bonding acidic solute, C(2.8)*, cation exchange at pH = 2.8; C(7.0)*, cation exchange at pH = 7.0; *H**, hydrophobic character, *S**, steric hindrance.

[a] A, Type A *silica;* B, Type B *silica;* CN, *cyano phase;* EP, *embedded phase;* Fluoro, *fluoronated phase;* Phenyl, *phenyl phase.*

[b] **Manufacturer**: *1-Dionex, 2-Thermo/Hypersil, 3-ACT (Advanced Chromatography Technologies Ltd.), 4-Waters, 5-SepaChrom, 6-Grace/Alltech, 7-Analytical Sales and Services, 8-Phenomenex, 9-Restek, 10-Grace/Jones, 11-Supelco, 12-CNW Technologies, 13-Bonna-Agela Technologies, Inc., 14-Dikma Technologies, 15-Imtakt, 16-Shiseido, 17-ES Industries, 18-Merck KGaA (EMD Millipore), 19-Higgins Analytical, 20-MicroSolv, 21-Genius Technologies, 22-Nacalai Tesque, 23-Grace/Vydac, 24-Nomura, 25-Macherey Nagel, 26-Bischoff, 27-no longer available, 28-Diamond Analytics, 29-Wesley Technologies Inc., 30-Fortis Technologies, 31-Orochem Technologies, 32-Grace/Grom, 33-Advanced Materials Technologies, 34-Hichrom, 35-Hitachi High-Tech, 36-Hamilton, 37-GL Sciences, 38-YMC, 39-Nanghavi Chromatography Solutions Ltd., 40-Kromasil by Nouryon, 41-Agilent/Varian, 42-Chromatopak, 43-Agilent Technologies, 44-Mac-Mod Analytical, 45-SIELC, 46-Sepax Technologies, 47-Shimadzu, 48-Showa Denko, 49-Chromanik, 50-Nanologica, 51-Eprogen, 52-Welch, 53-Tosoh Bioscience, 54-SEPSERV, 55-VDS Optilab, 56-ZirChrom, 57-Zodiac Life Sciences.*

Source: http://apps.usp.org/app/USPNF/columnsDB.html.

Appendix to chapter 11

APPENDIX 11.2.1 Several columns indicated as useable in HILIC mode on underivatized silica.

Brand name	Manufacturer	Phase type/pore size/particle size
Accucore	Thermo Scientific	Silica; 80 Å; 2.6 μm
Atlantis	Waters	Silica; 100 Å; 3.0 μm
Ascentis Express HILIC	Supelco	Core-shell silica; 2.7 μm
Betasil	Thermo Hypersil	Silica; 100 Å; 3.0 μm
Boltimate HILIC	Welch	Silica; 90 Å; 2.7 μm
ChromoLith	Merck	Silica monolith; mesopores (average pore size 13 nm)
COSMOSIL SL-II	Nacalai	Silica (silica gel); 120 Å; 3, 5, 15 μm
Halo; Halo-Penta HILIC	Hichrom	Silica; 90 Å; 2.7, 5.0 μm
Hypersil Gold HILIC	Thermo	Silica; 100 Å; 1.9, 3.0, 5.0 μm
Hypersil Silica	Thermo	Silica; 120 Å; 5 μm
Inspire Silica	Dikma	Silica; 100 Å; 3.0, 5.0, 10 μm
Kromasil	EKA Chemicals	Silica; 60 Å; 5.0, 10 μm
Nucleodur unmodified	Macherey-Nagel	Silica; 110 Å; 1.8, 3.0, 5.0 μm
Nucleosil (SIL) unmodified	Macherey-Nagel	Silica; 50, 100 Å; 5.0 μm
Promosil Silica	Bonna Agela	Silica; 100 Å; 5.0 μm
Silia Chrom XDB Si	Greyhound Chromatography	Silica; 100 Å; 3.0, 5.0, 10 μm
Silia Chrom XDB1 Si	Greyhound Chromatography	Silica; 100 Å; 3.0, 5.0, 10 μm
Silia Chrom XDB1 Si-300	Greyhound Chromatography	Silica; 300 Å; 3.0, 5.0, 10 μm
Spheri-5 Silica	Brownlee (Alltech)	Silica; 80 Å; 5.0 μm
Supelcosil LC-Sil	Supelco	Silica; 120 Å; 5.0 μm
Syncronis Silica	Thermo	Silica; 100 Å; 5.0 μm
Venusil XBP Silica	Bonna Agela	Silica; 100 Å; 3.0, 5.0, 10 μm
Venusil XBP-L Silica	Bonna Agela	Silica; 100 Å; 3.0, 5.0, 10 μm
YMC-Pack SIL	YMC	Silica; 60, 120, 200, 300 Å; 3.0, 5.0, 10 μm
Zorbax Rx-Sil	Agilent	Silica; 80 Å; 1.8, 5 μm

APPENDIX 11.2.2 Several neutral HILIC columns that are commercially available.

Brand name	Manufacturer	Support/pore size/ particle size	Nature of phase
Acclaim HILIC 10	Thermo (Dionex)	Silica; 120 Å; 3 μm	Proprietary polar group
Accucore 150 Amide HILIC	Thermo	Silica; 150 Å; 2.6 μm	Amide
Accucore Urea HILIC	Thermo	Silica; 80 Å; 2.6 μm	Urea
Alltima Cyano	Grace Alltech	Silica; 190 Å; 3, 5 μm	3-Cyanopropyl
Cogent Type C Silica	Microsolv	Silica; 100 Å; 4 μm	Silica hydride
COSMOSIL CN-MS	Nacalai	Silica; 120 Å; 5 μm	3-Cyanopropyl
Cyclobond I 2000	ASTEC	Silica; 100 Å; 5, 10 μm	β-Cyclodextrin
Epic Diol HILIC	ES Industries	Silica; 120 Å; 1.8 μm	Diol
Epic HILIC FL	ES Industries	Silica; 120 Å; 1.8 μm	Fluorinated
GlycoSep N	ProZyme	Silica; 10 μm	Amide
Hydrolyzed GMA-co-EDMA	Custom synthesis	Methacrylic copolymer;	2,3-Dihydroxypropyl
Inertsil Diol	GL Sciences	Silica; 100 Å; 3, 5 μm	2,3-Dihydroxypropyl
Inspire Diol	Dikma	Silica; 100 Å; 3, 5, 10 μm	Diol
Kinetex HILIC Core-shell diol	Phenomenex	Silica core-shell; 1.7, 2.6, 5 μm	Diol
Lichrospher Diol 100	Merck	Silica; 100 Å; 5 μm	2,3-Dihydroxypropyl
Luna HILIC diol	Phenomenex	Silica; 100 Å; 3, 5, 10 μm	Diol and ether embedded
Nucleodex b-OH a-PM, etc.	Macherey-Nagel	Silica; 100 Å; 5 μm	β-Cyclodextrin
Nucleodur CN/CN-RP	Macherey-Nagel	Silica; 110 Å; 3, 5 μm	3-Cyanopropyl
Nucleosil CN	Macherey-Nagel	Silica; 100, 120 Å; 5, 7, 10 μm	3-Cyanopropyl
Nucleosil OH (diol)	Macherey-Nagel	Silica; 100 Å; 5 μm	2,3-Dihydroxypropyl
Perhydroxyl-CB[6]uril silica	Custom synthesis	Silica	Perhydroxyl-cucurbit[6] uril
PolyGlycoplex	PolyLC	Silica (polysuccinimide); 5, 12 μm	Poly(succinimide)
PolyHydroxyethyl A	PolyLC	Silica (polyaspartic acid); 60–1500 Å; 5, 12 μm	Poly(2-hydroxyethyl-aspartamide)

APPENDIX 11.2.2 Several neutral HILIC columns that are commercially available.—cont'd

