Chapter 15

High-pressure liquid chromatography

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The phenomenal growth in chromatography is largely due to the introduction of the versatile technique called high-pressure liquid chromatography, which is frequently called high-performance liquid chromatography [1]. Both terms can be abbreviated as HPLC; see Section 15.1.1 for discussion as to which term is more appropriate. The technique offers major improvements over the classical column chromatography and some significant advantages over more recent techniques, such as supercritical fluid chromatography (SFC), capillary electrophoresis (CE), and capillary electrochromatography discussed in Chapters 16–17. The number of HPLC applications has increased enormously because a variety of complex samples have to be analyzed to solve numerous problems of scientific interest. Additionally, this demand is being continuously driven by the perpetual need to improve the speed of analysis.

The term liquid chromatography (LC) is applied to any chromatographic procedure in which the moving phase is liquid, as opposed to gas chromatography (GC) where a gas is utilized as a mobile phase (see discussion in Chapter 14). Classical column chromatography (see Section 15.1), paper chromatography—a forerunner of thin-layer chromatography (see Chapter 13), and HPLC are all examples of LC. This should clarify why it is inappropriate to further abbreviate HPLC to LC; unfortunately, it is still commonly done.

The major difference between HPLC and the old column chromatographic procedures is the pressure applied to drive the mobile phase through the column. This requires a number of improvements in the equipment, materials used for separation, and the application of the theory. HPLC offers major advantages in convenience, accuracy, speed, and the ability to carry out difficult separations. Comparisons to and relative advantages of HPLC over classical LC and GC are given below (see Sections 15.1 and 15.2) to provide the reader a better appreciation of this technique. The readers are encouraged to look up an excellent classical text on HPLC by Snyder and Kirkland [2]. A short review of

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this technique is provided below; for more detailed discussion see references [1–9].

15.1 EVOLUTION OF HPLC

The progress of chromatography remained relatively dormant since its discovery in the early twentieth century until the introduction of partition and paper chromatography in the 1940s, which was followed by development of gas and thin-layer chromatography in the 1950s and various gel or size exclusion methods in the early 1960s. Shortly, thereafter, the need for better resolution and high-speed analyses of nonvolatile samples led to the development of HPLC.

To better understand the evolution of HPLC, let us briefly review classical column chromatography, which is also called packed column or open-bed chromatography. In packed column chromatography, the column is gravity-fed with the sample or mobile phase; the column is generally used only once and then discarded. Therefore packing a column has to be repeated for each separation, and this represents significant expense in time and material. Sample application, if done correctly, requires some skill and time on the part of the operator. Solvent flow is achieved by gravity flow of the solvent through the column, and individual fractions are collected manually. Typical separation requires several hours. Detection and quantification are achieved by the analysis of each fraction. Generally, many fractions are collected, and their processing requires significant time and effort. The results are recorded as a bar graph of sample concentration versus fraction number.

15.1.1 High-pressure (or high-performance) liquid chromatography

Reusable columns, which have a frit at each end to contain the packing, are employed in HPLC so that numerous individual separations can be carried out on a given column. Since the cost of an individual column can be distributed over a large number of samples, it is possible to use more expensive column packing for obtaining high performance and also to spend more time on the careful packing of a column to achieve best results. Precise sample injection is achieved easily and rapidly in HPLC by using either syringe injection or a sample valve. High-pressure pumps are required to obtain the desired solvent flow through these relatively impermeable columns. This has a decided advantage in that controlled, rapid flow of the solvent results in more reproducible operation, which translates to greater accuracy and precision in HPLC

analysis. High-pressure operation leads to better and faster separation. Detection and quantification in HPLC are achieved with various types of on-line detectors. These detectors produce a final chromatogram without the operator intervention; thus producing an accurate record of the separation with a minimum effort.

A large number of separations can be performed by HPLC by simply injecting various samples and appropriate final data reduction, although the column and/or solvent may require a change for each new application. Based on these comments, it should be obvious that HPLC is considerably more convenient and less operator-dependent than classical LC. The greater reproducibility and continuous quantitative detection in HPLC allows more reliable qualitative analysis as well as more precise and accurate quantitative analysis than classical LC.

In contrast to classical column chromatography, HPLC requires high pressure for pumping liquids through more efficient columns, and this led to the name high-pressure liquid chromatography. At times, this technique is called high-performance liquid chromatography (which has the same abbreviation, viz., HPLC) because it provides high performance over classical LC. The use of this term should be made judiciously since all applications of HPLC are not necessarily based on high performance. As a result, the term high-pressure liquid chromatography is more appropriate for the majority of applications.

15.2 ADVANTAGES OVER GC, SFC, AND CE

GC provides separations that are faster and better in terms of resolution than the older chromatographic methods (see Chapter 14). It can be used to analyze a variety of samples. However, many samples simply cannot be handled by GC without derivatization, because they are insufficiently volatile and cannot pass through the column or they are thermally unstable and decompose under conditions used for GC separations. It has been estimated that only 20% of known organic compounds can be satisfactorily separated by GC without prior chemical modification of the sample.

15.2.1 Advantages over GC

HPLC is limited by sample volatility or thermal stability. It is also ideally suited for the separation of macromolecules and ionic species of biomedical interest, labile natural products, and a wide variety of other high molecular weight and/or less stable compounds (see Section 15.12).

HPLC offers a number of other advantages over GC and other separation techniques. A greater number of difficult separations are more readily achieved by HPLC than by GC because of the following reasons:

- More selective interactions are possible with the sample molecule in HPLC since both phases participate in the chromatographic process, as opposed to only one (stationary phase) in GC.
- A large variety of unique column packings (stationary phases) used in HPLC provide a wide range of selectivity.
- Lower separation temperatures frequently used in HPLC allow more effective intermolecular interactions.

Chromatographic separation results from the specific interactions of the sample molecules with the stationary and mobile phases. These interactions are essentially absent in the moving gas phase of GC, but they are present in the mobile liquid phase of HPLC, thus providing an additional variable for controlling and improving separation. A great variety of fundamentally different stationary phases have been found useful in HPLC, which further allows a wider variation of these selective interactions and greater possibilities for separation. The chromatographic separation is generally enhanced as the temperature is lowered, because intermolecular interactions become more effective. This works favorably for procedures such as HPLC that are usually carried out at ambient temperature. HPLC also offers a number of unique detectors that have found limited application in GC:

- Visible wavelength detectors
- Electrochemical detectors (ECD)
- Refractive index detectors (RI)
- UV detectors
- Fluorescent detectors
- Nuclear magnetic resonance (NMR) as on-line detector.

Even though detectors used for GC are generally more sensitive and provide unique selectivity for many types of samples, the available HPLC detectors offer unique advantages in a variety of applications. In short, it is a good idea to recognize the fact that HPLC detectors are favored for some samples, whereas GC detectors are better for others. It should be noted that mass spectrometric detectors have been used effectively for both GC and HPLC.

HPLC offers another significant advantage over GC in terms of the relative ease of sample recovery. Separated fractions are easily collected

in HPLC, simply by placing an open vessel at the end of the column. Recovery is quantitative, and separated sample components are readily isolated for identification by supplementary techniques. The recovery of separated sample bands in GC is also possible but is generally less convenient.

15.2.2 Advantages over SFC and CE

Both SFC and CE are emerging separation techniques that offer unique features and advantages (see Chapters 16 and 17). However, HPLC is unsurpassed for the wide variety of samples that can be analyzed by it. For example, it allows addition of a number of modifiers to the mobile phase that cannot be used with SFC. They offer unique selectivity.

Small sample size and sample introduction are significant problems with CE. Furthermore, CE is unable to offer the range of selective separations, including preparative separations, that are possible with HPLC.

15.3 SEPARATION PROCESS

In HPLC, the mobile phase is constantly passing through the column at a finite rate. The sample injections are made rapidly in the dynamic state in the form of a narrow band or plug (see Fig. 15.1), which provides a significant advantage over older stop-flow injection techniques in HPLC. After a sample has been injected as a narrow band, the separation can then be envisioned as a three-step process shown in the figure:

Step 1: As the sample band starts to flow through the column, a partial separation of compounds *X*, *Y*, and *Z* (the components of the sample) occurs.
Step 2: The separation improves as the sample moves further through the column.
Step 3: The three compounds are essentially separated from each other.

At step 3, we see two characteristic features of chromatographic separation.

1. Various compounds (solutes) in the sample migrate at different rates.
2. Each solute's molecules spread along the column.

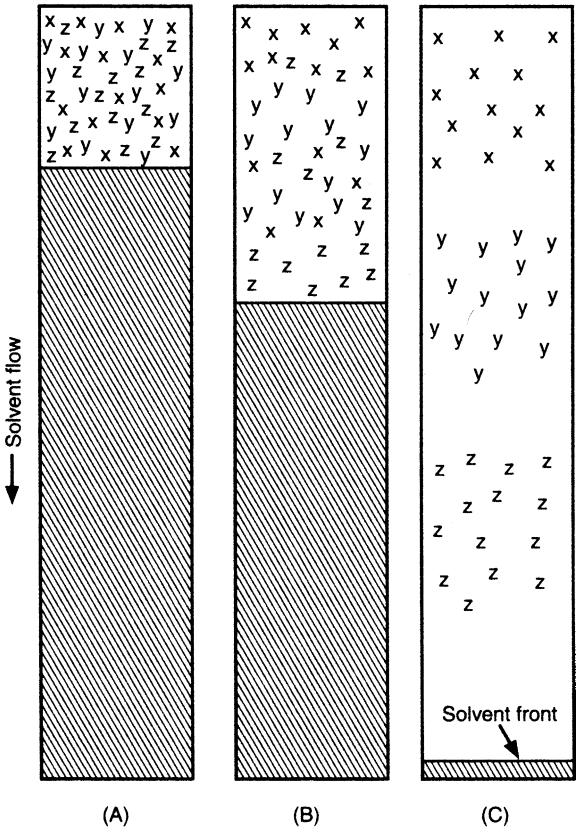


Fig. 15.1. Diagrammatic representation of separation.

Figure 15.1 shows that compound X moves most rapidly and leaves the column first, and compound Z moves the slowest and leaves the column last. As a result, compounds X and Z gradually separate as they move through the column.

The difference in movement rates of various compounds through a column is attributed to differential migration in HPLC. This can be related to the equilibrium distribution of different compounds such as X, Y, and Z between the stationary phase and the flowing solvent(s), or mobile phase. The speed with which each compound moves through the column (u_x) is determined by the number of molecules of that compound in the moving phase, at any moment, since sample molecules do not move through the column while they are in the stationary phase. The molecules of the solvent or mobile phase move at the fastest possible rate except in size exclusion chromatography, where molecular

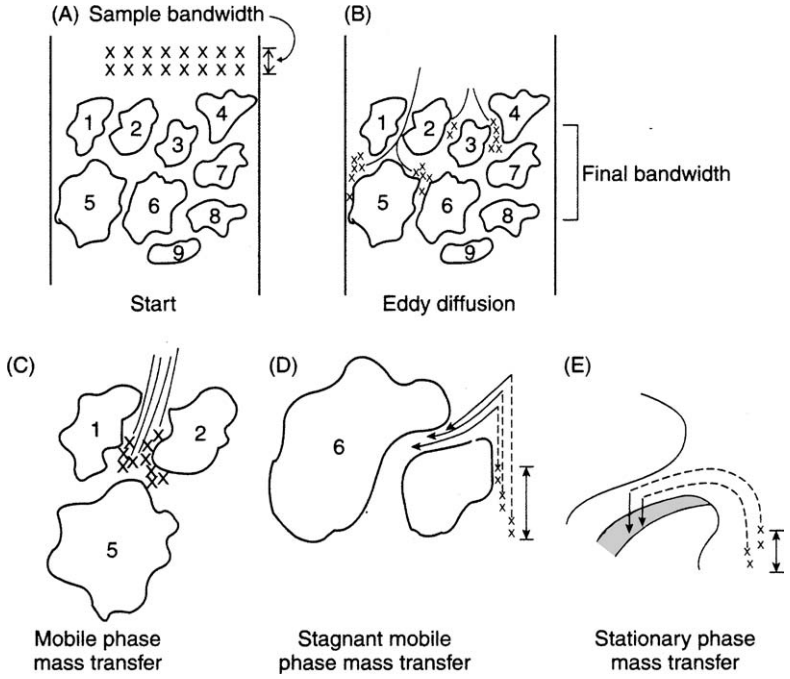


Fig. 15.2. Contributions to band spreading.

size is used to effect the separation. Compounds such as *X*, whose molecules are found in the mobile phase most of the time, move through the column more rapidly than the molecules of compounds such as *Z*, whose molecules spend most of the time in the stationary phase, and thus move slowly through the column.

Spreading of molecules such as *X* along the column is a less desirable characteristic of a chromatographic separation. The molecules start out as a narrow band (see Fig. 15.2A) that gradually broadens as it moves through the column. The differences in molecular migration arise from physical or rate processes. Some of the phenomena leading to molecular spreading on a chromatographic column are discussed below.

15.3.1 Eddy diffusion

One of the processes leading to molecular spreading is caused by multiple flow paths and is called eddy diffusion (see Fig. 15.2B). Within the column, eddy diffusion results from different microscopic flow streams that the solvent follows between different particles. As a result, sample

molecules take different paths through the packed bed, depending on which flow streams they follow.

15.3.2 Mass transfer

A second contribution to molecular spreading relating to mobile-phase mass transfer can be seen in Fig. 15.2C. This refers to varying flow rates for different parts of a single flow stream or path between surrounding particles. In Fig. 15.2C, where the flow stream between particles 1 and 2 is shown, it is seen that liquid adjacent to a particle moves slowly or not at all, whereas liquid in the center of the flow stream moves faster. As a result, at any given time, sample molecules near the particle move to a shorter distance, and those in the middle of the flow stream move to a longer distance. Again, this results in a spreading of molecules along the column.

Figure 15.2D shows the contribution of stagnant mobile-phase mass transfer to molecular spreading. With porous column-packing particles, the mobile phase contained within the pores of the particle is stagnant, i.e., it does not move (in Fig. 15.2D one such pore is shown for particle 6). Sample molecules move into and out of these pores by diffusion. Those molecules that happen to diffuse a short distance into the pore and then diffuse out, return to the mobile phase quickly, and move a certain distance down the column. Molecules that diffuse further into the pore, spend more time in the pore and less time in the external mobile phase. As a result, these molecules move to a shorter distance down the column. Again there is an increase in the molecular spreading.

Figure 15.2E shows the effect of stationary phase mass transfer. After molecules of sample diffuse into a pore, they migrate to the stationary phase (shaded region) or become attached to it in some fashion. If a molecule penetrates deep into the stationary phase, it spends a longer time in the particle and travels a shorter distance down the column, just as in Fig. 15.2D. Molecules that spend only a little time moving into and out of the stationary phase return to the mobile phase sooner, and move further down the column.

15.3.3 Longitudinal diffusion

An additional contribution to molecular spreading is provided by longitudinal diffusion. Whether the mobile phase within the column is moving or at rest, sample molecules tend to diffuse randomly in all

directions. Besides the other effects shown in Fig. 15.2, this causes a further spreading of sample molecules along the column. Longitudinal diffusion is often not an important effect, but is significant at low mobile-phase flow rates for small-particle columns.

15.4 RETENTION PARAMETERS IN HPLC

The retention parameters in HPLC are similar to those discussed in Chapter 14 on GC. To recap for HPLC, the average velocity of the sample band of X molecules (u_x) depends on M, the fraction of X molecules in the mobile phase, and the average velocity of solvent (u, cm/sec):

$$u_x = u\,M \tag{15.1}$$

When the fraction of molecules X in the moving phase is zero ($M = 0$), no migration occurs and u_x is zero. If the fraction of molecules X in the moving phase is 1 (i.e., all molecules of X are in the mobile phase, $M = 1$), then molecules X move through the column at the same rate as solvent molecules and $u_x = u$. As a result, M is also the relative migration rate of compound X.

