Chapter 8

Chromatography and electrophoresis

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Learning objectives

After reviewing this chapter, the reader will be able to:

- Define "chromatography" and describe common ways of categorizing different types of this method.
- Discuss the terms that are used to describe retention, band-broadening, and peak separations in chromatography.
- Explain the significance of the van Deemter equation and the general factors that affect the resolution of a separation in chromatography.
- Discuss the general concepts of gas chromatography and liquid chromatography, including the types of mobile phases, elution methods, supports, and stationary phases that are used in these techniques.
- Describe the design and applications of common detectors and detection systems that are used in gas chromatography and liquid chromatography.
- Define "electrophoresis" and describe general formats for this method.
- List the various factors that can affect the migration and band-broadening of chemicals in electrophoresis.
- Discuss the general concepts of gel electrophoresis and capillary electrophoresis, including the types of supports, application methods, and formats that are used in these techniques.
- Describe common detectors or detection systems that are used in gel electrophoresis and capillary electrophoresis.

Introduction to chromatography

General terms and concepts

The complexity of clinical samples such as blood, serum, and urine typically necessitates some separation prior to analysis. Chromatography is a method that is frequently employed for this purpose. Chromatography is a separation technique in which the components of a sample are separated based on how they distribute between two chemical or physical phases, a stationary phase and a more mobile phase that is allowed to travel through the separation system. This method is illustrated in Fig. 8.1, in which a sample is applied to the top of a tube, known as the "column." This column contains a fixed surface or layer, known as the "stationary phase," that is held in place by a solid support and that can interact with the various components of the sample. A second phase (called the "mobile phase") is used to apply samples to the column and pass their components through to the other end. Those substances that have the weakest interactions with the stationary phase will travel through more quickly than those with strong interactions, resulting in a separation of these chemicals [1–4].

There are many different types of chromatography. The main way of categorizing these techniques is based on their mobile phase. If a gas is used as the mobile phase, the method is called gas chromatography (GC); if the mobile phase is a liquid, the technique is known as liquid chromatography (LC). Both GC and LC can be divided into further subcategories based on their separation mechanism, which is determined by the type of stationary phase in the column. For instance, the use of underivatized solid particles in GC or LC as the stationary phase produces the methods of gas–solid chromatography (GSC) and liquid–solid chromatography (LSC).

Another way chromatographic methods can be grouped is according to the type of chromatographic bed or support that is present. A method in which a column contains the support and stationary phase is known as column chromatography. If the column is packed with support particles, this method is called packed bed chromatography. If the stationary phase is instead placed directly onto the interior wall of the column, the technique is known as open-tubular chromatography. It is also possible to have the support and stationary phase present on a flat plane, giving the technique of "planar chromatography." Specific examples of planar chromatography include paper chromatography, which uses paper as a support, and thin-layer chromatography, which generally uses a stationary phase coated on a glass or plastic sheet.

One way that chromatography can be utilized is for the isolation of a given chemical prior to its analysis by a

Contemporary Practice in Clinical Chemistry. DOI: https://doi.org/10.1016/B978-0-12-815499-1.00008-9

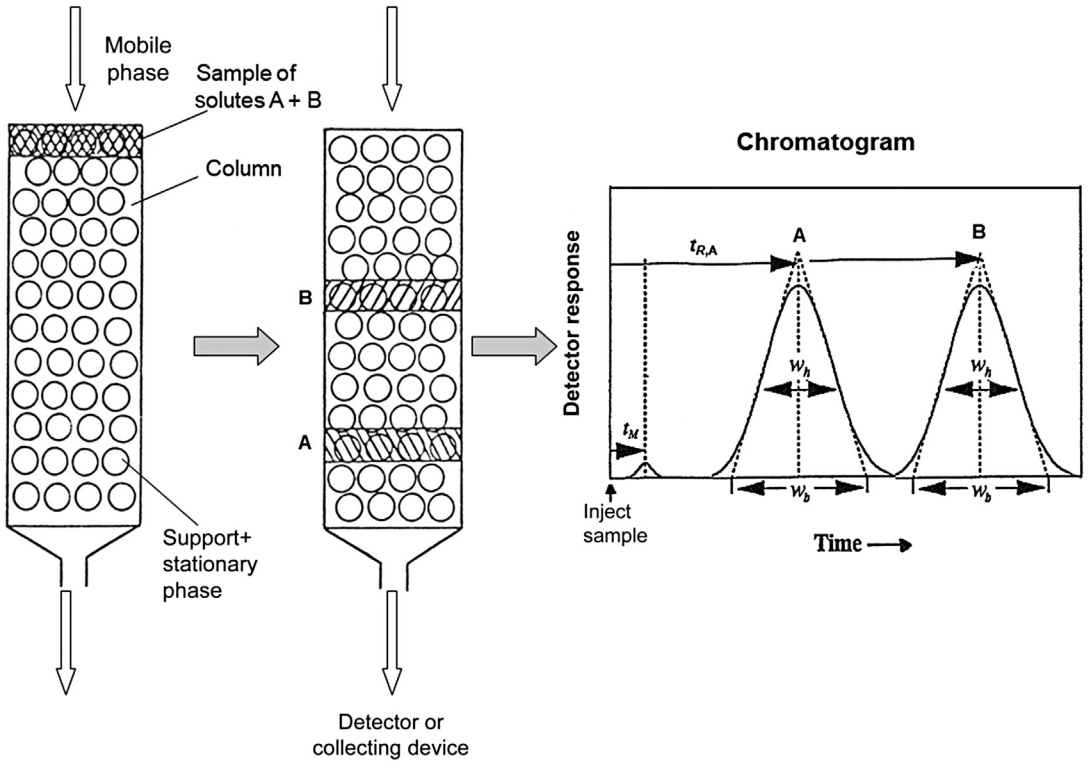

FIGURE 8.1 (A) General scheme for the separation of chemicals by chromatography and (B) a typical chromatogram for such a separation.

different method. However, chromatography can also be used directly for the identification and measurement of sample components if it is used along with a detector that can monitor these chemicals as they pass through the chromatographic system. The result is usually plotted by making a graph of the response measured by this detector as a function of the time or volume of mobile phase needed for the elution of each injected chemical. This plot, as shown in Fig. 8.1, is known as a "chromatogram."

Theory of chromatography

In chromatography, there is always a minimum amount of time required for any substance to pass through the system. This is known as the column "void time" (t_M). If a compound is bound (or "retained") by the stationary phase, it will travel more slowly through the column and exit at some later time. The average time required for this is known as that compound's "retention time" (t_R) (see Fig. 8.1). The length of this retention time is determined by the eluting substance's structure, as well as the type and composition of stationary and mobile phases being used in the chromatographic system. Elution volume can also be used to describe the movement of substances in chromatography. The average volume of mobile phase it takes to move a compound through the column is called that compound's "retention volume" (V_R). Similarly, the volume of mobile phase it takes to elute a totally nonretained substance is known as the column "void volume" (V_M).

Another way the retention of substances in chromatography can be described is by using the retention factor (k). The retention factor can be calculated by using one of the following relationships.

$$k = \frac{(t_R - t_M)}{t_M} \quad \text{or} \quad k = \frac{(V_R - V_M)}{V_M} \tag{8.1}$$

The retention factor is a more fundamental measure of retention than t_R and V_R, because it is related directly to the amount of stationary phase in the column and the extent to which solutes are interacting with this phase. As an example, Eq. (8.2) is another way of describing the retention factor for a chromatographic system in which a solute is partitioning between the mobile phase and the stationary phase.

$$k = K_D\left(\frac{V_S}{V_M}\right) \tag{8.2}$$

In this equation, (V_S/V_M) (i.e., the phase ratio) is the relative volume of stationary phase versus the volume of mobile phase in the column, and K_D is the distribution equilibrium constant for the solute in these two phases. Eq. (8.2) indicates that the degree of retention in chromatography will be related to both the degree of that

compound's interactions with the stationary phase (K_D) and the relative amount of stationary phase in the column (V_S/V_M).

As chemicals travel through a chromatographic system, the width of the region that contains each compound (i.e., the compound's peak or band) gradually becomes broader. This process is known as "band-broadening" and occurs even for substances that have little or no binding to the stationary phase. One way of describing band-broadening in chromatography is to measure the width of a peak at its baseline level (w_b) or its width at half-height (w_h) (Fig. 8.1). However, these values are highly dependent on the retention time or retention volume used for measurement. To compare the band-broadening for substances with different retention times, the "number of theoretical plates" or "plate number" (N) is utilized. The value of N can be calculated from w_b or w_h by one of the expressions given in Eq. (8.3) for a peak with a Gaussian shape.

$$N = 16\left(\frac{t_R}{w_b}\right)^2 \quad \text{or} \quad N = 5.545\left(\frac{t_R}{w_h}\right)^2 \tag{8.3}$$

A large value for N makes it easier for a chromatographic system to separate compounds. One way this parameter can be viewed is by having the value of N represent the number of times a substance will equilibrate between the mobile phase and stationary phase as it travels through the column. If a compound is able to undergo more of these equilibration steps, it becomes possible for the system to distinguish between compounds with smaller differences in their retention.