Brand name	Manufacturer	Support/pore size/ particle size	Nature of phase
Promosil CN	Bonna Agela	Silica; 100 Å; 5 μm	3-Cyanopropyl
Silasorb Diol	Chemapol	Silica	2,3-Dihydroxypropyl
Silia Cgrom XDB1 Diol-300	Greyhound Chromatography	Silica; 300 Å; 3, 5 μm	Diol
Silia Chrom HILIC	Greyhound Chromatography	Silica; 100 Å; 3, 5 μm	Urea
Silia Chrom HILIC-300	Greyhound Chromatography	Silica; 300 Å; 3, 5 μm	Urea
Silia Chrom XDB1 Diol	Greyhound Chromatography	Silica; 100 Å; 3, 5 μm	Diol
TSKgel Amide-80	Tosoh Bioscience	Silica; 100 Å; 2, 3, 5, 10 μm	Amide
Unisol Amide	Bonna Agela	Silica; 100 Å; 3, 5 μm	Amide
Venusil XBP CN	Bonna Agela	Silica; 100 Å; 5, 10 μm	3-Cyanopropyl
Venusil XBP Diol	Bonna Agela	Silica; 100 Å; 5 μm	Amide
XBridge amide	Waters	Polyetoxysilane (BEH); 130 Å; 3.5 μm	Amide
Xbridge HILIC	Waters	Polyetoxysilane (BEH); 130 Å; 1.7, 2.5, 3.5, 5, 10 μm	Diol
YMC-Pack CN	YMC	Silica; 120, 300 Å; 3, 5 μm	3-Cyanopropyl
YMC-pack Diol 120 NP	YMC	Silica; 120 Å; 1.9, 3, 5 μm	2,3-Dihydroxypropyl
YMC-Pack PVA-Sil	YMC	Silica support; 60, 120, 200, 300 Å; 3, 5 μm	Polyvinyl alcohol polymerized on silica

APPENDIX 11.2.3 Several HILIC columns with weak anion exchange properties that are commercially available.

Brand name	Manufacturer	Support/pore size/particle size	Nature of phase
Amino	Jordi	Silica; 120 Å; 3, 5 μm	Aminopropyl
Amino-bonded Zirconia	Custom synthesis	Zirconia	Mono-, di- and triamine
Asahipak NH_2 P	Shodex	Poly(vinyl alcohol) gel; 100 Å; 4, 5 μm	Amine
Astec apHera	ASTEC	PVA copolymer; 300 Å; 5 μm	Polyamine
COSMOSIL DEAE	Nacalai	Porous polymethacrylate	Diethylaminoethyl (DEAE)
COSMOSIL HILIC	Nacalai USA	Silica; 120 Å; 5 μm	Triazole
Durashell NH2	Bonna Agela	Silica; 100 Å; 5, 10 μm	Amino
EPIC-PI	ES Industries	Silica; 120 Å; 1.8 μm	Aromatic amine
GlycoSep C	ProZyme	Polymeric; 10 μm	DEAE
Hypersil APS2	Thermo Scientific	Silica; 120 Å; 3 μm	3-Aminopropyl
Hypersil GOLD PEI HILIC	Thermo Scientific	Silica; 175 Å; 1.9 μm	Polyethylene amine
Luna Amino	Phenomenex	Silica; 100 Å; 3, 5, 10 μm	3-Aminopropyl
Micropellicular AP Silica	Custom synthesis	Silica	3-Aminopropyl
Nucleodur NH2/NH2-RP	Macherey-Nagel	Silica; 110 Å; 3, 5, 7 μm	Amino
Nucleosil Carbohydrate	Macherey-Nagel	Silica; 10 μm	Amino
Nucleosil N(CH3)2	Macherey-Nagel	Silica; 100 Å; 5 μm	Tertiary amine
Nucleosil NH2	Macherey-Nagel	Silica; 100 Å; 3, 5, 10 μm	Amino
PolyWAX LPTM	PolyLC	Silica; 100, 300, 1000, 1500 Å; 3, 5, 12 μm	Linear polyethylenimine
Promosil NH2	Bonna Agela	Silica; 100 Å; 5 μm	Amino
Silia Chrom XDB1-Amino	Greyhound Chroma.	Silica; 100 Å; 3, 5 μm	Amino
Silia Chrom XDB1-Amino-300	Greyhound Chroma.	Silica; 300 Å; 3, 5 μm	Amino
Spherisorb NH_2	Waters	Silica; 80 Å; 5, 10 μm	3-Aminopropyl
TSK Gel NH2-100	Tosoh Bioscience	Silica (end-capped); 100 Å; 3 μm	Amino
Venusil XBP NH2	Bonna Agela	Silica; 120 Å; 5 μm	Amino
YMC-Pack PA	YMC	Silica; 120 Å; 5 μm	Amino
YMC-Pack Polyamine II	YMC	Silica; 120 Å; 5 μm	Polyamine (sec. and tert.)
Zorbax NH_2	Agilent	Silica; 70 Å; 3.5, 5, 7 μm	3-Aminopropyl

APPENDIX 11.2.4 Several HILC columns with cation exchange properties that are commercially available.

Brand name	Manufacturer	Support/pore size/particle size	Nature of phase	Type
Acrylamide CEC phase	Custom synthesis	4% Cross-linked polyacryl-amide	Dodecyl chains and sulfonic acid	SCX
COSMOSIL CM	Nacalai	Silica; 120 Å; 5 μm	Carboxymethyl	WCX
Excelpak CHA-P44	Yokogawa (Agilent)	Styrene/divinyl-benzene	Sulfonic acid	SCX
PolyCAT A	PolyLC	Silica; 300, 1000, 1500 Å; 3, 5, 12 μm	Poly(aspartic acid)	WCX
PolySulfoethyl A	PolyLC	Silica; 200, 300, 1000 Å; 3, 5, 12 μm	Poly(2-sulfonylethyl aspartamide)	SCX
Sulfonated S-DVB	Custom synthesis	Styrene/divinyl-benzene	Sulfonic acid	SCX
SynChropak CM 300	Eichrom (Eprogen)	Silica; 300 Å; 6 μm	Carboxymethyl	WCX

APPENDIX 11.2.5 Several HILC columns with zwitterionic phases that are commercially available.

Brand name	Manufacturer	Support/pore size/ particle size	Nature of phase
Nucleodur HILIC	Macherey-Nagel	Silica; 110 Å; 1.8, 3, 5 μm	Dimethylamino and sulfonic
Obelisc R	Sielc	Silica; 100 Å; 5, 10 μm	Zwitterionic negative charged groups
Obelisc N	Sielc	Silica; 100 Å; 5, 10 μm	Zwitterionic positive charged groups
PolyCAT A	PolyLC	Silica; 60, 100, 200, 300, 500, 1000, 1500 Å; 3, 5, 12 μm	Poly(aspartic acid)
Syncronis HILIC	Thermo Fisher	Silica; 100 Å, end-capped; 5 μm	Zwitterionic
ZIC-HILIC	SeQuant (Merck)	Silica with sulphobetaine; 100, 200 Å; 3.5, 5 μm	Polymeric sulfonylalkylbetaine
ZIC-pHILIC	SeQuant (Merck)	Polymer with sulphobetaine; 5 μm	Polymeric sulfonylalkylbetaine
ZIC-cHILIC	SeQuant (Merck)	Silica with phosphorylcholine; 100 Å; 3 μm	Polymeric phosphorylcholine

APPENDIX 11.2.6 Examples of HILIC columns with hydride-based stationary phase.

Brand name	Manufacturer	Particle size (μm)	Surface area (m^2/g)	Support/Pore size (Å)	pH range of use	Max temp (°C)
Cogent Diamond Hydride	HiChrom	4	350	Silica 100	2.0–8.0	80
Diamond Hydride	MicroSolv	4	390	Silica C 100	2.5–7.5	60
Diamond Hydride 2.0	MicroSolv	2.2	340	Silica C 120	2.5–7.5	60
Diamond Hydride	HiChrom	4	350	Silica C 100	2.5–7.0	60

APPENDIX 11.2.7 Several more recent HILIC columns.

