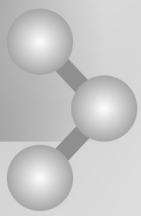


Chapter 12

Chromatographic and Electrophoretic Methods

Drawing from an arsenal of analytical techniques, many of which were the subject of the preceding four chapters, analytical chemists have designed methods for the analysis of analytes at increasingly lower concentrations and in increasingly more complex matrices. Despite the power of these techniques, they often suffer from a lack of selectivity. For this reason, many analytical procedures include a step to separate the analyte from potential interferents. Several separation methods, such as liquid–liquid extractions and solid-phase microextractions, were discussed in Chapter 7. In this chapter we consider two additional separation methods that combine separation and analysis: chromatography and electrophoresis.

12A Overview of Analytical Separations

In Chapter 7 we examined several methods for separating an analyte from potential interferents. For example, in a liquid–liquid extraction the analyte and interferent are initially present in a single liquid phase. A second, immiscible liquid phase is introduced, and the two phases are thoroughly mixed by shaking. During this process the analyte and interferents partition themselves between the two phases to different extents, affecting their separation. Despite the power of these separation techniques, there are some significant limitations.

12A.1 The Problem with Simple Separations

Suppose we have a sample containing an analyte in a matrix that is incompatible with our analytical method. To determine the analyte's concentration we first separate it from the matrix using, for example, a liquid–liquid extraction. If there are additional analytes, we may need to use additional extractions to isolate them from the analyte's matrix. For a complex mixture of analytes this quickly becomes a tedious process.

Furthermore, the extent to which we can effect a separation depends on the distribution ratio of each species in the sample. To separate an analyte from its matrix, its distribution ratio must be significantly greater than that for all other components in the matrix. When the analyte's distribution ratio is similar to that of another species, then a separation becomes impossible. For example, let's assume that an analyte, A, and a matrix interferent, I, have distribution ratios of 5 and 0.5, respectively. In an attempt to separate the analyte from its matrix, a simple liquid–liquid extraction is carried out using equal volumes of sample and a suitable extraction solvent. Following the treatment outlined in Chapter 7, it is easy to show that a single extraction removes approximately 83% of the analyte and 33% of the interferent. Although it is possible to remove 99% of A with three extractions, 70% of I is also removed. In fact, there is no practical combination of number of extractions or volume ratio of sample and extracting phases that produce an acceptable separation of the analyte and interferent by a simple liquid–liquid extraction.

12A.2 A Better Way to Separate Mixtures

The problem with a simple extraction is that the separation only occurs in one direction. In a liquid–liquid extraction, for example, we extract a solute from its initial phase into the extracting phase. Consider, again, the separation of an analyte and a matrix interferent with distribution ratios of 5 and 0.5, respectively. A single liquid–liquid extraction transfers 83% of the analyte and 33% of the interferent to the extracting phase (Figure 12.1). If the concentrations of A and I in the sample were identical, then their concentration ratio in the extracting phase after one extraction is

$$\frac{[\mathrm{A}]}{[\mathrm{I}]} = \frac{0.83}{0.33} = 2.5$$

Thus, a single extraction improves the separation of the solutes by a factor of 2.5. As shown in Figure 12.1, a second extraction actually leads to a poorer separation. After combining the two portions of the extracting phase, the concentration ratio decreases to

$$\frac{[\mathrm{A}]}{[\mathrm{I}]} = \frac{0.97}{0.55} = 1.8$$

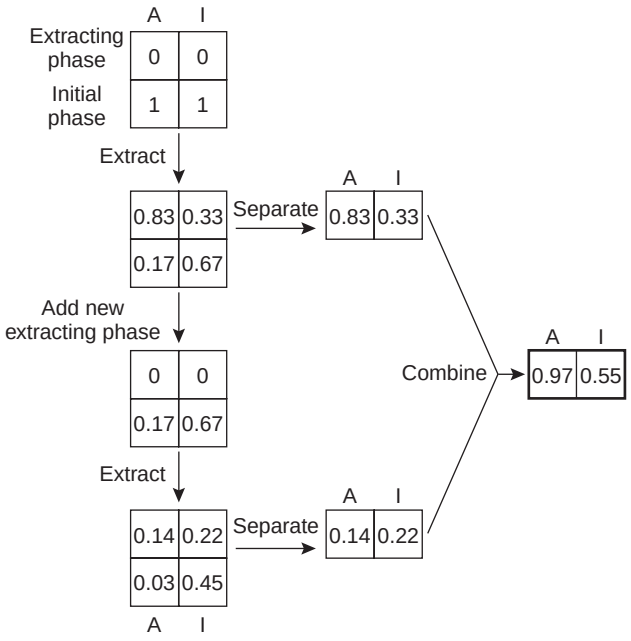


Figure 12.1
Progress of a liquid–liquid extraction using two identical extractions of a sample (initial phase) with fresh portions of the extracting phase. All numbers are fractions of solute in the phases; A = analyte, I = interferent.

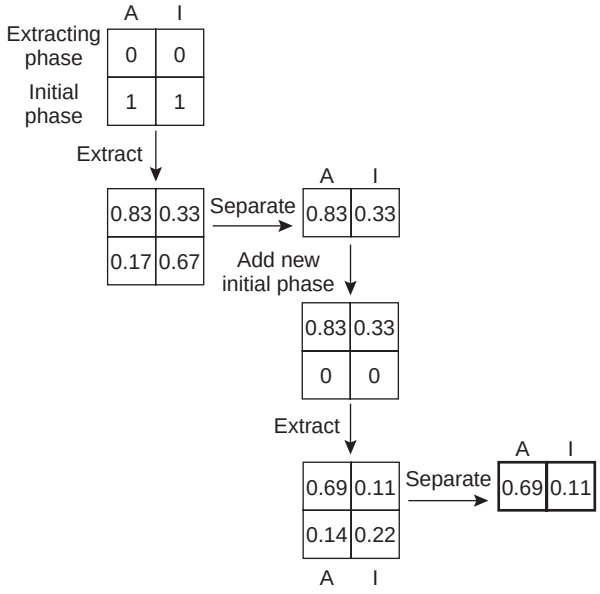


Figure 12.2
Progress of a liquid–liquid extraction in which the solutes are first extracted into the extracting phase and then extracted back into a fresh portion of the initial phase. All numbers are fractions of solute in the phases; A = analyte, I = interferent.

We can improve the separation by first extracting the solutes into the extracting phase, and then extracting them back into a fresh portion of the initial phase (Figure 12.2). Because solute A has the larger distribution ratio, it is extracted to a greater extent during the first extraction and to a lesser extent during the second extraction. In this case the final concentration ratio of

$$\frac{[A]}{[I]} = \frac{0.69}{0.11} = 6.3$$

countercurrent extraction
A liquid–liquid extraction in which solutes are extracted back and forth between fresh portions of two extracting phases.

mobile phase
In chromatography, the extracting phase that moves through the system.

stationary phase
In chromatography, the extracting phase that remains in a fixed position.

in the extracting phase is significantly greater. The process of extracting the solutes back and forth between fresh portions of the two phases, which is called a **countercurrent extraction,** was developed by Craig in the 1940s.[1*] The same phenomenon forms the basis of modern chromatography.

Chromatographic separations are accomplished by continuously passing one sample-free phase, called a **mobile phase,** over a second sample-free phase that remains fixed, or stationary. The sample is injected, or placed, into the mobile phase. As it moves with the mobile phase, the sample's components partition themselves between the mobile and **stationary phases.** Those components whose distribution ratio favors the stationary phase require a longer time to pass through the system. Given sufficient time, and sufficient stationary and mobile phase, solutes with similar distribution ratios can be separated.

The history of modern chromatography can be traced to the turn of the century when the Russian botanist Mikhail Tswett (1872–1919) used a column packed with a stationary phase of calcium carbonate to separate colored pigments from plant extracts. The sample was placed at the top of the column and carried through the stationary phase using a mobile phase of petroleum ether. As the sample moved through the column, the pigments in the plant extract separated into individual colored bands. Once the pigments were adequately separated, the calcium carbonate was removed from the column, sectioned, and the pigments recovered by extraction. Tswett named the technique **chromatography,** combining the Greek words for "color" and "to write." There was little interest in Tswett's technique until 1931 when chromatography was reintroduced as an analytical technique for biochemical separations. Pioneering work by Martin and Synge in 1941[2] established the importance of liquid–liquid partition chromatography and led to the development of a theory for chromatographic separations; they were awarded the 1952 Nobel Prize in chemistry for this work. Since then, chromatography in its many forms has become the most important and widely used separation technique. Other separation methods, such as electrophoresis, effect a separation without the use of a stationary phase.

chromatography
A separation in which solutes partition between a mobile and stationary phase.

12A.3 Classifying Analytical Separations

Analytical separations may be classified in three ways: by the physical state of the mobile phase and stationary phase; by the method of contact between the mobile phase and stationary phase; or by the chemical or physical mechanism responsible for separating the sample's constituents. The mobile phase is usually a liquid or a gas, and the stationary phase, when present, is a solid or a liquid film coated on a solid surface. Chromatographic techniques are often named by listing the type of mobile phase, followed by the type of stationary phase. Thus, in gas–liquid chromatography the mobile phase is a gas and the stationary phase is a liquid. If only one phase is indicated, as in gas chromatography, it is assumed to be the mobile phase.

Two common approaches are used to bring the mobile phase and stationary phase into contact. In **column chromatography,** the stationary phase is placed in a narrow column through which the mobile phase moves under the influence of gravity or pressure. The stationary phase is either a solid or a thin, liquid film coating on a solid particulate packing material or the column's walls. In **planar chromatography** the stationary phase coats a flat glass, metal, or plastic plate

column chromatography
A form of chromatography in which the stationary phase is retained in a column.

planar chromatography
A form of chromatography in which the stationary phase is immobilized on a flat surface.

*The theory behind countercurrent extractions is outlined in Appendix 6.

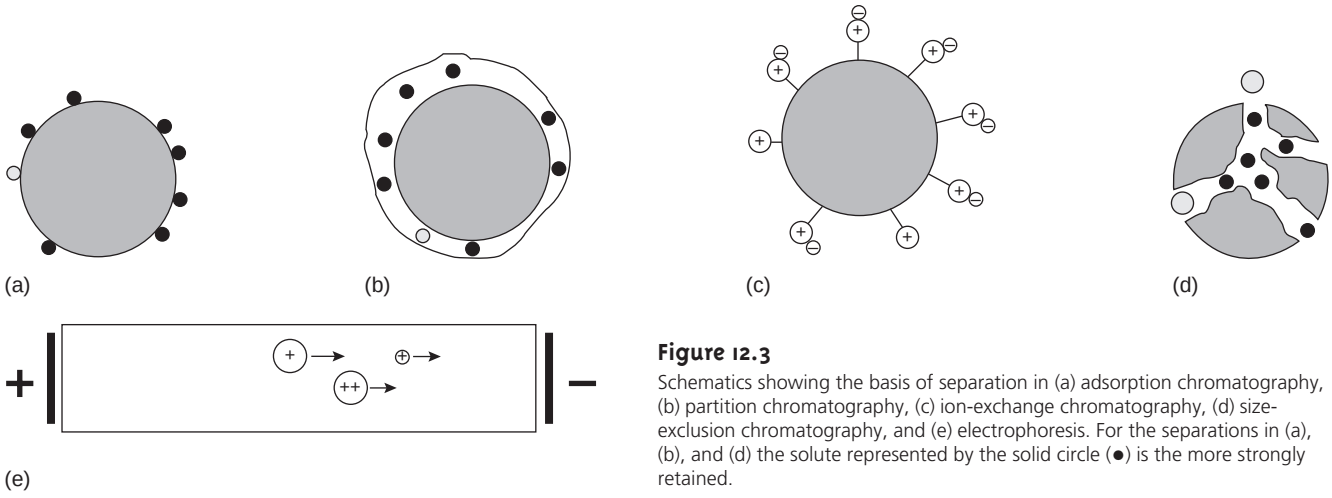


Figure 12.3
Schematics showing the basis of separation in (a) adsorption chromatography, (b) partition chromatography, (c) ion-exchange chromatography, (d) size-exclusion chromatography, and (e) electrophoresis. For the separations in (a), (b), and (d) the solute represented by the solid circle (●) is the more strongly retained.

and is placed in a developing chamber. A reservoir containing the mobile phase is placed in contact with the stationary phase, and the mobile phase moves by capillary action.

The mechanism by which solutes separate provides a third means for characterizing a separation (Figure 12.3). In adsorption chromatography, solutes separate based on their ability to adsorb to a solid stationary phase. In partition chromatography, a thin liquid film coating a solid support serves as the stationary phase. Separation is based on a difference in the equilibrium partitioning of solutes between the liquid stationary phase and the mobile phase. Stationary phases consisting of a solid support with covalently attached anionic (e.g., $-SO_3^-$) or cationic (e.g., $-N(CH_3)_3^+$) functional groups are used in ion-exchange chromatography. Ionic solutes are attracted to the stationary phase by electrostatic forces. Porous gels are used as stationary phases in size-exclusion chromatography, in which separation is due to differences in the size of the solutes. Large solutes are unable to penetrate into the porous stationary phase and so quickly pass through the column. Smaller solutes enter into the porous stationary phase, increasing the time spent on the column. Not all separation methods require a stationary phase. In an electrophoretic separation, for example, charged solutes migrate under the influence of an applied potential field. Differences in the mobility of the ions account for their separation.

12B General Theory of Column Chromatography

Of the two methods for bringing the stationary and mobile phases into contact, the more important is column chromatography. In this section we develop a general theory that we may apply to any form of column chromatography. With appropriate modifications, this theory also can be applied to planar chromatography.

A typical column chromatography experiment is outlined in Figure 12.4. Although the figure depicts a liquid–solid chromatographic experiment similar to that first used by Tswett, the design of the column and the physical state of the

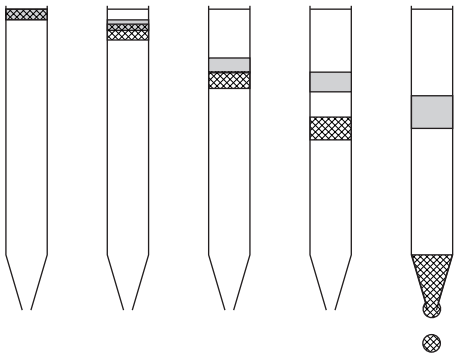


Figure 12.4
Progress of a column chromatographic separation showing the separation of two solute bands.

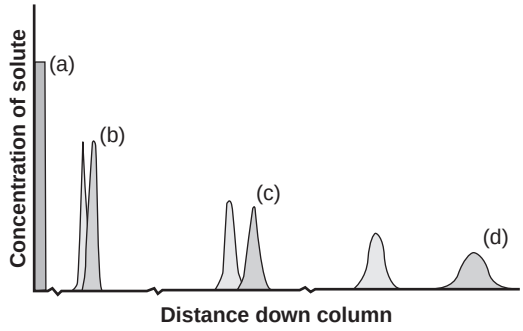


Figure 12.5
Another view of the progress of a column chromatographic separation showing the separation of two solute bands.

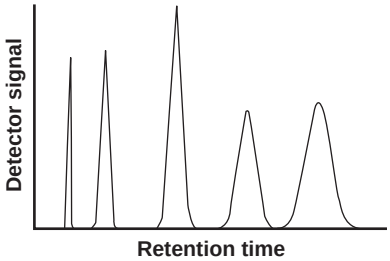


Figure 12.6
Typical chromatogram of detector response as a function of retention time.

chromatogram
A plot of the detector's signal as function of elution time or volume.

retention time
The time a solute takes to move from the point of injection to the detector (t_r).

retention volume
The volume of mobile phase needed to move a solute from its point of injection to the detector (V_r).

baseline width
The width of a solute's chromatographic band measured at the baseline (w).

stationary and mobile phases may vary. The sample is introduced at the top of the column as a narrow band. Ideally, the solute's initial concentration profile is rectangular (Figure 12.5a). As the sample moves down the column the solutes begin to separate, and the individual solute bands begin to broaden and develop a Gaussian profile (Figures 12.5b,c). If the strength of each solute's interaction with the stationary phase is sufficiently different, then the solutes separate into individual bands (Figure 12.5d). The progress of a chromatographic separation is monitored with a suitable detector situated at the end of the column. A plot of the detector's signal as a function of time or volume of eluted mobile phase is known as a **chromatogram** (Figure 12.6) and consists of a peak for each of the separated solute bands.

A chromatographic peak may be characterized in many ways, two of which are shown in Figure 12.7. The **retention time,** t_r, is the elapsed time from the introduction of the solute to the peak maximum. The retention time also can be measured indirectly as the volume of mobile phase eluting between the solute's introduction and the appearance of the solute's peak maximum. This is known as the **retention volume,** V_r. Dividing the retention volume by the mobile phase's flow rate, u, gives the retention time.

The second important parameter is the chromatographic peak's width at the baseline, w. As shown in Figure 12.7, **baseline width** is determined by the intersection with the baseline of tangent lines drawn through the inflection points on either side of the chromatographic peak. Baseline width is measured in units of time or volume, depending on whether the retention time or retention volume is of interest.

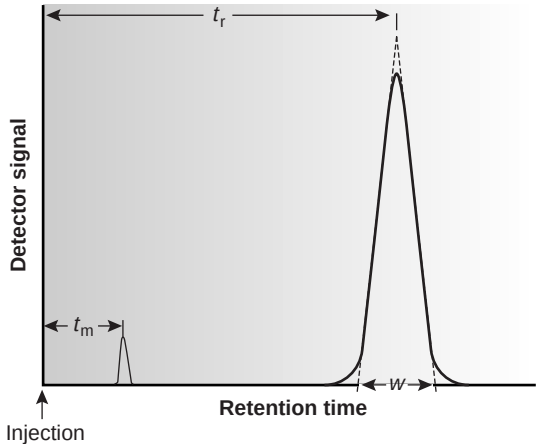


Figure 12.7
Measurement of the column's void time, t_m, and the retention time, t_r, and baseline width, w, for a solute.

Besides the solute peak, Figure 12.7 also shows a small peak eluted soon after the sample is injected into the mobile phase. This peak results from solutes that move through the column at the same rate as the mobile phase. Since these solutes do not interact with the stationary phase, they are considered nonretained. The time or volume of mobile phase required to elute nonretained components is called the column's **void time,** t_m, or **void volume.**

void time
The time required for unretained solutes to move from the point of injection to the detector (t_m).

void volume
The volume of mobile phase needed to move an unretained solute from the point of injection to the detector.

12B.1 Chromatographic Resolution

The goal of chromatography is to separate a sample into a series of chromatographic peaks, each representing a single component of the sample. **Resolution** is a quantitative measure of the degree of separation between two chromatographic peaks, A and B, and is defined as

$$R = \frac{t_{r,B} - t_{r,A}}{0.5(w_B + w_A)} = \frac{2\Delta t_r}{w_B + w_A} \qquad \textbf{12.1}$$

resolution
The separation between two chromatographic bands (R).

As shown in Figure 12.8, the degree of separation between two chromatographic peaks improves with an increase in R. For two peaks of equal size, a resolution of 1.5 corresponds to an overlap in area of only 0.13%. Because resolution is a quantitative measure of a separation's success, it provides a useful way to determine if a change in experimental conditions leads to a better separation.

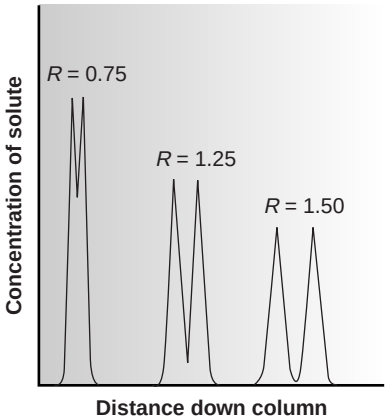


Figure 12.8
Three examples of chromatographic resolution.

Example

EXAMPLE 12.1

In a chromatographic analysis of lemon oil a peak for limonene has a retention time of 8.36 min with a baseline width of 0.96 min. γ-Terpinene elutes at 9.54 min, with a baseline width of 0.64 min. What is the resolution between the two peaks?

SOLUTION

Using equation 12.1, we find that the resolution is

$$R = \frac{2\Delta t_r}{w_B + w_A} = \frac{2(9.54 - 8.36)}{0.64 + 0.96} = 1.48$$

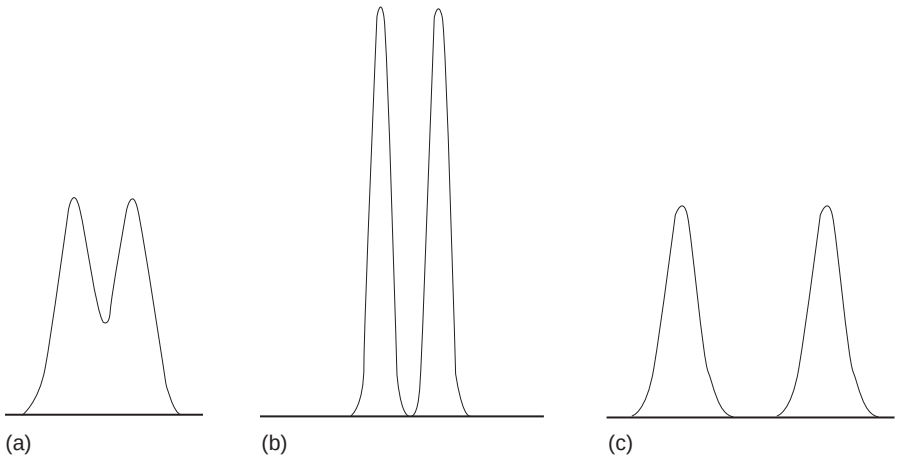


Figure 12.9
Two methods for improving chromatographic resolution: (a) Original separation showing a pair of poorly resolved solutes; (b) Improvement in resolution due to an increase in column efficiency; (c) Improvement in resolution due to a change in column selectivity.

From equation 12.1 it is clear that resolution may be improved either by increasing Δt_r or by decreasing w_A or w_B (Figure 12.9). We can increase Δt_r by enhancing the interaction of the solutes with the column or by increasing the column's selectivity for one of the solutes. Peak width is a kinetic effect associated with the solute's movement within and between the mobile phase and stationary phase. The effect is governed by several factors that are collectively called column efficiency. Each of these factors is considered in more detail in the following sections.

12B.2 Capacity Factor

The distribution of a solute, S, between the mobile phase and stationary phase can be represented by an equilibrium reaction

$$S_m \rightleftharpoons S_s$$

and its associated partition coefficient, K_D, and distribution ratio, D,

$$K_D = \frac{[S_s]}{[S_m]}$$

$$D = \frac{[S_s]_{tot}}{[S_m]_{tot}} \qquad \textbf{12.2}$$

where the subscripts m and s refer to the mobile phase and stationary phase, respectively. As long as the solute is not involved in any additional equilibria in either the mobile phase or stationary phase, the equilibrium partition coefficient and the distribution ratio will be the same.

Conservation of mass requires that the total moles of solute remain constant throughout the separation, thus

$$(\text{moles S})_{tot} = (\text{moles S})_m + (\text{moles S})_s \qquad \textbf{12.3}$$

Solving equation 12.3 for the moles of solute in the stationary phase and substituting into equation 12.2 gives

$$D = \frac{\{(\text{moles S})_{\text{tot}} - (\text{moles S})_{\text{m}}\}/V_{\text{s}}}{(\text{moles S})_{\text{m}}/V_{\text{m}}} = \frac{(\text{moles S})_{\text{tot}}V_{\text{m}} - (\text{moles S})_{\text{m}}V_{\text{m}}}{(\text{moles S})_{\text{m}}V_{\text{s}}}$$

where V_{m} and V_{s} are the volumes of the mobile and stationary phases. Rearranging and solving for the fraction of solute in the mobile phase, f_{m}, gives

$$f_{\text{m}} = \frac{(\text{moles S})_{\text{m}}}{(\text{moles S})_{\text{tot}}} = \frac{V_{\text{m}}}{V_{\text{m}} + DV_{\text{s}}} \qquad \textbf{12.4}$$

Note that this equation is identical to that describing the extraction of a solute in a liquid–liquid extraction (equation 7.25 in Chapter 7). Since the volumes of the stationary and mobile phase may not be known, equation 12.4 is simplified by dividing both the numerator and denominator by V_{m}; thus

$$f_{\text{m}} = \frac{1}{1 + D(V_{\text{S}}/V_{\text{m}})} = \frac{1}{1 + k'} \qquad \textbf{12.5}$$

where

$$k' = D\frac{V_{\text{s}}}{V_{\text{m}}} \qquad \textbf{12.6}$$

is the solute's **capacity factor.**

capacity factor
A measure of how strongly a solute is retained by the stationary phase (k').

A solute's capacity factor can be determined from a chromatogram by measuring the column's void time, t_{m}, and the solute's retention time, t_{r} (see Figure 12.7). The mobile phase's average linear velocity, u, is equal to the length of the column, L, divided by the time required to elute a nonretained solute.

$$u = \frac{L}{t_{\text{m}}} \qquad \textbf{12.7}$$

By the same reasoning, the solute's average linear velocity, v, is

$$v = \frac{L}{t_{\text{r}}} \qquad \textbf{12.8}$$

The solute can only move through the column when it is in the mobile phase. Its average linear velocity, therefore, is simply the product of the mobile phase's average linear velocity and the fraction of solute present in the mobile phase.

$$v = uf_{\text{m}} \qquad \textbf{12.9}$$

Substituting equations 12.5, 12.7, and 12.8 into equation 12.9 gives

$$\frac{L}{t_{\text{r}}} = \frac{L}{t_{\text{m}}}\left(\frac{1}{1 + k'}\right)$$

Finally, solving this equation for k' gives

$$k' = \frac{t_{\text{r}} - t_{\text{m}}}{t_{\text{m}}} = \frac{t'_{\text{r}}}{t_{\text{m}}} \qquad \textbf{12.10}$$

adjusted retention time
The difference between a solute's retention time and column's void time (t_{r}').

where t'_{r} is known as the **adjusted retention time.**

EXAMPLE 12.2

In a chromatographic analysis of low-molecular-weight acids, butyric acid elutes with a retention time of 7.63 min. The column's void time is 0.31 min. Calculate the capacity factor for butyric acid.

SOLUTION

$$k' = \frac{t_r - t_m}{t_m} = \frac{7.63 \text{ min} - 0.31 \text{ min}}{0.31 \text{ min}} = 23.6$$

12B.3 Column Selectivity

selectivity factor
The ratio of capacity factors for two solutes showing the column's selectivity for one of the solutes (α).

The relative selectivity of a chromatographic column for a pair of solutes is given by the **selectivity factor,** α, which is defined as

$$\alpha = \frac{k'_B}{k'_A} = \frac{t_{r,B} - t_m}{t_{r,A} - t_m} \qquad \textbf{12.11}$$

The identities of the solutes are defined such that solute A always has the smaller retention time. Accordingly, the selectivity factor is equal to 1 when the solutes elute with identical retention times, and is greater than 1 when $t_{r,B}$ is greater than $t_{r,A}$.

EXAMPLE 12.3

In the same chromatographic analysis for low-molecular-weight acids considered in Example 12.2, the retention time for isobutyric acid is 5.98 min. What is the selectivity factor for isobutyric acid and butyric acid?

SOLUTION

First we must calculate the capacity factor for isobutyric acid. Using the void time from Example 12.2, this is

$$k' = \frac{t_r - t_m}{t_m} = \frac{5.98 \text{ min} - 0.31 \text{ min}}{0.31 \text{ min}} = 18.3$$

The selectivity factor, therefore, is

$$\alpha = \frac{k'_{buty}}{k'_{iso}} = \frac{23.6}{18.3} = 1.29$$

12B.4 Column Efficiency

At the beginning of a chromatographic separation the solute occupies a narrow band of finite width. As the solute passes through the column, the width of its band

continually increases in a process called **band broadening.** Column efficiency provides a quantitative measure of the extent of band broadening.

band broadening
The increase in a solute's baseline width as it moves from the point of injection to the detector.

In their original theoretical model of chromatography, Martin and Synge[2] treated the chromatographic column as though it consists of discrete sections at which partitioning of the solute between the stationary and mobile phases occurs. They called each section a **theoretical plate** and defined column efficiency in terms of the number of theoretical plates, *N,* or the height of a theoretical plate, *H;* where

theoretical plate
A quantitative means of evaluating column efficiency that treats the column as though it consists of a series of small zones, or plates, in which partitioning between the mobile and stationary phases occurs.

$$N = \frac{L}{H} \qquad \textbf{12.12}$$

A column's efficiency improves with an increase in the number of theoretical plates or a decrease in the height of a theoretical plate.

Assuming a Gaussian profile, the extent of band broadening is measured by the variance or standard deviation of a chromatographic peak. The height of a theoretical plate is defined as the variance per unit length of the column

$$H = \frac{\sigma^2}{L} \qquad \textbf{12.13}$$

where the variance, σ^2, has units of distance squared. Because retention time and peak width are usually measured in seconds or minutes, it is more convenient to express the standard deviation in units of time, τ, by dividing σ by the mobile phase's average linear velocity.

$$\tau = \frac{\sigma}{u} = \frac{\sigma t_r}{L} \qquad \textbf{12.14}$$

When a chromatographic peak has a Gaussian shape, its width at the baseline, *w,* is four times its standard deviation, τ.

$$w = 4\tau \qquad \textbf{12.15}$$

Combining equations 12.13 through 12.15 gives the height of a theoretical plate in terms of the easily measured chromatographic parameters t_r and *w.*

$$H = \frac{Lw^2}{16t_r^2} \qquad \textbf{12.16}$$

The number of theoretical plates in a chromatographic column is obtained by combining equations 12.12 and 12.16.

$$N = 16\left(\frac{t_r}{w}\right)^2 \qquad \textbf{12.17}$$

Alternatively, the number of theoretical plates can be approximated as

$$N = 5.545\left(\frac{t_r}{w_{1/2}}\right)^2$$

where $w_{1/2}$ is the width of the chromatographic peak at half its height.

Example

EXAMPLE 12.4

A chromatographic analysis for the chlorinated pesticide Dieldrin gives a peak with a retention time of 8.68 min and a baseline width of 0.29 min. How many theoretical plates are involved in this separation? Given that the column used in this analysis is 2.0 meters long, what is the height of a theoretical plate?

SOLUTION

Using equation 12.17, the number of theoretical plates is

$$N = 16\left(\frac{t_r}{w}\right)^2 = 16\left(\frac{8.68 \text{ min}}{0.29 \text{ min}}\right)^2 = 14,300 \text{ plates}$$

Solving equation 12.12 for H gives the average height of a theoretical plate as

$$H = \frac{L}{N} = \frac{(2.0 \text{ m})(1000 \text{ mm/m})}{14,300 \text{ plates}} = 0.14 \text{ mm/plate}$$

It is important to remember that a theoretical plate is an artificial construct and that no such plates exist in a chromatographic column. In fact, the number of theoretical plates depends on both the properties of the column and the solute. As a result, the number of theoretical plates for a column is not fixed and may vary from solute to solute.

12B.5 Peak Capacity

peak capacity
The maximum number of solutes that can be resolved on a particular column (n_c).