15.4.1 Capacity factor

The capacity factor k' is equal to n_s/n_m where n_s is the total moles of X in stationary phase and n_m is the total moles of X in the mobile phase. Based on this relationship, we can formulate the following equations:

$$k' + 1 = \frac{n_s + n_m}{n_m n_m} \tag{15.2}$$

$$= \frac{n_s + n_m}{n_m} \tag{15.3}$$

M can then be related to capacity factor k' as follows:

$$\text{Since } M = \frac{n_m}{n_s + n_m} = \frac{1}{1 + k'} \tag{15.4}$$

Substituting for M in Eq. (15.1), we get

$$u_x = \frac{u}{1 + k'} \tag{15.5}$$

15.4.2 Retention time

Based on the following information, we can relate u_x to retention time t_R, the time, a component, is retained in the column, and column length L:

The retention time t_R is the time required for band X to travel the length of column and is generally given as seconds or minutes; the distance is the column length L (in centimeters), and the velocity is that of band X, u_x (cm/sec).

$$t_R = \frac{L}{u_x} \tag{15.6}$$

15.4.3 Zero retention time

The zero retention time t_0 for mobile phase or other unretained molecules to move from one end of the column to the other can be determined as follows:

$$t_0 = \frac{L}{u} \tag{15.7}$$

We can then eliminate L between these last two equations and get

$$t_R = \frac{ut_0}{u_x} \tag{15.8}$$

15.4.4 Relating capacity factor to retention time

The following equation can then be derived by substituting for the value of u_x from Eq. (15.5)

$$t_R = t_0(1 + k') \tag{15.9}$$

This expresses t_R as a function of the fundamental column parameters t_0 and k'; t_R can vary between t_0 (for $k' = 0$) and any larger value (for $k' > 0$). Since t_0 varies inversely with solvent velocity u, so does t_R. For a given column, mobile phase, temperature, and sample component X, k' is normally constant for sufficiently small samples. Thus, t_R is defined for a given compound X by the chromatographic system, and t_R can be used to identify a compound tentatively by comparison with a t_R value of a known compound.

TABLE 15.1
Solvent strength of useful solvents for HPLC

Solvent	Silica	Alumina	Selectivity group*
n-Hexane	0.01	0.01	—
Chloroform	0.26	0.40	VIII
Methylene chloride	0.32	0.42	V
Ethyl acetate	0.38	0.58	VI
Tetrahydrofuran	0.44	0.57	III
Acetonitrile	0.50	0.65	VI
Methanol	0.7	0.95	II

*See Section 15.11.1 for selectivity groups information.

On rearrangement, Eq. (15.9) gives an expression for k'

$$k' = \frac{t_R - t_0}{t_0} \tag{15.10}$$

It is generally necessary to determine k' for one or more bands in the chromatogram to plan a strategy for improving separation. Eq. (15.10) provides a simple, rapid basis for estimating k' values in these cases; k' is equal to the distance between t_0 and the band center, divided by the distance from injection to t_0.

The important column parameter t_0 can be measured in various ways. In most cases, the center of the first band or baseline disturbance, following sample injection, denotes t_0. If there is any doubt on the position of t_0, a weaker solvent (or other unretained compound) can be injected as sample, and its t_R value will equal t_0. A weaker solvent provides larger k' values and stronger sample retention than the solvent used as mobile phase (see Table 15.1 for a list of solvents according to strength).

For example, hexane (see Table 15.1) can be injected as a sample to determine t_0 if chloroform is being used as the mobile phase in liquid–solid chromatography. As long as the flow rate of the mobile phase through the column remains unchanged, t_0 is the same for any mobile phase. If flow rate changes by some factor x, t_0 will change by the factor $1/x$.

15.4.5 Retention volume

Retention in HPLC is sometimes measured in volume units (mL), rather than in time units (s or m). Thus, the retention volume V_R is the total volume of mobile phase required to elute the center of a given band X, i.e., the total solvent flow in time between sample injection and

appearance of the band center at the detector. The retention volume V_R is equal to retention time t_R multiplied by the volumetric flow rate F (mL/min.) of the mobile phase through the column.

$$V_R = t_R F \tag{15.11}$$

The total volume of solvent within the column (V_{m}):

$$V_{\mathrm{m}} = t_0 F \tag{15.12}$$

Substituting for F gives

$$V_R = V_{\mathrm{m}}(1 + k') \tag{15.13}$$

15.4.6 Peak width

The peak width relates to retention time of the peak and the efficiency of a chromatographic column. The efficiency of a column is directly related to the total number of theoretical plates (N) offered by it.

15.4.7 Total number of theoretical plates

Mathematically, the width of a chromatographic peak (t_w) can be related to the number of theoretical plates (N) of the column:

$$N = 16(t_R)^2 \tag{15.14}$$

The quantity N is approximately constant for different bands or peaks in a chromatogram for a given set of operating conditions (a particular column and mobile phase, with fixed mobile-phase velocity, and temperature). Hence N is a useful measure of column efficiency: the relative ability of a given column to provide narrow bands (small values of t_w) and improved separations.

Since N remains constant for different bands in a chromatogram, Eq. (15.14) predicts that peak width will increase proportionately with t_R, and this is generally found to be the case. Minor exceptions to the constancy of N for different bands exist [1], and in gradient elution chromatography, all bands in the chromatograms tend to be of equal width. Since HPLC peaks broaden as retention time increases, later-eluting bands show a corresponding reduction in peak height and eventually disappear into the baseline. The quantity N is proportional to column length L, so that other factors being equal, an increase in L results in an increase in N and better separation.

15.4.8 Height equivalent of a theoretical plate

The proportionality of N and L can be expressed in terms of the following equation

$$N = \frac{L}{H} \tag{15.15}$$

where H is the height equivalent of a theoretical plate (HETP).

The quantity H (equal to L/N) measures the efficiency of a given column (operated under a specific set of operating conditions) per unit length of the column (see van Deemter's equation in Chapter 14). Small H values mean more efficient columns and large N values. A very important goal in HPLC is to attain small H values that lead to maximum N and highest column efficiencies.

Based on various theoretical and practical observations, we know that the value of H decreases with

- small particles of column packing,
- low mobile-phase flow rates,
- less viscous mobile phases,
- separations at high temperatures, and
- small sample molecules.

15.5 RESOLUTION AND RETENTION TIME

A common but very important goal in HPLC is to obtain adequate separation of a given sample mixture. To achieve this goal, we need to have some quantitative measure of the relative separation or resolution achieved. The resolution, Rs, of two adjacent peaks 1 and 2 is defined as equal to the distance between the center of two peaks, divided by average peak width (see Fig. 15.3):

$$Rs = \frac{(t_2 - t_1)}{\frac{1}{2}(t_{w1} + t_{w2})} \tag{15.16}$$

The retention times t_1 and t_2 refer to the t_R values of peaks 1 and 2, and t_{w1} and t_{w2} are their peak width values. When $Rs = 1$, as in Fig. 15.3, the two peaks are reasonably well separated; that is, only 2% of one peak overlaps the other. Larger values of Rs mean better separation, and smaller values of Rs represent poorer separation. For a given value of Rs, peak overlap becomes more serious when one of the two peaks is much smaller than the other.

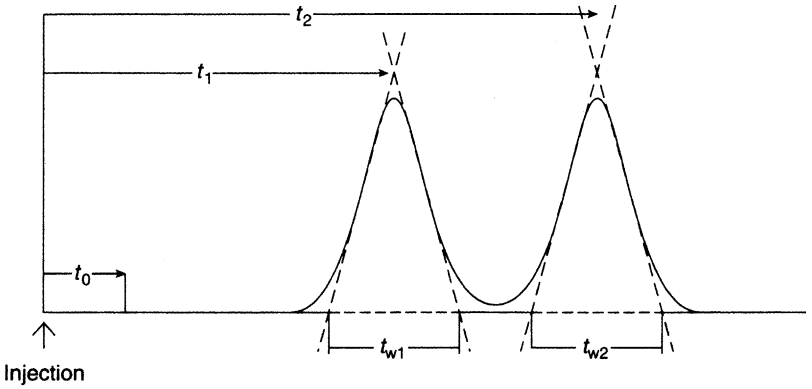


Fig. 15.3. A typical chromatogram.

The resolution value, Rs, of Eq. (15.16) serves to define a given separation. To control separation or resolution, we have to know how Rs varies with experimental parameters such as k' and N. We can derive a simplified relationship for two closely spaced (i.e., overlapping) peaks [1]. Based on Eq. (15.9), $t_1 = t_0\ (1+k_1)$ and $t_2 = t_0\ (1+k_2)$, where k_1 and k_2 are the k' values of bands 1 and 2.

Since t_1 is approximately equal to t_2, t_{w1} will be approximately equal to t_{w2}, based on Eq. (15.14) if we assume N is constant for both bands, Eq. (15.16) can be written as follows:

$$Rs = \frac{t_0(k_2 - k_1)}{t_{w1}} \tag{15.17}$$

From Eq. (15.14), we know $t_w = 4\ t_1/N(1/2)$ or $= 4\ t_0\ (1+k_1)/N(1/2)$

Substituting this value in Eq. (15.17) gives

$$Rs = \frac{(k_2 - k_1)N^{1/2}}{4(1 + k_1)}$$
$$= \frac{1}{4}\left(\frac{k_2}{k_1} - 1\right)N^{1/2}\left(\frac{k_1}{1 + k_1}\right) \tag{15.18}$$

15.5.1 Separation factor and resolution

The separation factor α is equal to k_2/k_1.

By inserting α and an average value of k' for k_2 and k_1, we can simplify the resolution equation as follows:

$$Rs = \frac{1}{4}(\alpha - 1)N\frac{1}{2}\left(\frac{k'}{1+k'}\right) \tag{15.19}$$

It is important to recognize that a number of assumptions were made in deriving Eq. (15.17) to arrive at the simplified Eq. (15.19). A more fundamental form of resolution expression is given below (see Eq. (15.22) in Section 15.10.1). To get a more accurate equation, the actual values of the peak widths and their respective capacity factors should be used; however, for most practical purposes the above equation or its original form, Eq. (15.16), is satisfactory.

15.6 EQUIPMENT

HPLC equipment has been designed and produced to assure correct volumetric delivery of the mobile phase, including the injected sample, and has low-noise detectors so that low concentrations of samples can be analyzed conveniently. Discussed below, briefly, are some of the important considerations for the HPLC equipment. More detailed discussion can be found in a recent text (see Chapter 3 of reference 3).

15.6.1 Modular versus integrated HPLC systems

A simple system is comprised of an isocratic pump, a manual injector, a UV detector, and a strip-chart recorder. A schematic diagram of an HPLC instrument is shown in Fig. 15.4. This simple configuration is rarely used in most modern laboratories. A typical HPLC system is likely to consist of a multi-solvent pump, an autosampler, an on-line degasser, a column oven, and a UV/Vis or photodiode array detector; all connected to and controlled by a data-handling workstation. Examples of modular and integrated systems are shown in Fig. 15.5. Some of the important instrumental requirements are summarized in Table 15.2.

HPLC systems can be classified as modular or integrated. In a modular system, separate modules are stacked and connected to function as a unit, whereas in an integrated system, modules are built inside a single housing and often share a common controller board. These built-in modules cannot function outside the system; solvent lines and electrical wires are inside the housing. Modular systems are considered easily serviceable since internal components are easily accessible, and the malfunctioning module can be swapped. Integrated systems provide

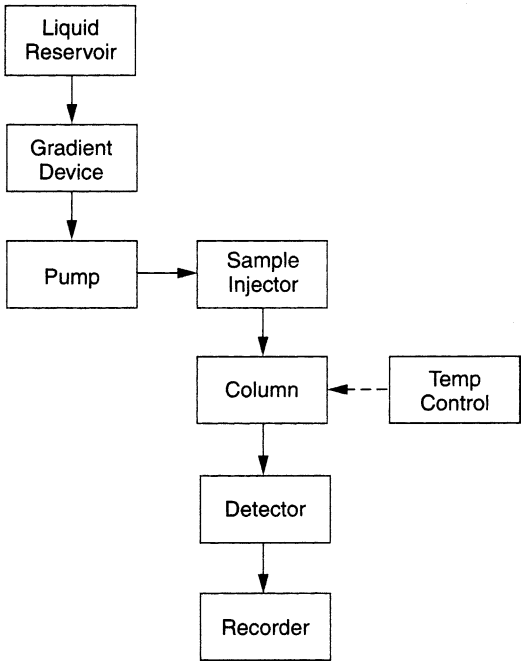


Fig. 15.4. A schematic of HPLC equipment.

better integration of modules; for example, an autosampler can be designed to inject samples right at the beginning of a pump stroke, thus yielding better precision in retention time.

15.6.2 Solvent delivery systems

A modern solvent delivery system consists of one or more pumps, solvent reservoirs, and a degassing system. HPLC pumps can be categorized in several ways: by flow range, driving mechanism, or blending method. A typical analytical pump has a flow range of 0.001–10 mL/min, which handles comfortably the flow rates required for most analytical work (e.g., 0.5–3 mL/min). Preparative pumps can have a flow range from 30 mL/min up to L/m.

Syringe pumps driven by screw mechanisms were popular in the 1960s because of their inherent precision and pulseless flow characteristics. Their disadvantages are higher manufacturing costs and the problems associated with syringe refill cycles. Syringe pumps are currently used in specialized systems for microbore and capillary HPLC.

Fig. 15.5. Modular and integrated HPLC systems [3].

Most HPLC pumps today use a reciprocating piston design, as shown in Fig. 15.6. Here, a motorized cam (or a direct screw-drive system) drives a piston back and forth to deliver solvent through a set of inlet and outlet check valves. All wettable components are made from inert materials; examples include stainless steel pump heads, ruby balls, sapphire seats in check valves, sapphire pistons, and fluorocarbon pump seals. Since liquid is delivered only during the inward stroke of the piston, the resulting sinusoidal liquid stream is usually passed through a pulse dampener to reduce pulsation. Another approach is to use a dual-piston in-parallel pump design, where a single motor drives two pistons in separate pump heads. Since the pistons are 180° out of phase, the combined output results in a steadier flow pattern. The digital stepper motor that drives the cam is controlled by a microprocessor

TABLE 15.2
HPLC instrumental requirements

Criteria	Characteristics	Instrumental requirements
Reproducibility	Controls various operational parameters	Controls precisely: mobile-phase composition temperature flow rate detector response
Detectability	High-detector response	Good signal-to-noise ratio
Sample variety	Narrow peaks	Efficient columns
Speed	Useful for a variety of samples	Variety of detectors and stationary phases
	Selective and efficient columns	Low dead-volume fittings
	High flow rate	High-pressure pumps
	Fast data output	Fast-response recorders and automatic data handling

that coordinates the piston speed with other components such as the proportioning valve and the pressure monitor.

Another way to classify pumping systems is based on the achievement of solvent blending; i.e., under low- or high-pressure mixing conditions.

A number of improvements have been made in pumps over the years. The life of the piston seal was improved by better designs, such as more durable spring-loaded seals, self-aligning piston mechanisms, and irrigation systems to eliminate any salt buildup (piston seal wash). Most pumps also have front panel access to the pump heads for easier maintenance. The pump performance often suffers at low flow rates. For example, since a typical piston size is about 100 μL, blending and pulse-free flow are difficult to achieve at a flow rate of <0.2 mL/min or at <2 pump strokes per min. High-end pumps use sophisticated mechanisms such as variable stroke length (20–100 μL), micro-pistons (5–30 μL), and hybrid dual-piston in-series designs to improve performance. Two pistons (often different sizes) are independently driven by separate motors in a dual-piston in-series design. The pre-piston is

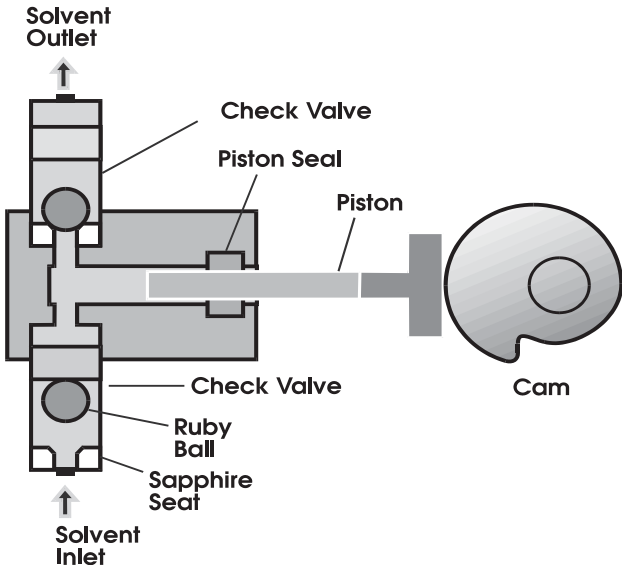


Fig. 15.6. A schematic of a reciprocating single-piston pump [3].

synchronized with the secondary piston to provide smoother flows and higher compositional accuracy. Variations of this design are used in many pumps such as Waters Alliance 2695, Agilent Series 1100, and Shimadzu LC10-AD.

The pumps specifically designed for HPLC are able to provide constant flow of the mobile phase against column pressure up to 10,000 psi. However, it is important to recognize that most HPLC separations are run at pressures lower than 6,000 psi. A comparison of various pumps used for HPLC is given in Table 15.3.

Most chromatographers limit themselves to binary or tertiary gradient systems; however, it should be noted that pumps capable of quaternary gradient are available (see reference 3, Chapter 3) and should be considered in the equipment selection process to allow greater versatility in method development.