Although N is useful for describing band-broadening in columns, it does have the disadvantage of being dependent on the column length. This problem can be overcome by using a related value known as the "height equivalent of a theoretical plate" or "plate height" (H). The value of H is calculated from N by using Eq. (8.4), where L is the length of column.

$$H = \frac{L}{N} \tag{8.4}$$

The value of H gives the length of column that corresponds to one theoretical plate, or one equilibration step of the analyte with the stationary phase. Because a large number of theoretical plates are desirable to provide a small degree of band-broadening, a small plate height is also desirable.

Plate heights are used not only to describe band-broadening and column efficiency, but also to evaluate how a particular experimental factor impacts band-broadening. One well-known relationship developed for this purpose is the van Deemter equation, which shows how a change in linear velocity (u, which is directly proportional to flow-rate) alters the measured plate height for a column (H).

$$H = A + \frac{B}{u} + Cu \tag{8.5}$$

In this equation, the terms A, B, and C are constants that represent the contributions to band-broadening due to several processes that occur as a solute passes through the column. These processes are eddy diffusion (A), longitudinal diffusion (B), and stagnant mobile phase mass transfer plus stationary phase mass transfer (C), where the effect of each process on H has a different dependence on the flow-rate. Eddy diffusion is produced by the presence of the large number of flow paths around support particles, with each path having a slightly different length. Longitudinal diffusion refers to the broadening of a compound's peak due to the diffusion of solutes along the length of the column. Stagnant mobile phase mass transfer is related to the rate of movement of solutes as they go from the outside of the support to the region in the support's pores or near the support's surface. Stationary phase mass transfer is related to the rate at which an analyte transfers between the stationary phase and the mobile phase.

A plot of the van Deemter equation gives a "U"-shaped curve in which there is a distinct linear velocity where H has its lowest optimum value. This type of graph is helpful in finding the flow-rate or linear velocity that gives the best possible efficiency for a column. Another way a van Deemter plot can be used is to compare the plate heights of columns that contain different supports or that are prepared by different processes. This information is useful in the development of new columns or in comparing existing columns.

Evaluating and optimizing chromatographic separations

The final success of any chromatographic separation will depend on how well the peaks of interest are separated. There are a number of ways to evaluate the extent of this separation. One way is to use the separation factor (α, also known as the "selectivity"). The separation factor is a measure of the relative difference in retention of two solutes as they pass through a column. The value of α is calculated as shown below, where k_1 is the retention factor for the solute exiting first from the column and k_2 is the retention factor for the second.

$$\alpha = \frac{k_2}{k_1} \tag{8.6}$$

The separation factor becomes larger as the relative difference in retention increases between two peaks. This

feature makes α useful in describing the effectiveness of a separation in chromatography. The separation factor can also be used to indicate whether it is feasible to resolve two compounds by a given column, in which a value for α greater than one is needed for any separation to occur.

Another approach for describing the separation of two peaks is to use the peak resolution (R_s). The value of R_s for two adjacent peaks can be calculated through the following formula.

$$R_s = \frac{t_{R2} - t_{R1}}{(w_{b2} + w_{b1})/2} \tag{8.7}$$

In this relationship, t_{R1} and w_{b1} are the retention time and baseline width (both in units of time) for the first eluting peak, while t_{R2} and w_{b2} are the retention time and baseline width of the second peak. An important advantage of using peak resolution instead of the separation factor to describe the extent of a separation is that R_s considers both the difference in retention between two compounds (as represented by $t_{R2} - t_{R1}$) and the degree of band-broadening (as represented by w_{b1} and w_{b2}), while α only considers the difference in retention for these substances.

The lowest possible value for R_s is zero, which occurs when two peaks have exactly the same degree of retention and are not separated. A value for R_s greater than zero represents some degree of separation between peaks, with the extent of this separation becoming larger as R_s increases. Ideally, it is desirable to have no significant overlap between two neighboring peaks. This situation usually occurs when R_s is greater than 1.5, a condition said to represent "baseline resolution." In some separations, peak resolution values between 1.0 and 1.5 are also adequate. The latter often occurs if the peaks are about the same size and can be measured by using peak heights, which are less affected by overlap than peak areas.

Because the resolution between two peaks is a measure of both the difference in compound retention and band-broadening, any items that affect retention or peak width will also affect R_s. This relationship is demonstrated in Eq. (8.8),

$$R_S = \frac{\sqrt{N}}{4}\frac{(\alpha - 1)}{\alpha}\frac{k_2}{(1 + k_2)} \tag{8.8}$$

where k_2 is the retention factor for the second peak, α is the separation factor between the first and second peaks, and N is the number of theoretical plates for the column. Eq. (8.8) is called the "resolution equation" of chromatography. This equation shows that the degree of a separation in chromatography will be affected by three factors: (1) the extent of band-broadening in the column (N); (2) the overall degree of peak retention (k); and (3) the selectivity of the column's stationary phase in binding to one compound versus another (α).

Some ways the band-broadening of a chromatographic system can be lowered to improve resolution include the use of a longer column, operation at a flow-rate producing a smaller plate height, or the use of a support with better mass transfer properties (e.g., smaller diameter support particles). An improvement in resolution by increasing the degree of retention generally involves changing the mobile phase or stationary phase. Altering the selectivity of the chromatographic system is usually performed last and is the most difficult of the options for improving resolution, because this requires more complete knowledge of the types of interactions that are taking place between the injected compounds and the stationary phase.

Gas chromatography

General terms and concepts

One of the most common types of chromatography is the method of GC [2–7]. As stated earlier, GC is a type of chromatography in which the mobile phase is a gas. Fig. 8.2

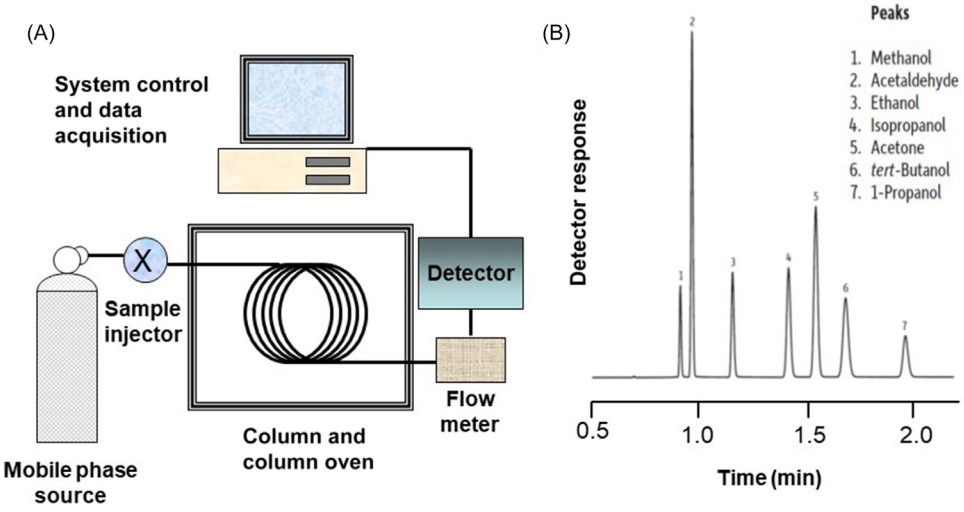

FIGURE 8.2 (A) A system for performing gas chromatography and (B) a typical separation performed by gas chromatography, using the separation of components in a blood alcohol standard as an example. *The chromatogram in (B) is adapted with permission from Restek.*

shows a typical GC system, which is known as a "gas chromatograph." The first major component of this system is the gas source that supplies the mobile phase. This source is usually a gas cylinder equipped with pressure regulators to deliver the mobile phase at a controlled rate. The second part of the gas chromatograph is the injection system, which usually consists of a heated loop or port into which the sample is placed and converted into a gaseous form. The next part of the system is the column, which contains the stationary phase and the support material. It is here that sample components are separated. This column is usually held in an enclosed area known as the "column oven" that keeps the temperature of the column and its surroundings at a well-defined level. The last part of the GC system is the detector (along with its recording device), which monitors the sample components as they leave the column.

One limitation of GC is that all analytes to be examined by this method must either be naturally volatile or capable of forming a volatile derivative. This requirement is needed so the analyte can be injected and passed through the GC system using a gas as the mobile phase. The volatility of a chemical will depend on such things as the temperature of the compound's surroundings, the size of the compound, and the types of functional groups in the compound's structure. As a rule, chemicals with molecular masses of below 600 g/mol and boiling points of below 500°C−550°C at a pressure of 1 atmosphere are usually suitable for GC. Along with good volatility, an analyte must also have good thermal stability, so that it does not decompose at the high temperatures often present in GC systems.

A common approach for increasing the volatility and thermal stability of analytes for GC is to use derivatization. This approach usually involves replacing one or more polar groups on an analyte (such as alcohol or amine groups) with less polar groups that have only weak interactions with their surrounding molecules. The result is a compound that is more volatile and easier to place into the gas phase. The same changes tend to make chemicals more thermally stable. The reaction between an alcohol-containing compound and the reagent trimethylchlorosilane is one example of derivatization. This particular reaction results in a product known as a "trimethylsilyl (TMS)-derivative" in which the hydrogen on the alcohol group is replaced with a bulkier and less polar TMS group.