Another important consideration is the number of solutes that can be baseline resolved on a given column. An estimate of a column's **peak capacity,** n_c, is

$$n_c = 1 + \frac{\sqrt{N}}{4} \ln \frac{V_{max}}{V_{min}} \qquad \textbf{12.18}$$

where V_{min} and V_{max} are the smallest and largest volumes of mobile phase in which a solute can be eluted and detected.[3] A column with 10,000 theoretical plates, for example, can resolve no more than

$$n_c = 1 + \frac{\sqrt{10,000}}{4} \ln \frac{30 \text{ mL}}{1 \text{ mL}} = 86 \text{ solutes}$$

if the minimum and maximum volumes of mobile phase in which the solutes can elute are 1 mL and 30 mL. This estimate provides an upper bound on the number of solutes that might be separated and may help to exclude from consideration columns that do not have enough theoretical plates to separate a complex mixture. Just because a column's theoretical peak capacity is larger than the number of solutes to be separated, however, does not mean that the separation will be feasible. In most situations the peak capacity obtained is less

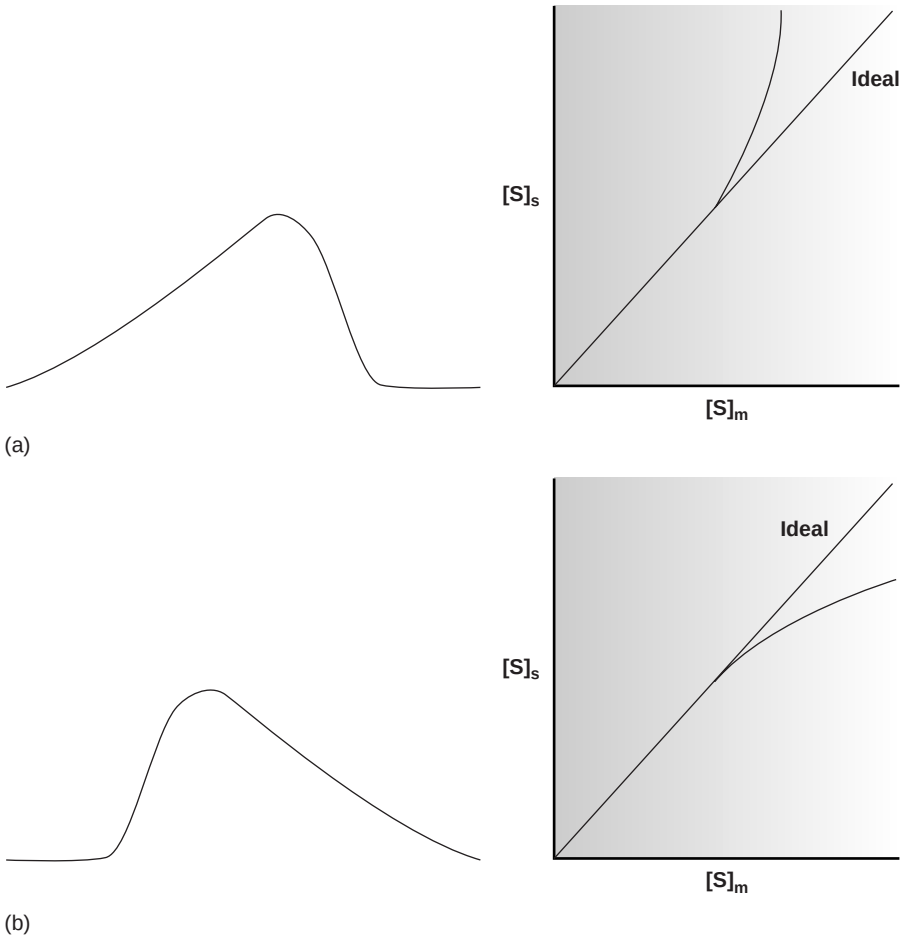


Figure 12.10
Nonideal asymmetrical chromatographic bands showing (a) fronting and (b) tailing. Also depicted are the corresponding sorption isotherms showing the relationship between the concentration of solute in the stationary phase as a function of its concentration in the mobile phase.

than the estimated value because the retention characteristics of some solutes are too similar to effect their separation. Nevertheless, columns with more theoretical plates, or a greater range of possible elution volumes, are more likely to separate a complex mixture.

12B.6 Nonideal Behavior

The treatment of chromatography outlined in Section 12B assumes that a solute elutes as a symmetrical band, such as that shown in Figure 12.7. This ideal behavior occurs when the solute's partition coefficient, K_D, is constant for all concentrations of solute. In some situations, chromatographic peaks show nonideal behavior, leading to asymmetrical peaks, similar to those shown in Figure 12.10. The chromatographic peak in Figure 12.10a is an example of "**fronting**" and is most often the result of overloading the column with sample. Figure 12.10b, which is an example of "**tailing,**" occurs when some sites on the stationary phase retain the solutes more strongly than other sites.

fronting
A tail at the beginning of a chromatographic peak, usually due to injecting too much sample.

tailing
A tail at the end of a chromatographic peak, usually due to the presence of highly active sites in the stationary phase.

12C Optimizing Chromatographic Separations

Now that we have defined capacity factor, selectivity, and column efficiency we consider their relationship to chromatographic resolution. Since we are only interested in the resolution between solutes eluting with similar retention times, it is safe to assume that the peak widths for the two solutes are approximately the same. Equation 12.1, therefore, is written as

$$R = \frac{t_{r,B} - t_{r,A}}{w_B} \qquad \textbf{12.19}$$

Solving equation 12.17 for w_B and substituting into equation 12.19 gives

$$R = \frac{1}{4}\sqrt{N_B}\left(\frac{t_{r,B} - t_{r,A}}{t_{r,B}}\right) \qquad \textbf{12.20}$$

The retention times for solutes A and B are replaced with their respective capacity factors by rearranging equation 12.10

$$t_r = k' t_m + t_m$$

and substituting into equation 12.20.

$$R = \frac{1}{4}\sqrt{N_B}\left(\frac{k'_B - k'_A}{1 + k'_B}\right)$$

Finally, solute A's capacity factor is eliminated using equation 12.11. After rearranging, the equation for the resolution between the chromatographic peaks for solutes A and B is

$$R = \frac{1}{4}\sqrt{N_B}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_B}{1 + k'_B}\right) \qquad \textbf{12.21}$$

Besides resolution, another important factor in chromatography is the amount of time required to elute a pair of solutes. The time needed to elute solute B is

$$t_{r,B} = \frac{16R^2H}{u}\left(\frac{\alpha}{\alpha - 1}\right)^2 \frac{(1 + k'_B)^3}{(k'_B)^2} \qquad \textbf{12.22}$$

Equations 12.21 and 12.22 contain terms corresponding to column efficiency, column selectivity, and capacity factor. These terms can be varied, more or less independently, to obtain the desired resolution and analysis time for a pair of solutes. The first term, which is a function of the number of theoretical plates or the height of a theoretical plate, accounts for the effect of column efficiency. The second term is a function of α and accounts for the influence of column selectivity. Finally, the third term in both equations is a function of k'_B, and accounts for the effect of solute B's capacity factor. Manipulating these parameters to improve resolution is the subject of the remainder of this section.

12C.1 Using the Capacity Factor to Optimize Resolution

One of the simplest ways to improve resolution is to adjust the capacity factor for solute B. If all other terms in equation 12.21 remain constant, increasing k'_B improves resolution. As shown in Figure 12.11, however, the effect is greatest when the

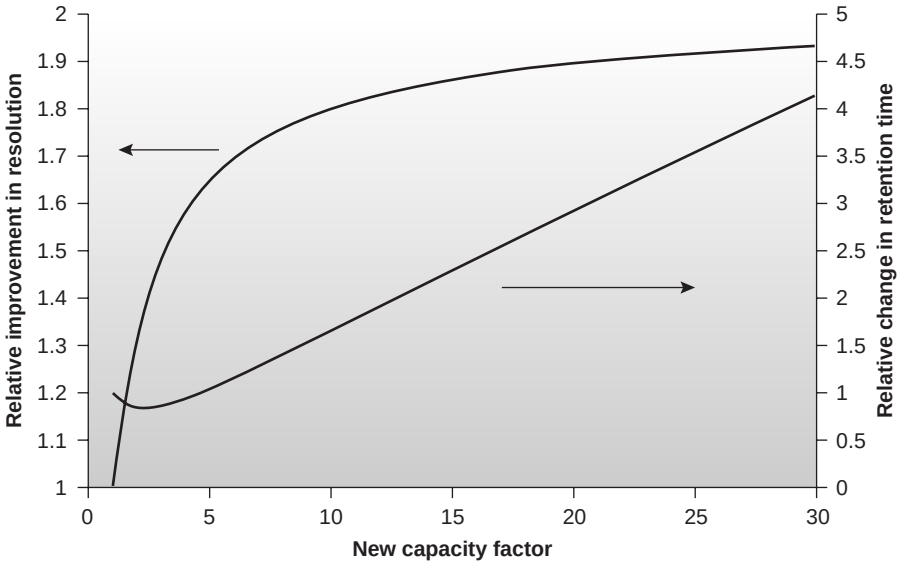


Figure 12.11
Effect of a change in k'_B on resolution and retention time. The original value of k'_B is assumed to be 1.

original capacity factor is small. Furthermore, large increases in k'_B do not lead to proportionally larger increases in resolution. For example, when the original value of k'_B is 1, increasing its value to 10 gives an 82% improvement in resolution; a further increase to 15 provides a net improvement in resolution of only 87.5%.

Any improvement in resolution obtained by increasing k'_B generally comes at the expense of a longer analysis time. This is also indicated in Figure 12.11, which shows the relative change in retention time as a function of the new capacity factor. Note that a minimum in the retention time curve occurs when k'_B is equal to 2, and that retention time increases in either direction. Increasing k'_B from 2 to 10, for example, approximately doubles solute B's retention time.

The relationship between capacity factor and analysis time can be advantageous when a separation produces an acceptable resolution with a large k'_B. In this case it may be possible to decrease k'_B with little loss in resolution while significantly shortening the analysis time.

A solute's capacity factor is directly proportional to its distribution ratio (equation 12.6), which, in turn, is proportional to the solute's equilibrium distribution constant. To increase k'_B without significantly changing α, which also is a function of k'_B, it is necessary to alter chromatographic conditions in a way that leads to a general, nonselective increase in the capacity factor for both solutes. In gas chromatography, this is usually accomplished by decreasing the column's temperature. At a lower temperature a solute's vapor pressure decreases, ensuring that it spends more time in the stationary phase increasing its capacity factor. In liquid chromatography, changing the mobile phase's solvent strength is the easiest way to change a solute's capacity factor. When the mobile phase has a low solvent strength, solutes spend proportionally more time in the stationary phase, thereby increasing their capacity factors. Additionally, equation 12.6 shows that the capacity factor is proportional to the volume of stationary phase. Increasing the volume of stationary phase, therefore, also leads to an increase in k'_B.

Adjusting the capacity factor to improve resolution between one pair of solutes may lead to an unacceptably long retention time for other solutes. For example, improving resolution for solutes with short retention times by increasing

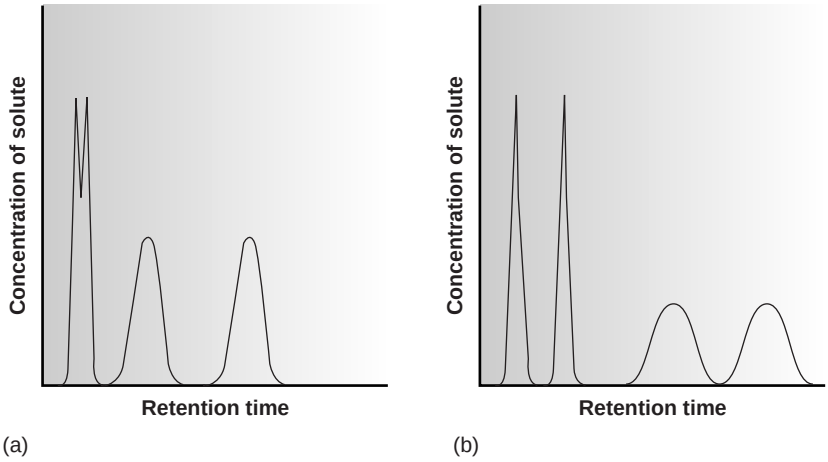


Figure 12.12
The general elution problem in chromatography. Improving the resolution of the overlapping bands in chromatogram (a) results in a longer analysis time for chromatogram (b).

k'_B may substantially increase the retention times for later eluting solutes. On the other hand, decreasing k'_B as a means of shortening the overall analysis time may lead to a loss of resolution for solutes eluting with shorter retention times. This difficulty is encountered so frequently that it is known as the general elution problem (Figure 12.12). One solution to the general elution problem is to make incremental adjustments to the capacity factor over time. Thus, initial chromatographic conditions are adjusted to enhance the resolution for solutes with short retention times. As the separation progresses, chromatographic conditions are changed in a manner that increases the elution rate (decreases the retention time) for later eluting solutes. In gas chromatography this is accomplished by **temperature programming.** The column's initial temperature is selected such that the first solutes to elute are fully resolved. The temperature is then increased, either continuously or in steps, to bring off later eluting components with both an acceptable resolution and a reasonable analysis time. In liquid chromatography the same effect can be obtained by increasing the solvent's eluting strength. This is known as a **gradient elution.**

temperature programming
The process of changing the column's temperature to enhance the separation of both early and late eluting solutes.

gradient elution
The process of changing the mobile phase's solvent strength to enhance the separation of both early and late eluting solutes.

12C.2 Using Column Selectivity to Optimize Resolution

A second approach to improving resolution is to adjust alpha, α. In fact, when α is nearly 1, it usually is not possible to improve resolution by adjusting k'_B or N. Changes in α often have a more dramatic effect on resolution than k'_B. For example, changing α from 1.1 to 1.5 improves resolution by 267%.

A change in α is possible if chromatographic conditions are altered in a manner that is more selective for one of the solutes. If a solute participates in a secondary equilibrium reaction in either the stationary or mobile phase, then it may be possible to alter that phase in a way that selectively changes the solute's capacity factor. For example, Figure 12.13a shows how the pH of an aqueous mobile phase can be used to control the retention times, and thus the capacity factors, for two substituted benzoic acids. The resulting change in α is shown in Figure 12.13b. In gas chromatography, adjustments in α are usually accomplished by changing the stationary phase, whereas changing the composition of the mobile phase is used in liquid chromatography.

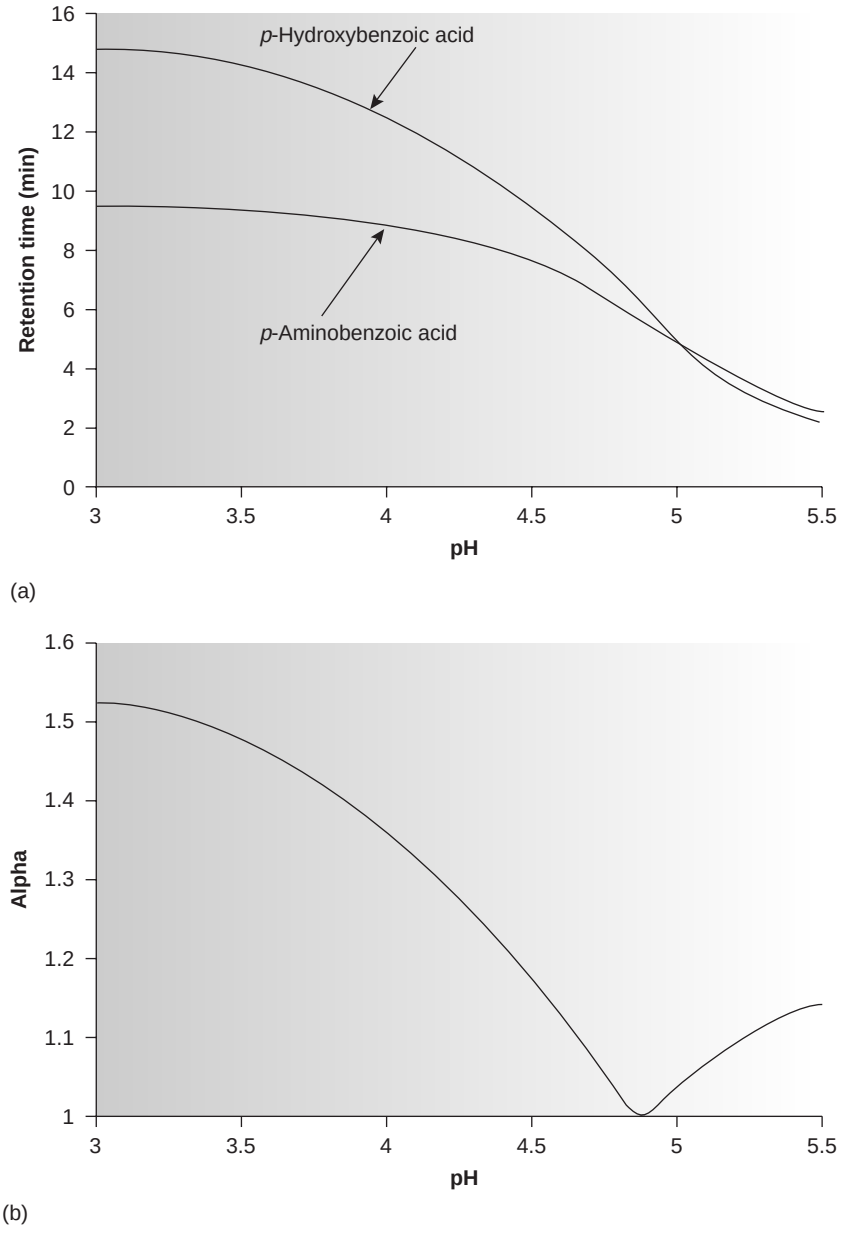


Figure 12.13
Use of column selectivity to improve chromatographic resolution showing: (a) the variation in retention time with mobile phase pH, and (b) the resulting change in alpha with mobile phase pH.

12C.3 Using Column Efficiency to Optimize Resolution

If the capacity factor and α are known, then equation 12.21 can be used to calculate the number of theoretical plates needed to achieve a desired resolution (Table 12.1). For example, given $\alpha = 1.05$ and $k'_B = 2.0$, a resolution of 1.25 requires approximately 24,800 theoretical plates. If the column only provides 12,400 plates, half of what is needed, then the separation is not possible. How can the number of theoretical plates be doubled? The easiest way is to double the length of the column; however, this also requires a doubling of the analysis time. A more desirable approach is to cut the height of a theoretical plate in half, providing the desired resolution without changing the analysis time. Even better, if H can be decreased by more than

Table 12.1 Number of Theoretical Plates Needed to Achieve Desired Resolution for Selected Values of k'_B and α

	$R = 1.00$		$R = 1.25$		$R = 1.50$	
k'_B	$\alpha = 1.05$	$\alpha = 1.10$	$\alpha = 1.05$	$\alpha = 1.10$	$\alpha = 1.05$	$\alpha = 1.10$
0.5	63,500	17,400	99,200	27,200	143,000	39,200
1.0	28,200	7,740	44,100	12,100	63,500	17,400
1.5	19,600	5,380	30,600	8,400	44,100	12,100
2.0	15,900	4,360	24,800	6,810	35,700	9,800
3.0	12,500	3,440	19,600	5,380	28,200	7,740
5.0	10,200	2,790	15,900	4,360	22,900	6,270
10.0	8,540	2,340	13,300	3,660	19,200	5,270

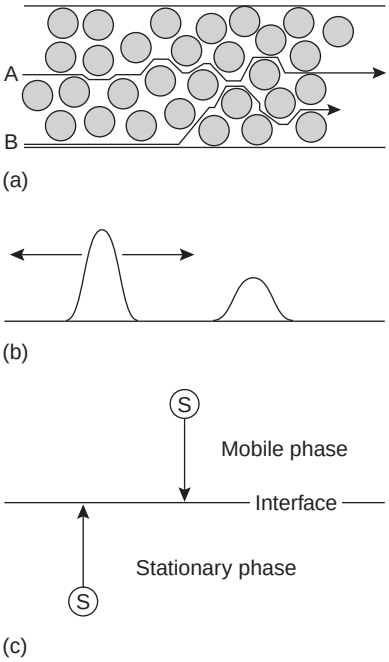


Figure 12.14
Schematics illustrating the contributions to band broadening due to (a) multiple paths, (b) longitudinal diffusion, and (c) mass transfer.

longitudinal diffusion
One contribution to band broadening in which solutes diffuse from areas of high concentration to areas of low concentration.

50%, it also may be possible to achieve the desired resolution with an even shorter analysis time by decreasing k'_B or α.

To determine how the height of a theoretical plate can be decreased, it is necessary to understand the experimental factors contributing to the broadening of a solute's chromatographic band. Several theoretical treatments of band broadening have been proposed. We will consider one approach in which the height of a theoretical plate is determined by four contributions: multiple paths, longitudinal diffusion, mass transfer in the stationary phase, and mass transfer in the mobile phase.

Multiple Paths Solute molecules passing through a chromatographic column travel separate paths that may differ in length. Because of these differences in path length, solute molecules injected simultaneously elute at different times. The principal factor contributing to this variation in path length is a nonhomogeneous packing of the stationary phase in the column. Differences in particle size and packing consistency cause solute molecules to travel paths of different length. Some solute molecules follow relatively straight paths through the column, but others follow a longer, more tortuous path (Figure 12.14a). The contribution of multiple paths to the height of a theoretical plate, H_p, is

$$H_p = 2\lambda d_p \qquad \textbf{12.23}$$

where d_p is the average diameter of the particulate packing material, and λ is a constant accounting for the consistency of the packing. A smaller range of particle sizes and a more consistent packing produce a smaller value for λ. Note that for an open tubular column, which does not contain packing material, H_p is 0.

Longitudinal Diffusion The second contribution to band broadening is the result of the solute's **longitudinal diffusion** in the mobile phase. Even if the mobile phase velocity is 0, solute molecules are constantly in motion, diffusing through the mobile phase. Since the concentration of solute is greatest at the center of a chromatographic band, more solute diffuses toward the band's forward and rear edges than diffuses toward the band's center. The net result is an increase in the band's width (Figure 12.14b). The contribution of longitudinal diffusion to the height of a theoretical plate, H_d, is

$$H_d = \frac{2\gamma D_m}{u} \qquad \textbf{12.24}$$

where D_m is the solute's diffusion coefficient in the mobile phase, u is the mobile phase velocity, and γ is a constant related to the column packing. The effect of H_d on the height of a theoretical plate is minimized by a high mobile-phase velocity. Because a solute's diffusion coefficient is larger in a gaseous mobile phase than in a liquid mobile phase, longitudinal diffusion is a more serious problem in gas chromatography.

Mass Transfer The final two contributions to band broadening result from the finite time required for a solute molecule to diffuse through the stationary phase and mobile phase. A chromatographic separation occurs because solutes move between the stationary and mobile phases. For a solute to move from one phase to the other, it must first diffuse to the interface between the two phases (Figure 12.14c)—a process called **mass transfer.** A contribution to band broadening occurs whenever the solute's movement to the interface is not fast enough to maintain a true equilibrium distribution of solute between the two phases. Thus, solute molecules in the mobile phase move farther down the column than expected before passing into the stationary phase. Solute molecules in the stationary phase, on the other hand, take longer than expected to cross into the mobile phase. The contributions of mass transfer in the stationary phase, H_s, and mass transfer in the mobile phase, H_m, are given by

mass transfer
One contribution to band broadening due to the time required for a solute to move from the mobile phase or the stationary phase to the interface between the two phases.

$$H_s = \frac{qk' d_f^2}{(1 + k')^2 D_s} u \qquad \textbf{12.25}$$

$$H_m = \frac{fn(d_p^2, d_c^2)}{D_m} u \qquad \textbf{12.26}$$

where d_f is the thickness of the stationary phase, d_c is the column's diameter, D_s is the solute's diffusion coefficient in the stationary phase, q is a constant related to the column packing material, and the remaining terms are as previously defined. As indicated in equation 12.26, the exact form of H_m is unknown, although it is a function of particle size and column diameter. The contribution of mass transfer to the height of a theoretical plate is smallest for slow mobile-phase velocities, smaller diameter packing materials, and thinner films of stationary phase.

Putting It All Together The net height of a theoretical plate is a summation of the contributions from each of the terms in equations 12.23–12.26; thus,

$$H = H_p + H_d + H_s + H_m \qquad \textbf{12.27}$$

An alternative form of this equation is the **van Deemter equation**

van Deemter equation
An equation showing the effect of the mobile phase's flow rate on the height of a theoretical plate.

$$H = A + \frac{B}{u} + Cu \qquad \textbf{12.28}$$

which emphasizes the importance of the mobile phase's flow rate. In the van Deemter equation, A accounts for multiple paths (H_p), B/u for longitudinal diffusion (H_d), and Cu for the solute's mass transfer in the stationary and mobile phases (H_s and H_m).

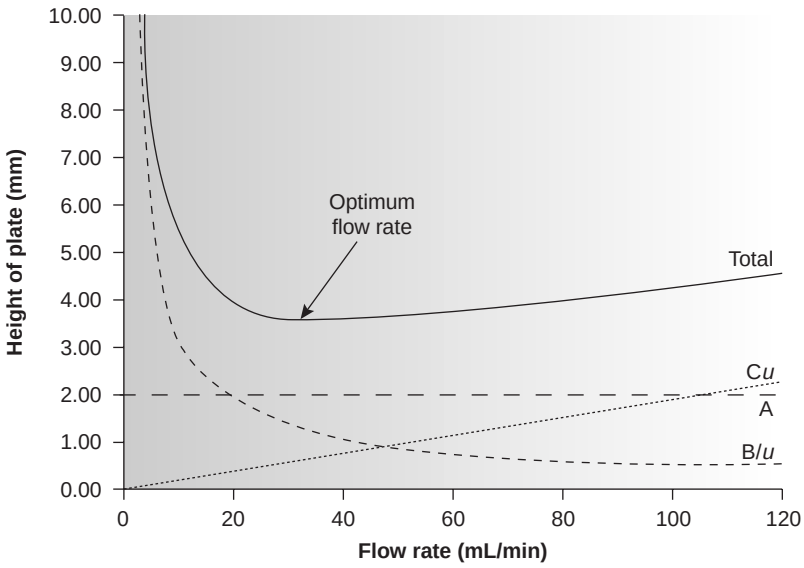


Figure 12.15
Plot of the height of a theoretical plate as a function of mobile-phase velocity using the van Deemter equation. The contributions to the terms *A, B/u,* and *Cu* also are shown.

There is some disagreement on the correct equation for describing the relationship between plate height and mobile-phase velocity.[4] In addition to the van Deemter equation (equation 12.28), another equation is that proposed by Hawkes

$$H = \frac{B}{u} + (C_s + C_m)u$$

where C_s and C_m are the mass transfer terms for the stationary and mobile phases respectively. A third equation was devised by Knox.

$$H = Au^{1/3} + \frac{B}{u} + Cu$$

All three equations, and others, have been used to characterize chromatographic systems, with no single equation providing the best explanation in every case.[5]

To increase the number of theoretical plates without increasing the length of the column, it is necessary to decrease one or more of the terms in equation 12.27 or equation 12.28. The easiest way to accomplish this is by adjusting the velocity of the mobile phase. At a low mobile-phase velocity, column efficiency is limited by longitudinal diffusion, whereas at higher velocities efficiency is limited by the two mass transfer terms. As shown in Figure 12.15 (which is interpreted in terms of equation 12.28), the optimum mobile-phase velocity corresponds to a minimum in a plot of H as a function of u.

The remaining parameters affecting the height of a theoretical plate are determined by the construction of the column and suggest how the column's design may be used to improve efficiency. For example, both H_p and H_m are a function of the size of the particles used for the packing material. Decreasing particle size, therefore, is one approach to improving efficiency. A decrease in particle size is limited, however, by the need for a greater pressure to push the mobile phase through the column.

capillary column
A narrow bored column that usually does not contain a particulate packing material.

One of the most important advances in column construction has been the development of open tubular, or **capillary columns** that contain no packing material ($d_p = 0$). Instead, the interior wall of a capillary column is coated with a thin film of the stationary phase. The absence of packing material means that the mobile phase

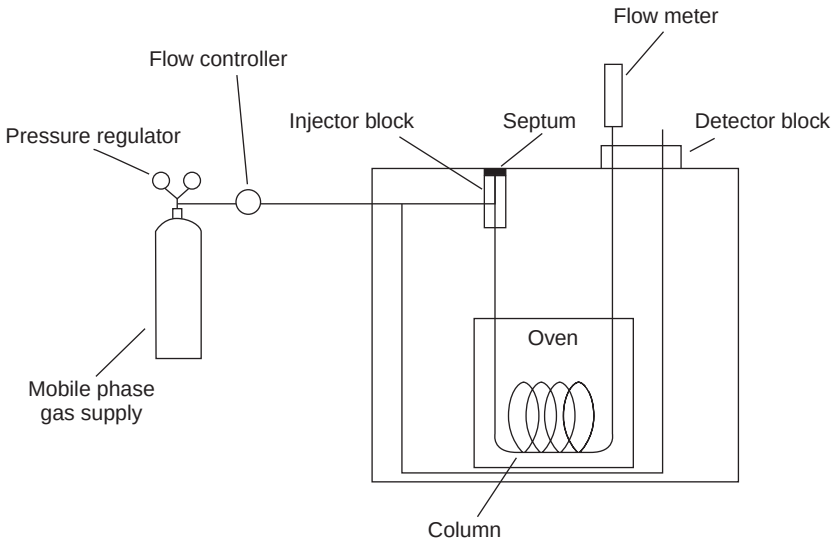


Figure 12.16
Schematic diagram for a typical gas chromatograph.

can move through the column with substantially less pressure. As a result, capillary columns can be manufactured with much greater lengths than is possible with a packed column. Furthermore, plate height is reduced because the H_p term in equation 12.27 disappears and the H_m term becomes smaller. The combination of a smaller height for a theoretical plate and a longer column leads to an approximate 100-fold increase in the number of theoretical plates. Capillary columns are not without disadvantages. Because capillary columns are much narrower than packed columns, they require a significantly smaller amount of sample. Difficulties with reproducibly injecting small samples complicates the use of capillary chromatography for quantitative work.

Another approach to improving resolution is to use thin films of stationary phase. Capillary columns used in gas chromatography and the bonded phases commonly used in HPLC provide a significant decrease in plate height due to the reduction of the H_s term in equation 12.27.

12D Gas Chromatography

In **gas chromatography** (GC) the sample, which may be a gas or liquid, is injected into a stream of an inert gaseous mobile phase (often called the carrier gas). The sample is carried through a packed or capillary column where the sample's components separate based on their ability to distribute themselves between the mobile and stationary phases. A schematic diagram of a typical gas chromatograph is shown in Figure 12.16.

gas chromatography
A chromatographic technique in which the mobile phase is a gas.

12D.1 Mobile Phase

The most common mobile phases for GC are He, Ar, and N_2, which have the advantage of being chemically inert toward both the sample and the stationary phase. The choice of which carrier gas to use is often determined by the instrument's detector. With packed columns the mobile-phase velocity is usually within the range of 25–150 mL/min, whereas flow rates for capillary columns are 1–25 mL/min. Actual flow rates are determined with a flow meter placed at the column outlet.

12D.2 Chromatographic Columns

A chromatographic column provides a location for physically retaining the stationary phase. The column's construction also influences the amount of sample that can be handled, the efficiency of the separation, the number of analytes that can be easily separated, and the amount of time required for the separation. Both packed and capillary columns are used in gas chromatography.

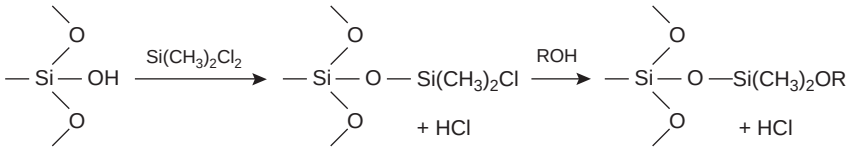
packed column
A wide-bore column containing a particulate packing material.

Packed Columns A **packed column** is constructed from glass, stainless steel, copper or aluminum and is typically 2–6 m in length, with an internal diameter of 2–4 mm. The column is filled with a particulate solid support, with particle diameters ranging from 37–44 μm to 250–354 μm.

The most widely used particulate support is diatomaceous earth, which is composed of the silica skeletons of diatoms. These particles are quite porous, with surface areas of 0.5–7.5 m^2/g, which provides ample contact between the mobile phase and stationary phase. When hydrolyzed, the surface of a diatomaceous earth contains silanol groups (–SiOH), providing active sites that absorb solute molecules in gas–solid chromatography.

gas–liquid chromatography
A chromatographic technique in which the mobile phase is a gas and the stationary phase is a liquid coated either on a solid packing material or on the column's walls.

In **gas–liquid chromatography** (GLC), separation is based on the partitioning of solutes between a gaseous mobile phase and a liquid stationary phase coated on the solid packing material. To avoid the adsorption of solute molecules on exposed packing material, which degrades the quality of the separation, surface silanols are deactivated by silanizing with dimethyldichlorosilane and washing with an alcohol (typically methanol) before coating with stationary phase.



More recently, solid supports made from glass beads or fluorocarbon polymers have been introduced. These supports have the advantage of being more inert than diatomaceous earth.

To minimize the multiple path and mass transfer contributions to plate height (equations 12.23 and 12.26), the packing material should be of as small a diameter as is practical and loaded with a thin film of stationary phase (equation 12.25). Compared with capillary columns, which are discussed in the next section, packed columns can handle larger amounts of sample. Samples of 0.1–10 μL are routinely analyzed with a packed column. Column efficiencies are typically several hundred to 2000 plates/m, providing columns with 3000–10,000 theoretical plates. Assuming V_{max}/V_{min} is approximately 50,[3] a packed column with 10,000 theoretical plates has a peak capacity (equation 12.18) of

$$n_c = 1 + \frac{\sqrt{10,000}}{4}\ln(50) \approx 100$$

open tubular column
A capillary column that does not contain a particulate packing material.