15.6.3 Detectors

An HPLC detector is often a modified spectrophotometer equipped with a small flow cell, which monitors the concentration (or mass) of eluting sample components. A number of detectors used in HPLC are discussed below. Most applications utilize absorbance detectors such as UV/Vis or

TABLE 15.3
Comparison of various HPLC pumps

	Reciprocating						Positive displacement		Pneumatic		
Pump characteristic	Simple single-head	Single-head smooth pulse	Simple dual-head	Dual-head, compressibility corrected, smooth pulse	Dual-head, closed loop flow control	Triple head low-volume	Syringe-type	Hydraulic amplifier	Simple	Amplifier	Amplifier with flow control
Resettable	+	+	++	++	++	++	++	++	–	–	+
Drift	+	+	++	++	++	++	++	+	–	+	+
Low Noise	–	+	++	++	++	++	++	++	+	+	++
Accurate	+	+	+	+	++	++	+	+	–	–	+
Versatile	–	+	++	++	++	++	–	+	–	++	+
Low Service	+	+	+	+	+	+	–	+	++	+	–
Durable	+	+	+	+	+	+	+	–	++	++	++
Coast	Low	Moderate	Moderate	High	Very high	Very high	Moderate to high	Moderate	Low	Moderate	High
Consistent flow	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
Consistent pressure	No	No	No	Yes	No	No	No	Yes	Yes	Yes	Yes

Note: Adapted from reference [2].
++ = optimum, + = satisfactory, – = needs improvement.

photodiode array detectors (PDA). Therefore, these detectors are covered in greater detail in this section.

15.6.3.1 UV/Vis absorbance detectors

These detectors monitor the absorption of UV or visible light by analytes in the HPLC eluent. A typical UV/Vis detector consists of a deuterium source and a monochromator (a movable grating controlled by stepper motor to select wavelength through an exit slit) to focus the light through a small flow cell. A dual-beam optical bench is typical for reducing drift. Two photodiodes are used to measure light intensities of sample and reference beams. The observed absorbance is controlled by Beer's Law (see Chapter 5):

$$\text{Absorbance (A)} = \text{molar absorptivity (a)} \times \text{path length (b)} \times \text{concentration (c)}$$

Most UV absorption bands correspond to transitions of electrons from $\pi \rightarrow \pi*$, $n \rightarrow \pi*$, or $n \rightarrow \sigma*$ molecular orbitals. Besides aromatic compounds, organic functional groups such as carbonyl, carboxylic, amido, azo, nitro, nitroso, and ketone groups have absorbance in the UV region.

Important performance characteristics of UV/Vis detectors are sensitivity, linearity, and band dispersion. These are controlled by design of the optics and the flow cell—more specifically by spectral bandpass, stray light characteristics, and the volume and path length of the flow cell.

Sensitivity is the most important requirement for any detector, and is influenced by baseline noise. The dual wavelength detection feature is useful for the simultaneous monitoring of active ingredients and preservatives in drug products. The baseline noise can be substantially higher in the dual wavelength mode because this feature is achieved by toggling the monochromator between the two wavelengths. While a wavelength range of 190–700 nm is typical, the sensitivity performance above 400 nm is lower because of lower energy of the deuterium source in the visible region. Some detectors have a secondary tungsten source to augment the deuterium source and extend the wavelength range to 190–1000 nm. A spectral bandwidth of 5–8 nm is typical in HPLC detectors. Spectral bandwidth is defined as the width in nm of the selected wavelength region, and is related to the optical slit width of the spectrometer. Increasing the bandwidth by widening the slit width improves detection sensitivity at the expense of linearity. Wavelength accuracy is an important requirement in instrument calibration. Wider linearity and lower baseline noise are critical for achieving acceptable limits of quantitation (LOQ). Flow-cell design

is a critical requirement for increasing sensitivity since signals are proportional to cell path lengths. However, increasing the path length by using a larger flow cell is often detrimental to system band dispersion and leads to extra-column band broadening. Advanced flow-cell designs such as tapered cells, or flow cells equipped with focusing lenses as cell windows, are often used to reduce gradient baseline shifts stemming from refractive index changes in the mobile phase. Most manufacturers offer a number of optional flow cells for applications such as semi-micro, microbore, semi-prep, or HPLC/MS.

Performance sensitivity has improved manyfold in the last two decades. The benchmark noise level of $\pm 1 \times 10^{-5}$ AU/cm, thought at one time to be the physical limit of UV/Vis detection imposed by short-term source fluctuations, thermal flow noise and electronic noise, is now surpassed by many detectors. Extending the linear dynamic range to >2 AU is possible by lowering stray lights in the optical bench. The typical lifetime of the deuterium source is now 1000–2000 h. Many modern detectors have dual- or multiple-wavelength detection and stop-flow scanning features. Most detectors have front panel access to self-aligned sources and flow cells for easy maintenance. Others have self-validation features such as power-up diagnostics, leak sensors, time logs for lamps, built-in holmium oxide filters for wavelength calibration, or filter-wheels for linearity verification.

15.6.3.2 Photodiode array (PDA) detector

A PDA detector provides UV spectra of eluting peaks in addition to monitoring the absorbance of the HPLC eluent. It is the detector of choice for method development and for monitoring impurities. A schematic of a PDA detector (Fig. 15.7) shows where the entire spectrum from the deuterium source or a selected portion passes through the flow cell and is dispersed onto a diode array element that measures the intensity of light at each wavelength. Most PDAs use a charge-coupled diode array with 512–1024 diodes, capable of spectral resolution of $\sim$1 nm. Sophisticated spectral evaluation software allows the convenient display of both chromatographic and spectral data along three axes (absorbance versus wavelength, versus retention time). With multiple windows, a system can display a contour map, a chromatogram at a specified wavelength, and a spectrum of the active ingredient peak in a sample. Most software also allows automated spectral annotations of λ_{max}, peak matching, library searches, and peak purity evaluation.

Further sensitivity enhancements of PDA are likely to stem from advanced flow-cell design using fiber-optic technology to extend the

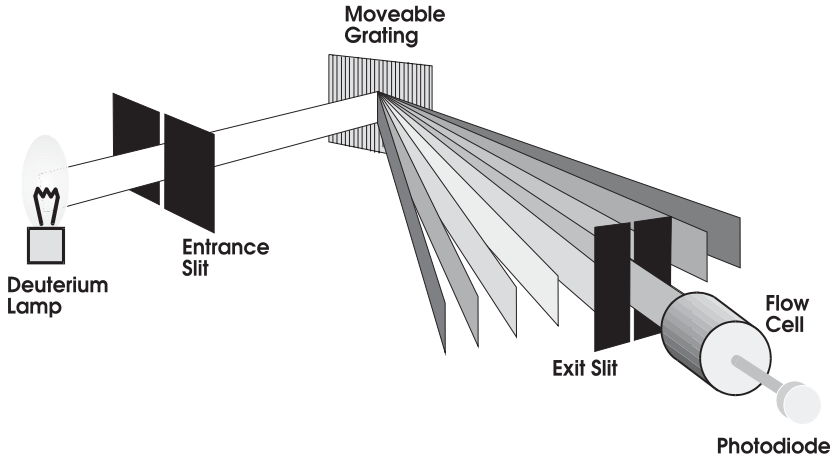


Fig. 15.7. A Schematic of a photodiode array detector [3].

path length without increasing noise or chromatographic band dispersion. This type of flow cell is especially important in micro HPLC for samples of limited availability. Linearity performance at high absorbance (up to 2.0 AU) is important in pharmaceutical purity testing because it allows the injection of a higher concentration of the active ingredient to enhance detection of trace impurities.

15.6.3.3 Fluorescence (Fl) detector

A fluorescence detector monitors the emitted fluorescent light of the HPLC eluent. It is selective and extremely sensitive (pg to fg) to highly fluorescent compounds. A detector consists of a xenon source, an excitation monochromator, an emission monochromator, a square flow cell, and a photomultiplier for amplifying the emitted photons. The xenon source can be a high-power continuous source (150 W) or a pulsed source ($<$20 W). The pulsed source is becoming popular because it requires less power, has more energy in the far UV region, and allows detection modes such as phosphorescence, chemiluminescence, and bioluminescence. All high-end units have a double monochromator for wavelength programmability. Filters are used in lower-cost units. Sensitivity specification is often quoted by the signal/noise ratio of the Raman band of water which ranges from $\sim$100 in older models to $>$300 in modern units. Sensitivity can be enhanced by widening the optical slits.

15.6.3.4 Refractive index (RI) detector

An RI detector measures the difference in RI between the sample cell containing the analyte in the eluting solvent and the reference cell (containing only eluting solvent). It offers universal detection but has lower sensitivity (0.01–0.1 μg) than UV/Vis absorbance detection, and is more prone to temperature and flow changes. RI detection is used for components of low chromophoric activities such as sugars, triglycerides, organic acids, pharmaceutical excipients, and polymers. It is the standard detector for polymer characterization in gel permeation chromatography. Modern RI detectors are generally the differential deflection type with a wide RI range of 1.00–1.75 RIU (refractive index unit). They have thermostatted flow-cell assemblies and allow unattended operation via auto-purging the reference flow cell. Sensitivity, baseline stability, and reliability have improved significantly in recent years. The greatest disadvantages are its low sensitivity and its incompatibility with gradient elution.

15.6.3.5 Evaporative light scattering detector (ELSD)

An ELSD converts the HPLC eluent into a particle stream and measures the scattered radiation. It offers universal detection for nonvolatile or semivolatile compounds and has higher sensitivity than the RI detector (in the low ng range) in addition to being compatible with gradient analysis. ELSD is routinely used in combinatorial screening. Response factors are less variable than that of other detectors. An ELSD consists of a nebulizer equipped with a constant temperature drift tube where a counter-current of heated air or nitrogen reduces the HPLC eluent into a fine stream of analyte particles. A laser or a polychromatic beam intersects the particle stream, and the scattered radiation is amplified by a photomultiplier. Manufacturers include Alltech, Polymer Laboratories, Shimadzu, Waters, Sedere, and ESA.

15.6.3.6 Electrochemical detector (ECD)

An ECD measures the current generated by electroactive analytes in the HPLC eluent between electrodes in the flow cell. It offers sensitive detection (pg levels) of catecholamines, neurotransmitters, sugars, glycoproteins, and compounds containing phenolic, hydroxyl, amino, diazo, or nitro functional groups. The detector can be the amperometric, pulsed-amperometric, or coulometric type, with the electrodes made from vitreous or glassy carbon, silver, gold, or platinum, operated in the oxidative or reductive mode. Manufacturers include BSA, ESA, and Shimadzu.

15.6.3.7 Conductivity detector
A conductivity detector measures the electrical conductivity of the HPLC eluent stream and is amenable to low-level determination (ppm and ppb levels) of ionic components such as anions, metals, organic acids, and surfactants. It is the primary detection mode for ion chromatography. Manufacturers include Dionex, Alltech, Shimadzu, and Waters.

15.6.3.8 Radioactivity detector
A radioactivity detector is used to measure radioactivity in the HPLC eluent, using a flow cell. The detection principle is based on liquid scintillation technology to detect phosphors caused by radiation, though a solid-state scintillator is often used around the flow cell [17,31]. This detector is very specific and can be extremely sensitive. It is often used for conducting experiments using tritium or C-14 radiolabeled compounds in toxicological, metabolic, or degradation studies.

15.6.3.9 Nuclear magnetic resonance and mass spectrometric detectors
HPLC/NMR and HPLC/MS are popular techniques that combine the versatility of HPLC with the identification power of NMR or MS (see Chapters 11, 12, and 18).

15.6.4 Data handling

Chromatographic data handling systems range from a strip-chart recorder, an integrator, a PC-based workstation to a server network system designed for the chromatographer's needs. Most PC-based data handling workstations also incorporate full instrumental control of the HPLC system from various manufacturers. Lately, major companies have installed centralized data network systems to ensure data security and integrity.

15.7 MODES OF SEPARATION IN HPLC

Discussed below are various modes of separations in HPLC. Included here is brief coverage of mobile-phase selection for various modes of chromatography and elementary information on mechanism, choice of solvents and columns, and other practical considerations. It should come as no surprise that reversed-phase HPLC is discussed at greater length in this section because it is the most commonly used technique in HPLC (more detailed discussion is provided in Section 15.8). Clearly,

a better understanding of separation mechanisms would enable the reader to make better decisions on mobile-phase optimizations (see Section 15.11 later in this chapter).

15.7.1 Adsorption chromatography

Separations in adsorption chromatography result largely from the interaction of polar functional groups with discrete adsorption sites on the stationary phase. The strength of these polar interactions (see Table 15.4) is responsible for the selectivity of the separation in adsorption chromatography. It should be noticed that alcholic, phenolic, and amino groups are highly retained. Adsorption chromatography with relatively nonpolar mobile phases and polar stationary phases is sometimes referred to as ''liquid–solid'' chromatography.

Adsorption chromatography is generally considered suitable for the separation of nonionic molecules that are soluble in organic solvents. Very polar compounds, those with high solubility in water and low solubility in organic solvents, interact very strongly with the adsorbent surface and result in peaks of poor symmetry and poor efficiency.

Solvent strength and selectivity can be controlled by using binary or higher-order solvent mixtures. The change in solvent strength as a function of the volume percent of the more polar component is not linear. At low concentrations of the polar solvent, small increases in concentration produce large increases in solvent strength; at the other extreme, relatively large changes in the concentration of the polar solvent affect

TABLE 15.4
Adsorption characteristics of various functional groups [39]

Retention	Type of compound
Nonadsorbed	Aliphatics
Weakly adsorbed	Alkenes, mercaptans, sulfides, aromatics, and halogenated aromatics
Moderately adsorbed	Polynuclear aromatics, ethers, nitrites, nitro compounds, and most carbonyls
Strongly adsorbed	Alcohols, phenols, amines, amides, imides, sulfoxides, and acids
Comparative	$F < Cl < Br < I$; cis compounds are retained more strongly than trans; equatorial groups in cyclohexane derivatives (and steroids) are more strongly retained than axial derivatives

the solvent strength of the mobile phase to a lesser extent. Once the optimal solvent strength has been determined for a separation, the resolution of the sample is improved by changing solvent selectivity at a constant solvent strength.

The uptake of water by the column adversely affects separation and leads to irreproducible separations and lengthy column regeneration times. The use of a 100% saturated solvent is undesirable because such liquid–solid chromatographic systems are often unstable. Apparently, under these conditions the pores of the adsorbent gradually fill with water, leading to changes in retention with time and possibly also to a change in the retention mechanism as liquid–liquid partition effects become more important. When silica is the adsorbent, 50% saturation of the mobile phase has been recommended for stable chromatographic conditions [10–12]. Solvents with 50% water saturation can be prepared by mixing dry solvent with a 100% saturated solvent or, preferably, by using a moisture-control system [13]. The latter consists of a water-coated thermostatted adsorbent column through which the mobile phase is recycled during the time required to reach the desired degree of saturation.

It should be noted that a column that has been deactivated with water no longer shows adequate separation properties. Restoring the activity of the column by pumping a large volume of dry mobile phase through the column is a slow and expensive process. Alternatively, reactivation can be accomplished chemically using the acid-catalyzed reaction between water and 2,2-dimethoxypropane, the products of which, acetone and methanol, are easily eluted from the column [14].

In addition to water, virtually any organic polar modifier can be used to control solute retention in liquid–solid chromatography. Alcohols, acetonitrile, tetrahydrofuran, and ethyl acetate in volumes of less than 1% can be incorporated into nonpolar mobile phases to control adsorbent activity. In general, column efficiency declines for alcohol-modulated eluents compared to water-modulated eluent systems.

The retention behavior of a sample solute is dominated largely by its functional groups. As a result, adsorption chromatography has been found useful for different classes of compounds. Isomeric and multifunctional compounds can generally be separated by adsorption chromatography since the relative position of the solute functional groups interacting with the spatial arrangement of the surface hydroxyl groups governs adsorption. This effect leads to a pronounced selectivity of silica gel for positional isomers.

Organic amine compounds can be separated successfully on silica gel columns with good peak symmetry using organic/aqueous mobile

phases [15–17]. Solute retention appears to involve both electrostatic and adsorption forces.