Column name	Manufacturer	Phase	Particle size	Type
Glycoprotein BEH Amide	Waters	Amide	1.7	porous
HALO Glycan	Advanced Materials Technol.	Proprietary	2.7	core-shell
HILICpak VG-50	Shodex	Amino	5	polymeric
HILICpak VT-50	Shodex	Quaternary ammonium	5	polymeric
iHILIC-Fusion	HILICON-AB	Hydroxyethyl amide, sulfate, phosphate, quaternary ammonium	1.8, 3.5, 5	porous
iHILIC-Fusion(+)	HILICON-AB	Hydroxyethyl amide, sulfate, quaternary ammonium	1.8, 3.5, 5	porous
SunShell HILIC Amide	ChromaNik Technologies Inc.	Amide	2.6	core-shell
Poroshell 120 HILIC-Z	Agilent Technologies	Zwitterionic	1.9, 4	Core-shell
SanArmor NH2	ChromaNik Technologies Inc.	Aminopropyl	3, 5	Porous
SunShell HILIC-S	ChromaNik Technologies Inc.	Bare silica	2.6	Core-shell
Raptor Polar X	Restek	Proprietary	2.7	Core-shell
Boltimate Core-Shell	Welch	Bare silica	2.7	Core-shell

Appendix to chapter 13

APPENDIX 13.2.1 Some commercial silica-based cation exchange columns.

Column	Manufacturer	Type	Dimensions (length × i.d., mm)	Capacity (mEquiv g^{-1})	Particle size (μm)	Pore size Å	Type of phase
BioBasic SCX	Thermo	Strong	Various	0.07	5	300	$-SO_3H$
IC YK-421	Shodex	Weak	125 × 4.6		5	20	Coated silica polymer-COOH
LiChrosil IC CA	Merck	Weak	100 × 4.6		5		Coated PBDMA[a]
Luna SCX	Phenomenex	Strong	150 × 4.6	0.15	5, 10	300	$-C_6H_4-SO_3H$
Nucleosil 5 SA	Machery-Nagel	Strong	125 × 4	0.5	5	100	$-SO_3H$
Nucleosil 5 SA	Machery-Nagel	Strong	Various	1	5, 10	100	$-C_3H_6-C_6H_4-SO_3H$
Partisil 10 SCX	Whatman	Strong	250 × 4.6	0.5	5, 10	85	$-C_6H_4-SO_3H$
PartiSphere	Whatman	Strong	125 × 4.6 250 × 4.6		5	120	
Phenosphere SCX	Phenomenex	Strong	Various	0.6	5, 10	80	$-C_6H_4-SO_3H$
PolyCAT A	PolyLC	Weak	Various		3, 5, 12	300, 1000, 1500	Poly(aspartic acid) bonded to silica
PolySULFOETHYL A	PolyLC	Strong	Various		3, 5, 12	300, 1000	Sulfoethyl-aspartamide
Spherisorb SCX	Waters	Strong	125 × 4		5	80	
Supelcosil LC-SCX	Sigma	Strong	250 × 4.6		5	120	$-C_3H_6-SO_3H$
SynChropak	Lab Unlimited	Weak			6	300	Carboxymethyl
TSK Gel IC Cation SW	Toyo Soda	Strong	50 × 4.6	0.5	5		$-SO_3H$
TSKgel CM-2SW	Tosoh	Weak	Various	0.3	5	125	Carboxymethyl
TSKgel SP-2SW	Tosoh	Strong	Various	0.3	5	125	$-C_3H_6-SO_3H$
Universal Cation	Alltech	Weak	100 × 4.6		7	180	Coated PBDMA
Vydac SC	Separation Group	Strong	250 × 4.6	0.1	30–44	300	$-SO_3H$

[a] *PBDMA, poly(butandiolmethacrylate).*

APPENDIX 13.2.2 Some cation exchange polymeric columns.

Column	Manufact.	Type	Dimensions (length × i.d., mm)[a]	Particle size/pore size	Capacity (mEquiv g^{-1})	Technol.	Type of phase[a]
CM-825	Shodex	Weak	75 × 8	8 μm/5000 Å	0.4		Carboxymethyl
Diaion	Mitsubishi	Strong	Various				PS-DVB-SO_3H
Diaion	Mitsubishi	Weak	Various			Highly porous	Acrylic acid-methacrylate
ES-502C 7C	Shodex	Weak	100 × 7.5	9 μm/2000 Å	0.55		PVA carboxymethyl
IC YS-50	Shodex	Weak	125 × 4.6				PVA
ICT-521	Shodex	Strong	150 × 4.6				PS-DVB-SO_3H
IonPac CS-10	Dionex	Strong	250 × 4	8.5 μm	0.08	Latex	PS-DVB-SO_3H
IonPac CS-11	Dionex	Strong	250 × 2	8 μm	0.035	Latex	PS-DVB-SO_3H
IonPac CS-12, 14, 16, 17, 18, 19	Thermo/ Dionex	Weak	250 × 4 or 250 × 2	8 μm/60 Å	0.7; 2.8	Latex	PS-DVB-COOH
IonPac CS-12A	Thermo/ Dionex	Medium	Various	5; 8 μm	0.7; 0.94; 2.8	Latex	PS-DVB-COOH +PS-DVB-PO_3H
IonPac CS-15	Thermo/ Dionex	Weak	250 × 4 or 250 × 2	8.5 μm	0.7; 2.8	Latex	PS-DVB-COOH, $-PO_3H$/crown ether
LCA K02	Sykam	Strong	125 × 4.6	5 μm	0.4		PMA
MAbPAC SCX-10	Thermo	Strong	Various	3; 5; 10 μm		Latex	PS-DVB-SO_3H
PL-SCX	Agilent	Strong	Various	10; 30 μm/1000, 4000 Å			PS-DVB-SO_3H
ProPac SCX	Thermo	Strong	250 × 4	10 μm		Latex	PS-DVB-SO_3H
ProPac WCX	Thermo	Weak	250 × 4	10 μm		Latex	PS-DVB-COOH
PRP-X100, X200, X400, etc.	Hamilton	Strong	250 × 4.6	100 Å	0.035–2.5		PS-DVB-SO_3H
Shimpack IC-C1	Shimadzu	Strong	150 × 4	10 μm			PS-DVB-SO_3H
SP-825	Shodex	Strong	75 × 8	8 μm/5000 Å	0.4		$-C_3H_6-SO_3H$
TSKgel BioAssist S	Tosoh	Strong	Various	7; 13 μm/1300 Å	0.1		$-C_3H_6-SO_3H$
TSKgel CM-STAT	Tosoh	Weak	Various	7; 10 μm	0.1		Carboxymethyl
TSKgel OApak-A	Tosoh	Weak	Various	5 μm	1.5		Methacrylate
TSKgel SP-5PW	Tosoh	Strong	Various	10, 13, 20 μm/ 1000 Å	>0.1		
TSKgel SP-STAT	Tosoh	Strong	Various	7; 10 μm	0.023		$-C_3H_6-SO_3H$
YS-50	Shodex	Weak	125 × 4.6	5 μm			PVA-COOH

[a] *Note: PS-DVB, polystyrene-divinylbenzene, PVA, polyvinyl alcohol, PMA, polymethylacrylate.*

APPENDIX 13.2.3 Some commercial silica-based anion exchange columns.

Column	Manufacturer	Type	Dimensions (length × i.d., mm)	Capacity (mEquiv g^{-1})	Particle size (μm)	Type of phase
Inertsil AX	GL Sciences	Strong	Various from 33 × 1 to 250 × 4.6	0.43	5	diethylamino group
Luna NH_2	Phenomenex	Weak	Various		3, 5, 10	$-NH_2$
Nucleosil 10 Anion	Machery-Nagel	Medium	250 × 4.0	0.06	10	trimethylamine methyldiethylamine
Partisil 10 SAX	Whatman	Strong	250 × 4.6	0.5	5, 10	$-NR_3^+$
Partisphere SAX	Whatman	Strong	150 × 4.6 250 × 4.6		5	
Phenosphere SAX	Phenomenex	Strong	Various	0.4	5, 10	$-NR_3^+$
SynChropak SAX	Lab Unlimited	Weak	100 × 4.6 250 × 4.6		6.5	polyethyleneimine
SynChropak SAX	Lab Unlimited	Strong	100 × 4.6 250 × 4.6		6.5	NR_3^+
TSKgel QAE-2SW	Tosoh	Strong	Various	>0.3	5	$-N(CH_3)_3^+$
TSKgel DEAE-2SW	Tosoh	Weak	Various	>0.3	5	diethylaminoethyl
TSKgel Q-STAT	Tosoh	Strong	Various	0.27	7; 10	$-NR_3^+$
TSKgel DNA-STAT	Tosoh	Strong	Various	0.27	5	Acrylate-NR_3^+
TSK Gel IC-SW	Toyo Soda	Strong	250 × 4.6	0.4	5	$-N(C_2H_5)_2CH_3^+$
Vydac 302 IC 4.6	Separation Group	Strong	50 × 4.6	0.1	10	Spherical particles with $-NR_3^+$
Vydac 300 IC 4.6	Separation Group	Strong	250 × 4.6	0.1	15	
Wescan 269-001	Wescan	Strong	250 × 4.6	0.08	13	$-NR_3^+$

APPENDIX 13.2.4 Some anion exchange polymeric columns.