Like any chromatographic method, the retention of a compound in GC will be determined by how much time this substance spends in the mobile phase versus the stationary phase. Due to the low density of gases, compounds passing through a GC column generally have no significant interactions with the mobile phase. As a result, the volatility of these chemicals is the main item that causes them to stay in the mobile phase during a GC separation. The most volatile compounds in a sample will tend to spend the most time in the mobile phase and elute most quickly from a GC column. Temperature also plays an important role in GC, because the volatility of a chemical will increase as the temperature of its surroundings increases. This is the reason why GC systems contain a column oven to maintain and control temperature during the separation process. Using a lower column temperature will produce longer retention times, because a lower temperature will cause the injected compounds to be less volatile and spend less time in the mobile phase. Increasing the temperature produces the opposite effect, with analytes becoming more volatile and passing through the column more quickly as they spend more time in the mobile phase.

Gas chromatography mobile phases and elution methods

One benefit of using a gas as the mobile phase in GC is it provides a chromatographic system with low band-broadening and narrow peaks. These narrow peaks make it easy to measure small quantities of analytes and allow GC to separate a large number of compounds in a single run. There are several reasons for the low band-broadening of GC systems. For instance, the low density of gases makes it possible for compounds in these gases to quickly move about by diffusion, which reduces many band-broadening processes. Another useful feature of gases is their low viscosity. A low viscosity for the carrier gas leads to small back pressures across GC columns, allowing the use of long columns for separations. The use of a longer column, in turn, provides a greater number of theoretical plates and better resolution.

Given that the main purpose of the mobile phase in GC is to simply move solutes through the column, the mobile phase in this technique is often referred to as the "carrier gas." Examples of common carrier gases used in GC are hydrogen, helium, nitrogen, and argon. All of these gases are relatively inexpensive, easy to obtain, inert (with the exception of hydrogen), and safe to use. These gases are usually provided by a standard gas cylinder, but sometimes they are supplied by a gas generator connected to the GC system. The carrier gas should always have high purity to avoid contamination or damage to the column and the GC system. Impurities like water, oxygen, organic substances, and particulate matter can be removed by passing the carrier gas through a series of traps and filters before it enters the column. The carrier gas source should also be equipped with regulators for pressure and flow-rate control. In some cases, it is necessary to use special devices to maintain a constant flow-rate as the temperature or pressure of the system is varied.

If a single temperature is used throughout a GC separation, this approach is called an "isothermal method." Similarly, if a constant pressure is being used, the technique is known as an "isobaric method." These elution methods work well if a sample is relatively simple or has only a few known compounds. However, these methods may not be feasible for complex samples that contain chemicals with a wide range of volatilities and/or interactions with the stationary phase. Such a situation causes some analytes to elute too quickly and others too slowly from the chromatographic system. This problem can be addressed by varying the elution conditions over time, giving an approach known as "gradient elution."

The most common way of performing gradient elution in GC is to vary the temperature of the column over time; this method is called "temperature programming." This elution method begins with a relatively low column temperature, which allows the most volatile compounds in an injected sample to better interact with the column for improved separation. Next, there is a step known as the "temperature ramp," during which the column temperature is gradually increased over time. It is during this step that analytes with intermediate volatilities are separated and eluted from the column. At the end of this ramp, the temperature is sometimes held at a high value for a short period of time to ensure that there are no low volatility substances remaining on the column from one sample injection to the next. There is then a cooling-down period in which the column is returned back to its initial temperature prior to the next sample injection.

Another type of gradient elution in GC is flow programming. In this method, the flow-rate of the carrier gas is changed during the separation. Because a change in pressure of the carrier gas is used to control its flow-rate, flow programming is sometimes referred to as "pressure programming." This method begins with a relatively slow flow-rate, which allows weakly retained components to have more time to contact the stationary phase and become better resolved. The pressure and the flow-rate are then increased to elute strongly retained compounds from the column. At the end of the elution program, the pressure and the flow-rate are adjusted back to their initial levels, and the next sample is injected. The result is a method that, like temperature programming, gives better resolution and shorter analysis times than can be obtained under constant chromatographic conditions.

Gas chromatography supports and stationary phases

GC columns can be placed into one of two major categories based on the type of support they employ: packed columns and open-tubular columns. A packed column is made up of a glass or metal tube that is usually 1−2-m long and a few millimeters in diameter; this tube is filled with small support particles that act as an adsorbent or are coated with the desired stationary phase. Packed GC columns are useful when a large amount of a sample must be separated, such as in preparative applications. This type of column can also be employed in analytical applications, but it typically has greater band-broadening than columns based on open-tubular supports. As a result, packed columns tend to be used only when a limited number of compounds are to be separated.

An open-tubular column (or "capillary" column) is used in most analytical applications of GC, including those used in clinical testing. This type of column generally ranges from 10 to 100 m in length, has an inner diameter of 0.1−0.75 mm, and possesses a stationary phase that is coated on or attached to its interior surface. A polymer such as polyimide is coated on the outside of the column to give it better strength and flexibility for handling and storage. The low band-broadening of this column allows it to provide better resolution, lower limits of detection, and/or faster rates of separation than a packed column. Three types of stationary phases are used with GC columns: (1) solid adsorbents; (2) liquids coated on solids; and (3) chemical phases bonded to a support. If a solid adsorbent is used as the stationary phase in GC, the resulting method is referred to as GSC. This technique involves the use of the same material as both the support and the stationary phase, with retention being based on the adsorption of analytes to the support's surface. An example of a support that is often used in GSC is a molecular sieve, which contains pores with well-defined sizes and binding regions. Molecular sieves are useful in retaining such things as small hydrocarbons and gases like hydrogen, oxygen, carbon monoxide, and nitrogen. Several other supports can also be used in GSC. These include organic polymers such as porous polystyrene and inorganic substances such as silica or alumina. The extent to which an analyte will bind to these materials will be determined by the support's total surface area, the size of pores in the support, and the functional groups located on the support's surface.

Another group of GC columns are those in which a chemical coating or layer is placed onto the support and used as the stationary phase. This format gives a technique known as gas−liquid chromatography (GLC), which is the most common type of GC. As shown in Table 8.1, there are many liquids used as stationary phases in this method. A common feature of all these liquids is they have high boiling points and low volatilities, which allows them to stay within the column even when high temperatures are used for sample injection and elution. Most of these stationary phases are based on polysiloxanes, which consist of a backbone of silicon and

TABLE 8.1 Typical stationary phases for gas–liquid chromatography.

Chemical name	Relative polarity[a]
100% Dimethylpolysiloxane	16 (Nonpolar)
5% Phenyl–95% methylpolysiloxane	33
14% Cyanopropylphenyl–86% methylpolysiloxane	67
50% Phenyl–50% methylpolysiloxane	119
50% Trifluoropropyl–50% methylpolysiloxane	146
50% Cyanopropylmethyl–50% phenylmethylpolysiloxane	228
Polyethylene glycol	322 (Polar)

[a]*These data are from Ewing [4]. The relative polarity is based on the McReynolds constant for benzene (X′) as a measure of stationary phase polarity.*

oxygen atoms attached in long strings of Si–O–Si bonds. The remaining two bonds on each silicon atom are bound to side groups that can have a variety of structures, ranging from nonpolar methyl groups to polar cyanopropyl groups. By altering the amount and type of these groups, it is possible to produce polysiloxanes with a variety of polarities.

One difficulty with using a liquid stationary phase is that the elevated temperatures often employed in GC will eventually cause this liquid to decompose or vaporize and leave the column over time. This process is known as "column bleeding" and changes the binding characteristics of the column. One approach for minimizing column bleeding is to use a stationary phase that is covalently attached to the support. The resulting material is known as a "bonded phase." One way a bonded phase can be produced is by reacting groups on the stationary phase with groups on the surface of the support. An alternative route is to cross-link the stationary phase to give a larger and more thermally stable structure. Either approach gives a column with less bleeding and that can be used at higher temperatures than columns that contain liquid stationary phases. It is for this reason that bonded phases are preferred in most clinical applications of GC.

Gas chromatography detectors

There are many detectors available for GC (see Table 8.2). These detectors can be divided into two categories: general detectors and selective detectors. The thermal conductivity detector (TCD) is a general detector that can be used for both organic and inorganic compounds. It produces a signal by measuring the thermal conductivity of the carrier gas/analyte mixture leaving the GC column. Hydrogen and helium are the two carrier gases that work best with a TCD, because they have the greatest difference in thermal conductivities from most analytes. The main advantage of a TCD is its ability to respond to any compound, provided that this substance is different from the carrier gas and is present in a sufficient quantity for monitoring. A disadvantage of a TCD is it will respond to impurities in the carrier gas, to stationary phase bleeding from the column, or to air leaking into the GC system. The TCD is also highly sensitive to changes in flow-rate, such as might occur during temperature programming or flow programming. Another disadvantage of a TCD is it has a relatively poor lower limit of detection compared with other common GC detectors.