15.7.2 Normal bonded phases

Polar bonded phases containing a diol, cyano, diethylamino, amino, or diamino functional group are commercially available; representative structures are shown in Table 15.5. The alkyl nitrile- and alkylamine-substituted stationary phases, when used with a mobile phase of low polarity, behave in a manner similar to the solid adsorbents discussed in the previous section, i.e., the retention of the sample increases with solute polarity, and increasing the polarity of the mobile phase reduces the retention of all solutes. The polar bonded-phase packings are generally less retentive than adsorbent packings, but are relatively free from the problems of chemisorption, tailing, and catalytic activity associated with silica and alumina. The bonded-phase packings respond rapidly to changes in mobile-phase composition and can be used in gradient elution analyses. Adsorbent packings respond slowly to changes in mobile-phase composition because of slow changes in surface hydration, making gradient elution analysis difficult. Because of

TABLE 15.5
Polar bonded phases [39]

Name	Structure	Application
Diol	$-(CH_2)_3\ OCH_2CH(OH)CH_2(OH)$	Surface modifying groups for silica packings used in SEC
Cyano	$-(CH_2)_3CN$	Partition or adsorption chromatography
Amino	$-(CH_2)_n\ NH_2$ $N = 3$ or 5	Adsorption, partition, or ion-exchange chromatography
Dimethyl-amino	$-(CH_2)_3N(CH_3)_2$	Ion-exchange chromatography
Diamino	$-(CH_2)_2NH(CH_2)_2\ NH_2$	Adsorption or ion-exchange chromatography

the above advantages, the polar bonded-phase packings have been proposed as alternatives to microporous adsorbents for separating the same sample type [18].

The alkyl nitrile-substituted phase is of intermediate polarity and is less retentive than silica gel but displays similar selectivity. It provides good selectivity for the separation of double bond isomers and ring compounds differing in either the position or number of double bonds [19]. With aqueous mobile phases, the alkyl nitrile-substituted stationary phases have been used for the separation of saccharides that are poorly retained on reversed-phase columns. The alkylamine-substituted phases provide a separation mechanism complementary to either silica gel or alkyl nitrile-substituted phases. The amino function imparts strong hydrogen bonding properties, as well as acid or base properties, to the stationary phase depending on the nature of the solute. The aminoalkyl-substituted stationary phase has been used for the class separation of polycyclic aromatic hydrocarbons [20,21]. Retention is based primarily on charge transfer interactions between the aromatic π-electrons of the polycyclic aromatic hydrocarbons and the polar amino groups of the stationary phase. Samples are separated into peaks containing those components with the same number of rings. Retention increases incrementally with increasing ring number, but is scarcely influenced by the presence of alkyl ring substituents. In contrast, reversed-phase separations show poor separation between alkyl-substituted polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbons of a higher ring number.

The diol- and diethyl amino-substituted stationary phases are used mainly in size exclusion and ion-exchange chromatography, respectively. The practice of normal-phase chromatography is similar to that described for adsorption chromatography. A polar solvent modifier, such as isopropanol at the 0.5–1.0% v/v level, is used in nonpolar solvents to improve peak symmetry and retention time reproducibility. It is believed that the polar modifier solvates the polar groups of the stationary phase, leading to an improvement in mass-transfer properties. For the separation of carboxylic acids or phenols, either glacial acetic acid or phosphoric acid is used at low levels as a tailing inhibitor. Likewise, propylamine is a suitable modifier for the separation of bases.

Certain specific problems arise with the use of alkylamine-substituted stationary phases. Since amines are readily oxidized, degassing the mobile phase and avoiding solvents that may contain peroxides, e.g., diethyl ether and tetrahydrofuran, are recommended. Samples or impurities in the mobile phase containing ketone or aldehyde groups may

react chemically with the amine group of the stationary phase, forming a Schiff's base complex [19]. This reaction will alter the separation properties of the column. The column may be regenerated by flushing with a large volume of acidified water [22].

15.7.3 Reversed-phase chromatography

Reversed-phase chromatography is performed on columns where the stationary phase surface is less polar than the mobile phase. The most commonly used column packings for reversed-phase separations have a ligand such as octadecyl (C-18), octyl (C-8), phenyl, or cyanopropyl chemically bonded to microporous silica particles. The silica particles can be either spherical or irregularly shaped, containing unreacted silanol groups. The unreacted silanol groups can be end-capped by silanization with a small silanizing reagent such as trimethylchlorosilane (TMCS).

It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed-phase mode. The reasons for this include the simplicity, versatility, and scope of the reversed-phase method [23]. The hydrocarbon-like stationary phases equilibrate rapidly with changes in mobile-phase composition and are therefore eminently suitable for use with gradient elution.

Retention in reversed-phase liquid chromatography (RPLC) occurs by nonspecific hydrophobic interactions of the solute with the stationary phase. The near-universal application of reversed-phase chromatography stems from the fact that virtually all organic molecules have hydrophobic regions in their structures and are capable of interacting with the stationary phase. Reversed-phase chromatography is thus ideally suited to separating the components of oligomers or homologues. Within a homologous series, the logarithm of the capacity factor is generally a linear function of the carbon number. Branched-chain compounds are generally retained to a lesser extent than their straight-chain analogues and unsaturated analogues. Since the mobile phase in reversed-phase chromatography is polar and generally contains water, the method is ideally suited to the separation of polar molecules that are either insoluble in organic solvents or bind too strongly to solid adsorbents for normal elution. Many samples with a biological origin fall into this category.

It is believed that retention in reversed-phase chromatography is a function of sample hydrophobicity, whereas the selectivity of the separation results almost entirely from specific interactions of the solute

with the mobile phase [24]. Generally, the selectivity may be conveniently adjusted by changing the type of organic modifier in the mobile phase. For ionic or ionizable solutes, appropriate pH is used to suppress ionization, or ion-pairing reagents are used to increase lipophilicity to assist the degree of solute transfer to the stationary phase and thus control selectivity [25]. Metal-ligand complexes and chiral reagents can be added to the mobile phase to separate optically active isomers.

The details of the mechanism governing retention in reversed-phase chromatography, using chemically bonded hydrocarbonaceous phases, are not completely understood [26]. Solute retention in RPLC can proceed either via partitioning between the hydrocarbonaceous surface layer of the nonpolar stationary phase and the mobile phase, or by adsorption of the solute to the nonpolar portion of the stationary phase. In this context, the partitioning mechanism seems less likely since the hydrocarbonaceous layer is only a monolayer thick and lacks the favorable properties of a bulk liquid for solubilizing solutes. However, the less polar solvent components of the mobile phase could accumulate near the apolar surface of the stationary phase, forming an essentially stagnant layer of mobile phase rich in the less polar solvent [27]. As a result, the solute could partition between this layer and the bulk mobile phase without directly interacting with the stationary phase proper. The balance of evidence favors the adsorption mechanism either with the stationary phase surface itself or by interaction with the ordered solvent molecule layer at the stationary phase surface [28].

Retention of a solute because it is forced by the solvent to go to the hydrocarbonaceous layer is called solvophobic effect. To provide a simple view of solvophobic theory, we will assume that solute retention occurs by adsorption of the solute to the stationary phase, without defining the stationary phase. The solvophobic theory assumes that aqueous mobile phases are highly structured because of the tendency of water molecules to self-associate by hydrogen bonding, and this structuring is perturbed by the presence of nonpolar solute molecules. As a result of this very high cohesive energy of the solvent, the less polar solutes are literally forced out of the mobile phases and are bound to the hydrocarbon portion of the stationary phase. Therefore, the driving force for solute retention is not because of the favorable affinity of the solute for the stationary phase, but because of the solvent's forcing the solute to the hydrocarbonaceous layer.

The most commonly used solvents for RPLC are methanol, acetonitrile, and tetrahydrofuran, used in binary, ternary, or quaternary combinations with water. The effect of solvent strengths can be seen in

Table 15.6. A significant difference in separation selectivity can be achieved by replacing a given solvent with a different solvent.

Changes in pH can change the separation selectivity for ionized or ionizable solutes, since charged molecules are distributed preferentially into the aqueous or more polar phase (for further discussion, see Chapter 6, reference 3.) In general, separation conditions that are used to vary α values of various peaks are summarized in Table 15.7.

TABLE 15.6

Solvent strength in reversed-phase HPLC [2]

Solvent	P'	k'*
Water	10.2	—
Dimethyl sulfoxide	7.2	1.5-fold
Ethylene glycol	6.9	1.5
Acetonitrile	5.8	2.0
Methanol	5.1	2.0
Acetone	5.1	2.2
Ethanol	4.3	2.3
Tetrahydrofuran	4.0	2.8
i-Propanol	3.9	3.0

* Decrease in k' for each 10% addition of solvent to water

TABLE 15.7

Separation conditions used to improve α values

Variable	Impact
Stationary phase	Choice of C-18, C-8, phenyl, cyano or trimethyl has varying impact, depending on type of sample
Organic solvent	Change from methanol to acetonitrile or THF commonly produces large change in α values
pH	Change in pH can have a major effect on α values of acidic or basic compounds
Solvent strength	Changes in % organic solvent often provides significant changes in values
Additives	Ion-pair reagents have great impact on α values. Other additives such as amine modifiers, buffers, and salts, including complexing agents, can be used to produce various effects
Temperature	Vary between 0–70°C to control α values

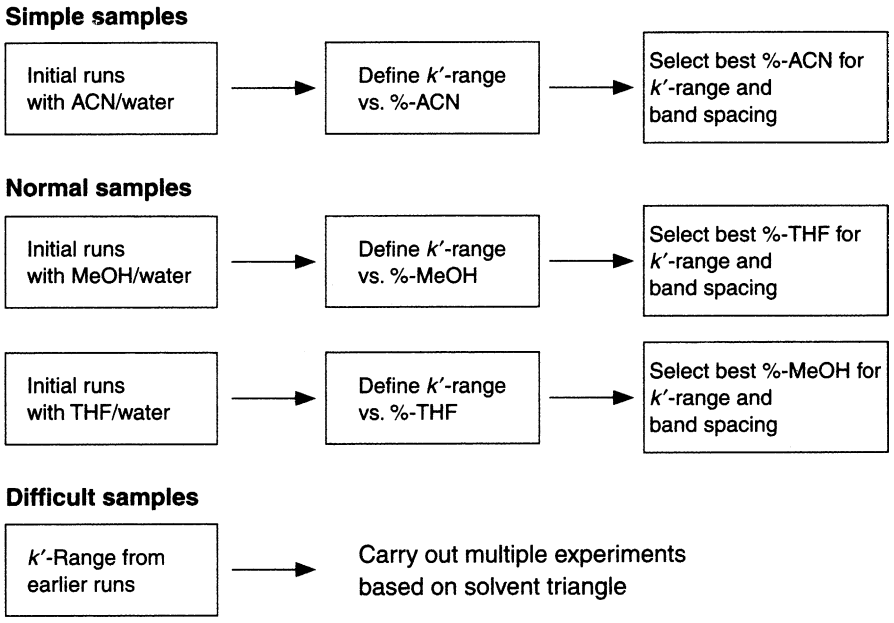


Fig. 15.8. A recommended approach for developing HPLC methods.

Frequently the choice of the mobile phase equates to methanol/water or acetonitrile mixtures in various proportions. The next step is to optimize the concentration of the organic solvent. Following that, low concentrations of tetrahydrofuran are explored to further improve separations (see Fig. 15.8).

Resolution can be mapped as a function of various proportions of acetonitrile, methanol, and THF in the mobile phase. Usually the k' range or run time is held constant during the process by varying the amount of water in the mobile-phase mixture so as to compensate for small differences in the strength of the three pure organic solvents. If further improvement in separations is needed, the additives given in Table 15.8 should be considered.

15.7.4 Ion-pair chromatography

Ionic or partially ionic compounds can be chromatographed on reversed-phase columns through the use of ion-pairing reagents. These reagents are typically long-chain alkyl anions or cations that, in dilute concentrations, can increase the retention of analyte ions. For cationic compounds, C5 to C10 alkyl sulfonates are commonly used; combinations may also be used

TABLE 15.8
Additives for RPLC [29]

Nature of Sample	Example	Additive concentration
Basic compounds	Amines	Phosphate buffer, triethylamine (buffered to pH 3.0)
Acidic compounds	Carboxylic	Phosphate buffer, 1% acetic acid (buffered to pH 3.0)
Mixture of acids and bases	Various	Phosphate buffer, 1% acetic acid (buffered to pH 3.0)
Cationic salts	Tetraalkyl quaternary ammonium compounds	Triethylamine, sodium nitrate
Anionic salts	Alkyl sulfonates	1% acetic acid, sodium nitrate

for difficult separations. In the case of anionic solutes, tetraalkyl ammonium salts (tetramethyl-, tetrabutyl-, etc. ammonium salts or (triethyl-, C5 to C8 alkyl ammonium salts) are generally used.

The following important observations can be made about ion-pair separations:

- In general, an increase in concentration of pairing reagent increases k' (capacity factor). Ion-pairing reagent concentrations range from 0.001 to 0.05 M; however higher concentrations have been recommended by some investigators.
- Increase in chain length increases k'. However, when k' is plotted versus surface concentration, different chain lengths show about the same increase in retention. Therefore, most changes of k' observed with increasing chain length can be reproduced with concentration changes of a single reagent [30].
- The k' changes little for neutral species with increases in concentration of the ion-pairing reagent [30].
- The k' for solutes having the same charge as the pairing reagent decreases with increases in concentration or chain length of pairing reagents [30].
- Removal of pairing reagents from the column by washing is more difficult as the chain length increases. This suggests that use of long-chain pairing reagents can change the nature of the column.

15.7.5 Ion-exchange chromatography

Ion-exchange chromatography utilizes the dynamic interactions between charged solute ions and stationary phases that possess oppositely charged groups. In separations of this type, sample ions and ions of like charge in the mobile phase, compete for sites (X) on the stationary phase:

$$A^- + X^+B^- \rightarrow B^- + X^+A^- \text{ (anion exchange)}$$
$$C^+ + X^-B^+ \rightarrow B^+ + X^-C^+ \text{ (anion exchange)}$$

The extent to which the ions compete with B for the charged sites (X) will determine their retention. In general, this type of chromatography may be used to separate ionic species, such as organic acids or bases, which can be ionized under certain pH conditions. Besides the reaction with ionic sites on the stationary phase, retention may also be affected by the partitioning of solutes between the mobile and stationary phases, as in reversed-phase chromatography. Thus, even nonionized solutes may be retained on ion-exchange columns.

As discussed before, ion-exchange chromatography is a flexible technique used mainly for the separation of ionic or easily ionizable species. The stationary phase is characterized by the presence of charged centers bearing exchangeable counterions. Both cations and anions can be separated by selection of the appropriate ion-exchange medium [31]. Ion exchange finds application in virtually all branches of chemistry. In clinical laboratories, it is used routinely to profile biological fluids and for diagnosing various metabolic disorders [32].

Ion exchange has long been used as the separation mechanism in automated amino acid and carbohydrate analyzers. More recently, ion-exchange chromatography has been used to separate a wide range of biological macromolecules using special wide-pore low-capacity packings designed for this purpose [33–35]. Ion exchange may be used to separate neutral molecules as their charged bisulfite or borate complexes and certain cations as their negatively charged complexes, e.g., $FeCl_4$. In the case of the borate complexes, carbohydrate compounds having vicinal diol groups can form stable charged adducts that can be resolved by anion-exchange chromatography [36]. Ligand-exchange chromatography has been used with cation exchangers (in the nickel or copper form) for the separation of amino acids and other bases. Ion-exchange packings may also be used to separate neutral and charged species by mechanisms not involving ion exchange.

Oligosaccharides and related materials can be separated by a partition mechanism on ion-exchange columns where water/alcohol mobile

phases are employed [37]. Ion-exclusion may be used to separate charged species from uncharged species and also charged species from one another on the basis of their degree of Donnan exclusion from the resin pore volume. An ion-exchange packing having the same charge as the sample ions is selected for this purpose. Retention is dependent on the degree of sample access to the packing pore volume. An example of this mechanism is the separation of organic acids with a cation-exchange packing in the hydrogen form [33,38]. Strong acids are completely excluded and elute early; weak acids are only partially excluded and have intermediate retention values; and neutral molecules are not influenced by the Donnan membrane potential and can explore the total pore volume.

The packings for ion-exchange chromatography are characterized by the presence of charge-bearing functional groups. As mentioned before, sample retention can be envisioned as a simple exchange between the sample ions and those counterions that are originally attached to the charge-bearing functional groups. However, this simple picture is a poor representation of the actual retention process. Retention in ion-exchange chromatography is known to depend on factors other than coulombic interactions. For organic ions, hydrophobic interactions between the sample and the nonionic regions of the support are important. Since the mobile phase in ion-exchange chromatography is often of high ionic strengths, hydrophobic interactions are favored because of the ''salting-out'' effect. From a qualitative standpoint, the retention of organic ions probably proceeds by a hydrophobic reversed-phase interaction with the support, followed by diffusion, of the sample ion to the fixed charge center where an ionic interaction occurs.