Capillary Columns Capillary, or **open tubular columns** are constructed from fused silica coated with a protective polymer. Columns may be up to 100 m in length with an internal diameter of approximately 150–300 μm (Figure 12.17). Larger bore columns of 530 μm, called megabore columns, also are available.

Figure 12.17
Photo of a capillary column.
Courtesy of Alltech Associates, Inc., Deerfield, Illinois.

Capillary columns are of two principal types. **Wall-coated open tubular columns** (WCOT) contain a thin layer of stationary phase, typically 0.25 μm thick, coated on the capillary's inner wall. In **support-coated open tubular columns** (SCOT), a thin layer of a solid support, such as a diatomaceous earth, coated with a liquid stationary phase is attached to the capillary's inner wall.

wall-coated open tubular column
An open tubular column in which the stationary phase is coated on the column's walls.

support-coated open tubular column
An open tubular column in which the stationary phase is coated on a solid support that is attached to the column's walls.

Capillary columns provide a significant improvement in separation efficiency. The pressure needed to move the mobile phase through a packed column limits its length. The absence of packing material allows a capillary column to be longer than a packed column. Although most capillary columns contain more theoretical plates per meter than a packed column, the more important contribution to their greater efficiency is the ability to fashion longer columns. For example, a 50-m capillary column with 3000 plates/m has 150,000 theoretical plates and, assuming V_{max}/V_{min} is approximately 50,[3] a peak capacity of almost 380. On the other hand, packed columns can handle larger samples. Due to its smaller diameter, capillary columns require smaller samples; typically less than 10^{-2} μL.

12D.3 Stationary Phases

Selectivity in gas chromatography is influenced by the choice of stationary phase. Elution order in GLC is determined primarily by the solute's boiling point and, to a lesser degree, by the solute's interaction with the stationary phase. Solutes with significantly different boiling points are easily separated. On the other hand, two solutes with similar boiling points can be separated only if the stationary phase selectively interacts with one of the solutes. In general, nonpolar solutes are more easily separated with a nonpolar stationary phase, and polar solutes are easier to separate using a polar stationary phase.

The main criteria for selecting a stationary phase are that it should be chemically inert, thermally stable, of low volatility, and of an appropriate polarity for the solutes being separated. Although hundreds of stationary phases have been developed, many of which are commercially available, the majority of GLC separations are accomplished with perhaps five to ten common stationary phases. Several of

Table 12.2 Selected Stationary Phases for Gas–Liquid Chromatography

Stationary Phase	Polarity	Trade Names	Temperature Limit (°C)	Applications
squalane	nonpolar	squalane	150	low-boiling aliphatic hydrocarbons
Apezion L	nonpolar	Apezion L	300	amides fatty acid methyl esters high-boiling aliphatic hydrocarbons terpenoids
polydimethyl siloxane	slightly polar	SE-30	300–350	alkaloids amino acid derivatives drugs pesticides phenols steroids
50% methyl-50% phenyl polysiloxane	moderately polar	OV-17	375	alkaloids drugs pesticides polyaromatic hydrocarbons polychlorinated biphenyls
50% trifluoropropyl-50% methyl polysiloxane	moderately polar	OV-210	275	alkaloids amino acid derivatives drugs halogenated compounds ketones phenols
50% cyanopropyl-50% phenylmethyl polysiloxane	polar	OV-225	275	nitriles pesticides steroids
polyethylene glycol	polar	Carbowax 20M	225	aldehydes esters ethers phenols

these are listed in Table 12.2, in order of increasing polarity, along with their physical properties and typical applications.

Many stationary phases have the general structure shown in Figure 12.18a. A stationary phase of polydimethyl siloxane, in which all the –R groups are methyl groups ($-CH_3$), is nonpolar and often makes a good first choice for a new separation. The order of elution when using polydimethyl siloxane usually follows the boiling points of the solutes, with lower boiling solutes eluting first. Replacing some of the methyl groups with other substituents increases the stationary phase's polarity, providing greater selectivity. Thus, in 50% methyl-50% phenyl polysiloxane, 50% of the –R groups are phenyl groups ($-C_6H_5$), producing a slightly polar stationary phase. Increasing polarity is provided by substituting trifluoropropyl ($-C_3H_6CF_3$) and cyanopropyl ($-C_3H_6CN$) functional groups or using a stationary phase based on polyethylene glycol (Figure 12.18b).

bleed
The tendency of a stationary phase to elute from the column.

An important problem with all liquid stationary phases is their tendency to "**bleed**" from the column. The temperature limits listed in Table 12.2 are those that minimize the loss of stationary phase. When operated above these limits, a column's useful lifetime is significantly shortened. Capillary columns with bonded or

cross-linked stationary phases provide superior stability. Bonded stationary phases are attached to the capillary's silica surface. Cross-linking, which is done after the stationary phase is placed in the capillary column, links together separate polymer chains, thereby providing greater stability.

Another important characteristic of a gas chromatographic column is the thickness of the stationary phase. As shown in equation 12.25, separation efficiency improves with thinner films. The most common film thickness is 0.25 μm. Thicker films are used for highly volatile solutes, such as gases, because they have a greater capacity for retaining such solutes. Thinner films are used when separating solutes of low volatility, such as steroids.

A few GLC stationary phases rely on chemical selectivity. The most notable are stationary phases containing chiral functional groups, which can be used for separating enantiomers.[6]

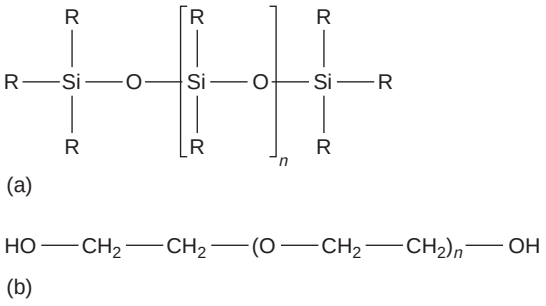


Figure 12.18
General structures of common stationary phases for gas chromatography.

12D.4 Sample Introduction

Three considerations determine how samples are introduced to the gas chromatograph. First, all constituents injected into the GC must be volatile. Second, the analytes must be present at an appropriate concentration. Finally, injecting the sample must not degrade the separation.

Preparing a Volatile Sample Gas chromatography can be used to separate analytes in complex matrices. Not every sample that can potentially be analyzed by GC, however, can be injected directly into the instrument. To move through the column, the sample's constituents must be volatile. Solutes of low volatility may be retained by the column and continue to elute during the analysis of subsequent samples. Nonvolatile solutes condense on the column, degrading the column's performance.

Volatile analytes can be separated from a nonvolatile matrix using any of the extraction techniques described in Chapter 7. Liquid–liquid extractions, in which analytes are extracted from an aqueous matrix into methylene chloride or other organic solvent, are commonly used. Solid-phase extractions also are used to remove unwanted matrix constituents.

An attractive approach to isolating analytes is a **solid-phase microextraction** (SPME). In one approach, which is illustrated in Figure 12.19, a fused silica fiber is placed inside a syringe needle. The fiber, which is coated with a thin organic film, such as polydimethyl siloxane, is lowered into the sample by depressing a plunger and is exposed to the sample for a predetermined time. The fiber is then withdrawn into the needle and transferred to the gas chromatograph for analysis.

Volatile analytes also can be separated from a liquid matrix using a purge and trap or by headspace sampling. In a purge and trap (see Figure 7.19 in Chapter 7), an inert gas, such as He or N_2, is bubbled through the sample, purging the volatile compounds. These compounds are swept through a trap packed with an absorbent material, such as Tenax, where they are collected. Heating the trap and back flushing with carrier gas transfers the volatile compounds to the gas chromatograph. In **headspace sampling** the sample is placed in a closed vial with an overlying air space. After allowing time for the volatile analytes to equilibrate between the sample and the overlying air, a portion of the vapor phase is sampled by syringe and injected into the gas chromatograph.

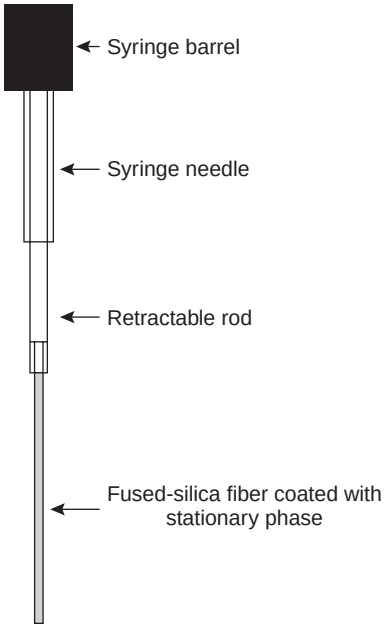


Figure 12.19
Schematic diagram of a device for solid-phase microextractions.

solid-phase microextraction
A solid-phase extraction in which the solid adsorbent is coated on a fused-silica fiber held within a syringe needle.

headspace sampling
The sampling of the vapor phase overlying a liquid phase.

Thermal desorption is used to release volatile analytes from solids. A portion of the solid is placed in a glass-lined, stainless steel tube and held in place with plugs of glass wool. After purging with carrier gas to remove O_2 (which could lead to oxidation reactions when heating the sample), the sample is heated. Volatile analytes are swept from the tube by the carrier gas and carried to the GC. To maintain efficiency the solutes often are concentrated at the top of the column by cooling the column inlet below room temperature, a process known as **cryogenic focusing.**

cryogenic focusing
The process of concentrating volatile solutes by cooling the column's inlet below room temperature.

Nonvolatile analytes must be chemically converted to a volatile derivative before analysis. For example, amino acids are not sufficiently volatile to analyze directly by gas chromatography. Reacting an amino acid with 1-butanol and acetyl chloride produces an esterfied amino acid. Subsequent treatment with trifluoroacetic acid gives the amino acid's volatile *N*-trifluoroacetyl-*n*-butyl ester derivative.

Adjusting the Analyte's Concentration Analytes present at concentrations too small to give an adequate signal need to be concentrated before analyzing. A side benefit of many of the extraction methods outlined earlier is that they often concentrate the analytes. Volatile organic materials isolated from aqueous samples by a purge and trap, for example, can be concentrated by as much as 1000-fold.

When an analyte is too concentrated, it is easy to overload the column, thereby seriously degrading the separation. In addition, the analyte may be present at a concentration level that exceeds the detector's linear response. Dissolving the sample in a volatile solvent, such as methylene chloride, makes its analysis feasible.

Injecting the Sample To avoid any precolumn loss in resolution due to band broadening, a sample of sufficient size must be introduced in a small volume of mobile phase. An example of a simple injection port for a packed column is shown in Figure 12.20. Injections are made through a rubber septum using a microliter syringe. The injector block is heated to a temperature that is at least 50 °C above the sample component with the highest boiling point. In this way rapid vaporization of the entire sample is ensured.

Capillary columns require the use of a special injector to avoid overloading the column with sample. Several capillary injectors are available, the most common of which is a split/splitless injector.[7] When used for a **split injection** only about 0.1–1% of the sample enters the column, with the remainder carried off as waste. In a **splitless injection,** which is useful for trace analysis, the column temperature is held 20–25 °C below the solvent's boiling point. As the solvent enters the column, it condenses, forming a barrier that traps the solutes. After allowing time for the solutes to concentrate, the column's temperature is increased, and the separation begins. A splitless injection allows a much higher percentage of the solutes to enter the chromatographic column.

split injection
A technique for injecting samples onto a capillary column in which only a small portion of the sample enters the column.

splitless injection
A technique for injecting a sample onto a capillary column that allows a higher percentage of the sample to enter the column.

For samples that decompose easily, an **on-column injection** may be necessary. In this method the sample is injected on the column without heating. The column temperature is then increased, volatilizing the sample with as low a temperature as is practical.

on-column injection
The direct injection of thermally unstable samples onto a capillary column.

12D.5 Temperature Control

As noted earlier, control of the column's temperature is critical to attaining a good separation in gas chromatography. For this reason the column is located inside a thermostated oven. In an isothermal separation the column is maintained at a constant temperature, the choice of which is dictated by the solutes. Normally, the tem-

perature is set slightly below that for the lowest boiling solute so as to increase the solute's interaction with the stationary phase.

One difficulty with an isothermal separation is that a temperature favoring the separation of low-boiling solutes may cause unacceptably long retention times for higher boiling solutes. Ovens capable of temperature programming provide a solution to this problem. The initial temperature is set below that for the lowest boiling solute. As the separation progresses, the temperature is slowly increased at either a uniform rate or in a series of steps.

12D.6 Detectors for Gas Chromatography

The final part of a gas chromatograph is the detector. The ideal detector has several desirable features, including low detection limits, a linear response over a wide range of solute concentrations (which makes quantitative work easier), responsiveness to all solutes or selectivity for a specific class of solutes, and an insensitivity to changes in flow rate or temperature.

Thermal Conductivity Detector One of the earliest gas chromatography detectors, which is still widely used, is based on the mobile phase's thermal conductivity (Figure 12.21). As the mobile phase exits the column, it passes over a tungsten–rhenium wire filament. The filament's electrical resistance depends on its temperature, which, in turn, depends on the thermal conductivity of the mobile phase. Because of its high thermal conductivity, helium is the mobile phase of choice when using a **thermal conductivity detector** (TCD).

When a solute elutes from the column, the thermal conductivity of the mobile phase decreases and the temperature of the wire filament, and thus its resistance, increases. A reference cell, through which only the mobile phase passes, corrects for any time-dependent variations in flow rate, pressure, or electrical power, all of which may lead to a change in the filament's resistance.

A TCD detector has the advantage of universality, since it gives a signal for any solute whose thermal conductivity differs from that of helium. Another advantage is that it gives a linear response for solute concentrations over a range of 10^4–10^5 orders of magnitude. The detector also is nondestructive, making it possible to isolate solutes with a postdetector cold trap. Unfortunately, the thermal

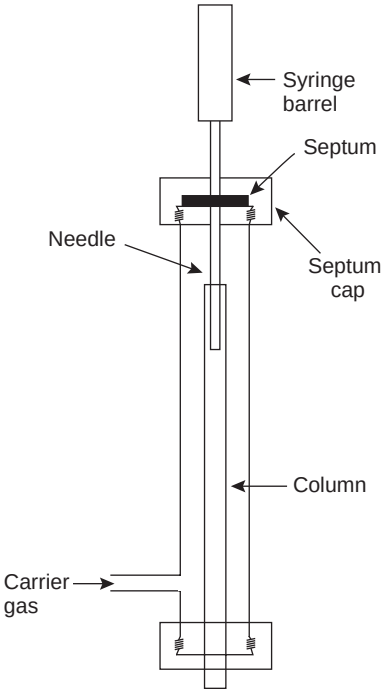


Figure 12.20
Schematic diagram of an injector for packed column gas chromatography.

thermal conductivity detector
A universal GC detector in which the signal is a change in the thermal conductivity of the mobile phase.

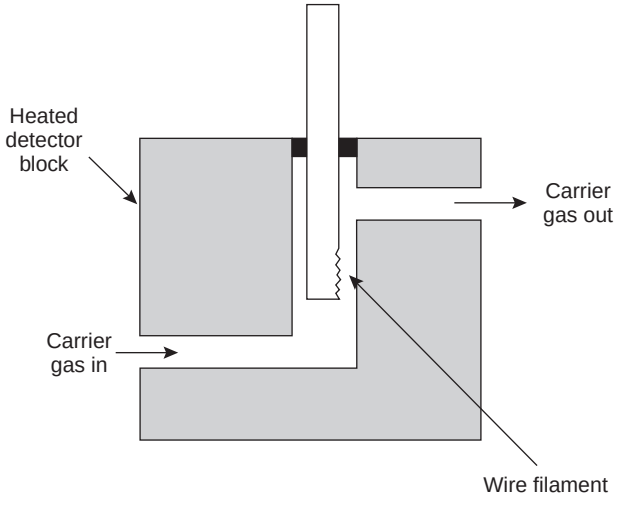


Figure 12.21
Schematic diagram of a thermal conductivity detector for gas chromatography.

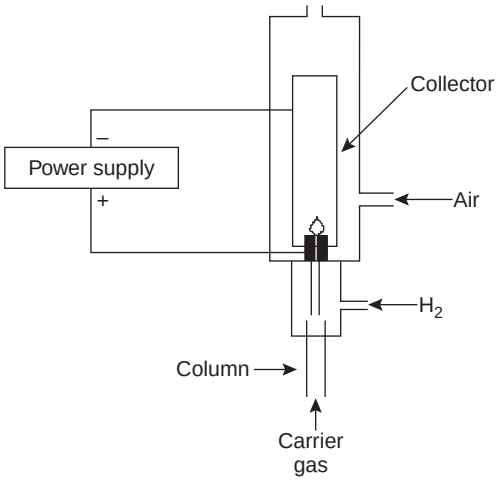


Figure 12.22
Schematic diagram of a flame ionization detector for gas chromatography.

conductivity detector's detection limit is poor in comparison with other popular detectors.

Flame Ionization Detector Combustion of an organic compound in an H_2/air flame results in a flame rich in electrons and ions. If a potential of approximately 300 V is applied across the flame, a small current of roughly 10^{-9}–10^{-12} A develops. When amplified, this current provides a useful analytical signal. This is the basis of the popular **flame ionization detector** (FID), a schematic of which is shown in Figure 12.22.

Most carbon atoms, except those in carbonyl and carboxylic groups, generate a signal, making the FID an almost universal detector for organic compounds. Most inorganic compounds and many gases, such as H_2O and CO_2, cannot be detected, making the FID detector ideal for the analysis of atmospheric and aqueous environmental samples. Advantages of the FID include a detection limit that is approximately two to three orders of magnitude smaller than that for a thermal conductivity detector and a linear response over 10^6–10^7 orders of magnitude in the amount of analyte injected. The sample, of course, is destroyed when using a flame ionization detector.

flame ionization detector
A nearly universal GC detector in which the solutes are combusted in an H_2/air flame, producing a measurable current.

Electron Capture Detector The **electron capture detector** is an example of a selective detector. The detector consists of a beta emitter (a beta particle is an electron) such as ^{63}Ni. The emitted electrons ionize the mobile phase, which is usually N_2, resulting in the production of additional electrons that give rise to an electric current between a pair of electrodes (Figure 12.23). When a solute with a high cross section for the capture of electrons elutes from the column, the electric current decreases. This decrease in electric current serves as the signal. The ECD is highly selective toward solutes with electronegative functional groups, such as halogens, and nitro groups and is relatively insensitive to amines, alcohols, and hydrocarbons. Although its detection limit is excellent, its linear range extends over only about two orders of magnitude.

electron capture detector
A detector for GC that provides selectivity for solutes with halogen and nitro functional groups.

Other Detectors Two additional detectors are similar in design to a flame ionization detector. In the flame photometric detector optical emission from phosphorus and sulfur provides a detector selective for compounds containing these elements. The thermionic detector responds to compounds containing nitrogen or phosphorus.

Two common detectors, which also are independent instruments, are Fourier transform infrared spectrophotometers (FT–IR) and mass spectrometers (MS). In GC–FT–IR, effluent from the column flows through an optical cell constructed

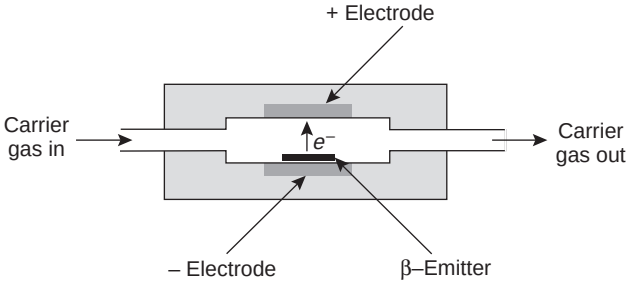


Figure 12.23
Schematic diagram of an electron capture detector for gas chromatography.

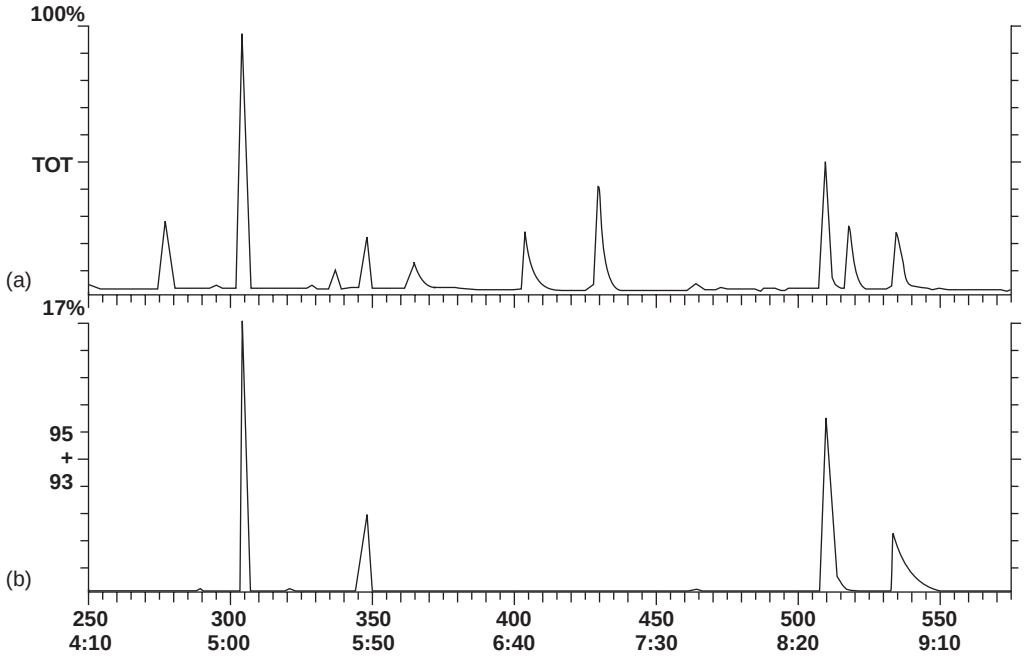


Figure 12.24
(a) Total ion chromatogram for a ten-component mixture; (b) Chromatogram recorded using selective ion monitoring for mass-to-charge ratios of 93 and 95, which are characteristic ions for the monoterpenes α-pinene (t_r = 5.08 min), β-pinene (t_r = 5.81 min), camphor (t_r = 8.51 min), and menthol (t_r = 8.93 min). (Chromatograms courtesy of Bryan Hanson and Sara Peters, DePauw University).

from a 10–40-cm Pyrex tube with an internal diameter of 1–3 mm. The cell's interior surface is coated with a reflecting layer of gold. Multiple reflections of the source radiation as it is transmitted through the cell increase the optical path length through the sample.

In GC–MS effluent from the column is introduced directly into the mass spectrometer's ionization chamber in a manner that eliminates the majority of the carrier gas. In the ionization chamber all molecules (remaining carrier gas, solvent, and solutes) are ionized, and the ions are separated by their mass-to-charge ratio. Because each solute undergoes a characteristic fragmentation into smaller ions, its **mass spectrum** of ion intensity as a function of mass-to-charge ratio provides qualitative information that can be used to identify the solute.

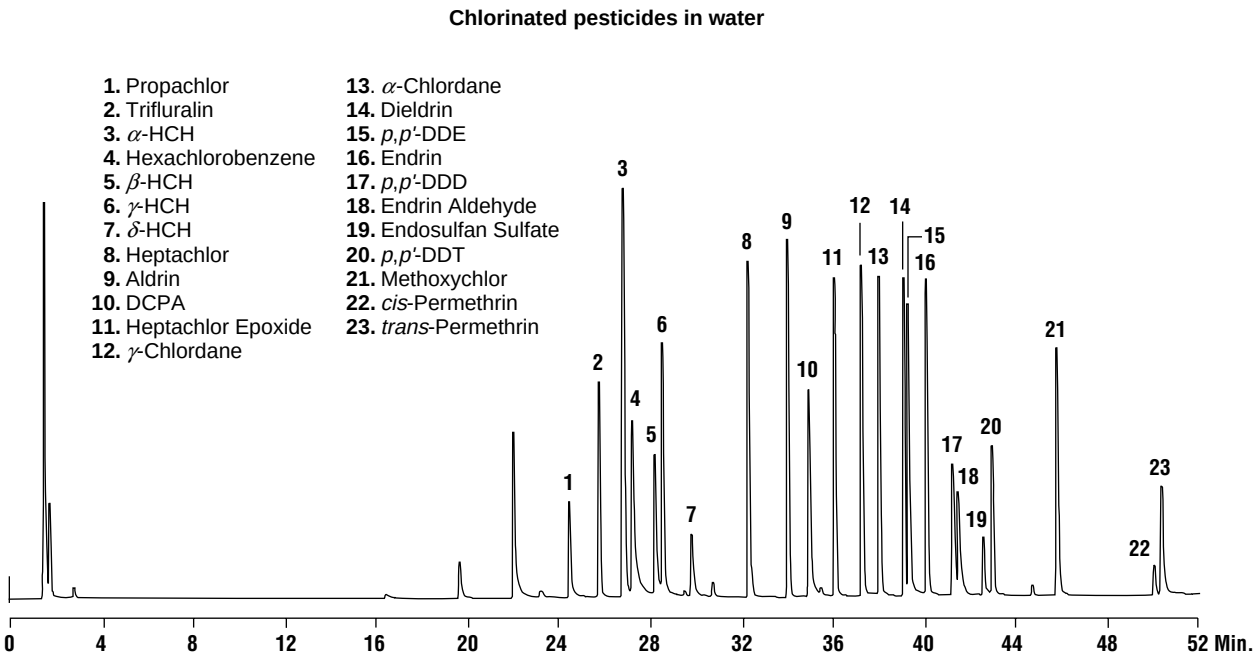
mass spectrum
A plot of ion intensity as a function of the ion's mass-to-charge ratios.

As a GC detector, the total ion current for all ions reaching the detector is usually used to obtain the chromatogram (Figure 12.24a). Selectivity can be achieved by monitoring only specific mass-to-charge ratios (Figure 12.24b), a process called selective ion monitoring. A mass spectrometer provides excellent detection limits, typically 25 fg to 100 pg, with a linear range spanning five orders of magnitude.

12D.7 Quantitative Applications

Gas chromatography is widely used for the analysis of a diverse array of samples in environmental, clinical, pharmaceutical, biochemical, forensic, food science, and petrochemical laboratories. Examples of these applications are discussed in the following sections.

Environmental Analysis One of the most important environmental applications of gas chromatography is for the analysis of numerous organic pollutants in air, water, and wastewater. The analysis of volatile organics in drinking water, for example, is accomplished by a purge and trap, followed by their separation on a capillary column with a nonpolar stationary phase. A flame ionization, electron capture, or



Column: Econo-Cap™ EC-5, 30m × 0.32mm ID × 0.25µm (Part No. **19646**)
Temp: 60°C to 300°C at 4°C/min
Carrier Gas: Helium, 1.45mL/min
Detector: ECD at 320°C
Injector: 250°C

(a)

Figure 12.25
Examples of the application of gas chromatography to the analysis of (a) chlorinated pesticides in water, (b) blood alcohols, (c) Scotch whiskey, and (d) unleaded gasoline. (Chromatograms courtesy of Alltech Associates, Inc. Deerfield, IL).

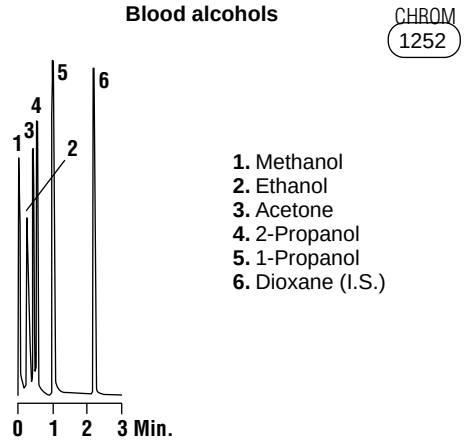
mass spectrometer can be used as a detector. Figure 12.25a shows a typical chromatogram for the analysis of chlorinated pesticides in water.

Clinical Analysis Clinical, pharmaceutical, and forensic labs make frequent use of gas chromatography for the analysis of drugs. Because the sample's matrix is often incompatible with the GC column, analytes generally must be isolated by extraction. Figure 12.25b shows how gas chromatography can be used in monitoring blood alcohol levels.

Consumer Goods Many flavors, spices, and fragrances are readily analyzed by GC, using headspace analysis or thermal desorption. Foods and beverages are analyzed either directly or following a suitable extraction. Volatile materials, such as those found in spices and fragrances, often can be obtained by headspace sampling. Figure 12.25c shows a typical analysis of a sample of Scotch whiskey.

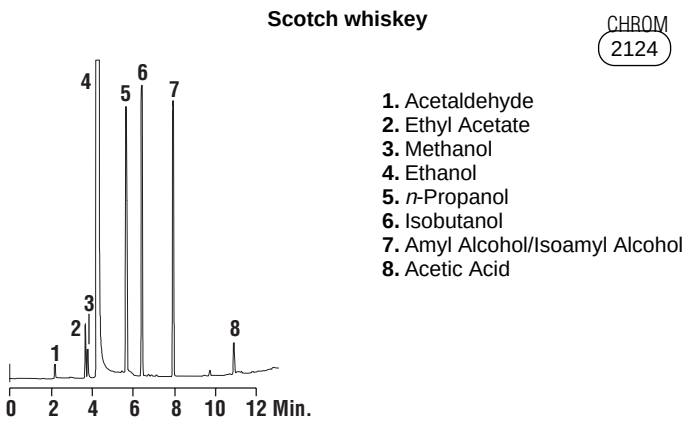
Petroleum Industry Gas chromatography is ideally suited for the analysis of petroleum products, including gasoline, diesel fuel, and oil. A typical chromatogram for the analysis of unleaded gasoline is shown in Figure 12.25d.

Quantitative Calculations In a quantitative analysis, the height or area of an analyte's chromatographic peak is used to determine its concentration. Although peak height is easy to measure, its utility is limited by the inverse relationship between the height and width of a chromatographic peak. Unless chromatographic conditions are carefully controlled to maintain a constant column efficiency, variations in



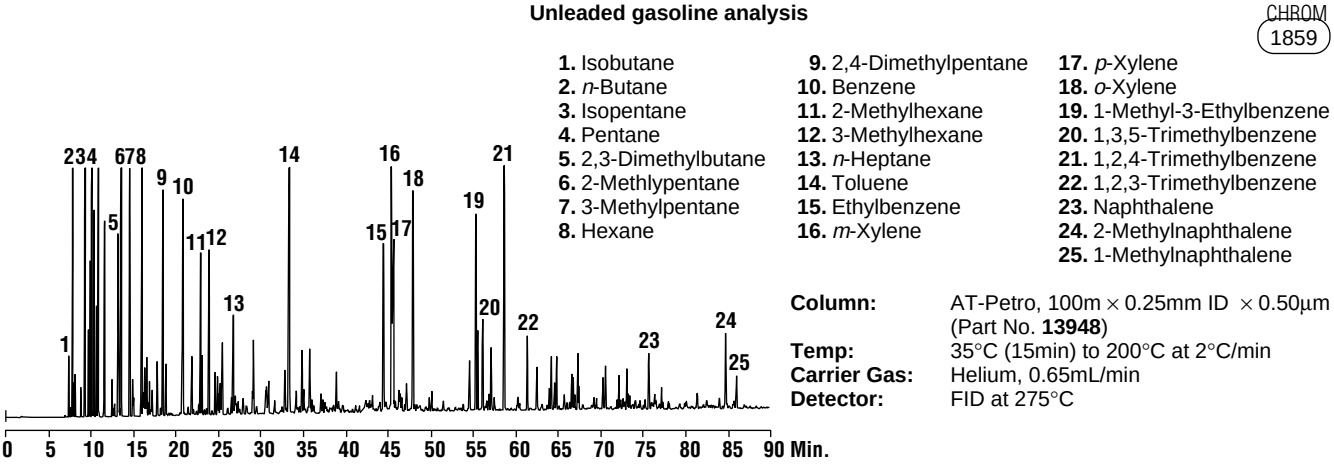
Column: Heliflex AT-1, 10m × 0.53mm ID × 5μm (Part No. **16842**)
Temp: 35°C (1min) to 130°C at 30°C/min
Carrier Gas: Helium, 6mL/min
Detector: FID

(b)



Column: Econo-Cap EC-WAX, 30m × 0.45mm × 1.0μm (Part No. **19663**)
Temp: 50°C (2min) to 200°C at 15°C/min
Carrier Gas: Helium, 34cm/sec **Split Ratio:** 20:1
Detector: FID/250°C **Injector:** 250°C

(c)



Column: AT-Petro, 100m × 0.25mm ID × 0.50μm (Part No. **13948**)
Temp: 35°C (15min) to 200°C at 2°C/min
Carrier Gas: Helium, 0.65mL/min
Detector: FID at 275°C

(d)

Figure 12.25—*Continued*

peak height may decrease the accuracy and precision of the quantitative analysis. A better choice is to measure the area under the chromatographic peak with an integrating recorder. Since peak area is directly proportional to the amount of analyte that was injected, changes in column efficiency will not affect the accuracy or precision of the analysis.