The flame ionization detector (FID) is another general GC detector. An FID detects organic compounds by measuring their ability to produce ions when they are burned in a flame. The flame in the FID is usually formed by burning the eluting compounds in a mixture of hydrogen and air. Positively charged ions produced by the combustion of organic compounds in the flame are collected by an electrode and produce a current that allows these compounds to be detected. An advantage of using an FID for the GC analysis of organic compounds is it gives little or no signal for many inorganic compounds, including carrier gases (e.g., He, Ar, and N_2) and common carrier gas contaminants (O_2, CO_2, and H_2O). The FID is also less sensitive to flow-rate changes than the TCD, which makes it easier to use with temperature programming and flow programming. One disadvantage of the FID is it is a destructive detector, which breaks down analytes during the process of their measurement, preventing this detector from being connected directly to other devices or techniques for compound analysis.

A nitrogen–phosphorus detector (NPD) is a selective detector used in GC for nitrogen- or phosphorus-containing compounds. The NPD is similar to an FID in that both are based on the measurement of ions produced from eluting compounds. The main difference from an FID is that, instead of a flame, an NPD uses thermal heating at or above a rubidium surface to form ions. This process is particularly efficient for nitrogen- or phosphorus-containing compounds, which makes the NPD selective for such chemicals. The greatest strength of the NPD is its good selectivity and low limits of detection for nitrogen- and phosphorus-containing compounds. Like an FID, the NPD does not detect many common carrier gases or impurities. It is necessary when using an NPD to periodically change the heated material, because it will slowly degrade over time.

The electron capture detector (ECD) is another selective detector used in GC. This device detects compounds

TABLE 8.2 Common detectors for gas chromatography.

Detector name	Compounds detected	Detection limit[a]
Thermal conductivity detector	General: all compounds	1 ng
Flame ionization detector	All organic compounds	1 pg
Nitrogen–phosphorus detector	N- and P-containing compounds	0.01–0.1 pg
Electron capture detector	Chemicals with electronegative groups	1–100 fg
Mass spectrometry	General: full-scan mode	0.1–1 ng
	Selective: SIM mode	1–10 pg

SIM, Selected ion monitoring.
[a]*This information is from Poole and Poole [2], Ewing [4], and manufacturers of these detectors.*

that have electronegative atoms or groups in their structure, such as halogen atoms (I, Br, Cl, and F) and nitro groups (NO_2). An ECD detects compounds based on the capture of electrons by such atoms or groups. These electrons are usually produced by a radioactive source, such as ^{3}H or ^{63}Ni, and in the presence of argon or nitrogen as the carrier gas. Although an ECD is selective and has good limits of detection, it does require a radioactive source that must be changed from time to time (now commonly done by a specially trained technician from the manufacturer of this device).

Another common detector used in GC is a mass spectrometer. This combination is known as gas chromatography/mass spectrometry (GC/MS). This is a powerful tool for both measuring and identifying a compound. For instance, this approach can provide information on a chemical's molecular mass (by using its molecular ion) or its structure (through the use of its fragment ions). Common ionization methods used in GC/MS are electron impact ionization and chemical ionization. The resulting ions are separated according to their mass and/or charge by using a device such as a quadrupole mass analyzer or ion trap. One way of viewing this information is to make a plot of the number of ions measured at each elution time, giving a mass chromatogram. Another way this information can be viewed is by looking at the mass spectrum for all ions that are produced for analytes eluting at a particular retention time.

A big advantage of GC/MS is it can be used to either universally or selectively detect compounds as they leave the column. When used as a universal detector, the total number of measured ions is used to plot the mass chromatogram. This method involves using the mass spectrometer to quickly scan through a wide range of mass-to-charge ratios while collecting information on each ion that occurs within this range. This detection format is also known as the "full-scan mode" of GC/MS. This mode is useful when the goal is to look for a broad range of compounds in a single analysis (e.g., during drug screening) or when a mass spectrum is desired to determine the identity of an unknown compound (e.g., determining the drug taken by a patient suffering from an overdose). A more selective mode for performing GC/MS is selected ion monitoring (SIM). In this approach, only a few ions characteristic of the compounds of interest are examined. This mode is employed when low detection limits are desired and when it is known in advance what compounds are to be analyzed.

Methods for sample injection and pretreatment in gas chromatography

Because GC requires that analytes be in the gas phase, gaseous analytes and samples are natural candidates for this technique. If the gaseous analytes are present at moderate-to-high concentrations, direct sampling and injection onto a GC system is possible. Direct injection can be performed by passing the sample through a gas-tight valve. Alternatively, a gas-tight syringe can be used to inject a known volume of gas into the GC system. For trace components in gases, it is often necessary to collect and concentrate these analytes for separation and measurement by GC. One way this collection and concentration can be accomplished is by passing a large volume of samples through a solid-phase extraction cartridge, a cold trap, or a liquid in which the analytes will dissolve.

Liquids are the type of sample most frequently encountered in GC. When using packed columns or wide-bore open-tubular columns, it is possible to use direct injection to place this liquid into the GC system. This direct injection involves the use of a calibrated microsyringe to apply the desired volume of liquid to the system through a gas-tight septum and into a chamber where it is vaporized for analysis. However, the volumes of liquid delivered by most microsyringes are too large for the

open-tubular columns used in many clinical applications of GC. This problem can be overcome by using split injection, in which only a small portion of the vaporized sample (0.01%−10%) is allowed to go into the column.

It is quite common for some pretreatment to be required before a liquid sample can be analyzed by GC. An example is the derivatization of a fatty acid or amino acid into a more volatile or thermally stable form prior to an analysis by GC. Another example is the transfer of analytes from their original sample into a solvent that is more suitable for testing by GC. The injection of water into most GC columns creates problems with the long-term behavior and reproducibility of these columns. These problems occur because water tends to bind strongly to such columns, changing their chromatographic properties from one injection to the next. In addition, the water may contain dissolved solids, salts, or other nonvolatile compounds that are not suitable for injection into a GC system. Because of these problems, aqueous-based samples are usually extracted before GC analysis. Either liquid−liquid or solid-phase extraction can be used to remove analytes from water and place them into a more volatile solvent, as well as to remove them from nonvolatile substances present in the sample. The use of solvent evaporation with these two methods also makes it possible to greatly reduce the sample volume, which allows a larger mass of analyte to be injected for easier detection.

Headspace analysis is another technique used to avoid the introduction of water and nonvolatile compounds into a GC system. This technique is based on the fact that volatile chemicals in a liquid or solid sample will also be present in the vapor phase located above the sample. By sampling this vapor (known as the "headspace"), a portion of the volatile substances can be collected without interference from other less volatile compounds. This approach can be performed in one of two ways: the static and dynamic methods. In the static method, the sample is placed in a closed container, and its contents are allowed to distribute between the sample and its vapor phase. After equilibrium has been reached, a portion of the vapor phase is injected into the GC system. In the dynamic method, an inert gas is passed through the liquid sample, which carries away volatile compounds. This gas is then passed through a cold trap or solid adsorbent, which collects and concentrates the volatile solutes for later analysis.

GC is not limited to gas and liquid samples but can be adapted for work with chemicals adsorbed or held within a solid sample. A common way of handling this type of analysis is to first extract the compounds of interest from the solid material by using liquid−liquid extraction, with the extracted analytes and their solvent then being treated as a liquid sample. It is also possible to analyze some solids without performing an extraction. For instance, headspace analysis might be used to examine the volatile compounds that are present in a solid. Another approach for examining solids is to use pyrolysis−GC. This approach is useful in forensic testing for solid substances like plastics and polymers that are not volatile and cannot be easily derivatized into a volatile form. Pyrolysis involves heating a solid sample in a controlled fashion to very high temperatures in order to break the solid apart into smaller, more volatile fragments that can be examined by GC. The result is a gas chromatogram which provides a fingerprint of the volatile compounds that are given off as the test substance is heated.

Liquid chromatography

General terms and concepts

LC is a chromatographic technique in which the mobile phase is a liquid [2−4,7−9]. There are many ways LC can be performed, but most LC methods for clinical analysis use a system like the one shown in Fig. 8.3. This

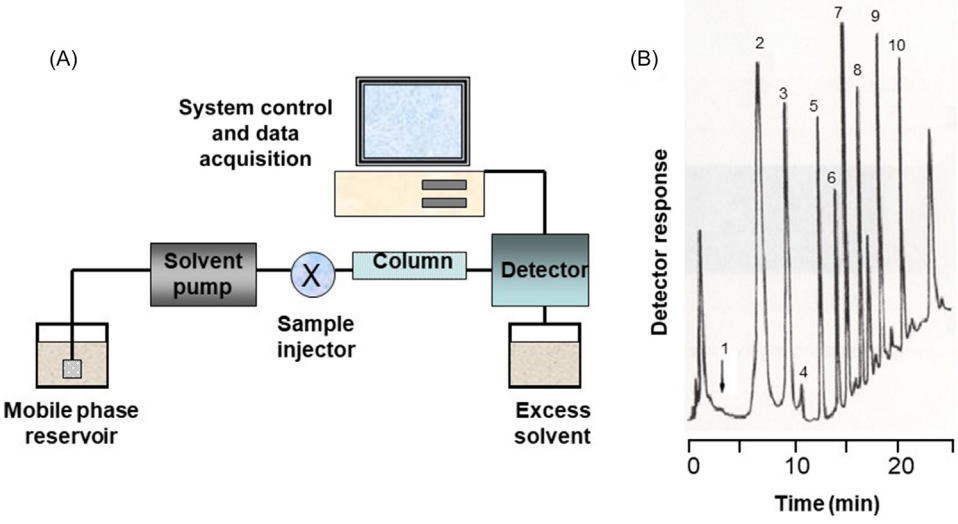

FIGURE 8.3 (A) A system for performing high-performance liquid chromatography and (B) a typical separation performed by high-performance liquid chromatography, using the analysis of basic drugs (peaks 1−10) in blood as an example. *The chromatogram in (B) is reproduced with permission from W.E. Lambert, D. Meyer, A.P. De Leenheer, Systematic toxicological analysis of basic drugs by gradient elution of an alumina-based HPLC packing material under alkaline conditions, J. Anal. Toxicol. 19 (1995) 73 [9]; courtesy Oxford University Press.*

system is known as a "liquid chromatograph," which includes a support and stationary phase enclosed in a column and a liquid mobile phase that is delivered to the column by means of a pump. An injection device is used to apply samples to the column, and a detector is usually present to detect and measure analytes as they leave the column.