Both sample retention and column efficiency are influenced by diffusion-controlled processes, of which the following steps are considered important [39]:

- Diffusion of ions through the liquid film surrounding the resin bead
- Diffusion of ions within the resin particle to the exchange sites
- The actual exchange of one counterion for another
- Diffusion of the exchanged ions to the surface of the resin bead
- Diffusion of the exchanged ions through the liquid film surrounding the resin bead into the bulk solution.

Slow diffusion of the sample ions within the resin beads contributes significantly to poor column performance. Reducing the particle size to less than 10 μm in diameter compensates for the poor mass-transfer

kinetics exhibited with conventional resin beads, by reducing the length of intraparticulate channels.

Because the column packings used in ion exchange contain charged functional groups, an equal distribution of mobile-phase ions inside and outside the resin bead develops in accordance with the Donnan membrane effects. The ion-exchange bead behaves like a concentrated electrolyte solution in which the resin charges are fixed, whereas the counterions are free to move. The contact surface between the resin bead and the mobile phase can be envisioned as a semipermeable membrane. When equilibration occurs between the external and internal solution, and one side of the membrane contains a nondiffusible ion, then a combination of the Donnan membrane effect and the need to preserve overall electrical neutrality results in a greater concentration of free electrolytes within the bead. Therefore, diffusion of sample ions and counterions across the Donnan membrane barrier is often the rate-controlling process in ion-exchange chromatography.

Selectivity series have been established for many counterions: $Li^+ < H^+ < Na^+ < NH4^+ < K^+ < Cs^+ < Ag^+ < Cu^{2+} < Cd^{2+} < Ni^{2+} < Ca^{2+} < Sr^{2+} < Pb^{2+} < Ba^{2+} < F^- < OH^- < CH3COO^- < HCOO^- < Cl^- < SCN^- < Br^- < I^- < NO_3^+ < SO_4^{2-}$

The absolute order depends on the individual ion exchanger, but deviations from the above order are usually only slight for different cation and anion exchangers. For weak-acid resins, H^+ is preferred over any common cation, while weak-base resins prefer OH^- over any of the common anions.

Once a selection of the column type has been made, sample resolution is optimized by adjusting the ionic strength, pH, temperature, flow rate, and concentration of buffer or organic modifier in the mobile phase [39a]. The influence of these parameters on solute retention is summarized in Table 15.9.

The temperature at which separations are performed is another variable that can markedly affect separations. Temperatures up to 50 or 60°C often result in improved separations due to decreased viscosity and better mass transfer. Solute stability at these elevated temperatures should be determined prior to use.

15.7.6 Ion chromatography

Ion chromatography has found widespread application for the analysis of inorganic and organic ions with pK_a values less than 7. It combines the techniques of ion-exchange separation, eluent suppression, and

TABLE 15.9
Factors influencing retention in ion-exchange chromatography [39]

Mobile-phase parameter	Influence on mobile-phase properties	Effect on sample retention
Ionic strength	Solvent strength	Solvent strength generally increases with an increase in ionic strength. Selectivity is little affected by ionic strength except for samples containing solutes with different valence charges. The nature of mobile-phase counterion controls the strength of the interaction with the stationary phase.
pH	Solvent strength	Retention increases in cation-exchange and decreases in anion-exchange chromatography with an increase in pH.
	Solvent selectivity	Small changes in pH can have a large influence on separation selectivity.
Temperature	Efficiency	Elevated temperatures increase the rate of solute exchange between the stationary and the mobile phases and also lower the viscosity of the mobile phase.
Flow rate	Efficiency	Flow rates may be slightly lower than in other HPLC methods to maximize resolution and improve mass-transfer kinetics.
Buffer salt	Solvent strength and selectivity	Solvent strength and selectivity are influenced by the nature of the counterion. A change in buffer salt may also change the mobile-phase pH.
Organic modifier	Solvent strength	Solvent strength generally increases with the volume percent of organic modifier. Its effect is most important when hydrophobic mechanisms contribute significantly to retention. In this case, changing

(*continued*)

TABLE 15.9 (*continued*)

Mobile-phase parameter	Influence on mobile-phase properties	Effect on sample retention
		the organic modifier can be used to adjust solvent selectivity as normally practiced in reversed-phase chromatography.
	Efficiency	Lowers mobile-phase viscosity and improves solute mass-transfer kinetics.

conductivity detection for the quantitative determination of a variety of ions such as mono- and divalent cations and anions, alkylamines, organic acids, etc. [40–45]. Its growth is due in part to the difficulty of determining these ions by other methods. Examples of ion chromatographic separations include common anions and the alkali earth elements [46].

The column packings are styrene-divinyl benzene bead-type resins that are surface functionalized to give low ion-exchange capacities of 0.001–0.05 M equiv/g [46,47]. These resins have good structural rigidity, allowing operation at high flow rates with only moderate back pressure. The mechanical strength of the column packings used limits pressures to about 2000 ψ. These resin beads are stable over the pH range of 1–14. The limited hydrolytic stability of silica-based packings curtails their use in ion chromatography compared to their dominant position in the modern practice of HPLC. For anion separations, a special packing that has an outer layer of fine (0.1–0.5 μm), aminated latex particles agglomerated to the surface of a surface-sulfonated resin bead is frequently used [48,49]. The latex layer is strongly attached to the surface-sulfonated core by a combination of electrostatic and van der Waals forces. The thinness of the exchange layer and the Donnan exclusion effect of the intermediate sulfonated layer provide excellent sample mass-transfer properties by ensuring that single penetration is confined to the outer latex layer.

In general, the efficiency of the columns used in ion chromatography is limited by the large-sized particles and broad particle size distributions of the resin packings. Resin beads are currently available in the ranges of 20–30, 37–74, and 44–57 μm.

The principal problem in the progress of ion chromatography was development of a suitable on-line detection system. Most common ions cannot be detected photometrically, and a method was needed that could detect the separated ions in the background of a highly conducting eluent. Since conductivity is a universal property of ions in solution and can be simply related to ion concentration, it was considered a desirable detection method provided that the contribution from the eluent background could be eliminated. The introduction of eluent suppressor columns for this purpose led to the general acceptance of ion chromatography [50].

The principal disadvantages of suppressor columns are the need to periodically replace or regenerate the suppressor column; the varying elution times for weak acid anions or weak base cations because of ion-exclusion effects in the unexhausted portion of the suppressor column; the apparent reaction of some ions such as nitrite with the unexhausted portion of the suppressor column, resulting in a varying response depending on the percentage exhaustion of the suppressor column; and interference in the baseline of the chromatogram by a negative peak characteristic of the eluent, which varies in elution time with the degree of exhaustion of the suppressor column [39]. Finally, there is some band spreading in the suppressor column that diminishes the efficiency of the separator column and reduces detection sensitivity.

Many of the problems encountered with conventional suppressor columns can be eliminated by using bundles of empty or packed ion-exchange hollow fibers for eluent suppression [51–53]. The sample anions in the column eluent do not permeate the fiber wall because of Donnan exclusion forces. The primary advantage of the hollow fiber ion-exchange suppressor is that it allows continuous operation of ion chromatography without varying interferences from baseline discontinuities, ion-exclusion effects, or chemical reactions. The main disadvantage is that the hollow fiber ion-exchange suppressors have approximately 2 to 5 times the void volume of conventional suppressor columns. This obviously leads to some loss of separation efficiency, particularly for ions eluting early in the chromatogram. Other limitations include restrictions on usable column flow rates to ensure complete suppression and the need for an excess of exchangeable counterions in the regeneration solution.

Common eluents in suppressor ion chromatography are dilute solutions of mineral acids or phenylenediamine salts for cation separations and sodium bicarbonate/sodium carbonate buffers for anion separations. These eluents are too highly conducting to be used without a suppressor column or conductivity detection. Fritz *et al*. [54–56] have

shown that if separator columns of very low capacity are used in conjunction with an eluent of high affinity of the separator resin but of low conductivity, a suppressor column is not required.

15.8 SEPARATION MECHANISM IN REVERSED-PHASE HPLC

Since reversed-phase HPLC is the most commonly used technique in HPLC, it would be a good idea to develop a better understanding of separation mechanisms. The reader would benefit from the basic review on physicochemical basis of retention in HPLC (see Chapter 2, reference 5).

As mentioned before, selectivity in HPLC involves both the mobile phase and the stationary phase; this distinguishes it from GLC where the stationary phase is primarily responsible for observed separation. It is also important to recognize that the stationary phase can be influenced by its environment. The reason for the erroneous assumption to the contrary arises from experiences in GLC where the stationary phase is minimally influenced by the gaseous mobile phase. In the early 1960s, it was demonstrated by Ahuja *et al*. [4] that even in GLC, the stationary phase can be affected by the composition of the mobile phase. Compounds such as water, when present in the carrier gas, can be retained by the stationary phase and then participate in the partition process when a sample is injected. These investigations showed that the stationary phase is a dynamic system that is prone to change with the composition of the mobile phase. It is much more important to remember this fact in HPLC, where multiple components are constantly bathing the stationary phase. Discussed below are various considerations that would lead to better understanding of separation mechanisms in reversed-phase HPLC.

15.8.1 Stationary phase effects

It is important to recognize that the following three distribution equilibria contribute to retention:

(1) Partition between the bulk phases
(2) Adsorption on the solid surface
(3) Adsorption on the liquid/liquid interface.

In a liquid/liquid chromatographic system, the interface between the phases can be involved in the distribution process. So, it is necessary to consider the distribution at the

- interface between solid support and the stationary liquid phase,
- stationary liquid phase,
- interface between the stationary liquid phase and the mobile phase,
- mobile liquid phase.

Reversed-phase (RP) silicas are extremely useful in solving many separation problems in modern LC. Nevertheless there are still problems with the reproducibility of chromatographic results obtained with these materials. One important obstacle to the improvement of RP silicas, with respect to constant properties and quality, might be the lack of reliable analytical methods giving detailed information about the silica matrix itself and about the ligand attached to the surface. Items of interest are the structure of the ligand and its purity, its degree of substitution, and its distribution on the surface of a mono-, di-, and trifunctional bound alkyl groups, and mono- and polymeric substitution [57]. Other important parameters are the proportion of unchanged silanol groups and the degree of end capping.

The interaction of amines with the chromatographic support is complex. The curvature in the k' versus amine concentration plot illustrates that more than one mechanism is operating. The behavior of amines can be compared to that of alcohols, phenols, and protonated thiols. The latter groups are readily chromatographed by silica-based RPLC. The fact that all these groups are poor hydrogen-bond acceptors but good donors implies that silanol groups are strong hydrogen-bond donors and poor acceptors, as would be expected from their Brønsted acidity. Thus, depending on the pK_a of the silanol groups and the mobile phase pH and ionic strength, both hydrogen bonding and ionic interactions can occur with the amine functional groups.

Since selectivity in HPLC involves both the stationary and mobile phases [5–9,58–60], it is important to note that the solvent strength of the mobile phase, as compared to the stationary phase, (composed of mobile-phase components reversibly retained by the bonded phase and silica support) determines the elution order or k' of the retained components. Unfortunately, the columns with the same stationary phase can exhibit significant variabilities from one manufacturer to another and even from the same manufacturer [5–8]. Based on discussions heard at various scientific meetings, this situation has not changed much. Variabilities can occur in the packing process even where all other conditions are supposedly constant. These factors have to be considered prior to developing an understanding as to how separations occur in HPLC.

The strategy employed for choosing a particular chromatographic separation generally entails maximizing the column efficiency and, at

the same time, choosing a stationary and/or mobile phase that is thought to be suitable for the sample at hand. There is a real lack of quantitative guidelines for optimizing selectivity decisions made pertaining to appropriate experimental conditions, and the techniques to achieve this end have generally been based primarily on the experience of the analyst as guided by literature [9]. An alternative approach to optimizing the system selectivity, one more rational than that of trial-and-error, would be understanding the physicochemical basis of separations, followed by modeling of the system at hand, and then optimizing the system and conditions within the boundaries of the required resolution. Several theories have been proposed to explain physicochemical interactions that take place between the solute and the mobile and stationary phases as well as those that may occur between the mobile and stationary phases themselves [9,58].

It is generally acknowledged that despite the enormous popularity of RPLC, which may be broadly classified as involving distribution of a nonpolar or moderately polar solute between a polar mobile phase and a relatively nonpolar stationary phase, there is a lack of understanding of details of solute retention and selectivity. Such an understanding is crucial for informed control and manipulation of separations that ultimately require a sufficiently detailed description of how retention and selectivity depend on the various mobile- and stationary-phase variables [58]. The nature of the solute distribution process in RPLC, i.e., the retention mechanism, has also been a topic of much study, discussion, and speculation. The most rigorous treatment to date is based on the solvophobic theory, with primary focus on the role of the mobile phase. Solute distribution is modeled by invoking ''solvophobic'' interactions, i.e., exclusion of the less polar solute molecule from the polar mobile phase with subsequent sorption by the nonpolar stationary phase. This implies that the mobile phase ''drives'' the solute toward the stationary phase in lieu of the solute's being driven by any inherently strong attraction between the solute and the stationary phase. The basic premise of the theory is reasonable, and agreement with experimental data is generally good; however, the description is incomplete in that it does not provide a sufficiently detailed explanation of the dependence of solute retention and selectivity on the stationary-phase variables. Moreover, it has been reported that under certain chromatographic conditions (very polar mobile-phase solvent and relatively long *n*-alkyl chains), solute distribution in RPLC appears to approach that of partitioning between two bulk liquid phases, suggesting quasi-liquidlike behavior of unswollen *n*-alkyl chains.

With just a few exceptions, there is a dearth of published information providing systematic studies of retention volumes as a function of composition of the eluent over the whole composition range of binary solvents. To rectify this situation, a general equation for HPLC binary solvent retention behavior has been proposed [59] that should help generate a chromatographic retention model to fit Eq. (15.20):

$$\frac{1}{V_{AB}} = \frac{\varphi \mathrm{A}}{V_A} + \frac{\mathrm{b}\varphi_B}{1 + b'\varphi_B} + \frac{\varphi_B}{V_B} \tag{15.20}$$

where

V_{AB} = the corrected elution volume of the solute (experimental elution volume-void volume) in the binary eluent A+B, with volume fractions φA and φB, respectively.
$V_A =$ the corrected elution volume for the solute with pure A as eluent.
V_B = the corrected elution volume for the solute with pure B as eluent.
Both b and b' are constants, and the term that includes them is reminiscent of the Langmuir adsorption isotherm.

15.8.2 Relationship of RPLC to partition in octanol

It is known that the RPLC retention parameters are often strongly correlated to the analyte's distribution coefficient in organic solvent/water. Generally, the relationship between liquid/liquid (LL) distribution and RPLC retention are of the form of the dimensionless Collander-type equations, e.g., see Eq. (15.21)

$$\log K_d = a_1 + b_1 \log k' \tag{15.21}$$

where

K_d = the solute's organic solvent:water distribution coefficient.
k' = chromatographic capacity ratio ($k' = t_r - t_0/t_0$, t_r and t_0 being solute retention time and mobile phase ''holdup'' time, respectively).
a and b = coefficients whose magnitudes depend on the LL distribution and RPLC systems.

The general validity of this type of relationships depends on

- the physicochemical state (temperature, degree of ionization) of solutes in both LL distribution and RPLC;

- the RPLC system used, with emphasis on the mobile phase, particularly on the type, and also to some extent on the concentration of organic cosolvents (modifiers) used; and
- The type of organic solvent used in the distribution system modeling and RPLC system.

It has been shown, e.g., that RPLC capacity factors of unionized solutes obtained using aqueous methanol mobile phases can be generally correlated with octanol/water distribution coefficients for neutral, acidic, and basic compounds, whereas no such overall correlation can be made with alkane/water distribution parameters [61,62]. HPLC can be used for the measurement of octanol/water partition coefficients even when a small amount of sample is available. Solutes are equilibrated between the two phases by using the conventional shake-flask method, which entails shaking them together and then analyzing by HPLC sampled portions of both phases [63]. The results thus obtained with a variety of substances having partition coefficients in the range of 10^{-3} to 10^{4} showed excellent agreement with literature data. The technique is rapid and has the advantage that small samples suffice, the substances need not be pure, and the exact volume of the phases need not be known. Furthermore, it could readily be developed into a micromethod for measurements with submicrogram quantities. Table 15.10 shows a comparison of data obtained by HPLC with the reported literature values by classical method.