Column	Manufacturer	Type	Particle size (μm)	Dim. (length × i.d., mm)	Type of phase
Allsep	Grace – Alltech	Strong	7 μm	Various	PMA gel-NR_3^+
AN1; AN300	Sarasep	Strong	9 μm	250 × 4.6 100 × 7.5	PS-DVB-amine
BioSuite DEAE	Waters	Strong	2.5 μm; 10 μm	35 × 4.6 75 × 7.5	PMA gel-NR_3^+
BioSuite Q-PEEK	Waters	Strong	10 μm	50 × 4.6	PMA gel-NR_3^+
CarboPac MA1	Thermo/ Dionex	Strong	7.5 μm	250 × 4.0	Resin with tertiary amine
CarboPac PA1[a]	Thermo/ Dionex	Strong	10 μm	250 × 2.0 250 × 4.0	Latex technology
CarboPac SA10	Thermo/ Dionex	Strong	6 μm	250 × 2.0 250 × 4.0	Latex nano beads
CarboPac PA1, PA10, PA20, PA100, PA200	Thermo/ Dionex	Strong	Various	Various	Pellicular, nanoporous beads, etc.
Cosmogel QA Cosmogel DEAE	Nacalai Tesque, Inc.	Strong	5 μm	75 × 8	PMA gel-NR_3^+ PMA gel-$NH(C_2H_5)^+$
Diaion	Mitsubishi Chemical	Strong	10 μm	75 × 7.5	Styrenic/acrylic amine
Discovery BIO PolyMA-WAX	Supelco	Strong	5 μm	50 × 4.6	PMA gel-NR_3^+
IC I-524A	Shodex	Strong	12 μm	100 × 4.6	Polyhydroxy-methacrylate-NR_3^+
IC NI-424	Shodex	Strong	5 μm	100 × 4.6	Polyhydroxy-methacrylate-NR_3^+
IC-Pak Anion	Waters	Strong	10 μm	75 × 4.6	PMA gel-NR_3^+
IC SI-35 4D; IC SI-50 4E; IC SI-52 4E; IC SI-90 4E; IC SI-91 4C	Shodex	Strong	3.5; 5; 9 μm	Various	PVA-NR_3^+
IEC DEAE-825	Shodex	Weak	12	75 × 8	PHMA-NR_3^+
Ion Swift Max 100 monolithic	Thermo/ Dionex	Medium		250 × 1 250 × 0.25	PS-DVB-alkanol–NR_3^+
Ion Swift Max 200 monolithic	Thermo/ Dionex	Medium		250 × 0.25	PS-DVB-alkanol–NR_3^+
IonPac AS10[a]	Thermo/ Dionex	Strong	8.5 μm	250 × 2 250 × 4	Latex technology
IonPac 15[a], 16[a], 20[a], etc.	Thermo/ Dionex	Various	9 μm	250 × 0.4 250 × 2 250 × 4	PS-DVB-NR_3^+ and alkanol
IonPac AS11[a]	Thermo/ Dionex	Strong	13 μm	250 × 2 250 × 4	Latex technology

APPENDIX 13.2.4 Some anion exchange polymeric columns.—*cont'd*

Column	Manufacturer	Type	Particle size (μm)	Dim. (length × i.d., mm)	Type of phase
IonPac AS11-HC[a]	Thermo/ Dionex	Strong	9 μm	250 × 0.2 250 × 2 250 × 4	Latex technology
IonPac AS12A[a]	Thermo/ Dionex	Strong	9 μm	200 × 2 200 × 4	Latex technology
IonPac AS14, 14A, 12A	Thermo/ Dionex	Medium	7; 9 μm	Various	PS-DVB-alkanol−NR_3^+
IonPac AS16[a]	Thermo/ Dionex	Strong	9 μm	250 × 0.4 250 × 2 250 × 4	Latex technology
IonPac AS17-C[a]	Thermo/ Dionex	Strong	10.5 μm	250 × 2 250 × 4	Latex technology
IonPac AS18	Thermo/ Dionex	Strong	7.5; 13 μm	Various	Polyethylvinylbenzene-DVB-NR_3^+
IonPac AS22, AS23	Thermo/ Dionex	Medium	6; 11 μm	250 × 0.4 250 × 2 250 × 4	PS-DVB-alkanol−NR_3^+
IonPac AS4A[a]	Thermo/ Dionex			Various	Latex technology
IonPac AS4A-SC[a]	Thermo/ Dionex	Strong		Various	Latex technology
IonPac AS5[a]	Thermo/ Dionex	Medium	15 μm	250 × 4	PS-DVB-alkanol-NR_3^+
IonPac AS7[a]	Thermo/ Dionex	Strong	10 μm	250 × 2 250 × 4	PS-DVB-NR_3^+
IonPac AS9 HC	Thermo/ Dionex	Strong	9 μm	250 × 2 250 × 4	PS-DVB-NR_3^+
IonPac AS9 SC, AS4A-SC	Thermo/ Dionex	Medium	9; 13 μm	250 × 2 250 × 4	PS-DVB-NR_3^+ and alkanol
IonPac AS9-HC[a]	Thermo/ Dionex	Strong			Latex technology
IonPac AS9-SC[a]	Thermo/ Dionex	Strong			Latex technology
LCA A01	Sykam	Strong		200 × 4.0	PS-DVB-amine
MetroSep Anion Dual 2	Metrohm-Peak, Inc.	Strong	8 μm	75 × 4.6	PMA gel-NR_3^+
MetroSep Anion Dual 3	Metrohm-Peak, Inc	Strong	6 μm	100 × 4.0	PMA gel-NR_3^+
MetroSep Anion Dual 4	Metrohm-Peak, Inc		–	100 × 4.6	Monolithic silica gel with 2 μm macropores and 13 nm mesopores

(Continued)

APPENDIX 13.2.4 Some anion exchange polymeric columns.—*cont'd*

Column	Manufacturer	Type	Particle size (μm)	Dim. (length × i.d., mm)	Type of phase
Metrosep A Supp 1	Metrohm-Peak, Inc	Strong	7 μm	50 × 4.6 250 × 4.6	PS-DVB-$N(CH_3)_3^+$
Metrosep A Supp 3	Metrohm-Peak, Inc	Strong	9 μm	250 × 4.6	PS-DVB-$N(CH_3)_3^+$
Metrosep A Supp 5	Metrohm-Peak, Inc	Strong	5 μm	50 × 4.0 100 × 4.0 150 × 4.0 250 × 4.0	PVA gel-NR_3^+
Metrosep A Supp 7	Metrohm-Peak, Inc	Strong	5 μm	150 × 4.0 250 × 4.0	PVA gel-NR_3^+
Metrosep A Supp 10 Metrosep A Supp 15 Metrosep A Supp 15	Metrohm-Peak, Inc	Strong	4.6 μm	50 × 4.0 75 × 4.0 100 × 4.0 250 × 4.0	PS-DVB-$N(CH_3)_3^+$
Nucleogel SAX	Machery-Nagel	Strong		250 × 4	PMA gel-NR_3^+
ProPac SAX	Thermo/ Dionex	Strong		250 × 4	$-NR_3^+$
ProPac WAX	Thermo/ Dionex	Strong		250 × 4	Tertiary amine
Protein-Pak Q 8HR	Waters	Strong		250 × 4	PMA gel-NR_3^+
PRP X100	Hamilton	Strong	10 μm	125 × 4.0 250 × 4	PS-DVB-$N(CH_3)_3^+$
PRP X500	Hamilton	Strong	5 μm	150 × 4.6	Poly(methacrylamido-propyl)-$N(CH_3)_3^+$
RCX-30	Hamilton	Strong	7 μm	150 × 4.6	PS-DVB-$N(CH_3)_3^+$
Shim-Pack WAX-1; WAX-2	Shimadzu	Strong	3; 5 μm	50 × 4	$-NR_3^+$
Shodex IEC QA-825	Shodex	Strong	12 μm	75 × 8	PMA gel-NR_3^+
Si-90 4E	Shodex	Strong	9 μm	250 × 4	PVA-NR_3^+
Star-Ion A300	Phenomenex	Strong	7 μm	100 × 4.6	PS-DVB-NR_3^+
Star-Ion A300 HC (high capacity)	Phenomenex	Strong	7 μm	100 × 10.0	PS-DVB-NR_3^+
Super-Sep IC Anion	Metrohm-Peak, Inc.	Strong	9 μm	250 × 4.6	PVA gel-NR_3^+
TSKgel BioAssist Q	Tosoh Bioscience	Strong	10; 13 μm	50 × 4.6	PMA gel-NR_3^+