Although LC and GC have many things in common, they also have some differences in terms of their sample and analyte requirements, their formats, and the role played by the mobile phase in these methods. The first requirement that must be met before a chemical can be examined by LC is that it must be possible to place this chemical into a liquid that can be injected into the mobile phase. This is a much less stringent requirement than the one presented in GC, where analytes must be naturally volatile or convertible to a volatile form. This feature makes LC valuable in separating many large biological compounds (e.g., proteins, peptides, and nucleic acids) that cannot be easily placed into the gas phase. The use of a liquid as the mobile phase also allows LC to be performed at much lower temperatures than GC, making it better suited for compounds that are thermally unstable.

A second requirement in LC is that there must be a difference in retention between the analytes to be separated. Although retention is altered in GC by adjusting the temperature and type of stationary phase within the column, retention in LC can also be varied by changing the mobile phase due to the higher density of liquids compared with gases. As a result, solute retention in LC depends on the interactions of sample components with both the mobile phase and the stationary phase, making LC more flexible than GC when optimizing a separation.

One disadvantage of LC is it tends to have greater band-broadening than GC. This greater band-broadening is due to the much slower diffusion coefficients of solutes in liquids versus gases. Thus, if it is possible to separate a series of chemicals by either LC or GC, GC is often the better choice, because it will provide narrower peaks and better separations. However, as discussed previously, the use of GC requires that the analytes be volatile or capable of being converted into a volatile derivative, which is not needed in LC. The greater density and viscosity of liquids compared with gases means that higher pressures are needed in LC than in GC to deliver mobile phase to the column. As a result, columns in LC are often much shorter than those used in GC, which also tends to make the number of theoretical plates lower for LC columns than for GC columns.

One result of the greater band-broadening and shorter columns in LC is that more emphasis is placed on obtaining more efficient supports with less band-broadening. Prior to the mid-1960s all LC columns contained supports made of relatively large and irregularly shaped particles. The use of such supports created broad peaks and separations with low resolution. This approach is sometimes called "classical LC" and is still commonly used for sample pretreatment because of its low cost and ease of use. However, a shift then occurred in LC toward the use of smaller and more uniform and rigid supports. This shift led to a technique known as high-performance liquid chromatography (HPLC). The presence of a more efficient support in this method produces narrower peaks, which in turn gives rise to better separations and lower limits of detection. These features make HPLC the current LC method of choice for clinical analysis. One consequence of using smaller support particles in HPLC is the need for greater pressures to pass the mobile phase through the column. Most modern HPLC columns require operating pressures of a few hundred to a few thousand pounds per square inch. This calls for special pumps and other system components that can be operated at such pressures (see Fig. 8.3). The sample in HPLC analysis is applied by using a closed system (e.g., an injection valve), and detection is typically performed by using an on-line detector.

Liquid chromatography mobile phases and elution methods

An important difference between LC and GC is that the retention of solutes in LC depends on interactions involving both the mobile and stationary phases. To describe how solutes are retained on an LC column in the presence of a given liquid, the terms "weak mobile phase" and "strong mobile phase" are used. A strong mobile phase is a solvent or a solution that quickly elutes a solute from a column. This situation occurs when the mobile phase is very similar to the stationary phase in the types of interactions that each can have with solutes. A weak mobile phase is a solvent or solution that elutes solutes slowly from a column, which occurs if the mobile phase is very different from the stationary phase in its interactions with solutes. It is important to note that whether a liquid is a weak or strong mobile phase will depend on the type of stationary phase in the column. For instance, a nonpolar solvent (such as hexane) will be a weak mobile phase on a polar stationary phase but a strong mobile phase for a nonpolar stationary phase. Thus it is the stationary phase that determines what liquids are strong or weak mobile phases for a given LC column.

Analytes can be eluted from an LC column by using either constant conditions or by using gradient elution. If a constant mobile phase composition is used for elution, this technique is referred to as "isocratic elution." Although this type of elution is simple and inexpensive, it does make it difficult to elute all solutes with good

resolution and in a reasonable amount of time. An alternative approach is to change the composition of the mobile phase with time. This approach is known as "solvent programming." This method is conducted by starting with a weak mobile phase, which allows early eluting solutes to be more strongly retained by the column. A switch is then made to a stronger mobile phase to allow highly retained solutes to come out in a reasonable amount of time. Solvent programming can be performed in one or more steps and by using a linear or nonlinear change in the mobile phase content, with linear gradients between two solvents being the most common method.

Temperature programming can also be used in LC, but it is much less common here than in GC. Temperature programming does improve resolution in some types of LC, such as size-exclusion chromatography (SEC) and ion-exchange chromatography (IEC). However, this method gives only marginal improvements in other types of LC and can cause degradation for some solutes. Flow programming can be used in LC as well. Advantages of flow programming include its ability to more quickly get the LC system ready for the next injection than solvent or temperature programming. A disadvantage of using flow programming in LC is the increased pressure across the column that occurs with an increase in flow-rate.

Types of liquid chromatography

Adsorption chromatography

A common way of grouping LC techniques is according to the mechanisms by which they separate solutes. The first of these is adsorption chromatography, which is a chromatographic technique that separates solutes based on their adsorption to the surface of a support. This method is also known as LSC. This technique is similar to GSC in that it uses the same material as both the stationary phase and the support. In fact, many of the supports used in GSC are also used in LSC.

The solid supports used in adsorption chromatography may be either polar or nonpolar in nature. Silica is the most popular support for adsorption chromatography and is a polar material with slightly acidic properties. Because silica is polar in nature, it will have the highest retention for polar compounds and will have a strong mobile phase that is polar in nature. Alumina is another popular support employed in adsorption chromatography. It is also polar and has the highest retention for polar compounds. Charcoal is also sometimes used as a support in adsorption chromatography. Unlike silica or alumina, charcoal is nonpolar and most strongly retains nonpolar solutes. The strong mobile phase on a charcoal column will also be nonpolar.

Adsorption chromatography is particularly useful in separating compounds with polar groups but that are soluble in nonpolar solvents; examples include steroids such as estrogens and testosterone. This method is also valuable in the separation of geometrical isomers and structurally similar chemicals. There are, however, problems in the use of adsorption chromatography. These problems include the heterogeneous nature of the surface on silica or alumina and the ability of these surfaces to act as catalysts for some chemical reactions. These supports can also give rise to nonreproducible retention for polar compounds and require the use of good quality solvents to give consistent mobile phase strengths.

Partition chromatography

The second major type of LC is partition chromatography. This is a chromatographic technique in which solutes are separated based on their partitioning between a liquid mobile phase and a stationary phase coated on a solid support. The support used in partition chromatography is usually silica but can also be other materials. Originally, partition chromatography involved coating the support with a liquid stationary phase that was immiscible with the mobile phase. However, most modern columns for partition chromatography employ stationary phases that are chemically bonded to the support.

There are two main types of partition chromatography. The first of these is normal-phase chromatography [also called "normal-phase liquid chromatography" (NPLC)], which is a type of LC in which the stationary phase is polar. These stationary phases contain polar regions based on cyano, amino, or diol groups that can form hydrogen bonds or undergo dipole interactions with solutes. Because NPLC has a polar stationary phase, it has the strongest retention for polar compounds. However, it may also be used for separating nonpolar compounds. The weak mobile phase in NPLC is a nonpolar liquid, such as an organic solvent, which is used as the injection solvent. A strong mobile phase is a polar liquid, such as water or methanol.

Reversed-phase chromatography [also known as "reversed-phase liquid chromatography" (RPLC)] is the second type of partition chromatography. It uses a nonpolar stationary phase, which is opposite or "reversed" in polarity from that utilized in normal-phase chromatography. This stationary phase usually consists of an alkane like an octyl group (C_8) or octadecyl group (C_{18}). RPLC is currently the most popular type of LC. The main reason for its popularity is that its weak mobile phase is a polar solvent, such as water. This feature makes RPLC ideal for the injection and separation of solutes in aqueous-based systems, such as drugs in clinical samples.