Calibration curves are usually constructed by analyzing a series of external standards and plotting the detector's signal as a function of their known concentrations. As long as the injection volume is identical for every standard and sample, calibration curves prepared in this fashion give both accurate and precise results. Unfortunately, even under the best of conditions, replicate injections may have volumes that differ by as much as 5% and often may be substantially worse. For this

reason, quantitative work requiring high accuracy and precision is accomplished using an internal standard.

Example

EXAMPLE 12.5

Marriott and Carpenter[8] report the following data for five replicate injections of a mixture of 1% v/v methylisobutylketone (peak 1) and 1% v/v *p*-xylene (peak 2).

Injection	Peak	Peak Area
A	1	49075
	2	78112
B	1	85829
	2	135404
C	1	84136
	2	132332
D	1	71681
	2	112889
E	1	58054
	2	91287

Assume that *p*-xylene is the analyte and that methylisobutylketone is the internal standard. Determine the 95% confidence interval for a single-point standardization, with and without using the internal standard.

SOLUTION

For a single-point external standard (omitting the internal standard) the relationship between peak area, A_2, and the concentration, C_2, of *p*-xylene is

$$A_2 = kC_2$$

Substituting the known concentration for the *p*-xylene standard and the appropriate peak areas, gives the following values for the constant *k*.

78112 135404 132332 112889 91287

The average value for *k* is 110,000, with a standard deviation of 25,100 (a relative standard deviation of 22.8%). The 95% confidence interval is 110,000 ± 31,200.

For an internal standardization, the relationship between the peak areas for the analyte, A_2, and the internal standard, A_1, and their respective concentrations, C_1 and C_2, is

$$\frac{A_2}{A_1} = k\frac{C_2}{C_1}$$

Substituting the known concentrations and the appropriate peak areas gives the following values for the constant *k*.

1.5917 1.5776 1.5728 1.5749 1.5724

The average value for *k* is 1.5779, with a standard deviation of 0.0080 (a relative standard deviation of 0.507%). The 95% confidence interval is 1.5779 ± 0.0099.

As this example clearly shows, the variation in individual peak areas between injections is substantial. The use of an internal standard, however, corrects for these variations, providing a means for accurate and precise calibration.

12D.8 Qualitative Applications

Gas chromatography also can be used for qualitative purposes. When using an FT–IR or a mass spectrometer as the detector, the available spectral information often can be used to identify individual solutes.

With conventional nonspectroscopic detectors, other methods must be used to identify the solutes. One approach is to spike the sample by adding an aliquot of a suspected analyte and looking for an increase in peak height. Retention times also can be compared with values measured for standards, provided that the operating conditions are identical. Because of the difficulty of exactly matching such conditions, tables of retention times are of limited utility.

Kovat's retention index provides one solution to the matching of retention times. Under isothermal conditions, the adjusted retention times of normal alkanes increase logarithmically. Kovat defined the retention index, *I,* for a normal alkane as 100 times the number of carbon atoms; thus, the retention index is 400 for butane and 500 for pentane. To determine the retention index for another compound, its adjusted retention time is measured relative to that for the normal alkanes eluting just before and after. For example, a compound eluting between butane and pentane has a retention index between 400 and 500. The exact value for the compound's retention index, I_{cpd}, is given as

Kovat's retention index
A means for normalizing retention times by comparing a solute's retention time with those for normal alkanes.

$$I_{cpd} = 100\left[\frac{(\log t_r')_{cpd} - (\log t_r')_x}{(\log t_r')_{x+1} - (\log t_r')_x}\right] + I_x \qquad \textbf{12.29}$$

where x is the normal alkane eluting before the compound, and x + 1 is the normal alkane eluting just after the compound.

Example

EXAMPLE 12.6

In a separation of a mixture of hydrocarbons, the following adjusted retention times were measured.

propane	2.23 min
isobutane	5.71 min
butane	6.67 min

What is the Kovat's retention index for each of these hydrocarbons?

SOLUTION

Kovat's retention index for a normal alkane is 100 times the number of carbons; thus

$$I_{propane} = 100 \times 3 = 300$$

$$I_{butane} = 100 \times 4 = 400$$

To find Kovat's retention index for isobutane, we use equation 12.29.

$$I_{isobutane} = 100\left[\frac{(\log t_r')_{isobutane} - (\log t_r')_{propane}}{(\log t_r')_{butane} - (\log t_r')_{propane}}\right] + I_{propane}$$

$$= 100\left[\frac{\log(5.71) - \log(2.23)}{\log(6.67) - \log(2.23)}\right] + 300 = 386$$

12D.9 Representative Method

Although each gas chromatographic method has its own unique considerations, the following description of the determination of trihalomethanes in drinking water provides an instructive example of a typical procedure.

Representative Methods

Method 12.1 Determination of Trihalomethanes in Drinking Water[9]

Description of Method. Trihalomethanes, such as chloroform ($CHCl_3$) and bromoform ($CHBr_3$), are found in most chlorinated waters. Since chloroform is a suspected carcinogen, the determination of trihalomethanes in public drinking water supplies is of considerable importance. In this method the trihalomethanes $CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$, and $CHBr_3$ are isolated by a liquid–liquid extraction with pentane and determined by gas chromatography using an electron capture detector. Because of its volatility and ubiquitous presence in most labs, chloroform from other sources is a significant interferent.

Procedure. Samples are collected in 40-mL vials with screw-caps lined with a Teflon septum. Fill the vial to overflowing, ensuring that there are no air bubbles. Add a reducing agent of ascorbic acid (25 mg/40 mL) to quench the further production of trihalomethanes, and seal the vial. Store samples at 4 °C, and analyze within 14 days.

Prepare a standard stock solution for each trihalomethane by placing 9.8 mL of methanol in a 10-mL volumetric flask. Let the volumetric flask stand for 10 min, or until all surfaces wetted with methanol are dry. Weigh the volumetric flask to the nearest ±0.1 mg. Using a 100-μL syringe, add 2 or 3 drops of the trihalomethane to the volumetric flask, allowing it to drop directly into the methanol. Reweigh the flask before diluting to volume and mixing. Transfer to a 15-mL screw-cap vial with Teflon liner, and report the concentration in micrograms per milliliter. Standard stock solutions are stable for 4 weeks when stored at 4 °C.

Prepare a single multicomponent working standard from the stock standards by making appropriate dilutions with methanol. Concentrations in the working standards should be at such a level that a 20-μL sample added to 100 mL of water gives a calibration standard whose response for each trihalomethane is within ±25% of that for the samples to be analyzed.

Samples and calibration standards are prepared for analysis using a 10-mL syringe. Add 10.00 mL of each sample and standard to separate 14-mL screw-cap vials containing 2.00 mL of pentane. Shake vigorously for 1 min to effect the separation. Wait 60 s for the phases to separate. Inject 3.0-μL aliquots of the pentane layer into a GC equipped with a 2-mm internal diameter, 2-m long glass column packed with a stationary phase of 10% squalane on a packing material of 80/100 mesh Chromosorb WAW. Operate the column at 67 °C and a flow rate of 25 mL/min.

Questions

1. **A simple liquid–liquid extraction rarely extracts 100% of the analyte. How does this method account for incomplete extractions?**

   Both the samples and standards are treated identically so their relative concentrations are unaffected by an incomplete extraction.

2. **This method uses a short, packed column that generally produces a poor resolution of chromatographic peaks. The liquid–liquid extraction used to extract the trihalomethanes is nonselective. Besides the trihalomethanes, a wide range of nonpolar and polar organic constituents, such as benzene and**

—Continued

phenol, also are extracted. Why does the presence of these other compounds not interfere with this analysis?

An electron capture detector is relatively insensitive to nonhalogenated compounds, providing the additional selectivity.

3. **Although chloroform is an analyte, it also can be interferent. Due to its volatility, chloroform present in the laboratory air may diffuse through the sample vial's Teflon septum, contaminating the samples. How can we determine whether samples have been contaminated in this manner?**

   A sample blank of trihalomethane-free water can be kept with the samples at all times. If the sample blank shows no evidence for chloroform, then we can safely assume that the samples also are free from contamination.

4. **Why is it necessary to collect samples such that there is no headspace (layer of air overlying the liquid) in the sample vial?**

   Due to the volatility of trihalomethanes, the presence of a headspace allows for the possible loss of analyte.

12D.10 Evaluation

Scale of Operation Analytes present at levels from major to ultratrace components have been successfully determined by gas chromatography. Depending on the choice of detector, samples with major and minor analytes may need to be diluted before analysis. The thermal conductivity and flame ionization detectors can handle larger amounts of analyte; other detectors, such as the electron capture detector or a mass spectrometer, require substantially smaller amounts of analyte. Although the volume of sample injected is quite small (often less than a microliter), the amount of available material from which the injection volume is taken must be sufficient to be a representative sample. For trace analytes, the actual amount of analyte injected is often in the picogram range. Using the trihalomethane analysis described in Method 12.1 as an example, a 3.0-μL injection of a water sample containing 1 μg/L of $CHCl_3$ corresponds to 15 pg of $CHCl_3$ (assuming a complete extraction of $CHCl_3$).

Accuracy The accuracy of a gas chromatographic method varies substantially from sample to sample. For routine samples, accuracies of 1–5% are common. For analytes present at very low concentration levels, for samples with complex matrices, or for samples requiring significant processing before analysis, accuracy may be substantially poorer. In the analysis for trihalomethanes described in Method 12.1, for example, determinate errors as large as ±25% are possible.[9]

Precision The precision of a gas chromatographic analysis includes contributions from sampling, sample preparation, and the instrument. The relative standard deviation due to the gas chromatographic portion of the analysis is typically 1–5%, although it can be significantly higher. The principal limitations to precision are detector noise and the reproducibility of injection volumes. In quantitative work, the use of an internal standard compensates for any variability in injection volumes.

Sensitivity In a gas chromatographic analysis, sensitivity (the slope of a calibration curve) is determined by the detector's characteristics. Of greater interest for quantitative work is the detector's linear range; that is, the range of concentrations over which a calibration curve is linear. Detectors with a wide linear range, such as a thermal conductivity detector and flame ionization detector, can be used to analyze samples of varying concentration without adjusting operating conditions. Other detectors, such as the electron capture detector, have a much narrower linear range.

Selectivity Because it combines separation with analysis, gas chromatography provides excellent selectivity. By adjusting conditions it is usually possible to design a separation such that the analytes elute by themselves. Additional selectivity can be provided by using a detector, such as the electron capture detector, that does not respond to all compounds.

Time, Cost, and Equipment Analysis time can vary from several minutes for samples containing only a few constituents to more than an hour for more complex samples. Preliminary sample preparation may substantially increase the analysis time. Instrumentation for gas chromatography ranges in price from inexpensive (a few thousand dollars) to expensive (more than $50,000). The more expensive models are equipped for capillary columns and include a variety of injection options and more sophisticated detectors, such as a mass spectrometer. Packed columns typically cost $50–$200, and the cost of a capillary column is typically $200–$1000.

12E High-Performance Liquid Chromatography

Although gas chromatography is widely used, it is limited to samples that are thermally stable and easily volatilized. Nonvolatile samples, such as peptides and carbohydrates, can be analyzed by GC, but only after they have been made more volatile by a suitable chemical derivatization. For this reason, the various techniques included within the general scope of liquid chromatography are among the most commonly used separation techniques. Although simple column chromatography, first introduced by Tswett, is still used in large-scale preparative work, the focus of this section is on **high-performance liquid chromatography** (HPLC).

high-performance liquid chromatography
A chromatographic technique in which the mobile phase is a liquid.

In HPLC, a liquid sample, or a solid sample dissolved in a suitable solvent, is carried through a chromatographic column by a liquid mobile phase. Separation is determined by solute/stationary-phase interactions, including liquid–solid adsorption, liquid–liquid partitioning, ion exchange and size exclusion, and by solute/mobile-phase interactions. In each case, however, the basic instrumentation is essentially the same. A schematic diagram of a typical HPLC instrument is shown in Figure 12.26. The remainder of this section deals exclusively with HPLC separations based on liquid–liquid partitioning. Other forms of liquid chromatography receive consideration later in this chapter.

12E.1 HPLC Columns

An HPLC typically includes two columns: an analytical column responsible for the separation and a guard column. The guard column is placed before the analytical column, protecting it from contamination.

Analytical Columns The most commonly used columns for HPLC are constructed from stainless steel with internal diameters between 2.1 mm and 4.6 mm, and

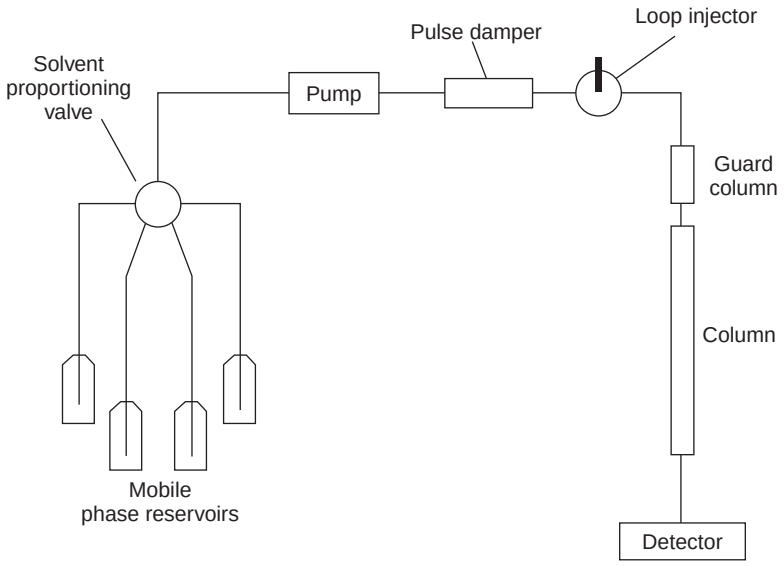


Figure 12.26
Schematic diagram of a high-performance liquid chromatograph.

lengths ranging from approximately 30 mm to 300 mm. These columns are packed with 3–10 μm porous silica particles that may have an irregular or spherical shape. Typical column efficiencies are 40,000–60,000 theoretical plates/m. Assuming V_{max}/V_{min} is approximately 50,[3] a 25-cm column with 50,000 plates/m has 12,500 theoretical plates and a peak capacity (equation 12.18) of 110.

Microcolumns use less solvent and, because the sample is diluted to a lesser extent, produce larger signals at the detector. These columns are made from fused silica capillaries with internal diameters of 44–200 μm and lengths of up to several meters. Microcolumns packed with 3–5-μm particles have been prepared with column efficiencies of up to 250,000 theoretical plates.[10]

Open tubular microcolumns also have been developed, with internal diameters of 1–50 μm and lengths of approximately 1 m. These columns, which contain no packing material, may be capable of obtaining column efficiencies of up to 1 million theoretical plates.[11] The development of open tubular columns, however, has been limited by the difficulty of preparing columns with internal diameters less than 10 μm.

Guard Columns Two problems tend to shorten the lifetime of an analytical column. First, solutes binding irreversibly to the stationary phase degrade the column's performance by decreasing the available stationary phase. Second, particulate material injected with the sample may clog the analytical column. To minimize these problems, a **guard column** is placed before the analytical column. Guard columns usually contain the same particulate packing material and stationary phase as the analytical column, but are significantly shorter and less expensive; a length of 7.5 mm and a cost one-tenth of that for the corresponding analytical column is typical. Because they are intended to be sacrificial, guard columns are replaced regularly.

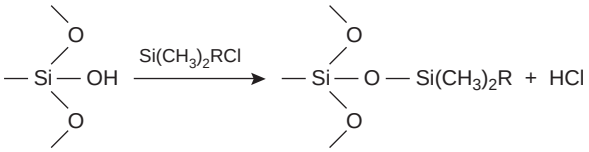
guard column
An inexpensive column used to protect a more expensive analytical column.

12E.2 Stationary Phases

In liquid–liquid chromatography the stationary phase is a liquid film coated on a packing material consisting of 3–10 μm porous silica particles. The stationary phase may be partially soluble in the mobile phase, causing it to "bleed" from the column

bonded stationary phase
A liquid stationary phase that is chemically bonded to a particulate packing material.

over time. To prevent this loss of stationary phase, it is covalently bound to the silica particles. **Bonded stationary phases** are attached by reacting the silica particles with an organochlorosilane of the general form $Si(CH_3)_2RCl$, where R is an alkyl or substituted alkyl group.



To prevent unwanted interactions between the solutes and any unreacted –SiOH groups, the silica frequently is "capped" by reacting it with $Si(CH_3)_3Cl$; such columns are designated as end-capped.

The properties of a stationary phase are determined by the nature of the organosilane's alkyl group. If R is a polar functional group, then the stationary phase will be polar. Examples of polar stationary phases include those for which R contains a cyano ($–C_2H_4CN$), diol ($–C_3H_6OCH_2CHOHCH_2OH$), or amino ($–C_3H_6NH_2$) functional group. Since the stationary phase is polar, the mobile phase is a nonpolar or moderately polar solvent. The combination of a polar stationary phase and a nonpolar mobile phase is called **normal-phase chromatography.**

normal-phase chromatography
Liquid chromatography using a polar stationary phase and a nonpolar mobile phase.

In **reverse-phase chromatography,** which is the more commonly encountered form of HPLC, the stationary phase is nonpolar and the mobile phase is polar. The most common nonpolar stationary phases use an organochlorosilane for which the R group is an *n*-octyl (C_8) or *n*-octyldecyl (C_{18}) hydrocarbon chain. Most reverse-phase separations are carried out using a buffered aqueous solution as a polar mobile phase. Because the silica substrate is subject to hydrolysis in basic solutions, the pH of the mobile phase must be less than 7.5.

reverse-phase chromatography
Liquid chromatography using a nonpolar stationary phase and a polar mobile phase.

12E.3 Mobile Phases

The elution order of solutes in HPLC is governed by polarity. In a normal-phase separation the least polar solute spends proportionally less time in the polar stationary phase and is the first solute to elute from the column. Retention times are controlled by selecting the mobile phase, with a less polar mobile phase leading to longer retention times. If, for example, a separation is poor because the solutes are eluting too quickly, switching to a less polar mobile phase leads to longer retention times and more opportunity for an acceptable separation. When two solutes are adequately resolved, switching to a more polar mobile phase may provide an acceptable separation with a shorter analysis time. In a reverse-phase separation the order of elution is reversed, with the most polar solute being the first to elute. Increasing the polarity of the mobile phase leads to longer retention times, whereas shorter retention times require a mobile phase of lower polarity.

Choosing a Mobile Phase Several indices have been developed to assist in selecting a mobile phase, the most useful of which is the **polarity index**.[12] Table 12.3 provides values for the polarity index, P', of several commonly used mobile phases, in which larger values of P' correspond to more polar solvents. Mobile phases of intermediate polarity can be fashioned by mixing together two or more of the mobile phases in Table 12.3. For example, a binary mobile phase made by combining solvents A and B has a polarity index, P'_{AB}, of

polarity index
A quantitative measure of a solvent's polarity.

Table 12.3 Properties of HPLC Mobile Phases

Mobile Phase	Polarity Index (P')	UV Cutoff (nm)
cyclohexane	0.04	210
n-hexane	0.1	210
carbon tetrachloride	1.6	265
i-propyl ether	2.4	220
toluene	2.4	286
diethyl ether	2.8	218
tetrahydrofuran	4.0	220
ethanol	4.3	210
ethyl acetate	4.4	255
dioxane	4.8	215
methanol	5.1	210
acetonitrile	5.8	190
water	10.2	—

$$P'_{AB} = \phi_A P'_A + \phi_B P'_B \qquad \textbf{12.30}$$

where P'_A and P'_B are the polarity indexes for solvents A and B, and ϕ_A and ϕ_B are the volume fractions of the two solvents.

Example

EXAMPLE 12.7

A reverse-phase HPLC separation is carried out using a mobile-phase mixture of 60% v/v water and 40% v/v methanol. What is the mobile phase's polarity index?

SOLUTION

From Table 12.3 we find that the polarity index is 10.2 for water and 5.1 for methanol. Using equation 12.30, the polarity index for a 60:40 water–methanol mixture is

$$P'_{AB} = (0.60)(10.2) + (0.40)(5.1) = 8.2$$

A useful guide when using the polarity index is that a change in its value of 2 units corresponds to an approximate tenfold change in a solute's capacity factor. Thus, if k' is 22 for the reverse-phase separation of a solute when using a mobile phase of water ($P' = 10.2$), then switching to a 60:40 water–methanol mobile phase ($P' = 8.2$) will decrease k' to approximately 2.2. Note that the capacity factor decreases because we are switching from a more polar to a less polar mobile phase in a reverse-phase separation.

Changing the mobile phase's polarity index, by changing the relative amounts of two solvents, provides a means of changing a solute's capacity factor. Such

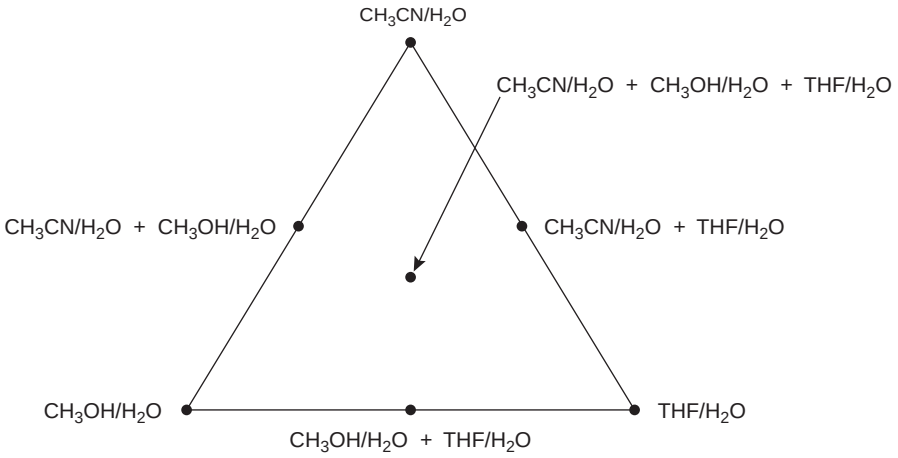


Figure 12.27
Solvent triangle for optimizing reverse-phase HPLC separations. Binary and ternary mixtures contain equal volumes of each of the aqueous mobile phases making up the vertices of the triangle.

changes, however, are not very selective; thus, two solutes that significantly overlap may continue to be poorly resolved even after making a significant change in the mobile phase's polarity.

To effect a better separation between two solutes it is often necessary to improve the selectivity factor, α. Two approaches are commonly used to accomplish this improvement. When a solute is a weak acid or a weak base, adjusting the pH of the aqueous mobile phase can lead to significant changes in the solute's retention time. This is shown in Figure 12.13a for the reverse-phase separation of *p*-aminobenzoic acid and *p*-hydroxybenzoic acid on a nonpolar C_{18} column. At more acidic pH levels, both weak acids are present as neutral molecules. Because they partition favorably into the stationary phase, the retention times for the solutes are fairly long. When the pH is made more basic, the solutes, which are now present as their conjugate weak base anions, are less soluble in the stationary phase and elute more quickly. Similar effects can be achieved by taking advantage of metal–ligand complexation and other equilibrium reactions.[13]

A second approach to changing the selectivity factor for a pair of solutes is to change one or more of the mobile-phase solvents. In a reverse-phase separation, for example, this is accomplished by changing the solvent mixed with water. Besides methanol, other common solvents for adjusting retention times are acetonitrile and tetrahydrofuran (THF). A common strategy for finding the best mobile phase is to use the solvent triangle shown in Figure 12.27. The separation is first optimized using an aqueous mobile phase of acetonitrile to produce the best separation within the desired analysis time (methanol or THF also could be chosen first). Table 12.4 is used to estimate the composition of methanol/H_2O and THF/H_2O mobile phases that will produce similar analysis times. These mobile phases are then adjusted, if necessary, establishing the three points of the solvent triangle. Four additional mobile phases are prepared using the binary and ternary mobile phases indicated in Figure 12.27. From these seven mobile phases it is possible to estimate how a change in the mobile-phase composition might affect the separation.

isocratic elution
The use of a mobile phase whose composition remains constant throughout the separation.

Isocratic Versus Gradient Elution When a separation uses a single mobile phase of fixed composition it is called an **isocratic elution.** It is often difficult, however, to find a single mobile-phase composition that is suitable for all solutes. Recalling the general elution problem, a mobile phase that is suitable for early eluting solutes may lead to unacceptably long retention times for later eluting solutes. Optimizing con-

Table 12.4 Organic Solvent/H_2O Mobile-Phase Compositions Having Approximately Equal Solvent Strength

%v/v CH_3OH	%v/v CH_3CN	%v/v THF
0	0	0
10	6	4
20	14	10
30	22	16
40	32	24
50	40	30
60	50	36
70	60	44
80	72	52
90	87	62
100	99	71

ditions for late eluting solutes, on the other hand, may provide an inadequate separation of early eluting solutes. Changing the composition of the mobile phase with time provides a solution to this problem. For a reverse-phase separation the initial mobile-phase composition is relatively polar. As the separation progresses, the mobile phase's composition is made less polar. Such separations are called gradient elutions.

12E.4 HPLC Plumbing

An important feature of HPLC instrumentation (see Figure 12.26) is the presence of several solvent reservoirs. As discussed in the previous section, controlling the mobile phase's polarity plays an important role in improving a liquid chromatographic separation. The availability of several solvent reservoirs allows the mobile phase's composition to be quickly and easily varied. This is essential when using a gradient elution, for which the mobile-phase composition is systematically changed from a weaker solvent to a stronger solvent.

Before they are used, mobile-phase solvents must be treated to remove dissolved gases, such as N_2 and O_2, and small particulate matter, such as dust. Dissolved gases often lead to the formation of gas bubbles when the mobile phase enters the detector, resulting in a distortion of the detector's signal. Degassing is accomplished in several ways, but the most common are the use of a vacuum pump or sparging with an inert gas, such as He, which has a low solubility in the mobile phase. Particulate material capable of clogging the HPLC tubing or column is removed by filtering. If the instrument is not designed to do so, degassing and filtering can be completed before the solvents are placed in their reservoirs.

The mobile-phase solvents are pulled from their reservoirs by the action of a pump. Most HPLC instruments use a reciprocating pump consisting of a piston whose back-and-forth movement is capable both of maintaining a constant flow rate of up to several milliliters per minute and of obtaining the high output pressure needed to push the mobile phase through the chromatographic column. A solvent proportioning valve controls the mobile phase's composition, making possible the

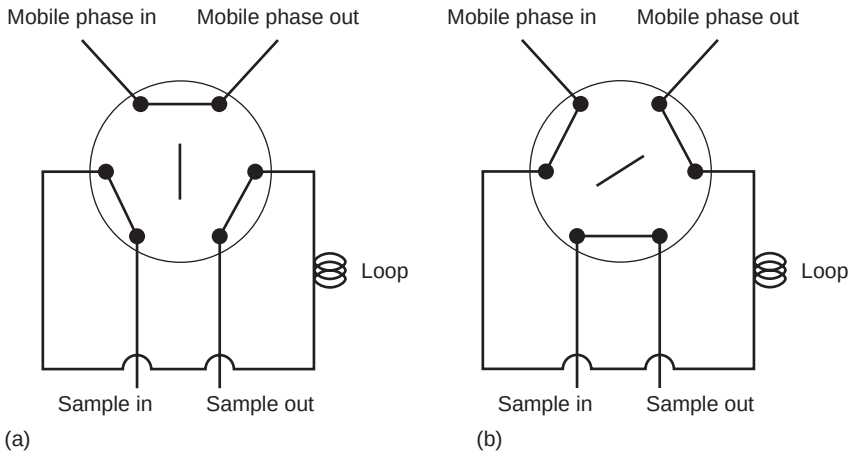


Figure 12.28
Schematic diagram of a loop injector in the (a) load and (b) inject positions.

necessary change in the mobile phase's composition when using a gradient elution. The back and forth movement of a reciprocating pump results in a pulsed flow that contributes noise to the chromatogram. To eliminate this problem a pulse damper is placed at the outlet of the pump.

12E.5 Sample Introduction

loop injector
A means for injecting samples in which the sample is loaded into a short section of tubing and injected onto the column by redirecting the mobile phase through the loop.

The typical operating pressure of an HPLC is sufficiently high that it is impossible to inject the sample in the same manner as in gas chromatography. Instead, the sample is introduced using a **loop injector** (Figure 12.28). Sampling loops are interchangeable, and available with volumes ranging from 0.5 μL to 2 mL.

In the load position the sampling loop is isolated from the mobile phase and is open to the atmosphere. A syringe with a capacity several times that of the sampling loop is used to place the sample in the loop. Any extra sample beyond that needed to fill the sample loop exits through the waste line. After loading the sample, the injector is turned to the inject position. In this position the mobile phase is directed through the sampling loop, and the sample is swept onto the column.

12E.6 Detectors for HPLC

As with gas chromatography, numerous detectors have been developed for use in monitoring HPLC separations.[14] To date, the majority of HPLC detectors are not unique to the method, but are either stand-alone instruments or modified versions of the same.

Colorplate 12 shows a photo of an HPLC equipped with a diode array detector.

Spectroscopic Detectors The most popular HPLC detectors are based on spectroscopic measurements, including UV/Vis absorption, and fluorescence. These detectors range from simple designs, in which the analytical wavelength is selected using appropriate filters, to essentially a modified spectrophotometer equipped with a flow cell. When using a UV/Vis detector, the resulting chromatogram is a plot of absorbance as a function of elution time. Instruments utilizing a diode array spectrophotometer record entire spectra, giving a three-dimensional chromatogram showing absorbance as a function of wavelength and elution time. Figure 12.29a shows a typical flow cell for HPLC when using a UV/Vis spectrophotometer as a detector. The flow cell has a volume of 1–10 μL and a path length of 0.2–1 cm. One limitation to using absorbance is that the mobile phase must not absorb strongly at

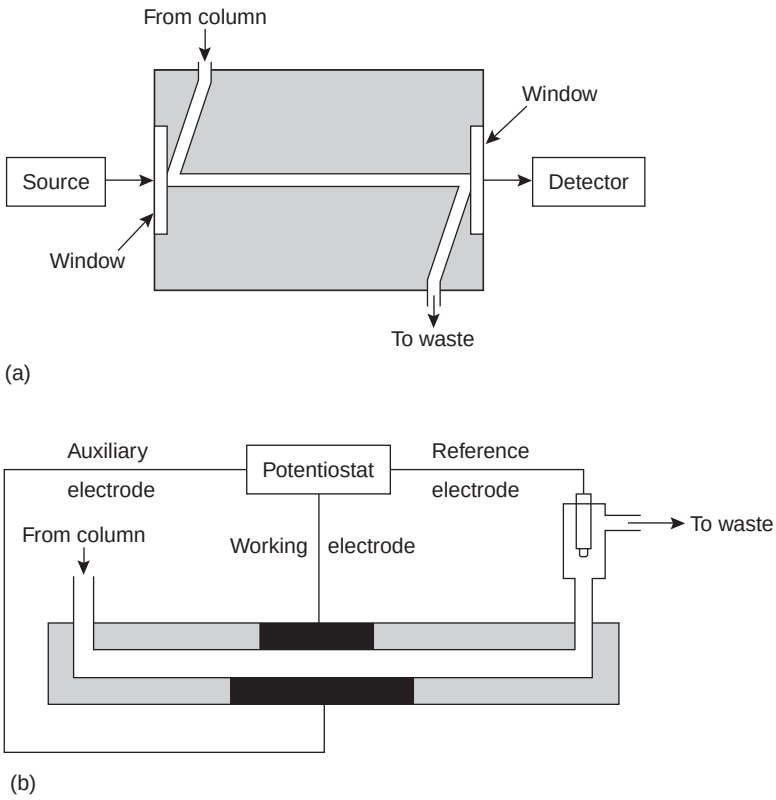


Figure 12.29
Schematic diagrams of flow cell detectors for HPLC using (a) UV/Vis absorption spectrophotometry and (b) amperometry.

the chosen wavelength. Table 12.3 lists the wavelengths below which UV/Vis absorbance cannot be used for different mobile phases. Detectors based on absorbance provide detection limits of as little as 100 pg–1 ng of injected analyte. Fluorescence detectors provide additional selectivity since fewer solutes are capable of fluorescing. The resulting chromatogram is a plot of fluorescence intensity as a function of time. Detection limits are as little as 1–10 pg of injected analyte.