APPENDIX 13.2.4 Some anion exchange polymeric columns.—*cont'd*

Column	Manufacturer	Type	Particle size (μm)	Dim. (length × i.d., mm)	Type of phase
TSKgel DNA-STAT	Tosoh Bioscience	Strong	5 μm	100 × 4.6	PMA gel-NR_3^+
TSKgel IC-Anion-PW	Tosoh Bioscience	Strong	10 μm	50 × 4.6	PMA gel-NR_3^+
TSKgel Q-STAT	Tosoh Bioscience	Strong	7; 10 μm	35 × 3	PMA gel-NR_3^+
TSKgel SuperQ-5PW	Tosoh Bioscience	Strong	10; 13 μm	75 × 7.5	PMA gel-NR_3^+
Zodiac IC Anion	Zodiac Life Sciences	Strong	7; 10 μm	250 × 4.6	PMA gel-NR_3^+

[a] *Note: Phases made using latex technology.*

APPENDIX 13.2.5 Some ion-moderated polymeric columns.

Column	Manufacturer	Type	Data about construction
ChromSpher Lipids	Agilent	Silica with sulfonic groups and Ag^+	pore size: 120Å; 5 μm particles; 250 × 4.6 mm
HC-75 Ca^{2+}	Hamilton	PS-DVB sulfonic Ca^{2+}	Pore size: 100 Å; exchange capacity: 5 meq/g
HC-75 H^+	Hamilton	PS-DVB sulfonic H^+	
HC-75 Pb^{2+}.	Hamilton	PS-DVB sulfonic Pb^{2+}.	
IonPac ICE-AS1	Thermo/Dionex	PS-DVB sulfonic H^+	27 meq/column for 250 × 9 mm; 5.3 meq/column for 250 × 4 mm; 7.5 μm bead diam.
IonPac ICE-AS6	Thermo/Dionex	PS-DVB sulfonic and carboxylic H^+	27 meq/column for 250 × 9 mm; 8 μm bead diam.
IonPac ICE-Borate	Thermo/Dionex	PS-DVB sulfonic H^+	27 meq/column for 250 × 9 mm; 7.5 μm bead diam.
MCI CK/CA Series	Mitsubishi Chem.	PS-DVB sulfonic with various ions Ca^{2+}, Ag^+, etc.	5—20 μm particle size; various dimensions
RCM-Monosaccharide (L19 packing)	Phenomenex	8 % cross-linked PS-DVB sulfonic Ca^{2+}	300 × 7.8 mm; 8 μm particle size
RHM-Monosaccharide (L17 packing)	Phenomenex	8 % cross-linked PS-DVB sulfonic H^+	"
RAM-Carbohydrate	Phenomenex	8 % cross-linked PS-DVB sulfonic Ag^+	"

(Continued)

APPENDIX 13.2.5 Some ion-moderated polymeric columns.—*cont'd*

Column	Manufacturer	Type	Data about construction
RSO-Oligosaccharide	Phenomenex	4 % cross-linked PS-DVB sulfonic Ag^{+}	200 × 10 mm; 12 μm particle size
RNO-Oligosaccharide	Phenomenex	4 % cross-linked PS-DVB sulfonic Na^{+}	"
RPM-Monosaccharide (L34 packing)	Phenomenex	8 % cross-linked PS-DVB sulfonic Pb^{2+}	300 × 7.8 mm; 8 μm particle size
RNM-Carbohydrate (L54 packing)	Phenomenex	8 % cross-linked PS-DVB sulfonic Na^{+}	"
ROA-Organic Acid (L22 packing)	Phenomenex	8 % cross-linked PS-DVB sulfonic H^{+}	"
RFQ-Fast Acid	Phenomenex	8 % cross-linked PS-DVB sulfonic H^{+}	100 × 7.8 mm; 8 μm particle size
RKP-Potassium	Phenomenex	8 % cross-linked PS-DVB sulfonic K^{+}	300 × 7.8 mm
RCU-USP Sugar Alcohols (L19 packing)	Phenomenex	8 % cross-linked PS-DVB sulfonic Ca^{2+}	250 × 4.0 mm; 8 μm particle size
SUGARSH1011, SUGARSC1011, etc.	Shodex	PS-DVB sulfonic H+; Ca^{2+}; Pb^{2+}; Zn^{2+}; Na^{+}	300 × 8 mm; 50 × 8 mm; 250 × 6 mm; 150 × 6 mm; 6–30 μm particles

Appendix to chapter 14

APPENDIX 14.2.1 Pirkle type stationary phases.

Chiral selector	Product line	Maker
(R)-Phenylglycine, 3,5-dinitrobenzoic acid, amide linkage, propyl handle	Chrex (R)-PGLY and DNB (3001)	Phenomenex
(R)-1-Naphtylglycine, 3,5-dinitrobenzoic acid, amide linkage, propyl handle	Chrex (R)-NGLY and DNB (3005)	Phenomenex
(S)-Valine, 3,5-dinitroaniline, urea linkage, propyl handle	Chrex 3010	Phenomenex
(S)-tert-Leucine, 3,5-dinitroaniline, urea linkage, propyl handle	Chrex 3011	Phenomenex
(R)-Phenylglycine, 3,5-dinitroaniline, urea linkage, propyl handle	Chrex 3012	Phenomenex
(S)-Valine, (R)-1-(α-naphthyl)ethylamine, urea linkage, propyl handle	Chrex 3014	Phenomenex
(S)-Proline, (R)-1-(α-naphthyl)ethylamine, urea linkage, propyl handle	Chrex 3018	Phenomenex
(S)-tert-Leucine, (S)-1-(α-naphthyl)ethylamine, urea linkage, propyl handle	Chrex 3019	Phenomenex
(S)-tert-Leucine, (R)-1-(α-naphthyl)ethylamine, urea linkage, propyl handle	Chrex 3020	Phenomenex
(S)-Indoline-2-carboxylic acid, (R)-1-(α-naphthyl)ethylamine, urea linkage, propyl handle	Chrex 3022	Phenomenex
(R) or (S)-Phenylglycine, 3,5-N-dinitrobenzoyl, amide linkage, propyl handle	ChiralCap (R) or (S)-DNBPG	Astec
(S)- Leucine 3,5-N-dinitrobenzoyl, urea linkage, propyl handle to silica	ChiralCap (S)-DNBLeu	Astec
(R)-Phenylmethylurea (propyl handle to silica)	Spherisorb Chiral 1	Waters
N-3, 5-Dinitrobenzoyl-3-amino-3-phenyl-2-(1,1-dimethylethyl)-propanoate bonded to silica by undecyl handle	(R,R)-, (S,S)-β-GEM 1	Regis Technologies
Dimethyl-N-3,5-dinitro-benzoyl-amino-2,2-dimethyl-4-pentenyl phosphonate silica	(R)-, (S)-α-Burke 2	Regis Technologies

(Continued)