NPLC has similar applications to adsorption chromatography performed with silica or alumina supports. These applications typically involve the use of NPLC for separating chemicals in organic solvents and that contain polar functional groups. Examples include steroids, pesticides, terpenoids, nonionic detergents, sugars, and metal complexes. RPLC is used to separate a broad range of substances in aqueous samples, including its use for both small molecules (such as drugs) and macromolecules (such as proteins).

Another type of partition chromatography is hydrophilic interaction liquid chromatography (HILIC). Like NPLC, HILIC uses a polar stationary phase. However, in HILIC, the components of a sample now distribute between an organic-rich region in the mobile phase and a more polar water-enriched layer located at or near the surface of the support. The polar groups on the support's surface, which can be neutral or charged, may also interact with chemicals as they enter the water-enriched layer on the support. HILIC has been of particular interest for the analysis of polar compounds in fields such as proteomics and glycomics.

Ion-exchange chromatography

Another major type of LC is IEC. This is a chromatographic technique in which solutes are separated by their adsorption onto a support containing fixed charges on its surface. IEC is a fairly common technique used for the removal or replacement of ionic compounds in samples. It is also used for the separation and analysis of charged compounds, including inorganic ions, organic ions, and biological compounds, such as amino acids, proteins, and nucleic acids.

There are two types of stationary phases used in IEC. The first type is a cation-exchanger, which has a negatively charged group and is used to separate positively charged ions. The second type is an anion-exchanger, which has a positively charged group and is used to separate negatively charged ions. These two types of stationary phases are used in the methods of cation-exchange chromatography and anion-exchange chromatography, respectively. There are several types of stationary phases used in these methods. For cation-exchange chromatography, the charged groups consist of either: (1) the conjugate base of a strong acid (e.g., a sulfonic acid group), which is ionized over a broad pH range; or (2) the conjugate base of a weak acid (e.g., a carboxylate group), which has a net charge over a moderate pH range that is above the pK_a of the weak acid. For anion-exchange chromatography, the stationary phase is either: (1) the conjugate acid of a strong base (e.g., a quaternary amine), which is ionized over a broad pH range; or (2) the conjugate acid of a weak base (e.g., a tertiary amine), which has a net charge over a moderate pH range that is below the pK_a of its conjugate acid.

Several supports can be used with these cation- or anion-exchange groups. One common support used in IEC for small inorganic and organic ions is polystyrene. Carbohydrate-based gels are another common type of support used in IEC. Examples of these include agarose, cross-linked dextran, and cellulose gels. Silica can also be used as a support in IEC. Each of these supports is modified prior to placing appropriate charged groups on their surface. The high efficiencies and rigid structure of silica make it useful for HPLC-based IEC. The main limitation of silica in these applications is the narrower range of pH values over which it is stable compared with polystyrene- or carbohydrate-based supports.

A strong mobile phase in IEC is a solution with a high concentration of competing ions. A change in ion concentration is the most common way of altering the retention of sample ions in IEC. However, the retention of charged analytes will also be affected by the type of competing ion, the type of ion-exchange site being used and the mobile phase pH, the latter of which can alter the charge on some analytes, competing ions, or ion-exchange sites. Adding a complexing agent to the mobile phase can also affect the charge of an analyte and alter its retention. For instance, complexation of the cation Fe^{+3} with excess Cl^- can be used to form a negatively charged $FeCl_4^-$ complex, which can then be retained and analyzed by anion-exchange chromatography.

As a preparative tool, IEC has been used for many years in biochemistry for purifying proteins, peptides, and nucleotides. Ion-exchange supports are also frequently employed for concentrating small inorganic and organic ions from food and environmental samples. In addition, IEC has been used for many years for direct chemical analysis. One common example in clinical chemistry is the use of IEC to separate amino acids and amines to look for metabolic disorders in patients, as illustrated in Fig. 8.4.

The use of a high concentration of competing ions makes it difficult to detect cations or anions as they leave ion-exchange columns. A way to overcome this problem is to use a special type of IEC known as "ion chromatography" (IC). In this method, the background signal due to competing ions is reduced by using a low number of charged sites for the stationary phase. The result is that a lower concentration of competing ions will be needed to elute sample ions. This method is often used with a second column or membrane separator (of opposite charge to the first ion-exchange column), in which competing ions that have high conductivity are replaced with chemicals or ions that have lower conductivity. The result is a lower signal due to the competing ions, providing better limits of detection for analyte ions.

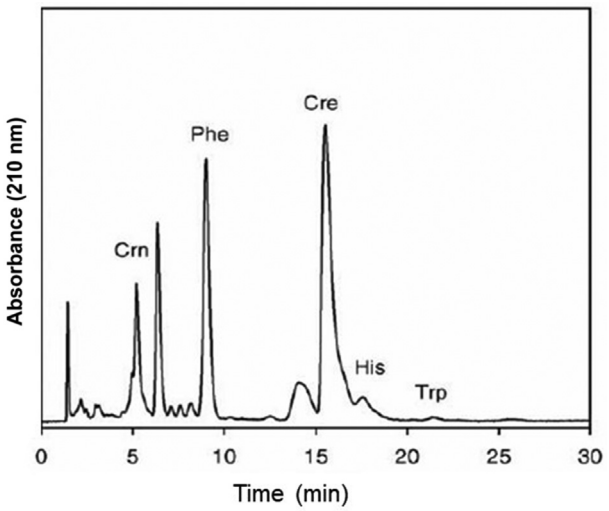

FIGURE 8.4 Use of ion-exchange chromatography to separate and analyze UV-absorbing amino acids in urine for an individual with phenylketonuria. Abbreviations: *Cre*, creatinine; *Crn*, creatine; *His*, histidine; *Phe*, phenylalanine; *Trp*, tryptophan. *Reproduced with permission from Y. Yokoyama, K. Yamasaka, H. Sato, Simultaneous determination of urinary creatinine and UV-absorbing amino acids using a novel low-capacity cation-exchange chromatography for the screening of inborn errors of metabolism, J. Chromatogr. B 816 (2005) 333 [10]; courtesy Elsevier.*

Size-exclusion chromatography

The fourth general type of LC is size-exclusion chromatography (SEC). This is a liquid chromatographic technique that separates substances according to their size. This technique is based on the different ability of analytes to access mobile phase within the pores of a support. No true stationary phase is present in this system. Instead, the mobile phase in the pores acts as the stationary phase. SEC uses a support that has a certain range of pore sizes. As solutes travel through this support, small molecules can enter the pores while large molecules cannot. Larger molecules can enter a smaller volume of the column, so they elute before smaller molecules. The result is a separation based on size or mass.

The ideal support in SEC consists of a porous material that does not interact directly with the injected solute. Carbohydrate-based supports like dextran and agarose can be used in SEC for biological compounds and aqueous-based samples. Polyacrylamide gel can also be employed for such samples. Similarly, polystyrene can be used for SEC when working with samples in organic solvents, and silica containing a diol-bonded phase can be utilized with aqueous samples.

The mobile phase in SEC can be either a polar or nonpolar solvent. Because there is no true stationary phase, there is also no weak or strong mobile phase in this method. Instead, the selection of mobile phase depends mostly on the solubility of the analytes and the support's stability. If an aqueous mobile phase is used in SEC, the technique is called "gel filtration chromatography." If an organic mobile phase is used, the technique is known as "gel permeation chromatography." As a preparative tool, SEC is often used with biological samples to remove small solutes from large agents like proteins. It can also be used to transfer large analytes from one solution to another or to remove salts from a sample. This approach can further be used to estimate the molecular weight of an analyte like a protein by comparing the retention of such an analyte to that of similar standard compounds with known masses.

Affinity chromatography

The fifth type of LC is affinity chromatography (AC). This method is based on biologically related interactions, like the binding of an enzyme with its substrate, or the binding of an antibody with an antigen. These reactions are used in AC by immobilizing one of a pair of interacting molecules onto a solid support and placing it into a column. This immobilized molecule is known as the "affinity ligand," and it is this agent that acts as the stationary phase in this method. The column containing the immobilized ligand can then be employed as a selective adsorbent for the complementary molecule.

The sample in AC is often applied to the column in the presence of an application buffer during an adsorption step. Because of the strong and selective nature of most biological interactions, the affinity ligand will bind to the analyte of interest during this step while allowing most other sample components to pass through as a nonretained peak. After these nonretained components have been washed from the column, an elution buffer is applied to release the retained analyte. This analyte is then detected as it leaves the column or collected for later use. The column and affinity ligand are then placed back into the application buffer, allowing them to be regenerated prior to injection of the next sample.

There are several types of ligands employed in AC, but all can be classified into one of two categories: (1) high-specificity ligands and (2) general (group-specific) ligands. High-specificity ligands are compounds that bind to only one or a few very closely related molecules. Examples include antibodies for binding to foreign agents (i.e., antigens) and single-stranded nucleic acids for separating and binding to complementary strands. General, or group-specific, ligands are molecules that bind to a family or class of related molecules. Examples of these include boronates for binding compounds with diol groups, and lectins for retaining compounds that have carbohydrate groups.