Electrochemical Detectors Another common group of HPLC detectors are those based on electrochemical measurements such as amperometry, voltammetry, coulometry, and conductivity. Figure 12.29b, for example, shows an amperometric flow cell. Effluent from the column passes over the working electrode, which is held at a potential favorable for oxidizing or reducing the analytes. The potential is held constant relative to a downstream reference electrode, and the current flowing between the working and auxiliary electrodes is measured. Detection limits for amperometric electrochemical detection are 10 pg–1 ng of injected analyte.

Other Detectors Several other detectors have been used in HPLC. Measuring a change in the mobile phase's refractive index is analogous to monitoring the mobile phase's thermal conductivity in gas chromatography. A refractive index detector is nearly universal, responding to almost all compounds, but has a poorer detection limit of 100 ng–1 μg of injected analyte. Furthermore, a refractive index detector is not useful for a gradient elution unless the mobile-phase components have identical refractive indexes. Another useful detector is a mass spectrometer. The advantages of using a mass spectrometer in HPLC are the same as for gas chromatography. Detection limits are quite good, typically 100 pg–1 ng of injected analyte, with values

as low as 1–10 pg in some situations. In addition, a mass spectrometer provides qualitative, structural information that can help identify the analytes. The interface between the HPLC and mass spectrometer is technically more difficult than that in a GC–MS because of the incompatibility of a liquid mobile phase with the mass spectrometer's high vacuum requirement. Recent developments in mass spectrometry, however, have led to a growing interest in LC–MS.

12E.7 Quantitative Applications

HPLC is routinely used for both qualitative and quantitative analyses of environmental, pharmaceutical, industrial, forensic, clinical, and consumer product samples. Figure 12.30 shows several representative examples.

Preparing Samples for Analysis Samples in liquid form can be analyzed directly, after a suitable clean-up to remove any particulate materials or after a suitable extraction to remove matrix interferents. In determining polyaromatic hydrocarbons (PAH) in wastewater, for example, an initial extraction with CH_2Cl_2 serves the dual purpose of concentrating the analytes and isolating them from matrix interferents. Solid samples must first be dissolved in a suitable solvent, or the analytes of interest must be brought into solution by extraction. For example, an HPLC analysis for the active ingredients and degradation products in a pharmaceutical tablet often begins by extracting the powdered tablet with a portion of mobile phase. Gases are collected by bubbling through a trap containing a suitable solvent. Organic isocyanates in industrial atmospheres can be determined in this manner by bubbling the air through a solution of 1-(2-methoxyphenyl)piperazine in toluene. Reacting the isocyanates with 1-(2-methoxyphenyl)piperazine serves the dual purposes of stabilizing them against degradation before the HPLC analysis while also forming a derivative that can be monitored by UV absorption.

Quantitative Calculations Quantitative analyses are often easier to conduct with HPLC than GC because injections are made with a fixed-volume injection loop instead of a syringe. As a result, variations in the amount of injected sample are minimized, and quantitative measurements can be made using external standards and a normal calibration curve.

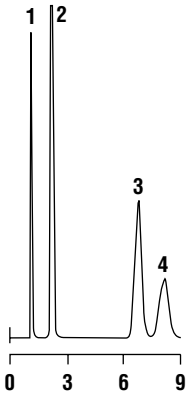
Example

EXAMPLE 12.8

The concentration of PAHs in soil can be determined by first extracting the PAHs with methylene chloride. The extract is then diluted, if necessary, and the PAHs are separated by HPLC using a UV/Vis or fluorescence detector. Calibration is achieved using one or more external standards. In a typical analysis, a 2.013-g sample of dried soil is extracted with 20.00 mL of methylene chloride. After filtering to remove the soil, a 1-mL portion of the extract is removed and diluted to 10 mL with acetonitrile. Injecting 5 μL of the diluted extract into an HPLC gives a signal of 0.217 (arbitrary units) for the PAH fluoranthene. When 5 μL of a 20.0-ppm fluoranthene standard is analyzed using the same conditions, a signal of 0.258 is measured. Report the parts per million of fluoranthene in the soil.

Acetaminophen, salicylic acid and caffeine

CHROM 7227



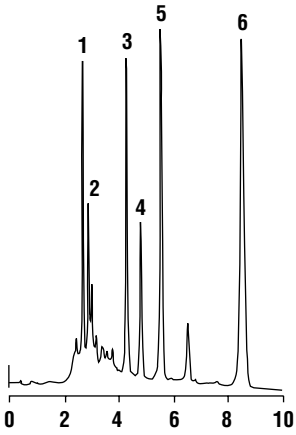
1. Acetaminophen (0.43mg/mL)
2. Caffeine (0.30mg/mL)
3. Benzoic Acid (Internal Std.) (1.45mg/mL)
4. Salicylic Acid (1.52mg/mL)

Column: Adsorbosil C18, 5µm, 100 × 4.6mm
Mobile Phase: Water:Methanol:Glacial Acetic Acid (69:28:3)
Flowrate: 1.5mL/min
Column Temp: 45°C
Detector: UV at 275nm

(a)

Chlorinated pesticides

CHROM 5006



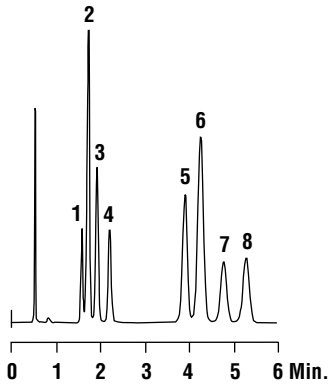
1. Aldrin
2. Heptachlor
3. Endrin
4. Dieldrin
5. TDE
6. Methoxychlor

Column: Adsorbosphere PAC. 5µm, 250 × 4.6mm
Mobile Phase: Isooctane:Ethyl Acetate (97.3:2.7)
Flowrate: 1.0mL/min
Detector: UV at 254nm

(b)

Tricyclic antidepressants

CHROM 7170



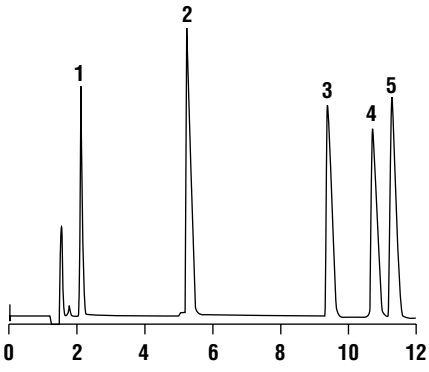
1. Trimipramine
2. Doxepin
3. Amitriptyline
4. Imipramine
5. Nordoxepin
6. Nortriptyline
7. Desipramine
8. Protriptyline

Column: Adsorbosphere CN-AQ, 5µm, 150 × 4.6mm
Mobile Phase: Acetonitrile:Methanol:0.01M K_2HPO_4, pH 7.0 (60:15:25)
Flowrate: 2.0mL/min
Detector: UV at 215nm

(c)

Peptides

CHROM 8761



1. Gly-Tyr
2. Val-Tyr-Val
3. Methionine Enkephalin
4. Angiotensin II
5. Leu-Enkephalin

Column: Alltima C18, 3µm, 100 × 4.6mm
Mobile Phase: **A:** 0.1%v/v TFA
B: 0.085%v/v TFA in Acetonitrile:Water (90:10)
Gradient:

Time:	0	20
%B:	15	45

Flowrate: 1.0mL/min
Detector: UV at 220nm

(d)

Figure 12.30

Examples of the application of HPLC to the analysis of (a) acetaminophen, salicylic acid, and caffeine; (b) chlorinated pesticides; (c) tricyclic antidepressants; and (d) peptides. (Chromatograms courtesy of Alltech Associates, Inc. Deerfield, IL).

SOLUTION

For a single-point external standard, the relationship between the signal, S, and the concentration, C, of fluoranthene is

$$S = kC$$

Substituting the known values for the standard's signal and concentration gives the value of k as

$$k = \frac{S}{C} = \frac{0.258}{20.0 \text{ ppm}} = 0.0129 \text{ ppm}^{-1}$$

Using this value for k and the signal obtained for the extracted and diluted soil sample gives a fluoranthene concentration of

$$C = \frac{S}{k} = \frac{0.217}{0.0129 \text{ ppm}^{-1}} = 16.8 \text{ ppm}$$

This, of course, is the concentration of fluoranthene in the sample injected into the HPLC. The concentration of fluoranthene in the soil is

$$\frac{(16.8 \text{ μg/mL}) \times (10 \text{ mL}/1 \text{ mL}) \times 20 \text{ mL}}{2.013 \text{ g}} = 1670 \text{ ppm}$$

12E.8 Representative Method

Although each HPLC method has its own unique considerations, the following description of the determination of the fluoxetine in serum provides an instructive example of a typical procedure.

Representative Methods

Method 12.2 Determination of Fluoxetine in Serum[15]

Description of Method. Fluoxetine, whose structure is shown in Figure 12.31a, is another name for the antidepressant drug Prozac. The determination of fluoxetine and its metabolite norfluoxetine, Figure 12.31b, in serum is an important part of monitoring its therapeutic use. The analysis is complicated by the complex matrix of serum samples. A solid-phase extraction followed by an HPLC analysis using a fluorescence detector provides the necessary selectivity and detection limits.

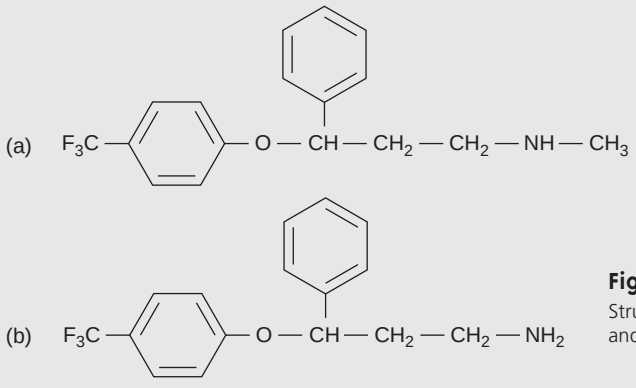


Figure 12.31
Structures of (a) fluoxetine and (b) norfluoxetine.

—Continued

Procedure. A known amount of the antidepressant protriptyline is added to a serum sample, serving as an internal standard. A 0.5-mL aliquot of the serum is passed through a solid-phase extraction cartridge containing silica particles with a bonded C_{18} phase. After washing to remove interfering constituents from the sample matrix, the remaining constituents, including both analytes and the internal standard, are removed by washing the cartridge with 0.25 mL of a 25:75 v/v mixture of 0.1 M $HClO_4$ and acetonitrile. A 20-μL aliquot is injected onto a 15-cm × 4.6-mm column packed with a 5-μm C_8-bonded stationary phase. An isocratic mobile-phase mixture of 37.5:62.5 v/v acetonitrile and water (containing 1.5 g of tetramethylammonium perchlorate and 0.1 mL of 70% v/v $HClO_4$) is used to elute the sample. Detection is with a fluorescence detector using an excitation wavelength of 235 nm and an emission wavelength of 310 nm.

Questions

1. **What is the purpose of including an initial solid-phase extraction?**

   A direct injection of serum is not advisable since the presence of particulate material in the sample matrix may clog the column. In addition, some of the sample's constituents may absorb too strongly to the stationary phase, thus degrading the column's performance. Finally, although an HPLC is capable of separating and analyzing complex mixtures, an analysis may still be difficult if there are too many constituents to provide adequate resolution of the analytes. The solid-phase extraction serves the purpose of cleaning up the sample before using the HPLC.

2. **One advantage of an HPLC analysis is that a loop injector often eliminates the need for an internal standard. Why is an internal standard used in this analysis? What assumption(s) must we make about the internal standard?**

   An internal standard is used because of difficulties introduced in the solid-phase extraction. For example, the volume of serum taken for the solid-phase extraction and the volume of solvent used to remove the analyte and internal standard are quite small (0.5 mL and 0.25 mL, respectively). The precision and accuracy with which these volumes can be measured are not as good as when using larger volumes. Using an internal standard compensates for any variations in these volumes. To be useful, the analytes and internal standard must be assumed to be retained to the same extent during the initial loading and washing of the cartridge, and they must be assumed to be extracted to the same extent during the final elution.

3. **If the peaks for fluoxetine and protriptyline are insufficiently resolved, how might you alter the mobile phase to improve their separation?**

   Decreasing the amount of acetonitrile and increasing the amount of water in the mobile will increase retention times, thereby providing a better resolution.

12E.9 Evaluation

When compared with gas chromatography, HPLC has only a few differences in the scale of operation; accuracy; precision; sensitivity; selectivity; and time, cost, and equipment necessary. Injection volumes in HPLC are usually significantly larger than in GC because of the greater capacity of HPLC columns. Precision in HPLC is often better due to the routine use of loop injectors. Because HPLC is not limited to volatile analytes, the range of compounds that can be analyzed is somewhat larger than for GC. Capillary GC columns, on the other hand, have more theoretical plates, providing greater resolving power for complex mixtures.

12F Liquid–Solid Adsorption Chromatography

liquid–solid adsorption chromatography
A form of liquid chromatography in which the stationary phase is a solid adsorbent.

In **liquid–solid adsorption chromatography** (LSC) the column packing also serves as the stationary phase. In Tswett's original work the stationary phase was finely divided $CaCO_3$, but modern columns employ porous 3–10-μm particles of silica or alumina. Since the stationary phase is polar, the mobile phase is usually a nonpolar or moderately polar solvent. Typical mobile phases include hexane, isooctane, and methylene chloride. The usual order of elution, from shorter to longer retention times, is

olefins < aromatic hydrocarbons < ethers < esters, aldehydes, ketones < alcohols, amines < amides < carboxylic acids

For most samples liquid–solid chromatography does not offer any special advantages over liquid–liquid chromatography (LLC). One exception is for the analysis of isomers, where LLC excels. Figure 12.32 shows a typical LSC separation of two amphetamines on a silica column using an 80:20 mixture of methylene chloride and methanol containing 1% NH_4OH as a mobile phase. Nonpolar stationary phases, such as charcoal-based absorbents, also may be used.

12G Ion-Exchange Chromatography

ion-exchange chromatography
A form of liquid chromatography in which the stationary phase is an ion-exchange resin.

In **ion-exchange chromatography** (IEC) the stationary phase is a cross-linked polymer resin, usually divinylbenzene cross-linked polystyrene, with covalently attached ionic functional groups (Figure 12.33). The counterions to these fixed charges are mobile and can be displaced by ions that compete more favorably for the exchange sites. Ion-exchange resins are divided into four categories: strong acid cation exchangers; weak acid cation exchangers; strong base anion exchangers; and weak base anion exchangers. Table 12.5 provides a list of several common ion-exchange resins.

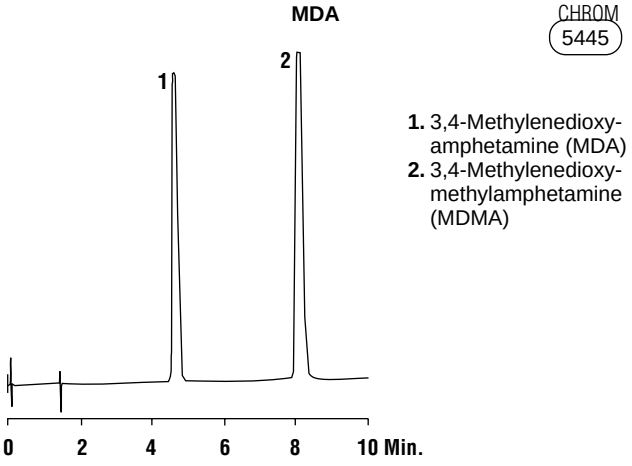


Figure 12.32
Example of the application of liquid–solid chromatography to the analysis of amphetamines. (Chromatogram courtesy of Alltech Associates, Inc. Deerfield, IL).

Column: Adsorbosphere silica, 3 μm, 150 × 4.6 mm
Mobile Phase: Methylene chloride:
Methanol with 1% NH_4OH (80:20)
Flowrate: 1.25 mL/min
Detector: UV at 280 nm

Strong acid cation exchangers include a sulfonic acid functional group that retains its anionic form, and thus its capacity for ion-exchange, in strongly acidic solutions. The functional groups for a weak acid cation exchanger, however, are fully protonated at pH levels less then 4, thereby losing their exchange capacity. The strong base anion exchangers are fashioned using a quaternary amine, therefore retaining a positive charge even in strongly basic solutions. Weak base anion exchangers, however, remain protonated only at pH levels that are moderately basic. Under more basic conditions, a weak base anion exchanger loses its positive charge and, therefore, its exchange capacity.

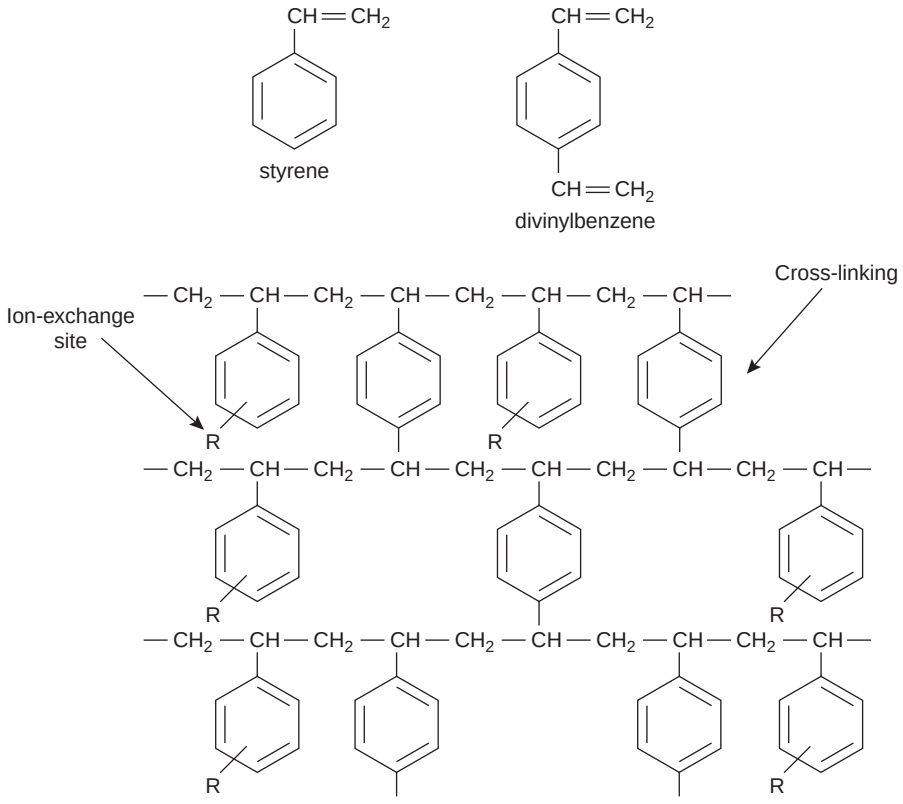


Figure 12.33
Structures of styrene, divinylbenzene, and a styrene-divinylbenzene co-polymer modified for use as an ion-exchange resin. The ion-exchange sites, indicated by R, are mostly in the *para* position and are not necessarily bound to all styrene units.

Table 12.5 Examples of Common Ion-Exchange Resins

Type	Functional Group	Examples
strong acid cation exchanger	sulfonic acid	$-SO_3^-$ $-CH_2CH_2SO_3^-$
weak acid cation exchanger	carboxylic acid	$-COO^-$ $-CH_2COO^-$
strong base anion exchanger	quaternary amine	$-CH_2N(CH_3)_3^+$ $-CH_2CH_2N(CH_2CH_3)_3^+$
weak base anion exchanger	amine	$-NH_3^+$ $-CH_2CH_2NH(CH_2CH_3)_2^+$

The ion-exchange reaction of a monovalent cation, M^+, at a strong acid exchange site is

$$-SO_3^-{-}H^+(s) + M^+(aq) \rightleftharpoons -SO_3^-{-}M^+(s) + H^+(aq)$$

The equilibrium constant for this ion-exchange reaction, which is also called the selectivity coefficient, is

$$K = \frac{\{-SO_3^-{-}M^+\}[H^+]}{\{-SO_3^-{-}H^+\}[M^+]} \qquad \textbf{12.31}$$

where the brackets { } indicate a surface concentration. Rearranging equation 12.31 shows that the distribution ratio for the exchange reaction

$$D = \frac{\text{amount of } M^+ \text{ in stationary phase}}{\text{amount of } M^+ \text{ in mobile phase}} = \frac{\{-SO_3^-{-}M^+\}}{[M^+]} = K\frac{\{-SO_3^-{-}H^+\}}{[H^+]}$$

is a function of the concentration of H^+ and, therefore, the pH of the mobile phase.

Ion-exchange resins are incorporated into HPLC columns either as micron-sized porous polymer beads or by coating the resin on porous silica particles. Selectivity is somewhat dependent on whether the resin includes a strong or weak exchange site and on the extent of cross-linking. The latter is particularly important because it controls the resin's permeability and, therefore, the accessibility of the exchange sites. An approximate order of selectivity for a typical strong acid cation exchange resin, in order of decreasing *D,* is

$$Al^{3+} > Ba^{2+} > Pb^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Ag^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+$$

Note that highly charged ions bind more strongly than ions of lower charge. Within a group of ions of similar charge, those ions with a smaller hydrated radius (Table 6.1 in Chapter 6) or those that are more polarizable bind more strongly. For a strong base anion exchanger the general order is

$$SO_4^{2-} > I^- > HSO_4^- > NO_3^- > Br^- > NO_2^- > Cl^- > HCO_3^- > CH_3COO^- > OH^- > F^-$$

Again, ions of higher charge and smaller hydrated radius bind more strongly than ions with a lower charge and a larger hydrated radius.

The mobile phase in IEC is usually an aqueous buffer, the pH and ionic composition of which determines a solute's retention time. Gradient elutions are possible in which the ionic strength or pH of the mobile phase is changed with time. For example, an IEC separation of cations might use a dilute solution of HCl as the mobile phase. Increasing the concentration of HCl speeds the elution rate for more strongly retained cations, since the higher concentration of H^+ allows it to compete more successfully for the ion-exchange sites.

Ion-exchange columns can be substituted into the general HPLC instrument shown in Figure 12.26. The most common detector measures the conductivity of the mobile phase as it elutes from the column. The high concentration of electrolyte in the mobile phase is a problem, however, because the mobile-phase ions dominate the conductivity. For example, if a dilute solution of HCl is used as the mobile phase, the presence of large concentrations of H_3O^+ and Cl^- produces a background conductivity that may prevent the detection of analytes eluting from the column.

ion-suppressor column
A column used to minimize the conductivity of the mobile phase in ion-exchange chromatography.

To minimize the mobile phase's contribution to conductivity, an **ion-suppressor column** is placed between the analytical column and the detector. This column selectively removes mobile-phase electrolyte ions without removing solute ions. For example, in cation ion-exchange chromatography using a dilute solution of HCl as

the mobile phase, the suppressor column contains an anion-exchange resin. The exchange reaction

$$H^+(aq) + Cl^-(aq) + Resin^+–OH^- \rightleftharpoons Resin^+–Cl^- + H_2O(\ell)$$

replaces the ionic HCl with H_2O. Analyte cations elute as hydroxide salts instead of as chloride salts. A similar process is used in anion ion-exchange chromatography in which a cation ion-exchange resin is placed in the suppressor column. If the mobile phase contains Na_2CO_3, the exchange reaction

$$2Na^+(aq) + CO_3^{2-}(aq) + 2Resin^-–H^+ \rightleftharpoons 2Resin^-–Na^+ + H_2CO_3(aq)$$

replaces a strong electrolyte, Na_2CO_3, with a weak electrolyte, H_2CO_3.

Ion suppression is necessary when using a mobile phase containing a high concentration of ions. **Single-column ion chromatography,** in which an ion-suppressor column is not needed, is possible if the concentration of ions in the mobile phase can be minimized. Typically this is done by using a stationary phase resin with a low capacity for ion exchange and a mobile phase with a small concentration of ions. Because the background conductivity due to the mobile phase is sufficiently small, it is possible to monitor a change in conductivity as the analytes elute from the column.

single-column ion chromatography
Ion-exchange chromatography in which conditions are adjusted so that an ion-suppressor column is not needed.

A UV/Vis absorbance detector can also be used if the solute ions absorb ultraviolet or visible radiation. Alternatively, solutions that do not absorb in the UV/Vis range can be detected indirectly if the mobile phase contains a UV/Vis-absorbing species. In this case, when a solute band passes through the detector, a decrease in absorbance is measured at the detector.

Ion-exchange chromatography has found important applications in water analysis and in biochemistry. For example, Figure 12.34a shows how ion-exchange chromatography can be used for the simultaneous analysis of seven common anions in approximately 12 min. Before IEC, a complete analysis of the same set of anions required 1–2 days. Ion-exchange chromatography also has been used for the analysis of proteins, amino acids, sugars, nucleotides, pharmaceuticals, consumer products, and clinical samples. Several examples are shown in Figure 12.34.

12H Size-Exclusion Chromatography

Two classes of micron-sized stationary phases have been encountered in this section: silica particles and cross-linked polymer resin beads. Both materials are porous, with pore sizes ranging from approximately 50 to 4000 Å for silica particles and from 50 to 1,000,000 Å for divinylbenzene cross-linked polystyrene resins. In **size-exclusion chromatography,** also called molecular-exclusion or gel-permeation chromatography, separation is based on the solute's ability to enter into the pores of the column packing. Smaller solutes spend proportionally more time within the pores and, consequently, take longer to elute from the column.

size-exclusion chromatography
A form of liquid chromatography in which the stationary phase is a porous material and in which separations are based on the size of the solutes.

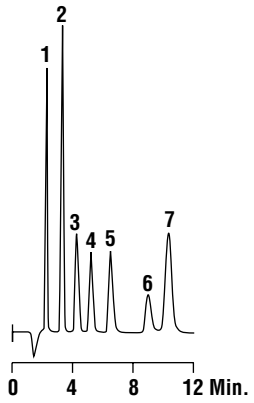
The size selectivity of a particular packing is not infinite, but is limited to a moderate range. All solutes significantly smaller than the pores move through the column's entire volume and elute simultaneously, with a retention volume, V_r, of

$$V_r = V_i + V_o \qquad \textbf{12.32}$$

where V_i is the volume of mobile phase occupying the packing material's pore space, and V_o is volume of mobile phase in the remainder of the column. The maximum size for which equation 12.32 holds is the packing material's **inclusion limit,**

inclusion limit
In size-exclusion chromatography, the smallest solute that can be separated from other solutes; all smaller solutes elute together.

Anion standards



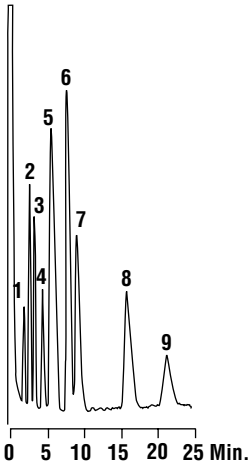
1. Fluoride, (2 ppm)
2. Chloride, (4 ppm)
3. Nitrite, (4 ppm)
4. Bromide, (4 ppm)
5. Nitrate, (4 ppm)
6. Phosphate, (6 ppm)
7. Sulfate, (6 ppm)

Column: Allsep™ Anion, 100 × 4.6 mm
Mobile Phase: 0.7 mM $NaHCO_3$:1.2 mM Na_2CO_3
Flowrate: 1.0 mL/min
Temperature: 40°C
Detector: Suppressed conductivity

(a)

Monovalent and divalent cations, and transition metals

CHROM 6355



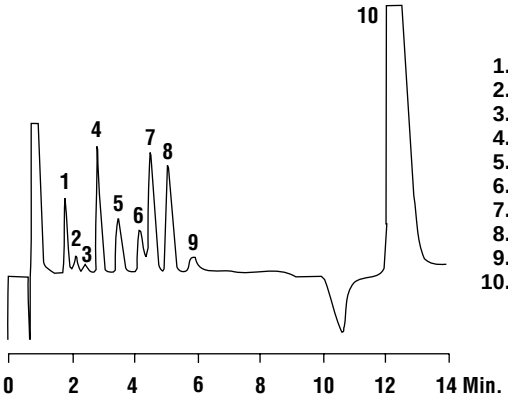
1. Lithium (0.5 ppm)
2. Sodium (0.5 ppm)
3. Ammonium (0.5 ppm)
4. Potassium (0.8 ppm)
5. Nickel (5 ppm)
6. Zinc (5 ppm)
7. Cobalt (5 ppm)
8. Magnesium (0.35 ppm) and Manganese (0.35 ppm)
9. Calcium (0.7 ppm)

Column: Universal cation 100 × 4.6 mm
Mobile Phase: 2 mM Tartaric acid/1 mM oxalic acid
Flowrate: 1.0 mL/min
Detector: Conductivity

(b)

Antifreeze analysis

CHROM 5885



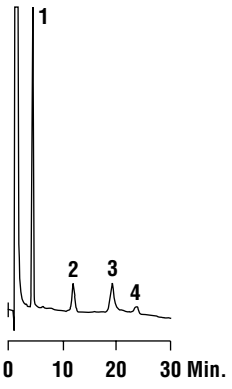
1. Glycolate
2. Phosphate
3. Formate
4. Chloride
5. Nitrite
6. Bromide
7. Chlorate
8. Nitrate
9. Benzoate
10. Sulfate

Column: Wescan Anion/S, 250 × 4.6 mm
Mobile Phase: 4 mM Phthalic Acid, pH 3.9
Flowrate: 3.4 mL/min
Detector: Conductivity

(c)

Carnitine and choline in vitamins

CHROM 6291

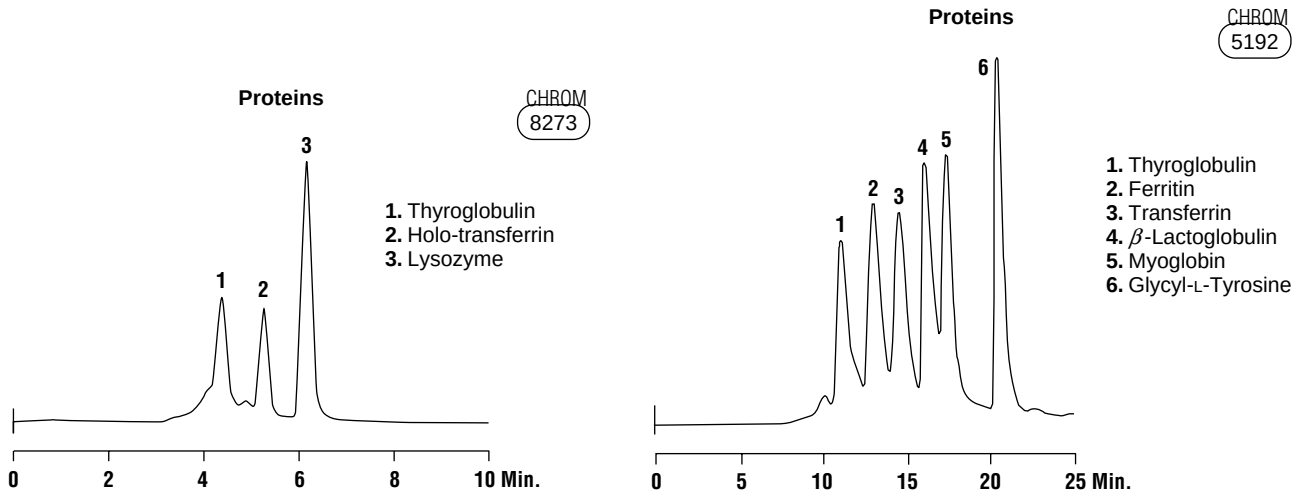


1. Sodium (1 ppm)
2. *L*-Carnitine (3 ppm)
3. Choline (3 ppm)
4. Calcium, trace

Column: Universal cation, 100 × 4.6 mm
Mobile Phase: 5 mM HCl
Flowrate: 1.0 mL/min
Detector: Conductivity

(d)

Figure 12.34
Examples of the application of ion-exchange chromatography to the analysis of (a) inorganic anions, (b) inorganic cations, (c) antifreeze, and (d) vitamins. (Chromatograms courtesy of Alltech Associates, Inc. Deerfield, IL).