APPENDIX 14.2.1 Pirkle type stationary phases.—*cont'd*

Chiral selector	Product line	Maker
N-(1-Propylsilica-2-oxo-4-phenylazetidin-3-yl)-3, 5- dinitrobenzamide	(3R,4S)-, (3S,4R) Pirkle 1-J	Regis Technologies
3,5-Dinitrobenzoyl derivative of diphenylethylenediamine bonded to silica by tetradecanoic acid handle	(R,R)-, (S,S)-ULMO	Regis Technologies
3,5-Dinitrobenzoyl derivative of 1,2-diaminocyclohexyl bonded to silica by ether containing handle	(R,R)-, (S,S)-DACH-DNB	Regis Technologies
3,5-Dinitro-N-(3-propyl-1,2,3,4-tetrahydrophenanthren-4-yl) benzamide (3-propyl handle to silica)	(R,R)-, (S,S)-Whelk 1	Regis Technologies
Similar to Whelk 1 using trifunctional bonding of propyl handle to silica	(R,R)-, (S,S)-Whelk 2	Regis Technologies
(6-Methoxyquinolin-4-yl)[6-(1H-1,2,3-triazol-4-yl)-1-azabicyclo[2.2.2]octan-2-yl]methyl- N-(3,5-dinitrophenyl) carbamate (propyl handle to silica)	Quinine carbamate (Q-Sell)	AZYP Separations and Analytics
Quinine group bonded by thioether	Chiralpak QN-AX	Experimental
1-N-[1-(Naphthalen-1-yl)ethyl]pyrrolidine-1,2-dicarboxamide (propyl handle to silica)	(S)-proline and (R)-1-(α-naphthyl)ethylamine-urea	Experimental
Naphthylleucine bonded to silica by tetradecyl handle	(R)-naphthylleucine	Experimental

APPENDIX 14.2.2 SUMICHIRAL[a] stationary phases pirkle type.

Phase	Phase	Chiral component	Mode[b]
OA-2000	OA-2000S	(R)-phenylglycine, dinitrobenzene	NP
OA-2500	OA-2500S	(R)-1-naphthylglycine, dinitrobenzene	NP
OA-3100	OA-3100R	(S)-valine, dinitrobenzene	NP,RP
OA-3200	OA-3200R	(S)-tert-leucine, dinitrobenzene	NP,RP
OA-3300	OA-3300S	(R)-phenylglycine, dinitrobenzene	NP-RP
OA-4000	OA-4000S	(S)-valine (S)-1-(α-naphthyl)ethylamine	NP
QA-4100	OA-4100R	(S)-valine (R)-1-(α-naphthyl)ethylamine	NP
OA-4400	OA-4400R	(S)-proline (S)-1-(α-naphthyl)ethylamine	NP
OA-4500	OA-4500R	(S)-proline (R)-1-(α-naphthyl)ethylamine	NP
OA-4600	OA-4600R	(S)-tert-leucine (S)-1-(α-naphthyl)ethylamine	NP
OA-4700	OA-4700R	(S)-tert-leucine (R)-1-(α-naphthyl)ethylamine	NP
OA-4800	—	(S)-indoline-2-carboxylic acid (S)-1-(α-naphthyl)ethylamine	NP
OA-4900	—	(S)-indoline-2-carboxylic acid (R)-1-(α -naphthyl)ethylamine	NP
OA-6000	OA-6000R	(L)-tartaric acid (S)-1-(α-naphthyl)ethylamine	RP
OA-6100	OA-6100R	(L)-tartaric acid, (S)-valine (R)-1-(α-naphthyl)ethylamine	RP

[a] *Note: manufactured by Sumika Chemical Analysis Services.*
[b] *Note: NP, similar to normal phase; RP, similar to reversed phase.*

APPENDIX 14.2.3 Chiral columns based on derivatized cellulose.

Name	Derivatization group	Applications
Chiralcel OA(ester)	-(CO)-CH_3	Small aliphatic compounds
Chiralcel CA-1(ester)	-(CO)-CH_3	Alcohols
Cellulose triacetate	-(CO)-CH_3	Various
Cellulose Cel-AC-40XF	-(CO)-CH_3	Various
Chiralcel OB (ester)	-(CO)-Ph	Small aliphatic and aromatic compounds
Chiralcel OB-H	-(CO)-Ph	High efficiency separations
Chiralcel OC (carbamate)	-CONH-Ph	Cyclopentenones
Chiralcel OJ (ester)	-(CO)-Ph-4-Me	Aromatic compounds
Chiralcel OD (carbamate)	-CONH-Ph-3,5-di-Me	Alkaloids, tropines, amines, beta blockers
Chiralcel OD-R	-CONH-Ph-3,5-di-Me	Pharmaceutical drugs
Kromasil CelluCoat	-CONH-Ph-3,5-di-Me	Pharmaceutical drugs
Chiral Art Cellulose C	-CONH-Ph-3,5-di-Me	Pharmaceutical drugs
Chiral Art Cellulose SB	-CONH-Ph-3,5-di-Me	Pharmaceutical drugs
Cellulose DMP (Astec)	-CONH-Ph-3,5-di-Me	Beta blockers
ChromegaChiral CCO F4 T3	-CO-Ph-4-F-3-trifluoromethyl	Small molecules
Lux Cellulose-1	-CONH-Ph-3,5-di-Me	Pharmaceutical drugs
Chiralcel OF	-CONH-Ph-*para*-Cl	Beta lactams, dihydroxypyridines, alkaloids
Chiralcel OG	-CONH-Ph-*para*-Me	Beta lactams, alkaloids
Chiralcel OK (ester)	-CO-(CH_2=CH_2)-Ph	Aryl methyl esters, aryl methoxy esters
Lux Cellulose-2	-CONH-Ph-3-Cl-4-Me	Pharmaceutical drugs
Sepapak-2	-CONH-Ph-3-Cl-4-Me	Chiral pesticides
Lux Cellulose-3	-(CO)-Ph-4-Me	Chiral pesticides
Lux Cellulose-4	-CONH-Ph-4-Cl-3-Me	Chiral pesticides
Sepapak-4	-CONH-Ph-4-Cl-3-Me	Chiral pesticides
ChiralCE-1	Phenylcarbamate-cellulose bonded phase	
ChiralCE-2	3,5-Dimethylphenylcarbamate-cellulose bonded phase	
ChiralCE-3	3-Chloro-4-methyl-phenylcarbamate-cellulose bonded phase	
Reflect C-Cellulose B	Cellulose tris(3,5-dimethylphenyl carbamate) bonded on silica	
Reflect I-Cellulose C	Cellulose tris(3,5-dichlorophenyl carbamate) bonded on silica	
Reflect I-Cellulose Z	Cellulose tris(3-chloro-4-methylphenyl carbamate) bonded on silica	

Note: Chiralcel (Daicel Chemical Industries).

APPENDIX 14.2.4 Chiral columns based on derivatized amylose.