Several types of supports are utilized in AC. Carbohydrate gels like agarose or cellulose are commonly

used with affinity ligands for the purification of biological molecules. Silica can also be used by first converting this support into a diol-bonded form. The affinity ligand is then attached to these supports by using an immobilization method. This process usually involves coupling the affinity ligand to the support through amine, carboxyl, or sulfhydryl groups. If appropriate immobilization conditions are not used, the ligand may be denatured or attached in a way that blocks its binding to the analyte. For the immobilization of small molecules, placement of a spacer arm between the ligand and the support may be necessary to reduce steric hindrance in the binding of larger molecules to this ligand.

A weak mobile phase in AC is one that allows strong binding between the analyte and the ligand. This weak mobile phase is usually a solvent that mimics the pH, ionic strength, and polarity of the affinity ligand in its natural environment. This mobile phase is known as the "application buffer." It is this solvent that is typically used during the application, washing, and regeneration steps. A strong mobile phase in AC is a solvent that can readily remove the analyte from the ligand. This is called the "elution buffer." This strong mobile phase is selected to either lower the association equilibrium constant for the analyte−ligand interaction or to displace the analyte from the affinity ligand through the use of a competing agent.

AC is frequently used as a large-scale purification method for enzymes and proteins. This application involves the use of columns that contain immobilized agents that can selectivity bind to and retain such substances in the presence of other sample components. However, AC is also commonly used as a method for sample preparation. Examples include the use of affinity columns containing antibodies for the isolation of cellular proteins or the use of immobilized metal ions to isolate recombinant proteins containing histidine tags as part of their structure. The selectivity of AC has also made it appealing for use in the direct analysis of complex biological samples. One example is the use of boronate affinity columns in the measurement of glycated hemoglobin, an indicator of long-term blood sugar levels in diabetes. Affinity columns have also been used with HPLC for the measurement of hormones, proteins, drugs, and other agents in clinical and biological samples.

Liquid chromatography detectors

As shown in Table 8.3, there are several types of detectors available for LC. These can again be classified as either general or selective detectors. An absorbance detector is an example of a general detector for LC. This type of device is similar to those used for other absorbance measurements but includes a special sample cell (known as a "flow cell"), which allows the mobile phase and analytes to pass through in a continuous manner. The simplest type of absorbance detector for LC is a fixed-wavelength absorbance detector. This device is set to always monitor a specific wavelength (usually 254 nm). A variable-wavelength absorbance detector has a more flexible design and allows the monitored wavelength to be varied over a wide range (e.g., 190−900 nm). A third design is a photodiode array detector, which uses an array of small detector cells to measure the change in absorbance at many wavelengths simultaneously. This array makes it possible to record an entire spectrum for an eluting compound. Absorbance detectors can detect any compound that absorbs light at the wavelength(s) monitored. They can also be used with gradient elution, provided that the weak and strong mobile phases do not have significant

TABLE 8.3 Common detectors for liquid chromatography.

Detector name	Compounds detected	Detection limit[a]
Refractive index detector	General: all compounds	0.1−1 μg
UV−vis absorbance detector	Compounds with chromophores	0.1−1 ng
Evaporative light scattering detector	Nonvolatile compounds	0.1 μg
Fluorescence detector	Fluorescent compounds	1−10 pg
Conductivity detector	Ionic compounds	0.5−1 ng
Electrochemical detector	Electrochemically active compounds	0.01−1 ng
Mass spectrometry	General: full-scan mode	0.1−1 ng
	Selective: SIM mode	

SIM, Selected ion monitoring.
[a]*This information is from Poole and Poole [2] and manufacturers of these detectors.*

differences in their absorbances at the detection wavelengths. The main disadvantage of these devices is they require a compound to have a chromophore that can absorb at the wavelengths being monitored or that can be derivatized into a form that does absorb.

A refractive index (RI) detector is one of the most universal detectors available for LC. This detector measures the ability of the mobile phase and analytes to refract or bend light. This property varies as the composition of the mobile phase changes, such as when analytes elute from a column. An RI detector will respond to any compound that has a different RI from the mobile phase, provided that enough solute is present to give a measurable signal. This property makes an RI detector useful in work where an analyte cannot be easily measured by other devices or where the nature or properties of an analyte are not yet known. However, an RI detector does not have limits of detection as low as absorbance detectors or other LC detectors. In addition, its signal is sensitive to changes in the mobile phase composition and temperature, making it difficult to use with gradient elution.

A third type of general detector for LC is an evaporative light scattering detector (ELSD). This device can be used for any solute that is less volatile than the mobile phase and detects analytes by first converting the eluting mobile phase into a spray of small droplets. As the solvent in these droplets evaporates, small particles of the nonvolatile sample components are left behind that can scatter light. The extent of this scattering will depend on the number and size of these particles and the original concentration of nonvolatile solutes in the mobile phase. An ELSD has a better limit of detection than an RI detector and can be used with gradient elution. Although absorbance detectors have better limits of detection, an ELSD does not require that a chromophore be present in the analyte. This feature gives an ELSD the ability to examine substances that cannot be easily detected based on their absorbance, such as lipids and carbohydrates.

An example of a selective detector for LC is a fluorescence detector. This device measures the ability of solutes to absorb and emit light at a particular set of wavelengths. Fluorescence can also be used to detect compounds that are first converted to a fluorescent derivative, as is often used for analytes such as alcohols, amines, amino acids, and proteins. Although fluorescence detectors can be used with gradient elution, extremely pure mobile phases must be employed, because even trace impurities can affect the background signal or quench the fluorescence of solutes.

A conductivity detector can monitor ionic compounds in LC. This detector measures the ability of the mobile phase and its contents to conduct a current when placed in an electrical field. Conductivity detectors can be used to detect any compound that is ionic, making them useful in IC. This device can be used with gradient elution as long as the ionic strength (and possibly pH) of the mobile phase is kept constant. It is also necessary that the background conductance of the mobile phase be sufficiently low so that sample ions can be detected.

An electrochemical detector is another device used to monitor specific compounds in LC. This combination is known as "LC/electrochemical detection" (LC/EC). An electrochemical detector can be used to measure the ability of an analyte to undergo either oxidation or reduction. Examples of compounds that may be detected by reduction in such a detector include aldehydes, ketones, esters, unsaturated compounds, and aromatics. Compounds that may be detected by their oxidation include phenols, mercaptans, aromatic amines, diamines, purines, and dihydroxy compounds, such as some carbohydrates. The response of an electrochemical detector depends on the extent of oxidation or reduction that occurs at the given potential of the electrode. For such compounds, the limit of detection can be quite low due to the accuracy with which electrical measurements (especially current) can be made.

Another type of detector that can be employed in LC is a mass spectrometer. The result is a technique known as "liquid chromatography/mass spectrometry" (LC/MS). The use of mass spectrometry with LC can be used to both measure the amount of a chemical and identify this chemical based on its molecular ion and/or fragment ions. By looking at all ions that are produced in the mass spectrometer (i.e., the full-scan mode), LC/MS can be used as a general detection method. If LC/MS is instead used to look at a few ion characteristics of a particular set of analytes (i.e., the SIM mode), this approach can also be used for selective detection.

The most common way of performing LC/MS is to use electrospray ionization (ESI). This combination can examine substances ranging from small polar compounds to proteins. It can also be utilized with gradient elution methods. The use of LC/MS with ESI is particularly useful in the analysis of proteins and peptides, which tend to give ions with mass-to-charge ratios that are outside the range of most common mass analyzers. In ESI, this problem is overcome by the fact that many charges are often placed on one biomolecule, giving ions with lower mass-to-charge ratios. One difficulty associated with this process is that a single protein or peptide can give rise to many molecular ions. However, this issue can be addressed by using computer programs that are designed to analyze such spectra and determine the true molecular masses from such information.

Introduction to electrophoresis

General terms and concepts

Another separation method often used in clinical testing is electrophoresis [11]. This is a technique in which

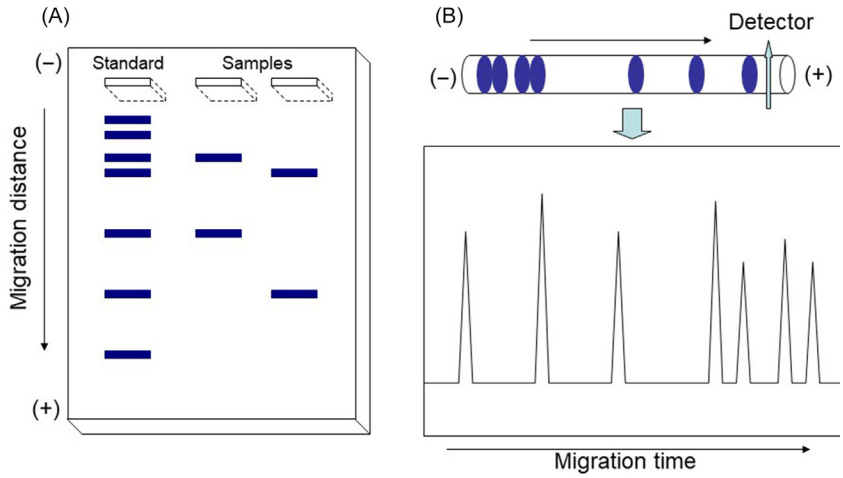

FIGURE 8.5 General scheme for the separation of chemicals by (A) gel electrophoresis and (B) capillary electrophoresis.

solutes are separated by their different rates of migration in an electric field. This concept is illustrated in Fig. 8.5, in which a sample is first placed in a container or support that also contains a background electrolyte (or "running buffer"). When an electric field is applied to this system, ions in the running buffer will flow from one electrode to the other and provide the current needed to maintain the applied voltage. At the same time, positively charged ions in the sample will move toward the negative electrode (the cathode), while negatively charged ions will move toward the positive electrode (the anode). The result is a separation of these ions based on their charge and size. Many biological compounds have charges or ionizable groups (e.g., DNA and proteins), which makes electrophoresis a common tool in clinical laboratories and medical research.