Column: Macrosphere GPC, 7.0μm, 300Å, 250 × 4.6mm
Mobile Phase: 0.05M Potassium Phosphate Dibasic & 0.15M Sodium Sulfate, pH7.0
Flowrate: 0.5mL/min
Detector: UV at 280nm

(a)

Column: Macrosphere GPC 150/300/500Å, 7μm, 250 × 4.6mm (3)
Mobile Phase: 0.05M KH_2PO_4 & 0.15M Na_2SO_4, pH7.0
Flowrate: 0.3mL/min
Detector: UV at 280nm

(b)

Figure 12.35

Examples of the application of size-exclusion chromatography to the analysis of proteins. The separation in (a) uses a single column; that in (b) uses three columns, providing a wider range of size selectivity. (Chromatograms courtesy of Alltech Associates, Inc. Deerfield, IL).

or permeation limit. All solutes too large to enter the pores elute simultaneously with a retention volume of

$$V_r = V_o \qquad \textbf{12.33}$$

Equation 12.33 defines the packing material's **exclusion limit.**

exclusion limit
In size-exclusion chromatography, the largest solute that can be separated from other solutes; all larger solutes elute together.

In between the inclusion limit and the exclusion limit, each solute spends an amount of time in the pore space proportional to its size. The retention volume for a solute is

$$V_r = V_o + DV_i \qquad \textbf{12.34}$$

where D is the solute's distribution ratio, which ranges from 0 at the exclusion limit to 1 at the inclusion limit. The validity of equation 12.34 requires that size exclusion be the only interaction between the solute and the stationary phase responsible for the separation. To this end, silica particles used for size exclusion are deactivated as described earlier, and polymer resins are synthesized without exchange sites.

Size-exclusion chromatography provides a rapid means for separating larger molecules, including polymers and biomolecules. Figure 12.35 shows the application of size-exclusion chromatography for the analysis of protein mixtures. In Figure 12.35a, a column packing with 300 Å pores, with an inclusion limit of 7500 g/mol and an exclusion limit of 1.2×10^6 g/mol, is used to separate a mixture of three proteins. Mixtures containing a wider range of formula weights can be separated by joining together several columns in series. Figure 12.35b shows an example spanning an inclusion limit of 4000 g/mol and an exclusion limit of 7.5×10^6 g/mol.

Another important application is for the determination of formula weights. Calibration curves of log(formula weight) versus V_r are prepared between the exclusion limit and inclusion limit (Figure 12.36). Since the retention volume is, to some

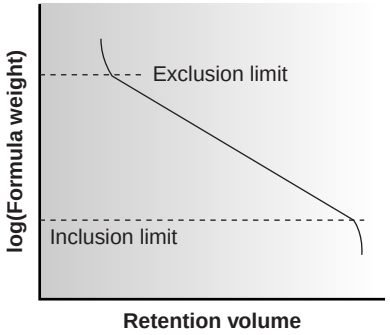


Figure 12.36
Calibration curve for the determination of formula weight by size-exclusion chromatography.

degree, a function of a solute's size and shape, reasonably accurate determinations of formula weight are possible only if the standards are carefully chosen to minimize the effect of shape.

Size-exclusion chromatography can be carried out using conventional HPLC instrumentation, replacing the HPLC column with an appropriate size-exclusion column. A UV/Vis detector is the most common means for obtaining the chromatogram.

12I Supercritical Fluid Chromatography

Despite their importance, gas chromatography and liquid chromatography cannot be used to separate and analyze all types of samples. Gas chromatography, particularly when using capillary columns, provides for rapid separations with excellent resolution. Its application, however, is limited to volatile analytes or those analytes that can be made volatile by a suitable derivatization. Liquid chromatography can be used to separate a wider array of solutes; however, the most commonly used detectors (UV, fluorescence, and electrochemical) do not respond as universally as the flame ionization detector commonly used in gas chromatography.

supercritical fluid chromatography
A separation technique in which the mobile phase is a supercritical fluid.

Supercritical fluid chromatography (SFC) provides a useful alternative to gas chromatography and liquid chromatography for some samples. The mobile phase in supercritical fluid chromatography is a gas held at a temperature and pressure exceeding its critical point (Figure 12.37). Under these conditions the mobile phase is neither a gas nor a liquid. Instead, the mobile phase is a supercritical fluid whose properties are intermediate between those of a gas and a liquid (Table 12.6). Specifically, supercritical fluids have viscosities that are similar to those of gases, which means that they can move through either capillary or packed columns without the need for the high pressures encountered in HPLC. Analysis time and resolution, although not as good as in GC, are usually better than that obtainable with conventional HPLC. The density of a supercritical fluid, however, is much closer to that of a liquid, accounting for its ability to function as a solvent. The mobile phase in SFC, therefore, behaves more like the liquid mobile phase in HPLC than the gaseous mobile phase in GC.

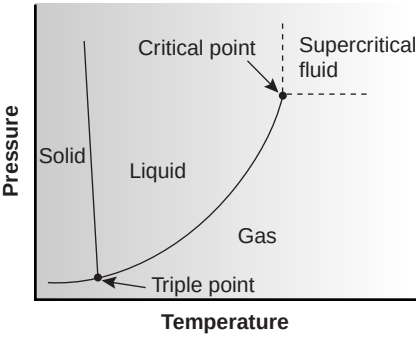


Figure 12.37
Phase diagram for a supercritical fluid.

Colorplate 11 shows the phase transition of liquid CO_2 to supercritical CO_2.

The most common mobile phase for supercritical fluid chromatography is CO_2. Its low critical temperature, 31 °C, and critical pressure, 72.9 atm, are relatively easy to achieve and maintain. Although supercritical CO_2 is a good solvent for nonpolar organics, it is less useful for polar solutes. The addition of an organic modifier, such as methanol, improves the mobile phase's elution strength. Other common mobile phases and their critical temperatures and pressures are listed in Table 12.7.

Table 12.6 Typical Properties of Gases, Liquids, and Supercritical Fluids[a]

Phase	Density (g cm^{-3})	Viscosity (g cm^{-1} s^{-1})	Diffusion coefficient (cm^2 s^{-1})
gas	$\approx 10^{-3}$	$\approx 10^{-4}$	$\approx 10^{-1}$
supercritical fluid	$\approx 0.1–1$	$\approx 10^{-4}–10^{-3}$	$\approx 10^{-4}–10^{-3}$
liquid	≈ 1	$\approx 10^{-2}$	$< 10^{-5}$

[a] Values are reported to the nearest factor of 10.

The instrumentation necessary for supercritical fluid chromatography is essentially the same as that for a standard GC or HPLC. The only important addition is the need for a pressure restrictor to maintain the critical pressure. Gradient elutions, similar to those in HPLC, are accomplished by changing the applied pressure over time. The resulting change in the density of the mobile phase affects its solvent strength. Detection can be accomplished using standard GC detectors or HPLC detectors.

Supercritical fluid chromatography has found many applications in the analysis of polymers, fossil fuels, waxes, drugs, and food products. Its application in the analysis of triglycerides is shown in Figure 12.38.

Table 12.7 Critical Point Properties for Selected Supercritical Fluids

Compound	Critical Temperature (°C)	Critical Pressure (atm)
carbon dioxide	31.3	72.9
ethane	32.4	48.3
nitrous oxide	36.5	71.4
ammonia	132.3	111.3
diethyl ether	193.6	36.3
isopropanol	235.3	47.0
methanol	240.5	78.9
ethanol	243.4	63.0
water	374.4	226.8

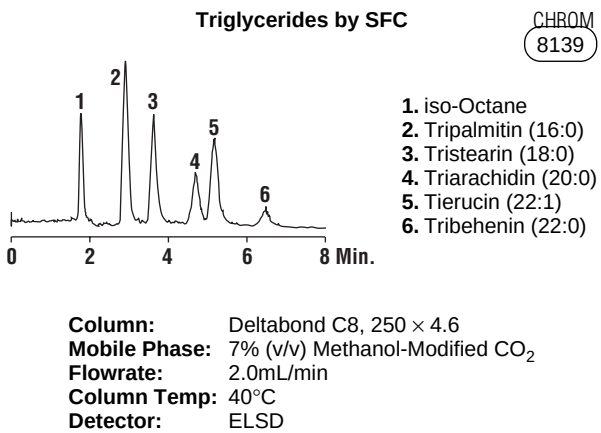


Figure 12.38
Example of the application of supercritical fluid chromatography to the analysis of triglycerides. (Chromatogram courtesy of Alltech Associates, Inc. Deerfield, IL).

12J Electrophoresis

Thus far all the separations we have considered involve a mobile phase and a stationary phase. Separation of a complex mixture of analytes occurs because each analyte has a different ability to partition between the two phases. An analyte whose distribution ratio favors the stationary phase is retained on the column for a longer time, thereby eluting with a longer retention time. Although the methods described in the preceding sections involve different types of stationary and mobile phases, all are forms of chromatography.

Electrophoresis is another class of separation techniques in which analytes are separated based on their ability to move through a conductive medium, usually an aqueous buffer, in response to an applied electric field. In the absence of other effects, cations migrate toward the electric field's negatively charged cathode, and anions migrate toward the positively charged anode. More highly charged ions and ions of smaller size, which means they have a higher charge-to-size ratio, migrate at a faster rate than larger ions, or ions of lower charge. Neutral species do not experience the electric field and remain stationary. As we will see shortly, under normal conditions even neutral species and anions migrate toward the cathode. In either case, differences in their rate of migration allow for the separation of complex mixtures of analytes.

electrophoresis
A separation technique based on a solute's ability to move through a conductive medium under the influence of an electric field.

There are several forms of electrophoresis. In slab gel electrophoresis the conducting buffer is retained within a porous gel of agarose or polyacrylamide. Slabs are formed by pouring the gel between two glass plates separated by spacers. Typical thicknesses are 0.25–1 mm. Gel electrophoresis is an important technique in biochemistry, in which it is frequently used for DNA sequencing. Although it is a powerful tool for the qualitative analysis of complex mixtures, it is less useful for quantitative work.

In **capillary electrophoresis** the conducting buffer is retained within a capillary tube whose inner diameter is typically 25–75 μm. Samples are injected into one end of the capillary tube. As the sample migrates through the capillary, its components separate and elute from the column at different times. The resulting **electropherogram** looks similar to the chromatograms obtained in GC or HPLC and provides

capillary electrophoresis
Electrophoresis taking place in a capillary tube.

electropherogram
The equivalent of a chromatogram in electrophoresis.

both qualitative and quantitative information. Only capillary electrophoretic methods receive further consideration in this text.

12J.1 Theory of Capillary Electrophoresis

In capillary electrophoresis the sample is injected into a buffered solution retained within a capillary tube. When an electric field is applied to the capillary tube, the sample's components migrate as the result of two types of mobility: electrophoretic mobility and electroosmotic mobility. **Electrophoretic mobility** is the solute's response to the applied electric field. As described earlier, cations move toward the negatively charged cathode, anions move toward the positively charged anode, and neutral species, which do not respond to the electric field, remain stationary. The other contribution to a solute's migration is **electroosmotic flow,** which occurs when the buffer solution moves through the capillary in response to the applied electric field. Under normal conditions the buffer solution moves toward the cathode, sweeping most solutes, even anions, toward the negatively charged cathode.

electrophoretic mobility
A measure of a solute's ability to move through a conductive medium in response to an applied electric field (μ_{ep}).

electroosmotic flow
The movement of the conductive medium in response to an applied electric field.

electrophoretic velocity
The velocity with which a solute moves through the conductive medium due to its electrophoretic mobility (ν_{ep}).

Electrophoretic Mobility The velocity with which a solute moves in response to the applied electric field is called its **electrophoretic velocity,** ν_{ep}; it is defined as

$$\nu_{ep} = \mu_{ep} E \qquad \textbf{12.35}$$

where μ_{ep} is the solute's electrophoretic mobility, and E is the magnitude of the applied electric field. A solute's electrophoretic mobility is defined as

$$\mu_{ep} = \frac{q}{6\pi\eta r} \qquad \textbf{12.36}$$

where q is the solute's charge, η is the buffer solvent's viscosity, and r is the solute's radius. Using equations 12.35 and 12.36, we can make several important conclusions about a solute's electrophoretic velocity. Electrophoretic mobility, and, therefore, electrophoretic velocity, is largest for more highly charged solutes and solutes of smaller size. Since q is positive for cations and negative for anions, these species migrate in opposite directions. Neutral species, for which q is 0, have an electrophoretic velocity of 0.

Electroosmotic Mobility When an electric field is applied to a capillary filled with an aqueous buffer, we expect the buffer's ions to migrate in response to their electrophoretic mobility. Because the solvent, H_2O, is neutral, we might reasonably expect it to remain stationary. What is observed under normal conditions, however, is that the buffer solution moves toward the cathode. This phenomenon is called the electroosmotic flow.

Electroosmosis occurs because the walls of the capillary tubing are electrically charged. The surface of a silica capillary contains large numbers of silanol groups (Si–OH). At pH levels greater than approximately 2 or 3, the silanol groups ionize to form negatively charged silanate ions (Si–O^-). Cations from the buffer are attracted to the silanate ions. As shown in Figure 12.39, some of these cations bind tightly to the silanate ions, forming an inner, or fixed, layer. Other cations are more loosely bound, forming an outer, or mobile, layer. Together these two layers are called the double layer. Cations in the outer layer migrate toward the cathode. Because these cations are solvated, the solution is also pulled along, producing the electroosmotic flow.

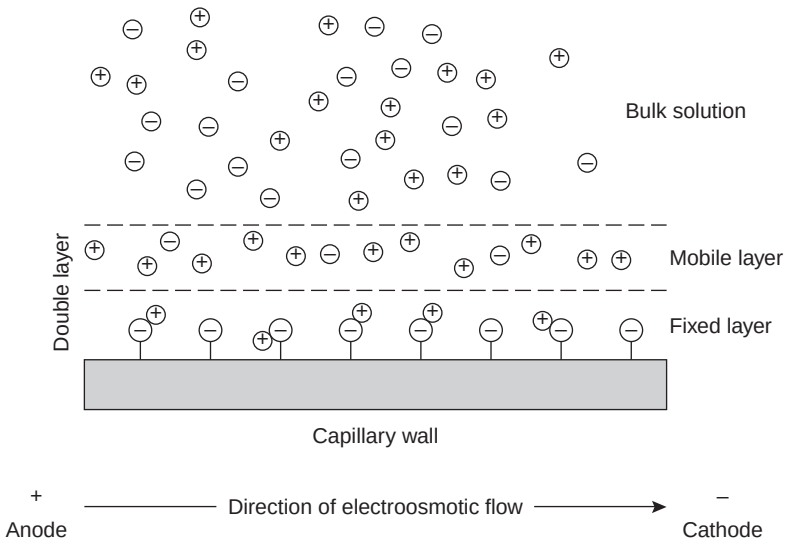


Figure 12.39
Schematic diagram showing the origin of electroosmotic flow.

Electroosmotic flow velocity, ν_{eof}, is a function of the magnitude of the applied electric field and the buffer solution's electroosmotic mobility, μ_{eof}.

electroosmotic flow velocity
The velocity with which the solute moves through the capillary due to the electroosmotic flow (ν_{eof}).

$$\nu_{eof} = \mu_{eof} E \qquad \textbf{12.37}$$

Electroosmotic mobility is defined as

$$\mu_{eof} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad \textbf{12.38}$$

where ε is the buffer solution's dielectric constant, ζ is the zeta potential, and η is the buffer solution's viscosity.

zeta potential
The change in potential across a double layer (ζ).

Examining equations 12.37 and 12.38 shows that the **zeta potential** plays an important role in determining the electroosmotic flow velocity. Two factors determine the zeta potential and, therefore, the electroosmotic velocity. First, the zeta potential is directly proportional to the charge on the capillary walls, with a greater density of silanate ions corresponding to a larger zeta potential. Below a pH of 2, for example, there are few silanate ions; thus, the zeta potential and electroosmotic flow velocity are 0. As the pH level is increased, both the zeta potential and the electroosmotic flow velocity increase. Second, the zeta potential is proportional to the thickness of the double layer. Increasing the buffer solution's ionic strength provides a higher concentration of cations, decreasing the thickness of the double layer.

The electroosmotic flow profile is very different from that for a phase moving under forced pressure. Figure 12.40 compares the flow profile for electroosmosis with that for hydrodynamic pressure. The uniform, flat profile for electroosmosis helps to minimize band broadening in capillary electrophoresis, thus improving separation efficiency.

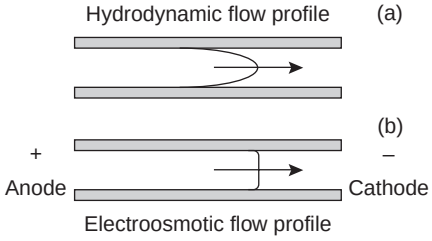


Figure 12.40
Schematic showing a comparison of the flow profiles for (a) GC and HPLC, and (b) electrophoresis.

Total Mobility A solute's net, or total velocity, ν_{tot}, is the sum of its electrophoretic velocity and the electroosmotic flow velocity; thus,

$$\nu_{tot} = \nu_{ep} + \nu_{eof}$$

and

$$\mu_{tot} = \mu_{ep} + \mu_{eof}$$

Under normal conditions the following relationships hold

$$(\nu_{tot})_{cations} > \nu_{eof}$$

$$(\nu_{tot})_{anions} < \nu_{eof}$$

$$(\nu_{tot})_{neutrals} = \nu_{eof}$$

Thus, cations elute first in an order corresponding to their electrophoretic mobilities, with small, highly charged cations eluting before larger cations of lower charge. Neutral species elute as a single band, with an elution rate corresponding to the electroosmotic flow velocity. Finally, anions are the last components to elute, with smaller, highly charged anions having the longest elution time.

Migration Time A solute's total velocity is given by

$$\nu_{tot} = \frac{l}{t_m}$$

where l is the distance the solute travels between its point of injection and the detector, and t_m is the migration time. Since

$$\nu_{tot} = \mu_{tot}E = (\mu_{ep} + \mu_{eof})E$$

we have, after rearranging,

$$t_m = \frac{l}{(\mu_{ep} + \mu_{eof})E} \qquad \textbf{12.39}$$

Finally, the magnitude of the electric field is

$$E = \frac{V}{L} \qquad \textbf{12.40}$$

where V is the applied potential, and L is the length of the capillary tube. Substituting equation 12.40 into equation 12.39 gives

$$t_m = \frac{lL}{(\mu_{ep} + \mu_{eof})V} \qquad \textbf{12.41}$$

Examining equation 12.41 shows that we can decrease a solute's migration time (and thus the total analysis time) by applying a higher voltage or by using a shorter capillary tube. Increasing the electroosmotic flow also shortens the analysis time, but, as we will see shortly, at the expense of resolution.

Efficiency The efficiency of capillary electrophoresis is characterized by the number of theoretical plates, N, just as it is in GC or HPLC. In capillary electrophoresis, the number of theoretic plates is determined by

$$N = \frac{(\mu_{ep} + \mu_{eof})V}{2D} \qquad \textbf{12.42}$$

where D is the solute's diffusion coefficient. From equation 12.42 it is easy to see that the efficiency of a capillary electrophoretic separation increases with higher voltages. Again, increasing the electroosmotic flow velocity improves efficiency, but at the expense of resolution. Two additional observations deserve comment.

First, solutes with larger electrophoretic mobilities (in the same direction as the electroosmotic flow) have greater efficiencies; thus, smaller, more highly charged solutes are not only the first solutes to elute, but do so with greater efficiency. Second, efficiency in capillary electrophoresis is independent of the capillary's length. Typical theoretical plate counts are approximately 100,000–200,000 for capillary electrophoresis.

Selectivity In chromatography, selectivity is defined as the ratio of the capacity factors for two solutes (equation 12.11). In capillary electrophoresis, the analogous expression for selectivity is

$$\alpha = \frac{\mu_{ep,1}}{\mu_{ep,2}}$$

where $\mu_{ep,1}$ and $\mu_{ep,2}$ are the electrophoretic mobilities for solutes 1 and 2, respectively, chosen such that $\alpha \geq 1$. Selectivity often can be improved by adjusting the pH of the buffer solution. For example, NH_4^+ is a weak acid with a pK_a of 9.24. At a pH of 9.24 the concentrations of NH_4^+ and NH_3 are equal. Decreasing the pH below 9.24 increases its electrophoretic mobility because a greater fraction of the solute is present as the cation NH_4^+. On the other hand, raising the pH above 9.24 increases the proportion of the neutral NH_3, decreasing its electrophoretic mobility.

Resolution The resolution between two solutes is

$$R = \frac{0.177(\mu_{ep,2} - \mu_{ep,1})V^{1/2}}{\sqrt{(\mu_{avg} + \mu_{eof})D}} \qquad \textbf{12.43}$$

where μ_{avg} is the average electrophoretic mobility for the two solutes. Examining equation 12.43 shows that increasing the applied voltage and decreasing the electroosmotic flow velocity improves resolution. The latter effect is particularly important because increasing electroosmotic flow improves analysis time and efficiency while decreasing resolution.

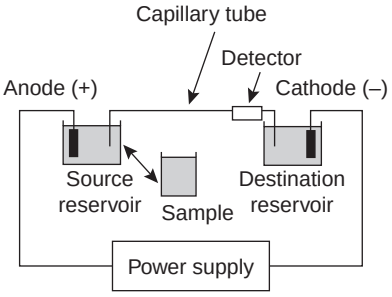


Figure 12.41
Schematic diagram for capillary electrophoresis. The sample and source reservoir are switched when making injections.

12J.2 Instrumentation

The basic instrumentation for capillary electrophoresis is shown in Figure 12.41 and includes a power supply for applying the electric field, anode and cathode compartments containing reservoirs of the buffer solution, a sample vial containing the sample, the capillary tube, and a detector. Each part of the instrument receives further consideration in this section.

Capillary Tubes Figure 12.42 shows a cross section of a typical capillary tube. Most capillary tubes are made from fused silica coated with a 20–35-μm layer of polyimide to give it mechanical strength. The inner diameter is typically 25–75 μm, which is smaller than that for a capillary GC column, with an outer diameter of 200–375 μm.

The narrow bore of the capillary column and the relative thickness of the capillary's walls are important. When an electric field is applied to a capillary containing a conductive medium, such as a buffer solution, current flows through the capillary. This current leads to **Joule heating,** the extent of which is proportional to the capillary's radius and the magnitude of the electric field. Joule heating is a problem because it changes the buffer solution's viscosity, with the solution at the center of the

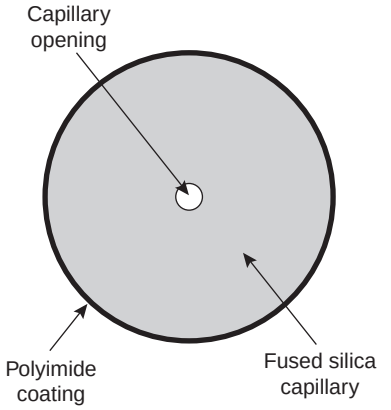


Figure 12.42
Schematic diagram showing a cross section of a capillary column for capillary electrophoresis.

Joule heating
The heating of a conductive solution due to the passage of an electric current through the solution.

capillary being less viscous than that near the capillary walls. Since the solute's electrophoretic mobility depends on the buffer's viscosity (see equation 12.36), solutes in the center of the capillary migrate at a faster rate than solutes near the capillary walls. The result is additional band broadening that degrades the separation. Capillaries with smaller inner diameters generate less Joule heating, and those with larger outer diameters are more effective at dissipating the heat. Capillary tubes may be placed inside a thermostated jacket to control heating, in which case smaller outer diameters allow a more rapid dissipation of thermal energy.

Injecting the Sample The mechanism by which samples are introduced in capillary electrophoresis is quite different from that used in GC or HPLC. Two types of injection are commonly used: hydrodynamic injection and electrokinetic injection. In both cases the capillary tube is filled with buffer solution. One end of the capillary tube is placed in the destination reservoir, and the other is placed in the sample vial.

hydrodynamic injection
An injection technique in capillary electrophoresis in which pressure is used to inject sample into the capillary column.

Hydrodynamic injection uses pressure to force a small portion of the sample into the capillary tubing. To inject a sample hydrodynamically a difference in pressure is applied across the capillary by either pressurizing the sample vial or by applying a vacuum to the destination reservoir. The volume of sample injected, in liters, is given by the following equation

$$V_{inj} = \frac{\Delta P d^4 \pi t}{128 \eta L} \times 10^3 \qquad \textbf{12.44}$$

where ΔP is the pressure difference across the capillary in pascals, d is the capillary's inner diameter in meters, t is the amount of time that the pressure is applied in seconds, η is the buffer solution's viscosity in kilograms per meter per second ($kg\ m^{-1}\ s^{-1}$), and L is the length of the capillary tubing in meters. The factor of 10^3 changes the units from cubic meters to liters.

Example

EXAMPLE 12.9

A hydrodynamic injection is made by applying a pressure difference of 2.5×10^3 Pa (approximately 0.02 atm) for 2 s to a 75-cm long capillary tube with an internal diameter of 50 μm. Assuming that the buffer solution's viscosity is 10^{-3} kg m^{-1} s^{-1}, what volume of sample is injected?

SOLUTION

Making appropriate substitutions into equation 12.44 gives the volume of injected sample as

$$V_{inj} = \frac{(2.5 \times 10^3\ \text{Pa})(50 \times 10^{-6}\ \text{m})^4 (3.14)(2\ \text{s})}{(128)(0.001\ \text{kg m}^{-1}\ \text{s}^{-1})(0.75\ \text{m})} \times 10^3 = 1 \times 10^{-9}\ \text{L} = 1\ \text{nL}$$

Since the injected sample plug is cylindrical, its length, l_{plug}, is easily calculated using the equation for the volume of a cylinder.

$$V = \pi r^2 l_{plug}$$

Thus,

$$l_{plug} = \frac{V}{\pi r^2} = \frac{(1 \times 10^{-9}\ \text{L})(10^{-3}\ \text{m}^3/L)}{(3.14)(25 \times 10^{-6}\ \text{m})^2} = 5 \times 10^{-4}\ \text{m} = 0.5\ \text{mm}$$

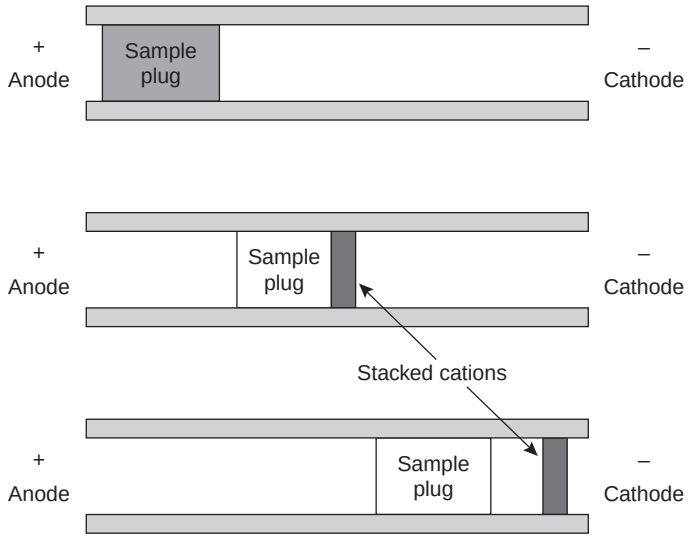


Figure 12.43
Schematic diagram demonstrating stacking.

Electrokinetic injections are made by placing both the capillary and the anode into the sample vial and briefly applying an electric field. The moles of solute injected into the capillary, n_{inj}, are determined using

electrokinetic injection
An injection technique in capillary electrophoresis in which an electric field is used to inject sample into the capillary column.

$$n_{inj} = \pi C t r^2 (\mu_{ep} + \mu_{eof}) E \frac{\kappa_{buf}}{\kappa_{samp}} \qquad \textbf{12.45}$$

where C is the solute's concentration in the sample, t is the amount of time that the electric field is applied, r is the capillary's radius, μ_{ep} is the solute's electrophoretic mobility, μ_{eof} is the electroosmotic mobility, E is the applied electric field, and κ_{buf} and κ_{samp} are the conductivities of the buffer solution and sample, respectively. An important consequence of equation 12.45 is that it is inherently biased toward sampling solutes with larger electrophoretic mobilities. Those solutes with the largest electrophoretic mobilities (smaller, more positively charged ions) are injected in greater numbers than those with the smallest electrophoretic mobilities (smaller, more negatively charged ions).

When a solute's concentration in the sample is too small to reliably analyze, it may be possible to inject the solute in a manner that increases its concentration in the capillary tube. This method of injection is called **stacking.** Stacking is accomplished by placing the sample in a solution whose ionic strength is significantly less than that of the buffering solution. Because the sample plug has a lower concentration of ions than the buffering solution, its resistance is greater. Since the electric current passing through the capillary is fixed, we know from Ohm's law

stacking
A means of concentrating solutes in capillary electrophoresis after their injection onto the capillary column.

$$E = iR$$

that the electric field in the sample plug is greater than that in the buffering solution. Electrophoretic velocity is directly proportional to the electric field (see equation 12.35); thus, ions in the sample plug migrate with a greater velocity. When the solutes reach the boundary between the sample plug and the buffering solution, the electric field decreases and their electrophoretic velocity slows down, "stacking" together in a smaller sampling zone (Figure 12.43).

Applying the Electric Field Migration in electrophoresis occurs in response to the applied electric field. The ability to apply a large electric field is important because

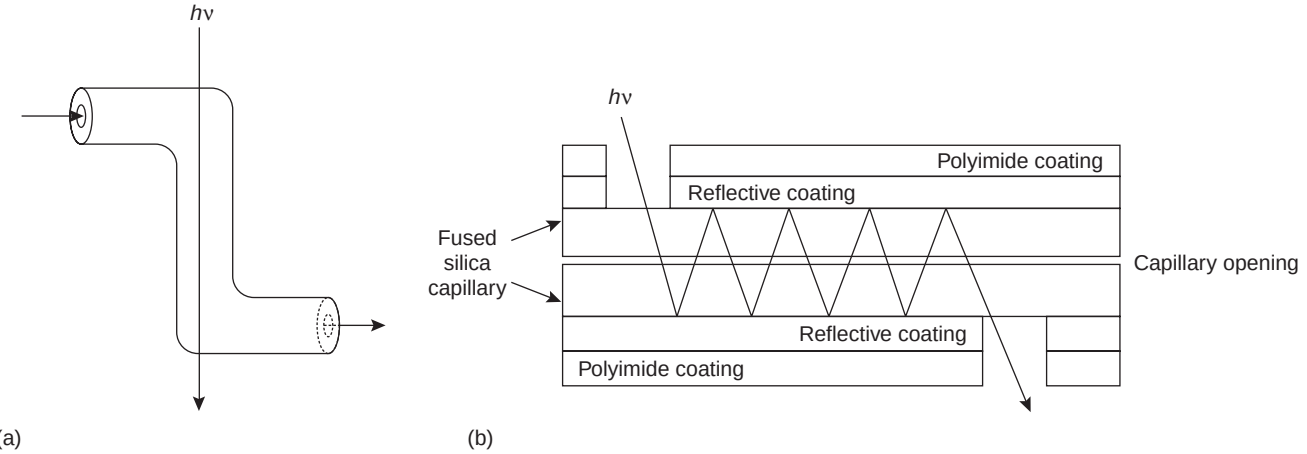


Figure 12.44
Schematic diagrams of two approaches to on-column detection using UV/Vis absorption spectroscopy.

higher voltages lead to shorter analysis times (see equation 12.41), more efficient separations (see equation 12.42), and better resolution (see equation 12.43). Because narrow-bore capillary tubes dissipate Joule heating so efficiently, voltages of up to 40 kV can be applied.

Detectors Most of the detectors used in HPLC also find use in capillary electrophoresis. Among the more common detectors are those based on the absorption of UV/Vis radiation, fluorescence, conductivity, amperometry, and mass spectrometry. Whenever possible, detection is done "on-column" before the solutes elute from the capillary tube and additional band broadening occurs.

UV/Vis detectors are among the most popular. Because absorbance is directly proportional to path length, the capillary tubing's small diameter leads to signals that are smaller than those obtained in HPLC. Several approaches have been used to increase the path length, including a Z-shaped sample cell or multiple reflections (Figure 12.44). Detection limits are about 10^{-7} M.

Better detection limits are obtained using fluorescence, particularly when using a laser as an excitation source. When using fluorescence detection, a small portion of the capillary's protective coating is removed and the laser beam is focused on the inner portion of the capillary tubing. Emission is measured at an angle of 90° to the laser. Because the laser provides an intense source of radiation that can be focused to a narrow spot, detection limits are as low as 10^{-16} M.