Data collected with a simple RPLC procedure has been found to be in good agreement with 1-octanol shake-flask partition or distribution coefficients over a 3.5 log range [64]. A chemically bonded octadecylsilane support is coated with 1-octanol. With 1-octanol-saturated buffers as mobile phases, a stable baseline (compared to 1-octanol absorbed on silica) is

TABLE 15.10
Comparison of log P values by HPLC with literature [63]

Name	HPLC	Literature
tert-Butylbenzene	4.07	4.11
Propylbenzene	3.44	3.62
Toluene	2.68	2.58
Acetophenone	1.58	1.66
Resorcinol	0.88	0.80
Hydroquinone	0.54	0.56
Caffeine	−0.05	−0.07

obtained rapidly, and the log relative retention times are highly correlated with unit slope to log distribution or partition coefficients obtained from the classical shake-flask procedures. Only relatively basic, unhindered pyridines deviate, probably because of binding with residual silanol sites.

In addition, if the apparent pK_a of an ionizable compound lies within the pH operating range of the column support, the apparent pK_a usually can be determined simultaneously with log P by measuring the log distribution coefficient at several pH values (Table 15.11). The main advantages of the procedure are that it gives rapid results, requires little material, and can tolerate impurities.

Using an unmodified commercial octadecylsilane column and a mobile phase consisting of methanol and an aqueous buffer, a linear relationship has been established between the literature log P values of 68 compounds and the logarithms of their k' values [65]. For the determination of the partition coefficients of unknowns, standards are used to calibrate the system, the choice being made on the basis of the hydrogen-bonding character of the compounds being evaluated. The overall method is shown to be rapid and widely acceptable and to give log P data that is comparable to results obtained by classical and other correlation methods.

The studies with barbiturates revealed that the logarithm of the retention time is linearly related to the octanol/water partition coefficients [66,67]. It has been observed that the retention index of the drug is linearly related to the octanol/water partition coefficient (log P), and that results are very close to that of the 2-keto alkane standard (solid line in Fig. 15.9).

TABLE 15.11

Comparison of log P and apparent pK_a determined by HPLC with literature values [64]

	HPLC		Literature	
Compound	pK_a	Log P	pK_a	Log P
Naproxen	4.28	3.21	4.53	3.18
	4.21	3.20	4.39	—
Benzoic acid	4.33	1.78	4.20	1.87
	4.38	1.77	4.18	
Salicylic acid	3.52	2.00	3.00	2.23
	3.29	2.18	—	—
p-Toluic acid	4.30	2.22	4.37	2.27
	4.41	2.26	—	—

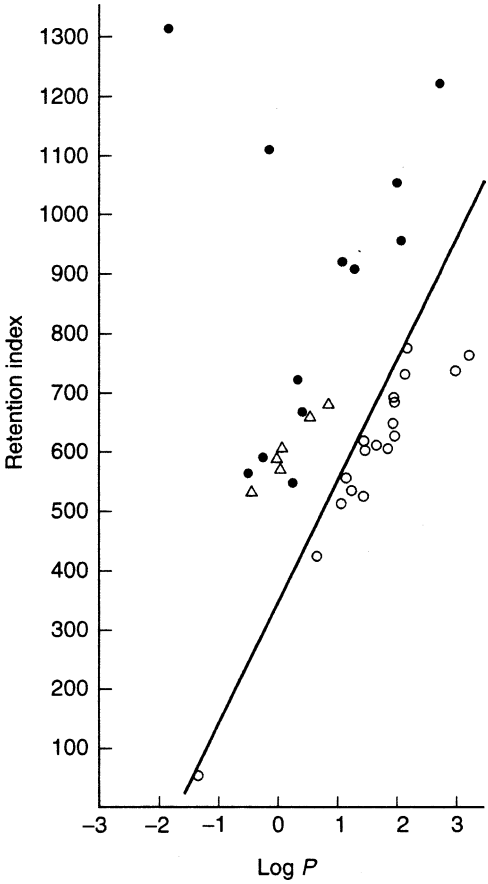


Fig. 15.9. Correlation between observed retention indices and octanol partition coefficients [66].

The retention data of catecholamine derivatives in reversed-phase chromatography with octadecyl-silica stationary phase and aqueous eluent has been analyzed. Good agreement is observed between the observed and predicted k' values [68]. Data obtained with different C-18 stationary phases at various temperatures suggest that quantitative structure-retention relationships can be transformed from one reversed-phase system to another as long as the eluent composition is the same. Kalizan [69] observed that C-18 columns without any additional coating give results that are precise enough for quantitative structure activity relationship (QSAR) purposes and are convenient over a wide range of operational conditions.

Tomlinson [70] suggests that a ''hydrophobic bond'' is formed when two or more nonpolar groups in an aqueous medium come into contact, thus decreasing the extent of interaction with the surrounding water molecules, and resulting in the liberation of water originally bound by the molecules. The hydrophobic bond is recognized as complex in nature, involving polar and apolar interactions; the hydrophobic bond concept has been useful in explaining association of organic and biologic molecules in aqueous solution. In QSAR models, the ability of a compound to partition between a relatively nonpolar solvent and water is normally used as a measure of its hydrophobic character.

Martin was the first to point out that a substituent changes the partition coefficient of a substance by a given factor that depends on the nature of the substituent and the two phases employed, but not on the rest of the molecule. Martin's treatment assumes that for any stated solvent system, the change in retention ($\Delta R_{\min}$ TLC) caused by the introduction of group X into a parent structure is of constant value, providing that its substitution into the parent structure does not result in any intramolecular interactions with other functions in the structure. On the other hand, it can be appreciated that if the introduction of a group into a structure causes a breakdown in the additivity principle, then intra- or intermolecular effects are likely to be more significant within the substituted structure. These effects are as follows:

- Steric effects (including ortho effect)
- Intramolecular hydrogen bonding
- Electronic effects
- Intramolecular hydrophobic bonding
- Chain branching.

As mentioned before, the hydrophobicity and the partition coefficient can be related to the solubility of the solutes in water (also see Section 15.8.4). The partition coefficient (P) between octanol and water can be described as the π-constant of Hansch or the $\log P$ values of Rekker. The $\log P$ values calculated from the fragmental constant are then used for the optimization of RPLC. However, this method is not adequate to develop an optimization system for the mixtures of different types of compounds [71].

15.8.3 Impact of other physicochemical parameters on retention

Discussed below is the impact of various other physicochemical parameters excluding solubility (see Section 15.8.4) on retention in HPLC.

The van der Waals volume can be related to the hydrophobicity of the solutes, and retention of molecular compounds can be predicted from their van der Waals volumes, π-energy, and hydrogen-bonding energy effects [72–74]. It should be noted that the isomeric effect of substituents cannot be predicted with good precision because this is not simply related to Hammett's σ or Taft's σ^* constants. On the other hand, the hydrophobicity is related to enthalpy [75]. Retention times of nonionizable compounds were measured in 70 and 80% acetonitrile/water mixtures on an octadecyl-bonded silica gel at 25–60°C and the enthalpy values obtained from these measurements.

Retention volumes of monosubstituted benzenes, benzoic acid, phenols, and anilines have been measured in RPLC [76]. Buffered acetonitrile/water and tetrahydrofuran/water eluents were used with an octadecylsilica adsorbent. From the net retention volumes, a substituent interaction effect was calculated and described with the linear free energy relationship developed by Taft. The data was interpreted in terms of hydrogen bonding between the solutes and the eluent.

Enthalpy–entropy compensation has been investigated in reversed-phase HPLC with octylsilica stationary phase [77]. The compensation temperatures were determined for this system, and the results show that their change with the composition of the mobile phase is almost similar to that with octadecylsilica stationary phase. It can be concluded that the retention mechanisms of the separation of alkyl benzenes is the same in both systems with the mobile phase exceeding 20% water content.

The separation of substituted benzene derivatives on a reversed-phase C-18 column has been examined [78]. The correlations between the logarithm of the capacity factor and several descriptors for the molecular size and shape and the physical properties of a solute were determined. The results indicated that hydrophobicity is the dominant factor to control the retention of substituted benzenes. Their retention in reversed-phase HPLC can be predicted with the help of the equations derived by multicombination of the parameters.

As mentioned before, retention in RPLC has been found to be related to the van der Waals volume, π-energy, and hydrogen-bonding energy effects. However, higher-molecular-weight compounds are retained more strongly than expected [79]. In order to investigate this effect more fully, the retention times of phenols were measured on an octadecyl-bonded silica gel in acidic acetonitrile/water mixtures at different temperatures. The enthalpies of phenols were then calculated from their $\log k'$ values. The magnitude of the enthalpy effect increases

with increasing molecular size, but the polarity of the molecule was the predominant factor in the enthalpy effect.

An increase in the number of methylene units in alkyl benzene did not significantly affect the π-energy effect on their retention, but the enthalpy effect increased dramatically [80]. This means that a hydrophobic compound can be adsorbed directly onto an octadecyl-bonded silica gel. The value of enthalpy effect of a methylene unit in alkyl benzene was calculated to be 500 cal/mol.

Schoenmakers *et al*. [81] investigated the relationship between solute retention and mobile-phase composition in RPLC over the entire range of composition, with emphasis on mobile phases with a high water content. It appears that a quadratic relationship between the logarithm of the capacity factor and the volume fraction of organic modifier is generally valid for mobile phases containing less than 90% water. When more water is added to the mobile phase, the quadratic equation turns out to be insufficient. An experimental study of ten solutes and three organic modifiers is used to show that an extension of the quadratic equation by a term proportional to the square root of the volume fraction leads to a description of the experimental retention data within approximately 10%.

The polarity values of binary acetonitrile/water and methanol/water mobile phases used in RPLC were measured and compared with methylene selectivity (α_{CH_2}) for both traditional siliceous bonded phases and for a polystyrene-divinylbenzene resin reversed-phase material [82]. The variation in methylene selectivity for both was found to correlate best with percent organic solvent in methanol/water mixtures, whereas the polarity value provided the best correlation in acetonitrile/water mixtures. The polymeric resin column was found to provide higher methylene selectivity than the siliceous-bonded phase at all concentrations of organic solvent.

The retention indices, measured on the alkyl aryl ketone scale, of a set of column test compounds (toluene, nitrobenzene, *p*-cresol, 2-phenyl ethanol, and *N*-methylaniline) were used to determine the changes in selectivity of a series of ternary eluents prepared from methanol/0.02 M phosphate buffer pH 7 (60:40), acetonitrile/0.02 M phosphate buffer pH 7 (50:50) and tetrahydrofuran/0.02 M phosphate buffer pH 7 (25:65). The analyses were carried out on a Spherisorb ODS reversed-phase column. The selectivity changes were often nonlinear between the binary composition [83].

Direct measurement of solute sorption-desorption kinetics in chromatographic systems provides some useful insights into the mechanism

of the sorption process and a sensitive means of measuring slight differences in those stationary phase–solvent interactions that are responsible for determining the chemical contributions of the stationary phase.

Variations in retention and selectivity have been studied in cyano, phenyl, and octyl reversed bonded phase HPLC columns. The retention of toluene, phenol, aniline, and nitrobenzene in these columns has been measured using binary mixtures of water and methanol, acetonitrile, or tetrahydrofuran mobile phases in order to determine the relative contributions of proton donor–proton acceptor and dipole–dipole interactions in the retention process. Retention and selectivity in these columns were correlated with polar group selectivities of mobile-phase organic modifiers and the polarity of the bonded stationary phases. In spite of the prominent role of bonded phase volume and residual silanols in the retention process, each column exhibited some unique selectivities when used with different organic modifiers [84].

The physicochemical framework has been examined by comparing the predictions of two models for the combined effects of the composition of the hydro-organic mobile phase and the column temperature on the retention of *n*-alkyl benzenes on hydrocarbonaceous bonded stationary phases. The ''well-mixed'' model leads to expressions for the dependence of retention on three factors that are equivalent to those derived previously from linear extra-thermodynamic relationships. The ''diachoric'' model stems from the assumption that the mobile phase is identical to the retention model most widely used in chromatography with polar sorbents and less polar solvents. Over limited ranges of mobile phase composition and temperature, each model describes retention behavior. However, only the well-mixed model describes retention well over the entire range of mobile-phase composition and temperature studied here. The success of the well-mixed model and the limits of the model give insights into the role of organic solvents in determining the magnitude of chromatographic retention on a nonpolar stationary phase with hydro-organic eluents [85].

It has been shown that when the intracolumn effect of mass transfer and diffusion is the main factor controlling band broadening, the column efficiency decreases with the increase of the viscosity of the methanol/water mixture; on the other hand, when the extra-column effect is the main factor, an increase in viscosity of the eluents will help in improving column efficiency. Column efficiency is also related to the properties of the sample [86].

15.8.4 Solubility and retention in HPLC

The solubility parameter concept was established in the 1930s by the work of Hildebrand and Scatchard. The original concept covers regular solutions, i.e., solutions that do not show an excess entropy effect on mixing. The solubility parameter concept offers the following interesting features:

- The concept is based on the assumption that the properties of mixtures can be described by the properties of pure components. As a result, the arithmetic expressions involved (regular mixing rule) are relatively simple.
- The solubility parameter concept relates to compounds rather than to molecules. Because it is a macroscopic approach, it relates to practical data more conveniently than a molecular statistical approach does.

In earlier work on the applicability of the solubility parameter theory to HPLC, attention was focused on quantitation; for this work, the model did not prove to be successful. Schoenmakers *et al*. [87] believe that the potential of the solubility parameter can aid in designing a genuine framework for retention behavior in LC. Based on their work, the following conclusions have been drawn:

- Reasonable retention times are obtained if the polarity of the solute is roughly intermediate between the polarities of the mobile and stationary phases.
- For higher members of a homologous series with approximately the same polarity, the logarithm of the capacity factor varies linearly with the molar volume or the carbon number.
- The absolute difference in polarity between the mobile phase and the stationary phase may be defined as the general selectivity of an HPLC system.
- There are two commonly used ways to elute a given compound in HPLC: the normal-phase mode ($\delta s > \delta m$) and the reversed-phase mode ($\delta m > \delta s$). Reversed-phase systems offer superior general selectivity. Solutes are eluted in ascending order of polarity in normal-phase systems and in descending order of polarity in reversed-phase systems.
- Although stationary phases of intermediate polarity (alumina, silica, carbon) provide only moderate general selectivity, they are potentially most powerful for very polar solutes when operated in the reversed-phase mode.

- Perfluorinated stationary phases offer superior selectivity in comparison to the current hydrocarbon bonded-stationary phases.
- Specific separation effects can be understood from the multicomponent solubility parameter theory. Specific effects for nonpolar compounds are predictable with perfluorinated and graphitized carbon black stationary phases. Specific selectivity for polar compounds in reversed-phase HPLC can be realized with polar additives to the mobile phase.
- Previously formulated transfer rules for binary mobile phases in reversed-phase HPLC can be explained by solubility parameter expressions.

15.9 MOLECULAR PROBES/RETENTION INDEX

A variety of compounds have been used as molecular probes to evaluate HPLC columns and characterize them.

- Nonpolar compounds, e.g., benzene, naphthalene
- Polar compounds, e.g., hydroquinone or steroids
- Chelating compounds, e.g., acetyl acetone
- Quaternary compounds, e.g., quaternary ammonium compounds
- Basic compounds, e.g., amines
- Acidic compounds, e.g., toluic acid.

Unfortunately, none of the commonly used molecular probes is adequate to evaluate column-to-column variabilities [88]. The absolute prediction of retention of any compound involves the use of a rather complex equation [89,90] that necessitates the knowledge of various parameters for both the solute and the solvent [91]. The relative prediction of retention is based on the existence of a calibration line describing the linearity between $\log k^*$ and interaction index. This second approach, although less general than the first, is simpler to use in practice, and it often gives more accurate results than the first. With a proper choice of calibration solutes, it is possible to take into account subtle mobile phase effects that cannot be included in the theoretical treatment.

However, certain conditions must be verified prior to using a prediction model based on a calibration of the chromatographic system. First, it is necessary to limit the number of calibration solutes. It is clear that the use of a large number of calibration standards can give a high degree of accuracy, but this is too time-consuming. Five to six solutes appear to offer a good compromise between accuracy and

convenience. Second, one must be able to use these calibration compounds in a rather large range of solvent composition. Finally, calibration compounds must be ''simple'' chemicals, stable, and easily available in any chemical laboratory.

Using steroids as solutes and 2-keto alkanes as reference compounds, simple linear equations have been developed for accurately predicting reversed-phase HPLC retention indices and resolution [92]. These equations have practical applications for predicting whether given pairs of compounds can be separated under given conditions, or for predicting the conditions that will separate mixtures of compounds in a minimum amount of time. The technique may be used to optimize isocratic or gradient separations of compound mixtures. The results of this study show that the Snyder-solvent-selectivity triangle concept for characterizing mobile-phase liquids fails to consistently group solvents according to selectivity for separating steroids. Contrary to theory, experimental separations often differ markedly within a given solvent group. Selctivity differences between solvents in the same group sometimes exceed those between solvents in different groups.