Name	Derivatization group	Support
Chiralpak AD and AD-3	-CONH-Ph-3,5-di-Me	Silica (coated on) 5 and 3 μm
Kromasil AmyCoat	-CONH-Ph-3,5-di-Me	Silica (coated on) 5 μm
Chiralpak AS	-CONH-C[a]H(Me)Ph	Silica (coated on)
Chiralpak AY-3	-CONH-Ph-5-Cl-2-Me	Silica (coated on) 3 μm
Chiralpak AZ-3, IF, IF-3	-CONH-Ph-3-Cl-4-Me	Silica (coated on) 3 μm
Chiralpak IA, IB	-CONH-Ph-3,5-di-Me	Silica (coated on) 5 μm (also used in SFC)
Chiralpak ID and ID3	-CONH-Ph-3-Cl	Silica (coated on) 5 and 3 μm
Chiralpak IE and IE3	-CONH-Ph-3,5-di-Cl	Silica (coated on) 5 and 3 μm
Lux Amylose-1	-CONH-Ph-3,5-di-Me	Silica (coated on) 5 and 3 μm
Lux Amylose-2	-CONH-Ph-5-Cl-2-Me	Silica (coated on) 5 and 3 μm
Chiral Art Amylose C	-CONH-Ph-3,5-di-Me	Silica (coated on) 5, 10, and 20 μm
Chiral Art Amylose SA	-CONH-Ph-3,5-di-Me	Silica (coated on) 10 and 20 μm
ChiralAM1 3 μm	-CONH-Ph	Silica bonded
ChiralAM2 3 μm	-CONH-Ph-2,3-di-Me	Silica bonded
ChiralAM3 3 μm	-CONH-Ph-3-Cl-4-Me	Silica bonded
ChiralAM5 3 μm	-CONH-Ph-3-Cl-5-Me	Silica bonded
ChiralAMCD1 3 μm	Phenylcarbamate-amylose-appended-beta-cyclodextrin bonded phase	
ChiralAMCD2 3 μm	3,5-Dimethylphenylcarbamate-amylose-appended-beta-cyclodextrin bonded phase	
ChiralAMCD3 3 μm	3-Chloro-4-methylphenylcarbamate-amylose-appended-beta-cyclodextrin bonded	
ChiralAMCE1 3 μm	Phenylcarbamate-amylose-appended-cellulose bonded phase	
ChiralAMCE2 3, 5, 10 μm	3,5-Dimethylphenylcarbamate-amylose-appended-cellulose bonded phase	
ChiralAMCE3 3 μm	3-Chloro-4-methylphenylcarbamate-amylose-appended-cellulose bonded phase	
ChiralAMCE5 3 μm	3-Chloro-5-methyl-phenylcarbamate-amylose-appended cellulose-bonded phase	
Reflect C-amylose A	-CONH-Ph-3,5-di-Me coated on silica	

[a] *Asymmetric carbon.*

APPENDIX 14.2.5 Cyclodextrins and cyclofructans on silica.

Product line	Chiral selector	Information about the column
Astec CYCLOBOND I 2000	β-cyclodextrin	Particles diameter d_p 5 or 10 μm; pore size: 100 Å; length x i.d.: various
Astec CYCLOBOND I 2000 AC	β-cyclodextrin peracetylated	
Astec CYCLOBOND I 2000 SP	β-cyclodextrin-hydroxypropyl	
Astec CYCLOBOND I 2000 DMP	tris-(3,5-dimethylphenyl) carbamate-β-cyclodextrin	
Astec CYCLOBOND I 2000 DM	dimethylated β-cyclodextrin	
Astec CYCLOBOND I 2000 DNP	tris-(3,5-dinitrophenyl) carbamate β-cyclodextrin	
Astec CYCLOBOND I 2000 RSP	(R,S)-hydroxypropyl modified beta-cyclodextrin	
Astec CYCLOBOND II	γ-cyclodextrin	
Astec CYCLOBOND II AC	γ-cyclodextrin peracetylated	
LARIHC	derivatized cyclofructan 6, cyclofructan 7	AZYP[a]
LARIHC CF6 RN	derivatized cyclofructan 6 (R-naphthylethyl)	AZYP
LARIHC CF6—P	derivatized cyclofructan 6	AZYP
LahricSell-P	cyclofructan 6 isopropyl	Core-Shell
FRULIC-C FRULIC-N	derivatized cyclofructan 6	d_p: 5 μm; length x i.d.: various
LARIHC CF7-DMP	derivatized cyclofructan 7 (3,5-dimethylphenyl)	AZYP
SUMICHRAL OA-7000	novel spacer to β-cyclodextrin (derivatized)	d_p: 5 μm; 150 x 4.6
CDShell-RSP	β-cyclodextrin hydroxypropyl modified	Core-shell
FructoShell-N	cyclofructan 6	Core-Shell
MaltoShell-DMP	Derivatized maltodextrin	Core-Shell
ChiralCD-1[b]	beta-cyclodextrin-1 bonded phase	
ChiralCD-2	beta-cyclodextrin-2 bonded phase	
ChiralCD-3	gamma-cyclodextrin-3 bonded phase	
ChiralCD-4	alpha-cyclodextrin-4 bonded phase	
ChiralCD-5	beta-cyclodextrin-5 bonded phase	
ChiralCD-6	beta-cyclodextrin-6 bonded phase	
ChiralCECD-1	Phenylcarbamate-cellulose-appended-beta-cyclodextrin bonded phase	

(Continued)

APPENDIX 14.2.5 Cyclodextrins and cyclofructans on silica.—*cont'd*

Product line	Chiral selector	Information about the column
ChiralCECD-2	3,5-Dimethylphenylcarbamate-cellulose-appended-beta-cyclodextrin bonded	
ChiralCECD-3	3-Chloro-4-methyl-phenylcarbamate-cellulose-appended-beta-cyclodextrin bonded	

[a] *Note: Commercialized by AZYP Separations and Analytics.*
[b] *Note: Commercialized by ChiralTec [https://www.hplc.eu/chiraltek.htm].*

APPENDIX 14.2.6 Proteins on silica used as chiral phases.

Product line	Chiral selector	Manufacturer
Bioptic AV-1	Avidin	GL Science
Chiral BSA	Bovine serum albumin (BSA)	Shandon
CHIRAL-HSA	Human serum albumin (HSA)	Shandon
CHIRALPAK-AGP	α1-acid glycoprotein	Regis (Sigma)
CHIRALPAK-CBH	Cellobiohydrolase	Regis (Sigma)
CHIRALPAK-HSA	Human serum albumin (HSA)	Regis (Sigma)
Chirobiotic V, V2, T, T2, TAG and R	Glycoproteins	Astec
Resolvosil BSA 7 and BSA-7PX	Bovine serum albumin (BSA)	Machery-Nagel
TSKgel Enantio-OVM	Ovomucoid	Tosoh
Ultron ES-BSA	Bovine serum albumin (BSA)	Shinwa Chem. Ind.
Ultron ES-OVM	Ovoglycoprotein	Shinwa Chem. Ind.

APPENDIX 14.2.7 Chiral synthetic polymers.

Chiral selector	Product line
poly(trans-1,2-cyclohexanediyl-bis-acrylamide)	Astec P-CAP
poly(diphenylethylenediamine-bis-acryloyl)	Astec P-CAP-DP
O,O′-bis (3,5-dimethylbenzoyl)-N,N′-diallyl-L-tartar diamide	Kromasil Chiral DMB
O,O′-bis (4-tert-butylbenzoyl)-N,N′-diallyl-L-tartar diamide	Kromasil Chiral TBB
polymethacrylate trimethylphenyl on silica	Chiralpak OT
polymethacrylate diphenyl(pyridyn-2-yl)methyl on silica	Chiralpak OP

Appendix to chapter 15

APPENDIX 15.2.1 Silica-based SEC commercial packings (for aqueous SEC or GFC).

Phase name (Supplier)	Pore size designation	Range of *Mw* use[a]	Particle size	Chemistry on silica
BioSuite	125; 250	5,000–150,000	10; 12; 17 μm	–
Ultrahydrogel	120; 250; 500; 1,000; 2,000 Å	5,000–7,000,000	6 μm	–
Shodex Protein KW (Showa Denko)	800 Series	1,000–10,000,000	5 μm	Diol; fully porous silica
TSK-gel SW, SW_{XL}, SuperSW (Tosoh)	Various G2000SW, G3000SW, $G3000SW_{XL}$, UP-SW3000	various 5,000–70,000	2 μm (for UP) 5 μm, 8 μm, 10 μm	Diol
TSK-gel QC-PAK	TSK 200, TSK 300	5,000 to 50,000	5 μm	Diol
TSK-gel BioAssist DS	125; 250; 300; 450	Various	Various: 2–15 μm	Diol
Zorbax GF Ser. (Agilent/Crawford Scientific)	GF-250, GF-450	4,000–400,000 10,000–900,000	4 μm 6 μm	Zr clad diol
Zorbax PSM (Agilent/ Thomas Scientific)	PMS 60, PMS 60S, PMS 300, PMS 1000, etc.	Various	5 μm	Silanized
LiChrosphere Si	Si60, Si100	Also normal phase	5 μm, 10 μm	Silica
UltraSpherogel (Grace/ Beckman)	2000, 4000, etc.	–	5 μm	Polyether
Bio-Sil, Bio-Select (Bio-Rad)	SEC-125-5, 250-5, 400-5	1000–1,000,000	5 μm, 10 μm	Silica
Protein-Pak (Waters)	300SW	1,000–20,000	10 μm	Diol
Bio-Sep (Phenomenex)	SEC-S2000, SEC-S3000, SEC-S4000	500–20,000,000	5 μm	Hydrophilic bonded silica
SynChropak (Lab Unlimited)	GPC Peptide, GPC 100 to GPC 4000, CATSEC 100 to 4000	Various ranges	5 μm, 10 μm	Diol

[a] *Units are Da.*

APPENDIX 15.2.2 Several commercially available phases used in nonaqueous SEC (GPC).