Solutes that do not absorb UV/Vis radiation or undergo fluorescence can be detected by other detectors. Table 12.8 provides a list of detectors used in capillary electrophoresis along with some of their important characteristics.

12J.3 Capillary Electrophoresis Methods

There are several different forms of capillary electrophoresis, each of which has its particular advantages. Several of these methods are briefly described in this section.

capillary zone electrophoresis
A form of capillary electrophoresis in which separations are based on differences in the solutes' electrophoretic mobilities.

Capillary Zone Electrophoresis The simplest form of capillary electrophoresis is **capillary zone electrophoresis** (CZE). In CZE the capillary tube is filled with a buffer solution and, after loading the sample, the ends of the capillary tube are placed in reservoirs containing additional buffer solution. Under normal conditions, the end of the capillary containing the sample is the anode, and solutes migrate toward

Table 12.8 Characteristics of Selected Detectors for Capillary Electrophoresis

Detector	Selectivity	Detection Limit: Moles Injected	Detection Limit: Molarity[a]	On-Column Detection?
UV/Vis absorbance	solute must have UV/Vis absorbing chromophore	10^{-13}–10^{-16}	10^{-5}–10^{-7}	yes
indirect absorbance	universal	10^{-12}–10^{-15}	10^{-4}–10^{-6}	yes
fluorescence	solute must have favorable fluorescent quantum efficiency	10^{-15}–10^{-17}	10^{-7}–10^{-9}	yes
laser fluorescence	solute must have favorable fluorescent quantum efficiency	10^{-18}–10^{-20}	10^{-13}–10^{-16}	yes
mass spectrometer	universal when monitoring all ions; selective when monitoring single ion	10^{-16}–10^{-17}	10^{-8}–10^{-10}	no
amperometry	solute must undergo oxidation or reduction	10^{-18}–10^{-19}	10^{-7}–10^{-10}	no
conductivity	universal	10^{-15}–10^{-16}	10^{-7}–10^{-9}	no
radiometric	solutes must be radioactive	10^{-17}–10^{-19}	10^{-10}–10^{-12}	yes

Source: Adapted from Baker, D. R. *Capillary Electrophoresis.* Wiley-Interscience: New York, 1995.[16]
[a]Concentration depends on the volume of sample injected.

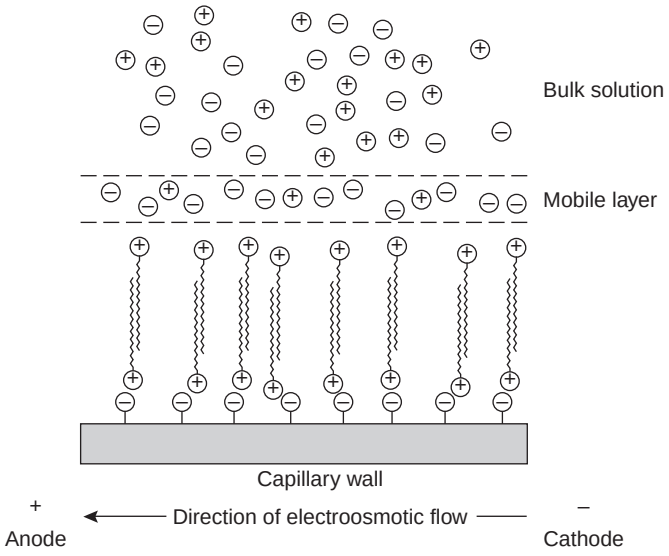


Figure 12.45
Schematic diagram showing the reversal of electroosmotic flow.

the cathode at a velocity determined by their electrophoretic mobility and the electroosmotic flow. Cations elute first, with smaller, more highly charged cations eluting before larger cations with smaller charges. Neutral species elute as a single band. Finally, anions are the last species to elute, with smaller, more negatively charged anions being the last to elute.

The direction of electroosmotic flow and, therefore, the order of elution in CZE can be reversed. This is accomplished by adding an alkylammonium salt to the buffer solution. As shown in Figure 12.45, the positively charged end of the alkylammonium ion binds to the negatively charged silanate ions on the capillary's walls. The alkylammonium ion's "tail" is hydrophobic and associates with the tail of another alkylammonium ion. The result is a layer of positive charges to which anions in the buffer solution are attracted. The migration of these solvated anions toward

the anode reverses the electroosmotic flow's direction. The order of elution in this case is exactly the opposite of that observed under normal conditions.

Capillary zone electrophoresis also can be accomplished without an electroosmotic flow by coating the capillary's walls with a nonionic reagent. In the absence of electroosmotic flow only cations migrate from the anode to the cathode. Anions elute into the source reservoir while neutral species remain stationary.

Capillary zone electrophoresis provides effective separations of any charged species, including inorganic anions and cations, organic acids and amines, and large biomolecules such as proteins. For example, CZE has been used to separate a mixture of 36 inorganic and organic ions in less than 3 minutes.[17] Neutral species, of course, cannot be separated.

micellar electrokinetic capillary chromatography
A form of capillary electrophoresis in which neutral solutes are separated based on their ability to partition into a charged micelle.

micelle
An agglomeration of molecules containing ionic "heads" and hydrophobic "tails," which form into a structure with a hydrophobic interior and a hydrophilic exterior.

Micellar Electrokinetic Capillary Chromatography One limitation to CZE is its inability to separate neutral species. **Micellar electrokinetic chromatography** (MEKC) overcomes this limitation by adding a surfactant, such as sodium dodecylsulfate (Figure 12.46a) to the buffer solution. Sodium dodecylsulfate, (SDS) has a long-chain hydrophobic "tail" and an ionic functional group, providing a negatively charged "head." When the concentration of SDS is sufficiently large, a micelle forms. A **micelle** consists of an agglomeration of 40–100 surfactant molecules in which the hydrocarbon tails point inward, and the negatively charged heads point outward (Figure 12.46b).

Because micelles are negatively charged, they migrate toward the cathode with a velocity less than the electroosmotic flow velocity. Neutral species partition themselves between the micelles and the buffer solution in much the same manner as they do in HPLC. Because there is a partitioning between two phases, the term "chromatography" is used. Note that in MEKC both phases are "mobile."

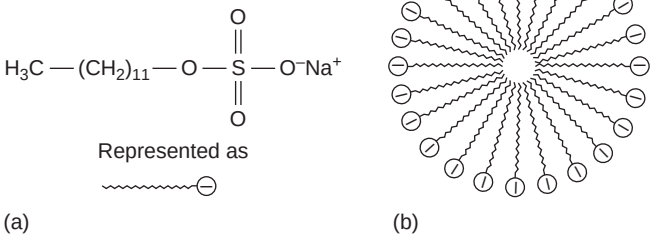


Figure 12.46
(a) Structure of sodium dodecylsulfate; (b) structure of a micelle.

The elution order for neutral species in MEKC depends on the extent to which they partition into the micelles. Hydrophilic neutrals are insoluble in the micelle's hydrophobic inner environment and elute as a single band as they would in CZE. Neutral solutes that are extremely hydrophobic are completely soluble in the micelle, eluting with the micelles as a single band. Those neutral species that exist in a partition equilibrium between the buffer solution and the micelles elute between the completely hydrophilic and completely hydrophobic neutrals. Those neutral species favoring the buffer solution elute before those favoring the micelles. Micellar electrokinetic chromatography has been used to separate a wide variety of samples, including mixtures of pharmaceutical compounds, vitamins, and explosives.

capillary gel electrophoresis
A form of capillary electrophoresis in which the capillary column contains a gel enabling separations based on size.

Capillary Gel Electrophoresis In **capillary gel electrophoresis** (CGE) the capillary tubing is filled with a polymeric gel. Because the gel is porous, solutes migrate through the gel with a velocity determined both by their electrophoretic mobility and their size. The ability to effect a separation based on size is useful when the solutes have similar electrophoretic mobilities. For example, fragments of DNA of varying length have similar charge-to-size ratios, making their separation by CZE difficult. Since the DNA fragments are of different size, a CGE separation is possible.

The capillary used for CGE is usually treated to eliminate electroosmotic flow, thus preventing the gel's extrusion from the capillary tubing. Samples are injected

electrokinetically because the gel provides too much resistance for hydrodynamic sampling. The primary application of CGE is the separation of large biomolecules, including DNA fragments, proteins, and oligonucleotides.

Capillary Electrochromatography Another approach to separating neutral species is **capillary electrochromatography** (CEC). In this technique the capillary tubing is packed with 1.5–3-μm silica particles coated with a bonded, nonpolar stationary phase. Neutral species separate based on their ability to partition between the stationary phase and the buffer solution (which, due to electroosmotic flow, is the mobile phase). Separations are similar to the analogous HPLC separation, but without the need for high-pressure pumps. Furthermore, efficiency in CEC is better than in HPLC, with shorter analysis times.

capillary electrochromatography A form of capillary electrophoresis in which a stationary phase is included within the capillary column.

12J.4 Representative Method

Although each capillary electrophoretic method has its own unique considerations, the following description of the determination of a vitamin B complex provides an instructive example of a typical procedure.

Representative Methods

Method 12.3 Determination of a Vitamin B Complex by CZE or MEKC[18]

Description of Method. The water-soluble vitamins B_1 (thiamine hydrochloride), B_2 (riboflavin), B_3 (niacinamide), and B_6 (pyridoxine hydrochloride) may be determined by CZE using a pH 9 sodium tetraborate/sodium dihydrogen phosphate buffer or by MEKC using the same buffer with the addition of sodium dodecylsulfate. Detection is by UV absorption at 200 nm. An internal standard of *o*-ethoxybenzamide is used to standardize the method.

Procedure. A vitamin B complex tablet is crushed and placed in a beaker with 20.00 mL of a 50% v/v methanol solution that is 20 mM in sodium tetraborate and contains 100.0 ppm of *o*-ethoxybenzamide. After mixing for 2 min to ensure that the B vitamins are dissolved, a 5.00-mL portion is passed through a 0.45-μm filter to remove insoluble binders. An approximately 4-nL sample is loaded into a 50-μm internal diameter capillary column. For CZE the capillary column contains a 20 mM pH 9 sodium tetraborate/sodium dihydrogen phosphate buffer. For MEKC the buffer is also 150 mM in sodium dodecylsulfate. A 40-kV/m electric field is used to effect both the CZE and MEKC separations.

Questions

1. **Methanol, which elutes at 4.69 min, is included as a neutral species to indicate the electroosmotic flow. When using standard solutions of each vitamin, CZE peaks are found at 3.41 min, 4.69 min, 6.31 min, and 8.31 min. Examine the structures and pK_a information in Figure 12.47, and determine the order in which the four B vitamins elute.**

   Vitamin B_1 is a cation and must, therefore, elute before the neutral species methanol; thus it elutes first at 3.41 min. Vitamin B_3 is a neutral species and should elute with methanol at 4.69 min. The remaining two B vitamins are weak acids that partially ionize in the pH 9 buffer. Of the two, vitamin B_6 is the stronger acid and is ionized (as the anion) to a greater extent. Vitamin B_6, therefore, is the last of the vitamins to elute.

—Continued

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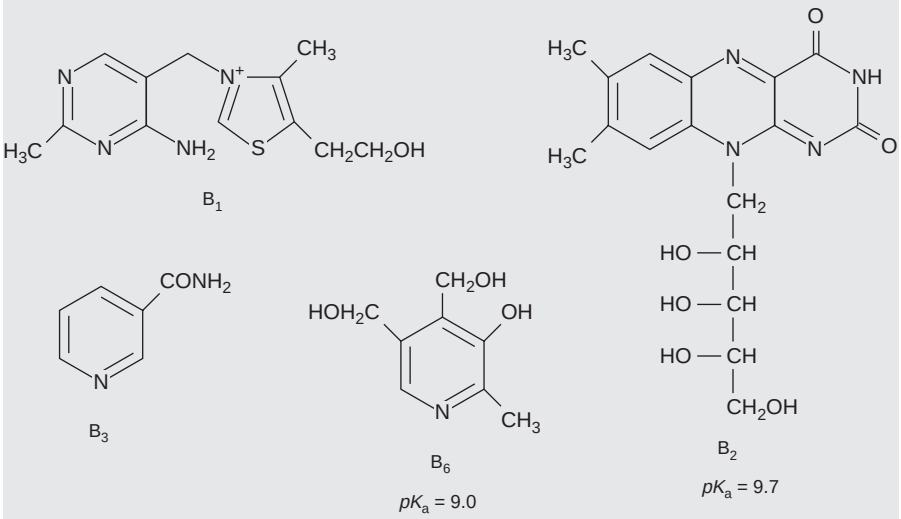

Figure 12.47
Structures of the vitamins B_1, B_2, B_3, and B_6.

2. **The order of elution when using MEKC is vitamin B_3 (5.58 min), vitamin B_6 (6.59 min), vitamin B_2 (8.81 min), and vitamin B_1 (11.21 min). What conclusions can you make about the solubility of the B vitamins in the sodium dodecylsulfate micelles?**

   The elution time for vitamin B_1 shows the greatest change, increasing from 3.41 min to 11.21 min. Clearly vitamin B_1 has the greatest solubility in the micelles. Vitamins B_2 and B_3 have a more limited solubility in the micelles, showing slightly longer elution times. Interestingly, the elution time for vitamin B_6 decreases in the presence of the micelles.

3. **A quantitative analysis for vitamin B_1 was carried out using this procedure. When a solution of 100.0 ppm B_1 and 100.0 ppm *o*-ethoxybenzamide was analyzed, the peak area for vitamin B_1 was 71% of that for the internal standard. The analysis of a 0.125-g vitamin B complex tablet gave a peak area for vitamin B_1 that was 1.82 times as great as that for the internal standard. How many milligrams of vitamin B_1 are in the tablet?**

   For an internal standardization the relevant equation is

$$\frac{S_A}{S_{IS}} = k\frac{C_A}{C_{IS}}$$

   where S_A and S_{IS} are, respectively, the signals for the analyte and internal standard, and C_A and C_{IS} are their respective concentrations. Making appropriate substitutions for the standard solution

$$\frac{71}{100} = k \times \frac{100.0\text{ ppm}}{100.0\text{ ppm}}$$

   gives k as 0.71. Substituting values for the sample

$$\frac{1.82}{1} = 0.71 \times \frac{C_A}{100.0\text{ ppm}}$$

   gives the concentration of vitamin B_1 as 256 ppm. This is the concentration in the sample as injected. To determine the number of milligrams of vitamin B_1, we must account for the sample's dissolution; thus

$$\frac{256\text{ mg}}{\text{L}} \times 0.0200\text{ L} = 5.1\text{ mg vitamin } B_1$$

12J.5 Evaluation

When compared with GC and HPLC, capillary electrophoresis provides similar levels of accuracy, precision, and sensitivity and a comparable degree of selectivity. The amount of material injected into a capillary electrophoretic column is significantly smaller than that for GC and HPLC; typically 1 nL versus 0.1 μL for capillary GC and 1–100 μL for HPLC. Detection limits for capillary electrophoresis, however, are 100–1000 times poorer than those for GC and HPLC. The most significant advantages of capillary electrophoresis are improvements in separation efficiency, time, and cost. Capillary electrophoretic columns contain substantially more theoretical plates ($\approx 10^6$ plates/m) than that found in HPLC ($\approx 10^5$ plates/m) and capillary GC columns ($\approx 10^3$ plates/m), providing unparalleled resolution and peak capacity. Separations in capillary electrophoresis are fast and efficient. Furthermore, the capillary column's small volume means that a capillary electrophoresis separation requires only a few microliters of buffer solution, compared with 20–30 mL of mobile phase for a typical HPLC separation.

12K KEY TERMS

adjusted retention time (*p. 551*)
band broadening (*p. 553*)
baseline width (*p. 548*)
bleed (*p. 566*)
bonded stationary phase (*p. 580*)
capacity factor (*p. 551*)
capillary column (*p. 562*)
capillary electrochromatography (*p. 607*)
capillary electrophoresis (*p. 597*)
capillary gel electrophoresis (*p. 606*)
capillary zone electrophoresis (*p. 604*)
chromatogram (*p. 548*)
chromatography (*p. 546*)
column chromatography (*p. 546*)
countercurrent extraction (*p. 546*)
cryogenic focusing (*p. 568*)
electrokinetic injection (*p. 603*)
electron capture detector (*p. 570*)
electroosmotic flow (*p. 598*)
electroosmotic flow velocity (*p. 599*)
electropherogram (*p. 597*)
electrophoresis (*p. 597*)
electrophoretic mobility (*p. 598*)
electrophoretic velocity (*p. 598*)
exclusion limit (*p. 595*)
flame ionization detector (*p. 570*)
fronting (*p. 555*)
gas chromatography (*p. 563*)
gas–liquid chromatography (*p. 564*)
gradient elution (*p. 558*)
guard column (*p. 579*)
headspace sampling (*p. 567*)
high-performance liquid chromatography (*p. 578*)
hydrodynamic injection (*p. 602*)
inclusion limit (*p. 593*)
ion-exchange chromatography (*p. 590*)
ion-suppressor column (*p. 592*)
isocratic elution (*p. 582*)
Joule heating (*p. 601*)
Kovat's retention index (*p. 575*)
liquid–solid adsorption chromatography (*p. 590*)
longitudinal diffusion (*p. 560*)
loop injector (*p. 584*)
mass spectrum (*p. 571*)
mass transfer (*p. 561*)
micellar electrokinetic capillary chromatography (*p. 606*)
micelle (*p. 606*)
mobile phase (*p. 546*)
normal-phase chromatography (*p. 580*)
on-column injection (*p. 568*)
open tubular column (*p. 564*)
packed column (*p. 564*)
peak capacity (*p. 554*)
planar chromatography (*p. 546*)
polarity index (*p. 580*)
resolution (*p. 549*)
retention time (*p. 548*)
retention volume (*p. 548*)
reverse-phase chromatography (*p. 580*)
selectivity factor (*p. 552*)
single-column ion chromatography (*p. 593*)
size-exclusion chromatography (*p. 593*)
solid-phase microextraction (*p. 567*)
split injection (*p. 568*)
splitless injection (*p. 568*)
stacking (*p. 603*)
stationary phase (*p. 546*)
supercritical fluid chromatography (*p. 596*)
support-coated open tubular column (*p. 565*)
tailing (*p. 555*)
temperature programming (*p. 558*)
theoretical plate (*p. 553*)
thermal conductivity detector (*p. 569*)
van Deemter equation (*p. 561*)
void time (*p. 549*)
void volume (*p. 549*)
wall-coated open tubular column (*p. 565*)
zeta potential (*p. 599*)

12L SUMMARY

Chromatography and electrophoresis are powerful analytical techniques that can separate a sample into its components while providing a means for determining their concentration. Chromatographic separations utilize the selective partitioning of the sample's components between a stationary phase that is immobilized within a column and a mobile phase that passes through the column.

The effectiveness of a separation is described by the resolution between the chromatographic bands for two components and is a function of the component's capacity factor, the column's efficiency, and the column's selectivity. A component's capacity factor is a measure of the degree to which it successfully partitions into the stationary phase, with larger capacity factors corresponding to more strongly retained components. The column's selectivity for two components is the ratio of the component's capacity factors, providing a relative measure of the column's ability to retain the two components. Column efficiency accounts for those factors that cause a component's chromatographic band to increase in width during the separation. Column efficiency is defined in terms of the number of theoretical plates and the height of a theoretical plate, the latter of which is a function of a number of parameters, most notably the mobile phase's flow rate. Chromatographic separations are optimized by increasing the number of theoretical plates, increasing the column's selectivity, or increasing the component's capacity factors.

In gas chromatography (GC) the mobile phase is an inert gas, and the stationary phase is a nonpolar or polar organic liquid that is either coated on a particulate material and packed into a wide-bore column or coated on the walls of a narrow-bore capillary column. Gas chromatography is useful for the analysis of volatile components.

In high-performance liquid chromatography (HPLC) the mobile phase is either a nonpolar solvent (normal phase) or a polar solvent (reverse phase). A stationary phase of opposite polarity, which is bonded to a particulate material, is packed into a wide-bore column. HPLC can be applied to a wider range of samples than GC; however, the separation efficiency for HPLC is not as good as that for GC.

Together, GC and HPLC account for the largest number of chromatographic separations. Other separation techniques, however, find specialized applications. Of particular importance are: ion-exchange chromatography, which is useful for separating anions and cations; size-exclusion chromatography, which is useful for separating large molecules; and supercritical fluid chromatography, which combines many of the advantages of GC and HPLC for the analysis of materials that are not easily analyzed by either of these methods.

In capillary zone electrophoresis a sample's components are separated based on their ability to move through a conductive medium under the influence of an applied electric field. Because of the effect of electroosmotic flow, positively charged solutes elute first, with smaller, more highly charged cationic solutes eluting before larger cations of lower charge. Neutral species elute without undergoing further separation. Finally, anions elute last, with smaller, more negatively charged anions being the last to elute. By adding a surfactant, neutral species also can be separated by micellar electrokinetic capillary chromatography. Electrophoretic separations also can take advantage of the ability of polymeric gels to separate solutes by size (capillary gel electrophoresis) and the ability of solutes to partition into a stationary phase (capillary electrochromatography). In comparison to GC and HPLC, capillary electrophoresis provides faster and more efficient separations.

12M *Suggested* EXPERIMENTS

Experiments

The following experiments may be used to illustrate the application of chromatography and electrophoresis to a number of different types of samples. Experiments are grouped by the type of technique, and each is briefly annotated.

The first set of experiments describes the application of gas chromatography. These experiments encompass a variety of different types of samples, columns, and detectors. Most experiments may be easily modified to use available equipment and detectors.

Elderd, D. M.; Kildahl, N. K.; Berka, L. H. "Experiments for Modern Introductory Chemistry: Identification of Arson Accelerants by Gas Chromatography," *J. Chem. Educ.* **1996,** *73,* 675–677.

Although aimed at the introductory class, this simple experiment provides a nice demonstration of the use of GC for a qualitative analysis. Students obtain chromatograms for several possible accelerants using headspace sampling and then analyze the headspace over a sealed sample of charred wood to determine the accelerant used in burning the wood. Separations are carried out using a wide-bore capillary column with a stationary phase of methyl 50% phenyl silicone and a flame ionization detector.

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Experiments

Graham, R. C.; Robertson, J. K. "Analysis of Trihalomethanes in Soft Drinks," *J. Chem. Educ.* **1988,** *65,* 735–737.

Trihalomethanes are extracted from soft drinks using a liquid–liquid extraction with pentane. Samples are analyzed using a packed column containing 20% OV-101 on 80/100 mesh Gaschrom Q equipped with an electron capture detector.

Kegley, S. E.; Hansen, K. J.; Cunningham, K. L. "Determination of Polychlorinated Biphenyls (PCBs) in River and Bay Sediments," *J. Chem. Educ.* **1996,** *73,* 558–562.

This somewhat lengthy experiment provides a thorough introduction to the use of GC for the analysis of trace-level environmental pollutants. Sediment samples are extracted by sonicating with 3 × 100-mL portions of 1:1 acetone:hexane. The extracts are then filtered and concentrated before bringing to a final volume of 10 mL. Samples are analyzed with a capillary column using a stationary phase of 5% phenylmethyl silicone, a splitless injection, and an ECD detector.

Quach, D. T.; Ciszkowski, N. A.; Finlayson-Pitts, B. J. "A New GC-MS Experiment for the Undergraduate Instrumental Analysis Laboratory in Environmental Chemistry: Methyl-*t*-butyl Ether and Benzene in Gasoline," *J. Chem. Educ.* **1998,** *75,* 1595–1598.

This experiment describes the determination of methyl-*t*-butyl ether and benzene in gasoline using the method of standard additions. Two compounds naturally present at high concentration (*o*-xylene and toluene) are used as internal standards to correct for variations in the amount of sample injected into the GC. Because of the complexity of gasoline, single-ion monitoring is used to determine the signals for the analytes and internal standards. Separations are carried out using a capillary column with a stationary phase of 5% diphenyl/95% dimethylsiloxane.

Rice, G. W. "Determination of Impurities in Whiskey Using Internal Standard Techniques," *J. Chem. Educ.* **1987,** *64,* 1055–1056.

An internal standard of 1-butanol is used to determine the concentrations of one or more of the following impurities commonly found in whiskey: acetaldehyde, methanol, ethyl acetate, 1-propanol, 2-methyl-1-propanol, acetic acid, 2-methyl-1-butanol and 3-methyl-1-butanol. A packed column using 5% Carbowax 20m on 80/120 Carbopak B and an FID detector were used.

Rubinson, J. F.; Neyer-Hilvert, J. "Integration of GC-MS Instrumentation into the Undergraduate Laboratory: Separation and Identification of Fatty Acids in Commercial Fats and Oils," *J. Chem. Educ.* **1997,** *74,* 1106–1108.

Fatty acids from commercial fats and oils, such as peanut oil, are extracted with methanolic NaOH and made volatile by derivatizing with a solution of methanol/BF_3. Separations are carried out using a capillary 5% phenylmethyl silicone column with MS detection. By searching the associated spectral library students are able to identify the fatty acids present in their sample. Quantitative analysis is by external standards.

Rudzinski, W. E.; Beu, S. "Gas Chromatographic Determination of Environmentally Significant Pesticides," *J. Chem. Educ.* **1982,** *59,* 614–615.

Students analyze samples of orange juice that have been spiked with diazinon, malathion, and ethion. Samples are extracted with acetonitrile and then extracted with pet ether. The pesticide residues are then purified using an activated magnesium silicate (Florisil) column, eluting the pesticides with mixtures of pet ether and ethyl ether. After removing most of the solvent, samples are analyzed by GC using a packed glass column containing 1.5% SP-2250/1.95% SP-2401 on 100/120 Supelcoport. Both electron capture and flame ionization detection are used.

Welch, W. C.; Greco, T. G. "An Experiment in Manual Multiple Headspace Extraction for Gas Chromatography," *J. Chem. Educ.* **1993,** *70,* 333–335.

The principle of headspace sampling is introduced in this experiment using a mixture of methanol, chloroform, 1,2-dichloroethane, 1,1,1-trichloroethane, benzene, toluene, and *p*-xylene. Directions are given for evaluating the distribution coefficient for the partitioning of a volatile species between the liquid and vapor phase and for its quantitative analysis in the liquid phase. Both packed (OV-101) and capillary (5% phenyl silicone) columns were used. The GC is equipped with a flame ionization detector.

Another experiment with the same focus is Ramachandran, B. R.; Allen, J. M.; Halpern, A. M. "Air-Water Partitioning of Environmentally Important Organic Compounds," *J. Chem. Educ.* **1996,** *73,* 1058–1061.

This experiment provides an alternative approach to measuring the partition coefficient (Henry's law constant) for volatile organic compounds in water. A OV-101 packed column and flame ionization detector are used.

Williams, K. R.; Pierce, R. E. "The Analysis of Orange Oil and the Aqueous Solubility of *d*-Limonene," *J. Chem. Educ.* **1998,** *75,* 223–226.

Two experiments are described in this paper. In the first experiment students determine the %w/w orange oil in a prepared sample by analyzing for *d*-limonene using anisole as an internal standard. Separations are accomplished using

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Experiments

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a megabore open tubular column with a 5% phenylmethyl silicone bonded stationary phase and a thermal conductivity detector. In the second experiment the solubility of *d*-limonene is determined by equilibrating different volumes of *d*-limonene with water and measuring the amount of *d*-limonene in the overlying vapor phase using headspace sampling.

Wong, J. W.; Ngim, K. K.; Shibamoto, T.; et al. "Determination of Formaldehyde in Cigarette Smoke," *J. Chem. Educ.* **1997,** *74,* 1100–1103.

Formaldehyde from cigarette smoke is collected by trapping the smoke in a 1-L separatory funnel and extracting into an aqueous solution. To aid in its detection, cysteamine is included in the aqueous extracting solution, leading to the formation of a thiazolidine derivative. Samples are analyzed using a DB-1 capillary column with a thermionic or flame photometric detector. Directions also are given for using an HPLC. Formaldehyde is derivatized using 2,4-dinitrophenylhydrazine, and samples are analyzed using a C_{18} column with a UV detector set to 365 nm.

Yang, M. J.; Orton, M. L., Pawliszyn, J. "Quantitative Determination of Caffeine in Beverages Using a Combined SPME-GC/MS Method," *J. Chem. Educ.* **1997,** *74,* 1130–1132.

Caffeine in coffee, tea, and soda is determined by a solid-phase microextraction using an uncoated silica fiber, followed by a GC analysis using a capillary SPB-5 column with an MS detector. Standard solutions are spiked with $^{13}C_3$ caffeine as an internal standard.

The second set of experiments describes the application of high-performance liquid chromatography. These experiments encompass a variety of different types of samples and a variety of common detectors.

Bidlingmeyer, B. A.; Schmitz, S. "The Analysis of Artificial Sweeteners and Additives in Beverages by HPLC," *J. Chem. Educ.* **1991,** *68,* A195–A200.

The concentrations of benzoic acid, aspartame, caffeine, and saccharin in a variety of beverages are determined in this experiment. A C_{18} column and a mobile phase of 80% v/v acetic acid (pH = 4.2) and 20% v/v methanol are used to effect the separation. A UV detector set to 254 nm is used to measure the eluent's absorbance. The ability to adjust retention times by changing the mobile phase's pH is also explored.

DiNunzio, J. E. "Determination of Caffeine in Beverages by High Performance Liquid Chromatography," *J. Chem. Educ.* **1985,** *62,* 446–447.

The concentration of caffeine in a typical serving of coffee and soda is determined in this experiment. Separations are achieved using a C_{18} column with a mobile phase of 30% v/v methanol in water, with UV detection at a wavelength of 254 nm.

Ferguson, G. K. "Quantitative HPLC Analysis of an Analgesic/Caffeine Formulation: Determination of Caffeine," *J. Chem. Educ.* **1998,** *75,* 467–469.

The %w/w caffeine in an analgesic formulation is determined in this experiment. The separation uses a C_{18} column with a mobile phase of 94.1% v/v water, 5.5% v/v acetonitrile, 0.2% v/v triethylamine, and 0.2% v/v acetic acid. A UV detector is set to 254 nm.

Ferguson, G. K. "Quantitative HPLC Analysis of a Psychotherapeutic Medication: Simultaneous Determination of Amitriptyline Hydrochloride and Perphenazine," *J. Chem. Educ.* **1998,** *75,* 1615–1618.

This experiment describes a quantitative analysis for the active ingredients in a prescription antipsychotic medication. The separation makes use of a cyanopropyl derivatized column and a mobile phase of 70% v/v acetonitrile, 5% v/v methanol, and 25% v/v 0.1 M aqueous KH_2PO_4. A UV detector set to 215 nm is used to measure the eluent's absorbance.

Haddad, P.; Hutchins, S.; Tuffy, M. "High Performance Liquid Chromatography of Some Analgesic Compounds," *J. Chem. Educ.* **1983,** *60,* 166–168.

This experiment focuses on developing an HPLC separation capable of distinguishing acetylsalicylic acid, paracetamol, salicylamide, caffeine, and phenacetin. A C_{18} column and UV detection are used to obtain chromatograms. Solvent parameters used to optimize the separation include the pH of the buffered aqueous mobile phase, the %v/v methanol added to the aqueous mobile phase, and the use of tetrabutylammonium phosphate as an ion-pairing reagent.

Mueller, B. L.; Potts, L. W. "HPLC Analysis of an Asthma Medication," *J. Chem. Educ.* **1988,** *65,* 905–906.

This experiment describes the quantitative analysis of the asthma medication Quadrinal for the active ingredients theophylline, salicylic acid, phenobarbital, ephedrine HCl, and potassium iodide. Separations are carried out using a C_{18} column with a mobile phase of 19% v/v acetonitrile, 80% v/v water, and 1% acetic acid. A small amount of triethylamine (0.03% v/v) is included to ensure the elution of ephedrine HCl. A UV detector set to 254 nm is used to record the chromatogram.

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Experiments

Remcho, V. T.; McNair, H. M.; Rasmussen, H. T. "HPLC Method Development with the Photodiode Array Detector," *J. Chem. Educ.* **1992,** *69,* A117–A119.

A mixture of methyl paraben, ethyl paraben, propyl paraben, diethyl phthalate, and butyl paraben is separated by HPLC. This experiment emphasizes the development of a mobile-phase composition capable of separating the mixture. A photodiode array detector demonstrates the coelution of the two compounds.

Siturmorang, M.; Lee, M. T. B.; Witzeman, L. K.; et al. "Liquid Chromatography with Electrochemical Detection (LC-EC): An Experiment Using 4-Aminophenol," *J. Chem. Educ.* **1998,** *75,* 1035–1038.