A study was conducted to utilize the 2-keto alkane system for calculating HPLC retention indices of a series of steroids, and then to utilize the retention index data to predict retention and resolution of the compounds as a function of solvent strength and selectivity. The main goal was to utilize this capability to predict resolution for optimizing the separation of steroid mixtures or pairs of individual steroids. A secondary objective was to study the selectivity characteristics of solvents grouped according to the solvent-selectivity triangle concept for their ability to separate given pairs of steroids.

These results contradict the theory of the solvent-selectivity triangle concept, which states that solvents in the same group should result in similar selectivity, while those in different groups should yield different selectivities [93,94]. A literature search showed a widespread usage of the solvent-selectivity triangle as a rationale for solvent selection. However, with the exception of a publication by Lewis *et al*. [95], definitive studies on the accuracy of the solvent groupings in the selectivity triangle appear to be lacking. They studied the separation of polystyrene oligomers using a total of 17 solvents representing all eight of the selectivity groups and concluded that the solvent triangle did not accurately predict selectivity for the separation being studied. These authors reported that the degree of the solute solubility in the pure mobile phase solvents was a better predictor of selectivity than were the groupings of the solvent triangle.

The studies with steroids and polystyrene oligomers have demonstrated that solvents classified within the same Snyder-solvent-selectivity group do not necessarily result in similar selectivity. This discrepancy might be due to the underlying assumptions of the solvent triangle theory, which assumes that selectivity is largely governed by the ability of the solvent to engage in hydrogen bonding and dipolar interactions and that dispersion interactions play an unimportant role in selectivity for solutions of polar solvents. The solvent triangle concept also fails to consider the role of the stationary phase and the nature of the solutes themselves in affecting a given separation. This concept further assumes that only three test solutes are needed to establish the primary selectivity characteristics of various solvents.

A method has been offered to characterize variations in the retention properties of RPLC by column–eluent combinations by using retention indices of a set of reference compounds, toluene, nitrobenzene, *p*-cresol, and 2-naphthylethanol [96]. These compounds were selected by multivariate analysis to give optimum discrimination between eluents and columns.

An interesting pair of compounds is caffeine and theophylline [97]; these compounds are relatively polar compounds with different functional groups (tertiary and secondary amine). In a few cases, more appropriate comparisons have been made such as between androstenedione/testosterone and methyl benzoate/anisole; these compounds are expected to be different in Snyder interaction groups.

A number of researchers have also used a proposed ASTM test mixture, benzaldehyde, acetophenone, methyl benzoate, dimethyl terphthalate, benzyl alcohol, and benzene to demonstrate separation on a column [98]. However the first four compounds are from the same interaction group and should behave in the same way on changing conditions. The first three have almost constant indices (respectively 760, 800, and 890) so that in effect they create an ''index scale'' with constant differences against which the last two compounds can be compared [96,99].

In order to determine the applicability of retention indices, based on the alkyl arylketone scale, as the basis of a reproducible method of reporting retentions, the separation of 10 barbiturates and a set of column test compounds were examined on an octadecylsilyl bonded silica (ODS-Hypersil) column with methanol-buffer of pH 8.5 as eluent [100]. The effects on the capacity factors and retention indices, on changing the eluent composition, pH, ionic strengthened temperature, showed that the retention indices of the barbiturates were much less susceptible to minor changes in the eluent than the capacity factors.

For nonionized compounds, the retention indices were virtually independent of the experimental conditions.

The silanophilic character of 16 reversed-phase high-performance liquid chromatographic columns was evaluated with dimethyl diphenycyclam, a cyclic tetraza macrocycle [101]. The method is rapid, does not require the removal of packing material, and uses a water-miscible solvent. The results demonstrate two points: first, cyclic tetraza macrocycles offer substantial benefits over currently used silanophilic agents; second, the method can easily differentiate the performance of various columns in terms of their relative hydrophobic and silanophilic contributions to absolute retention.

A mixture of acetyl acetone, 1-nitronaphthalene, and naphthalene has been proposed for evaluating reversed-phase packing material [102]. This reveals the usual optimum kinetic chromatographic parameters (the naphthalene peak), the degree of activity or end-capping status of the column (the ratio of the 1-nitronaphthalene and naphthalene retention times) and trace metal activity (the shape and intensity of the acetylacetone peak).

15.10 STRATEGY OF SEPARATION

A strategy to design a successful HPLC separation may involve the following steps:

a. Select the method most suitable for your sample based on solubility of sample and other relevant physical properties. For example, the preliminary choice of a method would include one of the following methods:
   - Normal phase
   - Reversed phase
   - Ion exchange
   - Size exclusion.

b. Select a suitable column based on the above-mentioned method for selection. For example, in reversed-phase HPLC, the choice may be to simply entail selecting C-8 or C-18.

c. Choose simple mobile phase for initial evaluations. For example, methanol: water (50:50) may be a good starting point in the case of RPLC.

d. Make desirable changes in the mobile phase in terms of proportions of solvent or vary solvents or include suitable additives.

e. Utilize specialized techniques such as gradient elution or derivatization to enhance detectability and/or improve separations.

15.10.1 Improving resolution

The primary goal of any separation process is to achieve optimum resolution of the components. Resolution can be improved by varying the three terms α, N, or k' in the resolution equation:

$$Rs = \frac{1}{4}\left(\alpha - \frac{1}{\alpha}\right)N^{1/2}\left(\frac{k'_2}{1+k'_2}\right) \tag{15.22}$$

The effect on sample resolution with changes in k', N or α values is shown in Fig. 15.10. For example, an increase in separation factor α results in a displacement of one band center relative to the other, and a

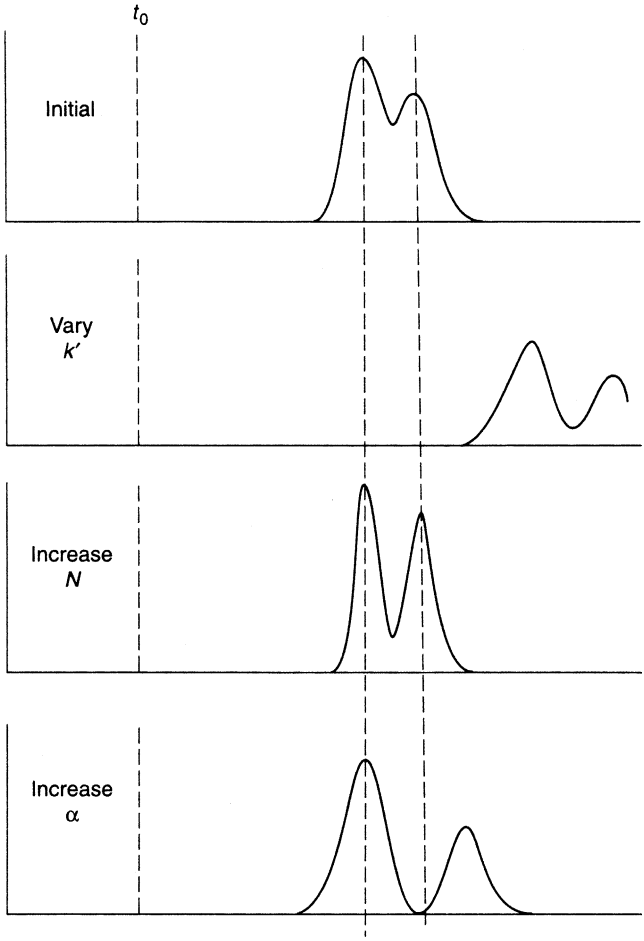


Fig. 15.10. Effect of α, k', or N on resolution.

rapid increase in Rs. The peak height or retention time is not significantly affected for a moderate change in α.

The increase in plate number N results in narrowing of the two bands and an increase in band height; the retention time is not affected if there is no change in weight ratio of the sample. The most dramatic effect on separation is caused by change in k' values. If k' for the initial separation falls within the range $0.5 < k' < 2$, a decrease in k' can make a separation look bad. On the other hand, an increase in k' can provide a significant increase in resolution. However, as k' is increased, band heights rapidly decrease and separation time increases.

When it is necessary to improve Rs, k' should be increased into the optimum range $1 < k' < 10$. When k' is < 1, Rs increases rapidly with increase in k'. For values of k' greater than 5, Rs increases very little with further increase in k' (see Table 15.12).

If k' were infinity, its proportionality would be 1.00. This means there is very small improvement over that seen with k' of 10 or 5 in the table. These observations suggest that the optimum k' is between 1 and 10.

It is important to note here that no other change in separation condition provides as large an increase in Rs value for very little effort.

The k' values in HPLC can be controlled by means of solvent strength. When it is necessary to increase k' value, a weaker solvent is used. For example, in reversed-phase separations, solvent strength is greater for pure methanol than for pure water. The right proportionality of these solvents has to be found to get optimum separation.

When k' is already within the optimum range of values and the resolution is still marginal, the best solution is to increase N. This is generally achieved by increasing column length or by decreasing the flow rate of the mobile phase.

TABLE 15.12
Impact of k' on Rs value*

k'	$K'/1+k'$
0	0
1	0.5
2	0.67
5	0.83
10	0.91

* See Eq. (15.20)

When Rs is still small even though k' is optimum, an increase in N would not be very helpful because it unusually prolongs separation time. In this case, increasing α value would be more desirable. However, it should be recognized that predicting the right conditions for the necessary change in α is not as simple procedure (for more details, see Section 15.8).

15.11 MOBILE-PHASE SELECTION AND OPTIMIZATION

The conventional approaches to mobile-phase selection and optimization are discussed here. The primary focus is on compounds with molecular weight less than 2000. More detailed information including coverage of macromolecules may be found in some basic texts [2,5,39,103]. As discussed earlier, various modes of chromatography utilized to separate these compounds can be classified as follows:

1. Adsorption chromatography
2. Normal-phase chromatography
3. Reversed-phase chromatography
4. Ion-pair chromatography
5. Ion-exchange chromatography
6. Ion chromatography.

Ion-pair chromatography is frequently performed in the reversed-phase mode and is therefore discussed in Section 15.7. Since ion chromatography is an offshoot of ion-exchange chromatography, it has been discussed right after ion-exchange chromatography (Section 15.7.6).

15.11.1 General considerations

Various means have been used to optimize separations for each chromatographic technique (see Chapters 5 through 8 in reference 5); the discussion here is limited to conventional approaches used to select the mobile phase.

These approaches are frequently based on intuitive judgment and know-how of the chromatographer. For the latter, it is important to emphasize that a knowledge of physicochemical basis of retention and a basic understanding of separation mechanism in HPLC, discussed above, will go a long way in helping to select the right mobile phase quickly and then to optimize it by the usual experimentation. These experiments can be logically conducted even when the operator is not present, by letting a computer select mobile-phase combinations based on certain preset

requirements. This aspect of method development is better appreciated once the conventional approaches have been mastered.

15.11.1.1 Properties of sample

The selection of an HPLC method should be made primarily from the properties of the sample (alternate terms are solute, analyte, or eluite) once it has been established that it has a sufficiently low molecular weight, i.e. <2000, to justify use of the techniques mentioned above. The decision could be based on the solubility of the sample, i.e., whether it is soluble in polar or nonpolar solvents (Chart 1).

From Chart 1, it is clear that compounds with molecular weight >2000 are better separated by using gel permeation chromatography or size exclusion chromatography (SEC). Of course SEC can be used for molecular weights below 2000 just as other modes of chromatography can be used for compounds with higher molecular weight. The discussion here is concerned primarily with compounds of molecular weight <2000.

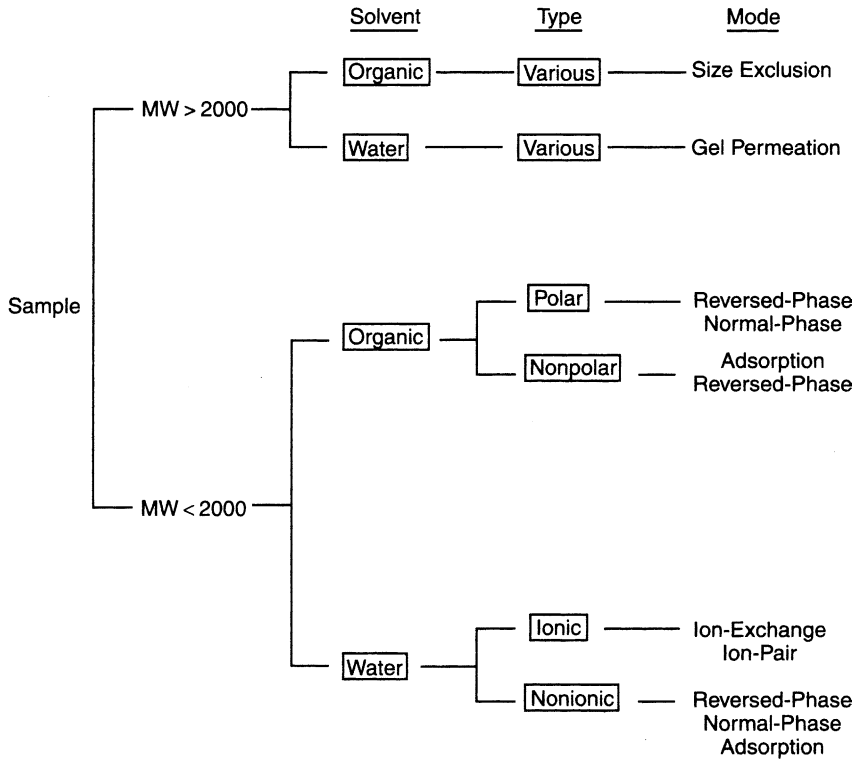


Chart 1. Selection of chromatographic method based on solubility.

Information other than solubility that can help select the suitable mode of chromatography, whether or not the sample is ionic. In this regard, the dissociation constant of the compound is of great value because with appropriate adjustment of pH, one can select a desirable percent ionization of the compound of interest, remembering when $pH = pK_a$, the compound is 50% ionized.

15.11.1.2 Column selection

The choice of column should be made after careful consideration of mode of chromatography, column-to-column variability, and a number of other considerations [3–5]. A short discussion on columns and column packings is given below. The column packings may be classified according to the following features [2]:

1. Rigid solids or hard gels
2. Porous or pellicular and superficially porous particles
3. Spherical or irregular particles
4. Particle size (*dp*).

Rigid solids based on a silica matrix are most commonly used as HPLC packings. Such packings can withstand the relatively high pressures (10,000–15,000 psi). The silica particles can be obtained in a variety of sizes, shapes, and varying degrees of porosity. Furthermore, various functional groups or polymeric layers can readily be attached to the silica surface, thus extending the utility of these particles for applications to any individual HPLC method.

Hard gels are generally based on porous particles of polystyrene cross-linked with divinyl benzene. Depending on how they are prepared, the resulting particles may vary in rigidity and porosity over fairly wide limits. They still find use in ion exchange and SEC; however, rigid solids are gradually replacing hard gels.

Packings for HPLC can be further described as either pellicular or porous. Pellicular particles are made from spherical glass beads, which are then coated with a thin layer of stationary phase. For example, a porous layer can be deposited onto the glass bead to produce a porous layer or a superficially porous particle. The porous layer can in turn be coated with liquid stationary phase or reacted to give a bonded stationary phase. Pellicular particles are generally less efficient than the porous layer of superficially porous particles.

As the particle size (*dp*) decreases, column plate height decreases and the column becomes less permeable. As a result, for small values of *dp*, a column of some fixed length generates higher plate count; i.e., higher

efficiency, but greater pressure drop across the column is required for a given value of linear flow. This suggests that columns of small particles are more efficient, but require higher operating pressures.

For columns of similar efficiency, the maximum sample size allowed is generally smaller for pellicular particles than for porous particles. The reason is that a less stationary phase is available per unit volume of column. Roughly five times as much sample can be charged to a porous column before there is a significant decrease in k'. Since larger samples can be injected onto a porous-particle column, the resulting bands are larger and more easily detected. The columns of small porous particles give good detection sensitivities and are preferred for ultratrace analysis [6].

For comparison of similar columns, it is important that experimental conditions for the test chromatogram are faithfully reproduced and sufficient time is allowed for column equilibration before starting the test. The expected changes in column performance parameters that are due to changes in the experimental conditions are summarized in Table 15.13.

The columns commonly used in HPLC can be classified based on mode of separation, selected backbone, particle size, and functionalities (Table 15.14).

15.11.1.3 Column evaluations

As mentioned earlier, columns should be thoroughly evaluated prior to use [5]. Some of the desirable properties of test solutes are given below.