Phase name (Supplier)	Pore size designation	Range of *Mw* use[a]	Particle size
Jordi Labs GPC DVB Fluorinated	Various from 100 to 100,000 Å	Various between 100 and 10,000,000	5 μm
PLgel (Polymer Lab. Ltd./Agilent)	Various from 50 Å to 1,000,000 Å	Various from 100−2,000 to 100,000−20,000,000	3, 5, 10, 20 μm
PLgel multipore bed	Various from MIXED-A to MIXED-E	Various from 200 −400,000 to 1,000 −40,000,000	5, 10, 20 μm
PolySep (Phenomenex)	Various from 1000 to 6000 and linear	Various from 100−2,000 to 100,000−20,000,000	5, 10, 20 μm
Shodex K and KF, KD, KL (Showa Denko)	Various indicated as 801, 802, ... 807	Various from 70,000 average to 200,000,000 average	Nominally 7 μm
Shodex K multipore bed	Various indicated as 803L to 807L	Various from 1500 average to 200,000,000 average	Various depending on range (6, 10, 17 μm)
TSK-Gel H_{XL}, SuperHZ (Toyo Soda)	Various indicated as G1000 to G7000	Various from 1,000 average to 400,000,000 average	Various depending on range (5−9 μm)
TSK-GEL H_{HR} and H_{XL} (Aldrich)	G1000H to G7000H, GMH-H, GMH-L, GMH-M	Various from 1,500 to 1,000,000	5 μm, 13 μm
TSK-Gel H_{XL}, SuperHZ multipore bed	GMHXL, GMHXL-HT, GMHXL-L	400,000,000 average	9, 13, 6 μm
Styragel HR (Waters)	Various indicated as HR 0.5, HR 1 to HR 4	Various from small-1,000 to 5,000−600,000	5 μm
Styragel HR multipore bed	HR 4E HR 5E	50−10,000 2,000−4,000,000	5 μm
Styragel HT, Ultrastyragel (Waters)	Various indicated as HT 3 to HT 6, Ultrastyragel	Various from 500 −30,000 to 200,000 −10,000,000	10 μm
Styragel HMW (Waters)	HMW 7	500,000−100,000,000	20 μm
Styragel HT, Styragel HMW multipore bed	HT 6E HMW 6E	5,000−10,000,000	10 μm 20 μm
Bio-beads, S-X beads Bio-Rad	−	400−14,000	Soft gel
Hydrocell (Biochrom Labs.)	GPC 3000, 3000HS	20,000−1,000,000	5 μm
Phenogel (Phenomenex)	50 Å, 100 Å, 500, 10^3 Å, 10^4 Å, 10^5 Å, 10^6 Å, and linear	Various	5, 10, 20 μm

[a] *Note: the range of* Mw *use is based on polystyrene as analyte.*

APPENDIX 15.2.3 Several commercially available phases used in aqueous or mixed polar solvents SEC (GFC).

Phase name (Supplier)	Pore size designation	Range of *Mw* use	Particle size	Chemistry
Jordi BGR	100 Å, 500 Å, 10^3 Å, 10^4 Å, 10^5 Å	Various polymers between 10^2–10^7	5 μm	PSDVB[a] functionalized
Jordi Hydroxylated DVB	100 Å, 500 Å, 10^3 Å, 10^4 Å, 10^5 Å	Smaller molecules, not proteins	5 μm	PSDVB functionalized
Jordi Polar Pac Wax	100 Å, 500 Å, 10^3 Å, 10^4 Å, 10^5 Å	Various poly-hydroxy 10^2–10^7	5 μm	PSDVB functionalized
PL aquagel-OH (Polymer Lab. Ltd./Agilent	AOH 30, 40, 50, 60	100,000, 1,000,000, 20,000,000	5 μm, 8μm, 15 μm	Polyhydroxyl surface
PL aquagel-OH mixed bed	AOH Mixed H, Mixed M	20,000,000, up to 600,000, respectively	8 μm,	Polymer
PolarGel	M, L	Up to 500,000	5, 10, 20 μm	–
Shodex OHpak (Showa Denko), Protein KW	Various indicated as KB-802, to KB-806, KB-80M, SB-401, etc.	Various from 4,000 to 20,000,000	Various	HPMMA[b]
TSK-GEL PW, PW_{XL}, PW_{XL}-CP (Toyo Soda)	Various G1000 to G6000, GM, PW_{XL} G5000PW, GMPW, etc.	Various from 1,000 to 8,000,000	Various	HPMMA
TSK-GEL H_{HR} and H_{XL} (Aldrich)	G1000H to G7000H, GMH-H, GMH-L, GMH-M	Various from 1,500 to 1,000,000	7 μm	HPMMA
Toyopearl HW	40S, 40F, 40C, 50S, 50F, 55S, 55F, 65S, 65F, 75F (five pore sizes)	100–50,000,000	20–40 Å, 30–60 Å, 50–100 Å	HPMA[c]
TSK-GEL Alpha and Super AW,	Alpha-3000, Alpha-5000, Super AW2500 to Super AW6000	Various up to 10,000,000	Various	HPMMA
TSK-GEL Super-Multipore PW	PW-N, PW-M, PW-H	300–50,000 to 1,000 –10,000,000	4 μm, 5μm, 8 μm	HPMMA
Ultrahydrogel (Waters)	Various 120 Å–2,000 Å indicated as 250, 500, etc., linear	Various from 5,000 to 7,000,000	10 μm	HPMMA
Asahipak (Asahi Chemical/ Phenomenex)	Various GS-220, GS-320, GS520, GS-620, GS-710, GFA-30, GFA-7M, GF ser.	Various from 3,000 to 10,000,000	Various	PVA[d] copolymer
Asahipak (Phenomenex)	GF-310 HQ to GF710 HQ, and multimode	Various	5, 6, 9 μm	PVA copolymer
Suprema (PSS)	30 Å, 100 Å, 300 Å, 1000 Å, Linear S, M, XL	Various from 20,000 to 10,000,000	Various	HPMA
MCI Gel CQP (Mitsubishi Chemical)	Various indicated as CQO06, CQP06G, CQP10, CQP30, etc.	Various from 1,000 to 1,000,000	10 μm, 30 μm	HPMA

(Continued)

APPENDIX 15.2.3 Several commercially available phases used in aqueous or mixed polar solvents SEC (GFC).—*cont'd*

Phase name (Supplier)	Pore size designation	Range of *Mw* use	Particle size	Chemistry
Polyhydroxyethyl aspartamide; Polyhydroxyethyl A (PolyLC Inc.)	200 Å, 1000 Å	100–1,000,000	3 μm	–
Bio-Prep SE (Bio-Rad)	SE100/17, SE1000/17	5,000–1,000,000	17 μm	Agarose
PolySep-GFC-P (Phenomenex)	1,000–6,000 and Linear	Various ranges	–	Highly hydrophilic

[a] *PSDVB, Polystyrenedivinylbenzene.*
[b] *HPPMA, Hydroxylated poly(methylmethacrylate).*
[c] *HPMA, Hydroxylated poly(methacrylate).*
[d] *PVA, Polyvinyl alcohol.*

APPENDIX 15.2.4 Several more recent SEC columns.

Column name	Manufacturer	Phase	Particle size	Type
AdvancedBio SEC	Agilent	Proprietary	2.7	Core-shell
Yarra 1.8μ SEC-X150	Phenomenex	Proprietary	1.8	Porous
OHpak LB-803 and LB-806M	Shodex	Proprietary	6, 13 (LB-806M)	Polymeric
TSKGel UP-SW3000	Tosoh Bioscience	Diol	2	Porous

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