The use of an amperometric detector is emphasized in this experiment. Hydrodynamic voltammetry (see Chapter 11) is first performed to identify a potential for the oxidation of 4-aminophenol without an appreciable background current due to the oxidation of the mobile phase. The separation is then carried out using a C_{18} column and a mobile phase of 50% v/v pH 5, 20 mM acetate buffer with 0.02 M $MgCl_2$, and 50% v/v methanol. The analysis is easily extended to a mixture of 4-aminophenol, ascorbic acid, and catechol, and to the use of a UV detector.

Tran, C. D.; Dotlich, M. "Enantiomeric Separation of Beta-Blockers by High Performance Liquid Chromatography," *J. Chem. Educ.* **1995,** *72,* 71–73.

This experiment introduces the use of a chiral column (a β-cyclodextrin-bonded C_{18} column) to separate the beta-blocker drugs Inderal LA (*S*-propranolol and *R*-propranolol), Tenormim (DL-atenolol) and Lopressor (DL-metaprolol). The mobile phase was 90:10 (v/v) acetonitrile and water. A UV detector set to 254 nm is used to obtain the chromatogram.

Van Arman, S. A.; Thomsen, M. W. "HPLC for Undergraduate Introductory Laboratories," *J. Chem. Educ.* **1997,** *74,* 49–50.

In this experiment students analyze an artificial RNA digest consisting of cytidine, uridine, thymidine, guanosine, and adenosine using a C_{18} column and a mobile phase of 0.4% v/v triethylammonium acetate, 5% v/v methanol, and 94.6% v/v water. The chromatogram is recorded using a UV detector at a wavelength of 254 nm.

Wingen, L. M.; Low, J. C.; Finlayson-Pitts, B. J. "Chromatography, Absorption, and Fluorescence: A New Instrumental Analysis Experiment on the Measurement of Polycyclic Aromatic Hydrocarbons in Cigarette Smoke," *J. Chem. Educ.* **1998,** *75,* 1599–1603.

The analysis of cigarette smoke for 16 different polyaromatic hydrocarbons is described in this experiment. Separations are carried out using a polymeric bonded silica column with a mobile phase of 50% v/v water, 40% v/v acetonitrile, and 10% v/v tetrahydrofuran. A notable feature of this experiment is the evaluation of two means of detection. The ability to improve sensitivity by selecting the optimum excitation and emission wavelengths when using a fluorescence detector is demonstrated. A comparison of fluorescence detection with absorbance detection shows that better detection limits are obtained when using fluorescence.

The third set of experiments provides a few representative applications of ion chromatography.

Bello, M. A.; Gustavo González, A. "Determination of Phosphate in Cola Beverages Using Nonsuppressed Ion Chromatography," *J. Chem. Educ.* **1996,** *73,* 1174–1176.

In this experiment phosphate is determined by single-column, or nonsuppressed, ion chromatography using an anionic column and a conductivity detector. The mobile phase is a mixture of *n*-butanol, acetonitrile, and water (containing sodium gluconate, boric acid, and sodium tetraborate).

Kieber, R. J.; Jones, S. B. "An Undergraduate Laboratory for the Determination of Sodium, Potassium, and Chloride," *J. Chem. Educ.* **1994,** *71,* A218–A222.

Three techniques, one of which is ion chromatography, are used to determine the concentrations of three ions in solution. The combined concentrations of Na^+ and K^+ are determined by an ion exchange with H^+, the concentration of which is subsequently determined by an acid–base titration using NaOH. Flame atomic absorption is used to measure the concentration of Na^+, and K^+ is determined by difference. The concentration of Cl^- is determined by ion-exchange chromatography on an anionic column using a mobile phase of HCO_3^- and CO_3^{2-} with ion suppression. A conductivity detector is used to record the chromatogram.

Koubek, E.; Stewart, A. E. "The Analysis of Sulfur in Coal," *J. Chem. Educ.* **1992,** *69,* A146–A148.

Sulfur in coal is converted into a soluble sulfate by heating to 800 °C in the presence of MgO and Na_2CO_3. After dissolving in water, the concentration of sulfate is determined by single-column ion chromatography, using an anionic column and a mobile phase of 1 mM potassium hydrogen phthalate. A conductivity detector is used to record the chromatogram.

Luo, P.; Luo, M. A.; Baldwin, R. P. "Determination of Sugars in Food Products," *J. Chem. Educ.,* **1993,** *70,* 679–681.

—Continued

Experiments

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The concentrations of nine sugars (fucose, methylglucose, arabinose, glucose, fructose, lactose, sucrose, cellobiose, and maltose) in beer, milk, and soda are determined using an anionic column and a mobile phase of 0.1 M NaOH. Detection is by amperometry at a Cu working electrode.

The last set of experiments provides examples of the application of capillary electrophoresis. These experiments encompass a variety of different types of samples and include examples of capillary zone electrophoresis and micellar electrokinetic chromatography.

Conradi, S.; Vogt, C.; Rohde, E. "Separation of Enatiomeric Barbiturates by Capillary Electrophoresis Using a Cyclodextrin-Containing Run Buffer," *J. Chem. Educ.* **1997,** *74,* 1122–1125.

In this experiment the enantiomers of cyclobarbital and thiopental, and phenobarbital are separated using MEKC with cyclodextran as a chiral selector. By adjusting the pH of the buffer solution and the concentration and type of cyclodextran, students are able to find conditions in which the enantiomers of cyclobarbital and thiopental are resolved.

Conte, E. D.; Barry, E. F.; Rubinstein, H. "Determination of Caffeine in Beverages by Capillary Zone Electrophoresis," *J. Chem. Educ.* **1996,** *73,* 1169–1170.

Caffeine in tea and coffee is determined by CZE using nicotine as an internal standard. The buffer solution is 50 mM sodium borate adjusted to pH 8.5 with H_3PO_4. A UV detector set to 214 nm is used to record the electropherograms.

Hage, D. S.; Chattopadhyay, A.; Wolfe, C. A. C.; et al. "Determination of Nitrate and Nitrite in Water by Capillary Electrophoresis," *J. Chem. Educ.* **1998,** *75,* 1588–1590.

In this experiment the concentrations of NO_2^- and NO_3^- are determined by CZE using IO_4^- as an internal standard. The buffer solution is 0.60 M sodium acetate buffer adjusted to a pH of 4.0. A UV detector set to 222 nm is used to record the electropherogram.

Janusa, M. A.; Andermann, L. J.; Kliebert, N. M.; et al. "Determination of Chloride Concentration Using Capillary Electrophoresis," *J. Chem. Educ.* **1998,** *75,* 1463–1465.

Directions are provided for the determination of chloride in samples using CZE. The buffer solution includes pyromellitic acid which allows the indirect determination of chloride by monitoring absorbance at 250 nm.

McDevitt, V. L.; Rodríguez, A.; Williams, K. R. "Analysis of Soft Drinks: UV Spectrophotometry, Liquid Chromatography, and Capillary Electrophoresis," *J. Chem. Educ.* **1998,** *75,* 625–629.

Caffeine, benzoic acid, and aspartame in soft drinks are analyzed by three methods. Using several methods to analyze the same sample provides students with the opportunity to compare results with respect to accuracy, volume of sample required, ease of performance, sample throughput, and detection limit.

Thompson, L.; Veening, H.; Strain, T. G. "Capillary Electrophoresis in the Undergraduate Instrumental Analysis Laboratory: Determination of Common Analgesic Formulations," *J. Chem. Educ.* **1997,** *74,* 1117–1121.

Students determine the concentrations of caffeine, acetaminophen, acetylsalicylic acid, and salicylic acid in several analgesic preparations using both CZE (70 mM borate buffer solution, UV detection at 210 nm) and HPLC (C_{18} column with 3% v/v acetic acid mixed with methanol as a mobile phase, UV detection at 254 nm).

Vogt, C.; Conradi, S.; Rhode, E. "Determination of Caffeine and Other Purine Compounds in Food and Pharmaceuticals by Micellar Electrokinetic Chromatography," *J. Chem. Educ.* **1997,** *74,* 1126–1130.

This experiment describes a quantitative analysis for caffeine, theobromine, and theophylline in tea, pain killers, and cocoa. Separations are accomplished by MEKC using a pH 8.25 borate–phosphate buffer with added SDS. A UV detector set to 214 nm is used to record the electropherogram. An internal standard of phenobarbital is included for quantitative work.

Weber, P. L.; Buck, D. R. "Capillary Electrophoresis: A Fast and Simple Method for the Determination of the Amino Acid Composition of Proteins," *J. Chem. Educ.* **1994,** *71,* 609–612.

This experiment describes a method for determining the amino acid composition of cyctochrome c and lysozyme. The proteins are hydrolyzed in acid, and an internal standard of α-aminoadipic acid is added. Derivatization with naphthalene-2,3-dicarboxaldehyde gives derivatives that absorb at 420 nm. Separation is by MEKC using a buffer solution of 50 mM SDS in 20 mM sodium borate.

12N PROBLEMS

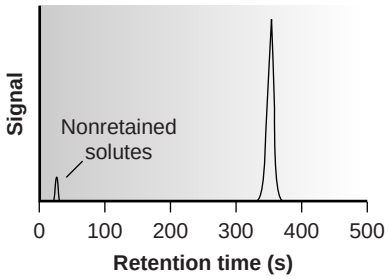
1. The following data were obtained for four compounds separated on a 20-m capillary column.

Compound	t_r (min)	w (min)
A	8.04	0.15
B	8.26	0.15
C	8.43	0.16

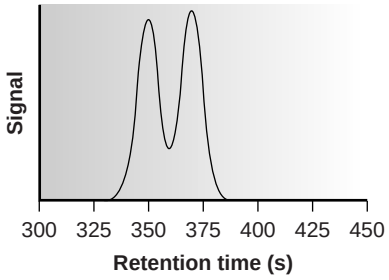
(a) Calculate the number of theoretical plates for each compound and the average number of theoretical plates for the column. (b) Calculate the average height of a theoretical plate. (c) Explain why it is possible for each compound to have a different number of theoretical plates.

2. Using the data from Problem 1, calculate the resolution and selectivity factors for each pair of adjacent compounds. For resolution, use both equations 12.1 and 12.21, and compare your results. Discuss how you might improve the resolution between compounds B and C. The retention time for an unretained solute is 1.19 min.

3. Using the chromatogram shown here, which was obtained on a 2-m column, determine values for t_r, w, t_r', k', N, and H.



4. Using the partial chromatogram shown here, determine the resolution between the two solute bands.



5. The chromatogram in Problem 4 was obtained on a 2-m column with a column dead time of 50 s. How long a column is needed to achieve a resolution of 1.5? What height of a theoretical plate is needed to achieve a resolution of 1.5 without increasing the length of the column?

6. Complete the following table.

N_B	α	k_B'	R
100,000	1.05	0.50	
10,000	1.10		1.50
10,000		4.0	1.00
	1.05	3.0	1.75

7. Moody[19] studied the efficiency of a GC separation of 2-butanone on a dinonyl phthalate column. Evaluating the plate height as a function of flow rate gave a van Deemter equation for which A is 1.65 mm, B is 25.8 mm · mL min^{-1}, and C is 0.0236 mm · min mL^{-1}. (a) Prepare a graph of H versus u for flow rates in the range of 5–120 mL/min. (b) For what range of flow rates does each term in the van Deemter equation have the greatest effect? (c) What are the optimum flow rate and the height of a theoretical plate at that flow rate? (d) For open tubular columns the A term is no longer needed. If the B and C terms remain unchanged, what are the optimum flow rate and the height of a theoretical plate at that flow rate? (e) How many more theoretical plates will there be in the open tubular column compared with the packed column? (f) Equation 12.28 is written in terms of the linear velocity (centimeters per second), yet we have evaluated it in this problem using the flow rate (milliliters per minute). Why can we do this?

8. Hsieh and Jorgenson[20] prepared 12–33-μm HPLC columns packed with 5.44-μm spherical stationary phase particles. To evaluate these columns they measured reduced plate height, h,

$$h = \frac{H}{d_p}$$

as a function of reduced flow rate, v,

$$v = \frac{ud_p}{D_m}$$

where d_p is the particle diameter, and D_m is the solute's diffusion coefficient in the mobile phase. The data were analyzed using van Deemter plots, with a portion of their results summarized in the following table for the solute norepinephrine.

Column Internal Diameter (μm)	A	B	C
33	0.63	1.32	0.10
33	0.67	1.30	0.08
23	0.40	1.34	0.09
23	0.58	1.11	0.09
17	0.31	1.47	0.09
17	0.40	1.41	0.11
12	0.22	1.53	0.11
12	0.19	1.27	0.12

(a) Construct separate van Deemter plots using the data in the first and last rows for flow rates in the range 0.7–15. Determine the optimum flow rate and plate height for each case, given $d_p = 5.44$ μm and $D_m = 6.23 \times 10^{-6}$ cm^2 s^{-1}. (b) The *A* term in the van Deemter equation appears to be strongly correlated with the column's inner diameter, with smaller diameter columns providing smaller values of *A*. Explain why this effect is seen (*Hint:* Consider how many particles can fit across a capillary of each diameter).

9. Method 12.1 describes the analysis of the trihalomethanes $CHCl_3$, $CHBr_3$, $CHCl_2Br$, and $CHClBr_2$ in drinking water using a packed column with a nonpolar stationary phase. Predict the order in which these four trihalomethanes will elute.

10. A mixture of *n*-heptane, tetrahydrofuran, 2-butanone, and *n*-propanol elutes in this order when using a polar stationary phase such as Carbowax. The elution order is exactly the opposite when using a nonpolar stationary phase such as polydimethyl siloxane. Explain the order of elution in each case.

11. The analysis of trihalomethanes in drinking water is described in Method 12.1. A single standard gives the following results when carried through the described procedure.

Trihalomethane	Concentration (ppb)	Peak Area
$CHCl_3$	1.30	1.35×10^4
$CHCl_2Br$	0.90	6.12×10^4
$CHClBr_2$	4.00	1.71×10^4
$CHBr_3$	1.20	1.52×10^4

Analysis of water from a drinking fountain gives areas of 1.56×10^4, 5.13×10^4, 1.49×10^4, and 1.76×10^4 for $CHCl_3$, $CHCl_2Br$, $CHClBr_2$, and $CHBr_3$, respectively. Determine the concentration of each of the trihalomethanes in the sample of water.

12. Zhou and colleagues determined the %w/w H_2O in methanol by GC, using a capillary column coated with a nonpolar stationary phase and a thermal conductivity detector.[21] A series of calibration standards gave the following results.

% w/w H_2O	Peak Height (arb. units)
0.00	1.15
0.0145	2.74
0.0472	6.33
0.0951	11.58
0.1757	20.43
0.2901	32.97

(a) What is the %w/w H_2O in a sample giving a peak height of 8.63? (b) The %w/w H_2O in a freeze-dried antibiotic is determined in the following manner. A 0.175-g sample is placed in a vial along with 4.489 g of methanol. Water in the vial extracts into the methanol. Analysis of the sample gave a peak height of 13.66. What is the %w/w H_2O in the antibiotic?

13. Loconto and co-workers describe a method for determining trace levels of water in soil.[22] The method takes advantage of the reaction of water with calcium carbide, CaC_2, to produce acetylene gas, C_2H_2. By carrying out the reaction in a sealed vial, the amount of acetylene produced may be determined by sampling the headspace. In a typical analysis a sample of soil is placed in a sealed vial with CaC_2. Analysis of the headspace gave a blank-corrected signal of 2.70×10^5. A second sample is prepared in the same manner except that a standard addition of 5.0 mg H_2O/g solid is added, giving a blank-corrected signal of 1.06×10^6. Determine the number of milligrams of H_2O/g soil in the soil sample.

14. Van Atta and Van Atta used gas chromatography to determine the %v/v methyl salicylate in rubbing alcohol.[23] A set of standard additions was prepared by transferring 20.00 mL of rubbing alcohol to separate 25-mL volumetric flasks and pipeting 0.00 mL, 0.20 mL, and 0.50 mL of methyl salicylate to the flasks. All three flasks were then diluted to volume using isopropanol. Analysis of the three samples gave peak heights for methyl salicylate of 57.00 mm, 88.5 mm, and 132.5 mm, respectively. Determine the %v/v methyl salicylate in the rubbing alcohol.

15. The amount of camphor in an analgesic ointment can be determined by GC using the method of internal standards.[24] A standard sample was prepared by placing 45.2 mg of camphor and 2.00 mL of a 6.00 mg/mL internal standard solution of terpene hydrate in a 25-mL volumetric flask and diluting to volume with CCl_4. When an approximately 2-μL sample of the standard was injected, the FID signals for the two components were measured (in arbitrary units) as 67.3 for camphor and 19.8 for terpene hydrate. A 53.6-mg sample of an analgesic ointment was prepared for analysis by placing it in a 50-mL Erlenmeyer flask along with 10 mL of CCl_4. After heating to 50 °C in a water bath, the sample was cooled to below room temperature and filtered. The residue was washed with two 5-mL portions of CCl_4, and the combined filtrates were collected in a 25-mL volumetric flask. After adding 2.00 mL of the internal standard solution, the contents of the flask were diluted to volume with CCl_4. Analysis of an approximately 2-μL sample gave FID signals of 13.5 for the terpene hydrate and 24.9 for the camphor. Report the %w/w camphor in the analgesic ointment.

16. The concentration of pesticide residues on agricultural products, such as oranges, may be determined by GC-MS.[25] Pesticide residues are extracted from the sample using methylene chloride, and the concentrations of the extracted pesticides are concentrated by evaporating the methylene chloride to a smaller volume. Calibration is accomplished using anthracene-d_{10} as an internal standard. In a study to determine the parts per billion of heptachlor epoxide on oranges, a 50.0-g sample of orange rinds was chopped and extracted with 50.00 mL of methylene chloride. After removing any insoluble material by filtration, the methylene chloride was reduced in volume, spiked with a known amount of the internal standard, and diluted to 10 mL in a volumetric flask. Analysis of the sample gives a peak–area ratio ($A_{anal}/A_{int\ stan}$) of 0.108. A series of calibration standards, each

containing the same amount of anthracene-d_{10} as the sample, give the following results.

ppb Heptachlor Epoxide	$A_{anal}/A_{int\ stan}$
20.0	0.065
60.0	0.153
200.0	0.637
500.0	1.554
1000.0	3.198

Report the concentration of heptachlor epoxide residue (in nanograms per gram) on the oranges.

17. The adjusted retention times for octane, toluene, and nonane on a particular GC column are 15.98 min, 17.73 min, and 20.42 min, respectively. What is the retention index for all three compounds?

18. The following data were collected for a series of normal alkanes using a stationary phase of Carbowax 20M.

Alkane	t_r' (min)
pentane	0.79
hexane	1.99
heptane	4.47
octane	14.12
nonane	33.11

What is the retention index for a compound whose adjusted retention time is 9.36 min?

19. The following data have been reported for the gas chromatographic analysis of *p*-xylene and methylisobutylketone (MIBK) on a capillary column.[8]

Injection Mode	Compound	t_r (min)	Peak Area	Peak Width (min)
split	MIBK	1.878	54285	0.028
	p-xylene	5.234	123483	0.044
splitless	MIBK	3.420	2493005	1.057
	p-xylene	5.795	3396656	1.051

Explain the difference in the retention times, the peak areas, and the peak widths when switching from a split injection to a splitless injection.

20. Otto and Wegscheider report the following capacity factors for the reverse phase separation of 2-aminobenzoic acid on a C_{18} column when using 10% v/v methanol as a mobile phase.[26]

pH	k'
2.0	10.5
3.0	16.7
4.0	15.8
5.0	8.0
6.0	2.2
7.0	1.8

Explain the changes in capacity factor.

21. Haddad and associates report the following capacity factors for the reverse-phase separation of salicylamide (k_{sal}') and caffeine (K_{caff}').[27]

%v/v methanol	30%	35%	40%	45%	50%	55%
k_{sal}'	2.4	1.6	1.6	1.0	0.7	0.7
k_{caff}'	4.3	2.8	2.3	1.4	1.1	0.9

Explain the changes in capacity factor. What is the advantage of using a mobile phase with a smaller %v/v methanol? Are there any disadvantages?

22. Suppose that you are to separate a mixture of benzoic acid, aspartame, and caffeine in a diet soda. The following information is available to you.

	t_r in Aqueous Mobile Phase Buffered to a pH of			
Compound	3.0	3.5	4.0	4.5
benzoic acid	7.4	7.0	6.9	4.4
aspartame	5.9	6.0	7.1	8.1
caffeine	3.6	3.7	4.1	4.4

(a) Explain the change in retention time for each compound. (b) Plot retention time versus pH for each compound on the same graph, and identify a pH level that will yield an acceptable separation.

23. The composition of a multivitamin tablet is conveniently determined using an HPLC with a diode array UV/Vis detector. A 5-μL standard sample containing 170 ppm vitamin C, 130 ppm niacin, 120 ppm niacinamide, 150 ppm pyridoxine, 60 ppm thiamine, 15 ppm folic acid, and 10 ppm riboflavin is injected into the HPLC, giving signals (in arbitrary units) of, respectively, 0.22, 1.35, 0.90, 1.37, 0.82, 0.36, and 0.29. The multivitamin tablet is prepared for analysis by grinding into a powder and transferring to a 125-mL Erlenmeyer flask containing 10 mL of 1% v/v NH_3 in dimethyl sulfoxide. After sonicating in an ultrasonic bath for 2 min, 90 mL of 2% acetic acid is added, and the mixture is stirred for 1 min and sonicated at 40 °C for 5 min. The extract is then filtered through a 0.45-μm membrane filter. Injection of a 5-μL sample into the HPLC gives signals of 0.87 for vitamin C, 0.00 for niacin, 1.40 for niacinamide, 0.22 for pyridoxine, 0.19 for thiamine, 0.11 for folic acid, and 0.44 for riboflavin. Report the number of milligrams of each vitamin present in the tablet.

24. The amount of caffeine in an analgesic tablet was determined by HPLC using a normal calibration curve. Standard solutions of caffeine were prepared and analyzed using a 10-μL fixed-volume injection loop. Results for the standards are summarized in the following table.

Concentration of Standards (ppm)	Signal (arbitrary units)
50.0	8354
100.0	16925
150.0	25218
200.0	33584
250.0	42002

The sample was prepared by placing a single analgesic tablet in a small beaker and adding 10 mL of methanol. After allowing the sample to dissolve, the contents of the beaker, including the insoluble binder, were quantitatively transferred to a 25-mL volumetric flask and diluted to volume with methanol. The sample was then filtered, and a 1.00-mL aliquot was transferred to a 10-mL volumetric flask and diluted to volume with methanol. When analyzed by HPLC, the signal for the caffeine was found to be 21469. Report the number of milligrams of caffeine in the analgesic tablet.

25. Kagel and Farwell report a reverse-phase HPLC method for determining the concentration of acetylsalicylic acid (ASA) and caffeine (CAF) in analgesic tablets using salicylic acid (SA) as an internal standard.[28] A series of standards was prepared by adding known amounts of acetylsalicylic acid and caffeine to 250-mL Erlenmeyer flasks and adding 100 mL of methanol. A 10.00-mL aliquot of a standard solution of salicylic acid was then added to each. The following results are obtained for a typical set of standard solutions.

Standard	Milligrams ASA	Milligrams CAF	Peak Height Ratio ASA/SA	Peak Height Ratio CAF/SA
1	200.0	20.0	20.5	10.6
2	250.0	40.0	25.1	23.0
3	300.0	60.0	30.9	36.8

A sample of an analgesic tablet was placed in a 250-mL Erlenmeyer flask and dissolved in 100 mL of methanol. After adding a 10.00-mL portion of the internal standard, the solution was filtered. Analysis of the sample gave a peak height ratio of 23.2 for ASA and 17.9 for CAF.
(a) Determine the number of milligrams ASA and CAF in the tablet. (b) Why was it necessary to filter the sample? (c) The directions indicate that approximately 100 mL of methanol is used to dissolve the standards and samples. Why is it not necessary to measure this volume more precisely? (d) In the presence of moisture, ASA decomposes to SA and acetic acid. What complication might this present for this analysis? How might you evaluate whether this is a problem?

26. Bohman and colleagues described a reverse-phase HPLC method for the quantitative analysis of vitamin A in food using the method of standard additions.[29] In a typical example, a 10.067-g sample of cereal is placed in a 250-mL Erlenmeyer flask along with 1 g of sodium ascorbate, 40 mL of ethanol, and 10 mL of 50% w/v KOH. After refluxing for 30 min, 60 mL of ethanol is added, and the solution is cooled to room temperature. Vitamin A is extracted using three 100-mL portions of hexane. The combined portions of hexane are evaporated, and the residue containing vitamin A is transferred to a 5-mL volumetric flask and diluted to volume with methanol. A standard addition is prepared in a similar manner using a 10.093-g sample of the cereal and spiking it with 0.0200 mg of vitamin A. Injecting the sample and standard addition into the HPLC gives peak areas of 6.77×10^3 and 1.32×10^4, respectively. Report the vitamin A content of the sample in milligrams/100 g cereal.

27. Ohta and Tanaka reported a method for the simultaneous analysis of several inorganic anions and the cations Mg^{2+} and Ca^{2+} in water by ion-exchange chromatography.[30] The mobile phase includes 1,2,4-benzenetricarboxylate, which absorbs strongly at 270 nm. Indirect detection of the analytes is possible because their presence in the detector leads to a decrease in absorbance. Unfortunately, Ca^{2+} and Mg^{2+}, which are present at high concentrations in many environmental waters, form stable complexes with 1,2,4-benzenetricarboxylate that interfere with the analysis.
(a) Adding EDTA to the mobile phase eliminates the interference caused by Ca^{2+} and Mg^{2+}; explain why. (b) A standard solution containing 1.0 M $NaHCO_3$, 0.20 mM $NaNO_2$, 0.20 mM $MgSO_4$, 0.10 mM $CaCl_2$, and 0.10 mM $Ca(NO_3)_2$ gives the following typical peak areas (arbitrary units).

Ion	HCO_3^-	Cl^-	NO_2^-	NO_3^-
Peak Area	373.5	322.5	264.8	262.7
Ion	Ca^{2+}	Mg^{2+}	SO_4^{2-}	
Peak Area	458.9	352.0	341.3	

Analysis of a river water sample (pH of 7.49) gives the following results.

Ion	HCO_3^-	Cl^-	NO_2^-	NO_3^-
Peak Area	310.0	403.1	3.97	157.6
Ion	Ca^{2+}	Mg^{2+}	SO_4^{2-}	
Peak Area	734.3	193.6	324.3	

Determine the concentration of each ion in the sample of rain water. (c) The detection of HCO_3^- actually gives the total concentration of carbonate in solution ($[CO_3^{2-}] + [HCO_3^-] + [H_2CO_3]$). Given that the pH of the water is 7.49, what is the actual concentration of HCO_3^-? (d) An independent analysis gives the following additional concentrations.

$[Na^+] = 0.60$ mM $\quad [NH_4^+] = 0.014$ mM $\quad [K^+] = 0.046$ mM

A solution's ionic balance is defined as the ratio of the total cation charge to the total anion charge. Determine the ion balance for this sample of water, and comment on whether the result is reasonable.

28. The concentrations of Cl^-, NO_3^-, and SO_4^{2-} may be determined by ion chromatography. A 50-μL standard sample of 10.0-ppm Cl^-, 2.00-ppm NO_3^-, and 5.00-ppm SO_4^{2-} gave signals (in arbitrary units) of 59.3, 16.1, and 6.08, respectively. A sample of effluent from a wastewater treatment plant was diluted tenfold, and a 50-μL portion gave signals of 44.2 for

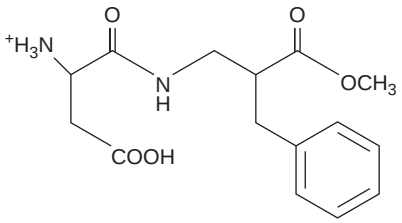
Cl^-, 2.73 for NO_3^-, and 5.04 for SO_4^{2-}. Report the parts per million of each anion in the effluent sample.

29. A series of polyvinylpyridine standards of different molecular weight were analyzed by size-exclusion chromatography, yielding the following results.

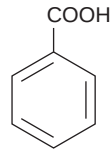
Formula Weight	Retention Volume (mL)
600,000	6.42
100,000	7.98
20,000	9.30
3,000	10.94

When a preparation of polyvinylpyridine of unknown formula weight was analyzed the retention volume was found to be 8.45. Report the average formula weight for the preparation.

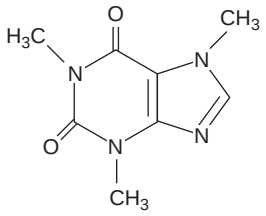
30. Diet soft drinks contain appreciable quantities of aspartame, benzoic acid, and caffeine. What is the expected order of elution for these compounds in a capillary zone electrophoresis separation using a pH 9.4 buffer solution, given that aspartame has pK_a values of 2.964 and 7.37, benzoic acid's pK_a is 4.2, and the pK_a for caffeine is less than 0.



aspartame



benzoic acid



caffeine

31. Janusa and co-workers report the determination of chloride by CZE.[31] Analysis of a series of external standards gives the following calibration curve.

$$\text{Area} = -883 + 5590(\text{ppm } Cl^-)$$

A standard sample of 57.22% w/w Cl^- was analyzed by placing 0.1011 g in a 100-mL volumetric flask and diluting to volume. Three unknowns were prepared by pipeting 0.250 mL, 0.500 mL, and 0.750 mL of the bulk unknown into separate 50-mL volumetric flasks and diluting to volume. Analysis of the three unknowns gave areas of 15310, 31546, and 47582, respectively. Evaluate the accuracy of this analysis.

32. The analysis of NO_3^- in aquarium water was carried out by CZE using IO_4^- as an internal standard. A standard solution of 15.0-ppm NO_3^- and 10.0-ppm IO_4^- gives peak heights (arbitrary units) of 95.0 and 100.1, respectively. A sample of water from an aquarium is diluted 1:100, and sufficient internal standard added to make its concentration 10.0 ppm. Analysis gives signals of 29.2 and 105.8 for NO_3^- and IO_4^-, respectively. Report the parts per million of NO_3^- in the sample of aquarium water.

33. Suggest conditions for separating a mixture of 2-aminobenzoic acid (pK_{a1} = 2.08, pK_{a2} = 4.96), benzylamine (pK_a = 9.35), and 4-methylphenol (pK_a = 10.26) by capillary zone electrophoresis.

34. McKillop and associates have examined the electrophoretic separation of alkylpyridines by CZE.[32] Separations were carried out using either 50-μm or 75-μm inner diameter capillaries, with a total length of 57 cm and a length of 50 cm from the point of injection to the detector. The run buffer was a pH 2.5 lithium phosphate buffer. Separations were achieved using an applied voltage of 15 kV. The electroosmotic flow velocity, as measured using a neutral marker, was found to be 6.398×10^{-5} cm^2 V^{-1} s^{-1}. The diffusion coefficient, D, for the alkylpyridines may be taken to be 1.0×10^{-5} cm^2 s^{-1}.(a) Calculate the electrophoretic mobility for 2-ethylpyridine, given that its elution time is 8.20 min. (b) How many theoretical plates are there for 2-ethylpyridine? (c) The electrophoretic mobilities for 3-ethylpyridine and 4-ethylpyridine are 3.366×10^{-4} cm^2 V^{-1} s^{-1} and 3.397×10^{-4} cm^2 V^{-1} s^{-1}, respectively. What is the expected resolution between these two alkylpyridines? (d) Explain the trends in electrophoretic mobility shown in the following table.

Alkylpyridine	μ_{ep} (cm^2 V^{-1} s^{-1})
2-methylpyridine	3.581×10^{-4}
2-ethylpyridine	3.222×10^{-4}
2-propylpyridine	2.923×10^{-4}
2-pentylpyridine	2.534×10^{-4}
2-hexylpyridine	2.391×10^{-4}

(e) Explain the trends in electrophoretic mobility shown in the following table.

Alkylpyridine	μ_{ep} (cm^2 V^{-1} s^{-1})
2-ethylpyridine	3.222×10^{-4}
3-ethylpyridine	3.366×10^{-4}
4-ethylpyridine	3.397×10^{-4}

(f) The pK_a for pyridine is 5.229. At a pH of 2.5 the electrophoretic mobility of pyridine is 4.176×10^{-4} cm^2 V^{-1} s^{-1}. What is the expected electrophoretic mobility if the run buffer's pH is 7.5?