TABLE 15.13
HPLC parameters affecting column efficiency

Parameter	Change in efficiency (N)
Flow rate	Low flow rate generally gives high value of N
Particle size	Small particle size gives high value of N
Column length, L	N is proportional to L
Mobile-phase viscosity	Low value gives high value of N
Temperature, T	High values reduce viscosity and give high values of N
Capacity factor, k'	Low k' (<2) give low values of N; for high k' values (>2), N is influenced
Dead volume	N is decreased due to band-broadening contributions to peak width
Sample of size	Large amounts (mg) or large volumes decrease N

TABLE 15.14
HPLC columns

Mode	Material	Particle size (μM)	Treatment
Adsorption	Silica, irregular	2–20	Unreacted
	Silica, spherical	5–10	Unreacted
	Alumina, irregular	3–12	Unreacted
	Alumina	5–20	Unreacted
Reversed phase	Silica with long C chain	3–15	C-18
	Silica with intermediate C chain	5–10	C-8
	Silica with short C chain	5–10	C-1,C-3
Normal phase	Silica(weak)	5–15	Ester, ether, diester
	Silica(medium)	5–15	$N0_2$, CN
	Silica(high)	5–15	Alkylamino, amino
Ion exchange	Silica(anion)	5–15	NMe^{+3}, NR^{+3}, NH_2
	Resin(anion)	7–20	NMe^{+3}, $-NH^{+3}$
	Silica(cation)	5–10	$\_SO^{3}\_(H^{+})$, $\_SO^{3}\_$, $(NH_4)^{+}$
	Resin	5–20	$\_SO^{3}\_$

1. Test solutes should be of low-molecular weight to ensure rapid diffusion and easy access to the packing pore structure.
2. The solute should include components that
   - characterize the column in terms of both kinetic and thermodynamic performance;
   - determine the column dead volume, i.e., of the test mixture as an unretained solute; and
   - differentiate retention with k' values between 2 and 10.
3. Test solutes should have strong absorbance, preferably at 254 nm.

15.11.1.4 Mobile phase selection in HPLC

Retention in HPLC depends on the strength of the solute's interaction with both the mobile and stationary phases as opposed to GC, where the mobile phase does not contribute to the selectivity. An intelligent selection of the type of stationary phase for the separation is made and

selectivity is adjusted by modifying the mobile phase. The selection of the mobile phase for a particular separation is thus a very important consideration in HPLC.

For HPLC, some fairly broad generalizations can be made about the selection of certain preferred solvents from the large number available. A suitable solvent will preferably have low viscosity, be compatible with the detection system, be readily available in pure form, and if possible have low flammability and toxicity. In selecting organic solvents for use in mobile phases, several physical and chemical properties of the solvent should be considered. From the standpoint of detection, the refractive index or UV cutoff values are also important.

The term polarity refers to the ability of a sample or solvent molecule to interact by combination of dispersion, dipole, hydrogen bonding, and dielectric interactions (see Chapter 2 in reference 5). The combination of these four intermolecular attractive forces constitutes the solvent polarity, which is a measure of the strength of the solvent. Solvent strength increases with polarity in normal phase, and adsorption HPLC decreases with polarity in reversed-phase HPLC. Thus, polar solvents preferentially attract and dissolve polar solute molecules.

Common HPLC solvents with adequate purity are commercially available. Halogenated solvents may contain traces of acidic impurities that can react with stainless steel components of the HPLC system. Mixtures of halogenated solvents with water should not be stored for long periods, as they are likely to decompose. Mixtures of halogenated solvents with various ethers, e.g., diethyl ether, react to form products that are particularly corrosive to stainless steel. Halogenated solvents such as methylene chloride react with other organic solvents such as acetonitrile and, on standing, form crystalline products.

15.11.1.5 Solvent selection

Snyder (for more details, see Chapter 2, reference 5) has described a scheme for classifying common solvents according to their polarity or chromatographic strength (P' values) based on their selectivity or relative ability to engage in hydrogen bonding or dipole interactions. Various common solvents classified into eight groups (I–VIII) showing significantly different selectivities (Fig. 15.11).

Group	Common Solvents
I	Aliphatic ethers, tetramethylguanidine
II	Aliphatic alcohols
III	Tetrahydrofuran, pyridine derivatives, glycol ethers, sulfoxides
IV	Acetic acid, formamide, benzyl alcohol, glycols
V	Methylene chloride, ethylene chloride
VIa	Aliphatic ketones and esters, dioxane, tricresyl phosphate
VIb	Sulfones, nitriles
VII	Aromatic hydrocarbons, halo-substituted aromatic hydrocarbons, nitro compounds, aromatic ethers
VIII	Water, *m*-cresol, fluoroalcohols, chloroform

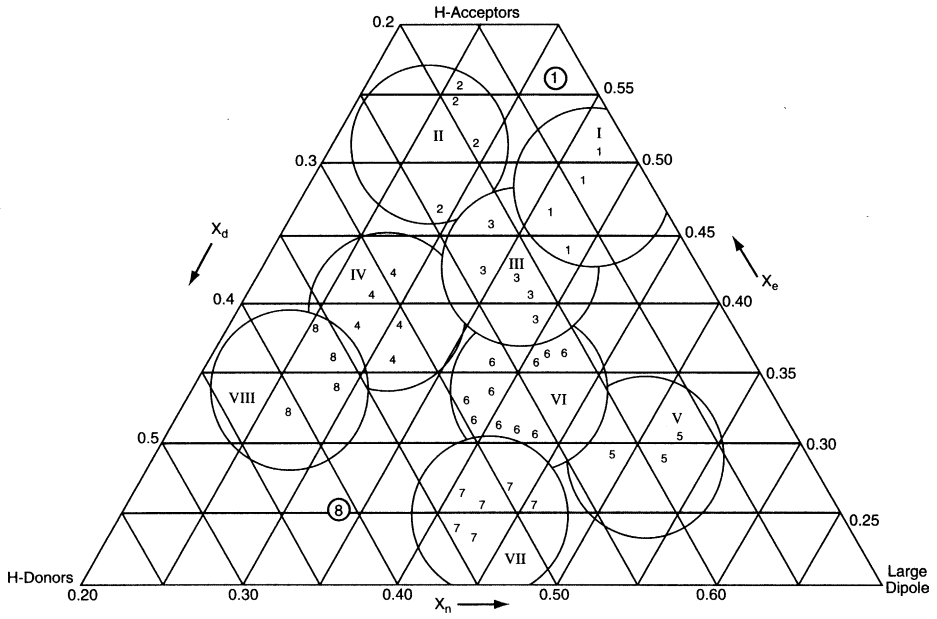


Fig. 15.11. Solvent-selectivity triangle [5].

The P' (polarity index) values and selectivity group classifications for some solvents commonly used in LC are given in Table 15.15.

The values of P' and selectivity factors are calculated from the experimentally derived solute polarity distribution coefficient for the test solutes ethanol, dioxane, and nitromethane. The solute distribution

TABLE 15.15
Polarity of some common solvents [5]

Solvent	δ	P'	ε
n-Hexane	7.3	0.1	0.00
Ethyl ether	7.4	2.8	0.43
Triethylamine	7.5	1.9	—
Cyclohexane	8.2	0.2	0.03
Carbon tetrachloride	8.6	1.6	0.11
Ethyl acetate	8.9	4.4	0.48
Tetrahydrofuran	9.1	4.0	0.53
Chloroform	9.3	4.1	0.26
Methylene chloride	9.6	3.1	0.30
Acetone	9.7	5.1	0.53
Dioxane	10.1	4.8	0.51
Dimethyl formamide	11.8	6.4	—
Isopropanol	12.0	3.9	0.60
Acetonitrile	12.1	5.8	0.52
Ethanol	12.7	4.3	—
Methanol	14.5	5.1	0.70
Formamide	19.2	9.6	—
Water	23.4	10.2	—

δ = Hildebrand solubility parameter
P' = Polarity index
ε = Solvent strength for silica adsorbent

coefficients are corrected for effects that are due to solute molecular size, solute/solvent dispersion interactions, and solute/solvent induction due to solvent polarizability. The resultant parameters P' and solvent selectivity should reflect only the selective interaction properties of the solvent. The test solutes ethanol, dioxane, and nitromethane are used to measure the strengths of solvent proton acceptor, proton donor, and strong dipole interactions, respectively.

Changes in the mobile phase can result in significant selectivity changes for various sample analytes. The greatest change in mobile phase selectivity can be obtained when the relative importance of the various intermolecular interactions between solvent and solute molecules is markedly changed. The changes in selectivity can be affected by making use of the following solvent properties:

- Proton donors: alcohols, carboxylic acids, phenols, and chloroform
- Proton acceptors: amines, ethers, sulfoxides, amides, esters, and alcohols

- Large dipole solvents: methylene chloride, nitrites, sulfoxides, and ketones.

Substitution of methanol by another alcohol such as propanol would not be expected to radically change selectivity because in both cases a proton donor solvent is present. However, a greater change in selectivity can be expected by using ethyl ether (proton acceptor) or methylene chloride (large dipole moment).

The solvent classification scheme is helpful in identifying solvents with different chromatographic selectivities. It is generally preferable to use mixtures of solvents rather than a single pure solvent as the mobile phase. For binary solvents, mixing a strength-adjusting solvent with various volume fractions of a strong solvent enables the complete polarity or solvent strength range between the extremes represented by the pure solvents themselves to be covered. The strength-adjusting solvent is usually a nonselective solvent, such as water for reversed-phase chromatography, and hexane for normal-phase applications. The solvent strength of a binary solvent mixture is the arithmetic average of the solvent strength weighting factors adjusted according to the volume fraction of each solvent. For normal-phase chromatography, the solvent strength weighting factor, S_i, is the same as the polarity index, P'. In reversed-phase chromatography, a different set of experimentally weighting factors is used [104].

The solvent strength for any solvent mixture can be calculated from this equation:

$$S_{\mathrm{T}} = \sum\nolimits_{\mathrm{i}} S_{\mathrm{i}}\theta_{\mathrm{i}} \tag{15.23}$$

where

S_{T} = total solvent strength of the mixture
S_i = solvent strength weighting factor
θ_i = volume fraction of solvent in the mixture

Binary solvent mixtures provide a simple means of controlling solvent strength but limited opportunities for controlling solvent selectivity. With ternary and quaternary solvent mixtures, it is possible to fine-tune solvent selectivity while maintaining a constant solvent strength [105–107]. In addition, there are only a small number of organic modifiers that can be used as binary mixtures with water.

The Snyder-solvent-selectivity triangle concept can be combined with a mixture-design statistical technique to define the optimum mobile-phase composition for a particular separation A feature of this mixture-design

technique is that it leads to the selection of a quaternary mobile-phase system for most separations. The selection process can be controlled by a microprocessor in an interactive way if the solvent delivery system can pump four solvents simultaneously (for more details, see Chapter 11, reference 5).

15.11.1.6 Mobile-phase additives

At times it is necessary to add reagents such as buffers, ion-pairing reagents, or other modifiers such as triethylamine to the mobile phase to improve reproducibility, selectivity, or peak shape.

Buffers are used mainly to control the pH and the acid-base equilibrium of the solute in the mobile phase. They can also be used to influence the retention times of ionizable compounds. The buffer capacity should be maximum and should be uniform in the pH range of 2–8 commonly used in HPLC. The buffers should be soluble, stable, and compatible with the detector employed, e.g., citrates are known to react with certain HPLC hardware components.

Addition of compounds such as long-chain alkyl compounds in reversed-phase separations will alter the retention of ionic compounds but will have no effect on nonionic compounds unless the concentration is high enough to form micelles (for additional information, please refer to Chapter 8, reference 5).

Competing amines such as triethylamine and di-*n*-butylamine have been added to the mobile phase in reversed-phase separations of basic compounds. Acetic acid can serve a similar purpose for acidic compounds. These modifiers, by competing with the analyte for residual active sites, cause retention time and peak tailing to be reduced. Other examples are the addition of silver ions to separate geometric isomers and the inclusion of metal ions with chelating agents to separate racemic mixtures.

15.12 APPLICATIONS

HPLC can be used in virtually all fields. Listed below are the types of compounds that can be resolved by HPLC [7].

- Amino acids
- Dyes
- Explosives
- Nucleic acids
- Pharmaceuticals, excluding impurities and metabolites

- Pharmaceutical impurities
- Pharmaceutical metabolites
- Plant active ingredients
- Plant pigments
- Polar lipids
- Polysaccharides
- Proteins and peptides
- Recombinant products
- Surfactants
- Synthetic polymers.

It is clearly not possible to cover all these applications within the scope of this chapter. Detailed applications can be found in the literature [1–3,5,7]. Reproduced below is a case study to show how one may select a mode of separation in HPLC and at the same time develop a better understanding of separation mechanism.

15.12.1 A case study

Ionizable compounds such as baclofen (I), 4-amino-3-(p-chlorophenyl)butyric acid, can be chromatographed by several modes in high-pressure liquid chromatography (HPLC). It can be chromatographed as a cation or an anion on cationic or anionic exchange columns respectively, or as an ion-pair with the oppositely charged counterion by RPLC. Ionization suppression techniques may also be used in RPLC. The separation mechanisms involved in chromatography of baclofen (I) from its transformation product were investigated by comparison of ion-exchange and ion-pair reversed-phase HPLC. These investigations were aimed primarily at the amino group, which was protonated for the cation-exchange chromatography or ion-paired with pentane sulfonated anion for reversed-phase chromatography. Model compounds were used to evaluate selectivity for ionic and nonionic compounds. Table 15.16 ows selectivity of the ion-exchange method. These experiments clearly show that retention in ion-exchange chromatography can be considerably influenced by the nonionized portion of the molecule, e.g., see retention data of Compounds I, III, and VI.

A combination of different modes of chromatography can provide excellent resolution for those components that chromatograph poorly with a single mode [108,109]. The chromatographic separation of baclofen (I) from its potential transformation product (II) with dual mode chromatography entailing ion-pair reversed-phase chromatography and

TABLE 15.16
Selectivity of ion-exchange method

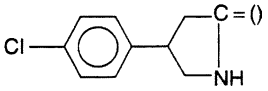
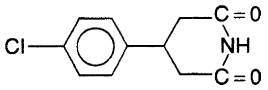
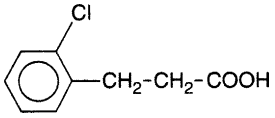
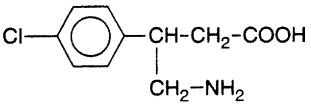
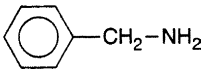
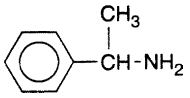
Compound	Structure	Retention volume
Lactam		1.97
4-(*p*-Chlorophenyl) glutarimid		1.97
o-Chlorohydrocinnamic acid		1.97
Baclofen		5.91
Benzylamine		9.84
dl-α-Methylbenzylamine		18.6

TABLE 15.17
Effect of sulfonic acid on retention time of baclofen

	Retention time (min)	
Concentration of C-5 reagent	Baclofen (I)	Lactam (II)
0.0 m	5.6 (asymmetrical peak)	22.0
0.007 m	10.1 (symmetrical peak)	24.0

reversed-phase chromatography, respectively, demonstrates this point clearly (Table 15.17).

Ion pairing between the amino group of baclofen and pentane sulfonic acid is primarily responsible for the chromatographic behavior of baclofen on a reversed-phase octadecylsilane column. However, the transformation

TABLE 15.18
Effect of increasing concentration of C-5 sulfonic acid on retention

Concentration of C-5 reagent	t_R Baclofen (min)	Calc. Δ t_R /mM
0.0	5.97	—
0.22	6.04	0.32
2.2	9.25	1.49
5.4	10.87	0.91
10.8	12.08	0.57
16.2	12.90	0.43
21.6	13.43	0.34
32.3	14.23	0.26
43.1	15.06	0.21

product (II) does not form an ion pair with pentane sulfonic acid and is, therefore, separated primarily by a reversed-phase partition process. It was noted that peak symmetry and analysis time of (I) can be significantly influenced by the concentration of C-5 sulfonic acid (Table 15.18). These experiments show the importance of investigations on stationary phase dynamics in separations by ion-pair RPLC.

CONCLUSIONS

The phenomenal growth in chromatography is largely due to the introduction of the technique called HPLC. It allows separations of a large variety of compounds by offering some major improvements over the classical column chromatography, thin-layer chromatography, GC, and it presents some significant advantages over more recent techniques such as SFC, CE, and electrokinetic chromatography. New developments in HPLC include utilization of smaller particle size and ultrahigh pressures or miniaturization to combine nanoLC with MS.

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REVIEW QUESTIONS

1. Discuss various modes of chromatography.
2. Which mode of chromatography is most commonly used in HPLC?
3. How can you vary α values in RPLC?
4. List some of the common additives for RPLC.
5. Describe various approaches to solvent optimization in RPLC.