Electrophoresis can be performed in a variety of formats. One way is to apply small amounts of a sample to a support (usually a gel) and allow analytes to travel in a running buffer through the support when an electric field is applied. This approach is known as "gel electrophoresis." It is also possible to separate the components of a sample in a narrow capillary filled with a running buffer and placed into an electric field. This second method is called "capillary electrophoresis" (CE).

Depending on the type of electrophoresis being performed, the result of the separation can be viewed in one of two ways. In the case of gel electrophoresis, the separation is stopped before the analytes have traveled off the support. The result is a series of bands in which the migration distance characterizes the extent these substances have interacted with the electric field. Because the migration distance of an analyte through an electrophoresis gel will depend on the exact voltage and time used for the separation, it is common to include standard samples on the support to help in analyte identification. The intensities of the resulting bands are then used to measure the amounts of analytes in the sample.

In CE, all analytes travel the same distance, from the point of injection to the opposite end, where a detector is located. However, the analytes now differ in the time it takes to travel this distance. In this case, it is the migration time for each analyte that is measured and recorded. The resulting plot of detector response versus migration time is called an "electropherogram." This migration time is used for analyte identification, while the peak height or peak area is utilized for measurement. An internal standard is usually injected along with the sample to correct for variations during injection or for small fluctuations in the experimental conditions during the separation.

Theory of electrophoresis

The separation of analytes by electrophoresis requires two items. First, there must be a difference in how the analytes will interact with the electric field. This requirement means the analytes must have a difference in their migration times or migration distances. The second requirement is that the bands or peaks for the analytes must be sufficiently narrow to allow them to be resolved.

The overall rate of travel of a charged solute in electrophoresis will depend on two opposing forces. The first of these is the attraction of a charged solute toward the electrode of opposite charge. This force depends on the strength of the applied electric field (E) and the charge on the solute (z). However, the solute will also experience resistance to its movement by the surrounding medium. The force of this resistance depends on the size of the solute (as described by the solvated radius r), the viscosity of the medium (η), and the velocity of the solute (v). When an electric field is applied, a solute will accelerate toward the electrode of opposite charge until these two

opposing forces become equal in size but opposite in direction. At this point, a steady-state situation is produced in which the solute moves at a constant velocity, as given by Eq. (8.9).

$$v = \frac{(Ez)}{(6\pi r\eta)} \tag{8.9}$$

To see how this velocity will be affected by only the strength of the electric field, other terms in Eq. (8.9) can be combined to give a single constant (μ),

$$v = \mu E \tag{8.10}$$

where $\mu = z/(6\pi r\eta)$. This combination of terms is known as the "electrophoretic mobility." The value of μ is constant for a given analyte under a particular set of temperature and solvent conditions. However, this value will depend on the apparent size and charge of the solute, as represented by the ratio z/r in Eq. (8.9). As a result, any two solutes with different charge-to-size ratios can, in theory, be separated by electrophoresis.

It is also possible to have movement of the running buffer in an electric field. This movement can occur if there are any fixed charges present within the system, such as on the surface of a support. The presence of these fixed charges attracts ions of opposite charge from the running buffer and creates an electrical double layer at the support's surface. In the presence of an electric field, this double layer acts like a piston that causes a net movement of the buffer toward the electrode of opposite charge to the fixed ionic groups. This process is known as "electroosmosis" and results in a net flow of the buffer within the system.

The extent to which electroosmosis affects the buffer and analytes in electrophoresis is described by the electroosmotic mobility (μ_{eo}). This term depends on such things as the size of the electric field, the type of running buffer, and the charge on the support. Depending on the direction of buffer flow, electroosmosis can work either with or against the natural migration of an analyte through the electrophoretic system. The overall observed electrophoretic mobility (μ_{Net}) for an analyte will equal the sum of its own electrophoretic mobility (μ) and the mobility of the running buffer due to electroosmotic flow (μ_{eo}).

$$\mu_{Net} = \mu + \mu_{eo} \tag{8.11}$$

In gel electrophoresis, electroosmotic flow is usually small compared with the inherent rate of analyte migration. However, this is not usually the case in CE, where the support has a relatively large charge and high surface area compared with the volume of running buffer.

The same terms used to describe band-broadening in chromatography (e.g., the number of theoretical plates N and the height equivalent of a theoretical plate H) can be used in electrophoresis. However, chromatography and electrophoresis differ in which processes are most important in producing this band-broadening. For instance, one important band-broadening process in many types of electrophoresis is *Joule heating*. This is heating that occurs whenever an electric field is applied to the system. According to *Ohm's law*, placing a voltage V across a medium with a resistance of R requires that a current of I be present to maintain this voltage.

$$V = IR \tag{8.12}$$

However, as current flows through the system, heat is generated. This heat production depends on the voltage, current, and time t that current passes through the system, as shown below.

$$\text{Heat} = VIt \tag{8.13}$$

As heat is produced, the temperature of the electrophoretic system will begin to rise and increase the rate of diffusion. In addition, if the heat is not distributed uniformly, the temperature will not be the same throughout the system. This effect leads to regions with different densities, which causes mixing and results in even more band-broadening.

One way Joule heating can be decreased is by using a lower voltage for the separation. However, using a lower voltage will also lower the migration velocities of analytes and give longer separation times. An alternative approach is to use more efficient cooling, which allows higher voltages to be employed and provides shorter separation times. A third possibility is to add a support to minimize the effects of uneven heat distribution and density gradients in the running buffer. Another factor that affects Joule heating is the ionic strength of the running buffer, where a lower ionic strength buffer will decrease heat production.

Wick flow is a source of band-broadening that occurs in gel electrophoresis. In such a system, the gel is kept in contact with the electrodes and buffer reservoirs through the use of wicks. The gel is often open to air, which means Joule heating will cause evaporation of solvent in the running buffer at the surface of this support. As this solvent is lost, it is replenished by the flow of more solvent through the wick and from the buffer reservoir. This process leads to a net movement of buffer toward the center of the support. Because the rate of this flow depends on the rate of evaporation, it increases with high voltages and high currents.

Gel electrophoresis

General terms and concepts

One of the most common types of electrophoresis is the method of gel electrophoresis. This is an electrophoretic method that is performed by applying a sample to a gel

support that is then placed into an electric field. In this type of system, several samples are usually applied to the gel and allowed to migrate in the presence of an electric field. The separation is stopped before analytes leave the gel, with the location and intensities of their bands then being determined.

Supports and sample application in gel electrophoresis

A system for performing gel electrophoresis may involve a support held in either a vertical or horizontal position. The type of support used will depend on the analytes and samples being examined. Cellulose acetate and filter paper are valuable for work with small molecules like amino acids and nucleotides. Starch is also sometimes used as a support in electrophoresis. However, a more common support is agarose. In addition to its low nonspecific binding for many biological compounds and its low inherent charge, agarose gels have relatively large pores. This property makes agarose useful in dealing with large molecules like DNA, as occurs in DNA sequencing.

Probably, the most common support used in gel electrophoresis is polyacrylamide. This combination is often referred to as "polyacrylamide gel electrophoresis" (PAGE). Polyacrylamide is a synthetic polymer that can be made with a variety of pore sizes. These pores are generally smaller than those in agarose and of a size more suitable for the separation of proteins and peptide mixtures. Like agarose, polyacrylamide has low nonspecific binding for many biological compounds and does not have any inherent charged groups in its structure.

The samples in gel electrophoresis are applied by a micropipette to small wells that are made in the gel during its preparation. This process usually involves applying sample volumes in the range of 10−100 μL. These sample volumes help provide a sufficient amount of analytes for later detection and collection. However, there is also a danger of introducing band-broadening by creating a large sample band at the beginning of the separation. A common approach to create narrow sample bands is to employ two types of gels in the system: a stacking gel and a running gel. The stacking gel has a low degree of cross-linking (giving it large pores) and is located in the section of the support in which the sample wells are located. The running gel has a higher degree of cross-linking (i.e., smaller pores) and is used during the actual separation of analytes. After a sample has been placed in the wells in the stacking gel, analytes will travel quickly through this medium until they reach its boundary with the running gel. These substances will then travel much more slowly, allowing other parts of the sample to catch up and form a narrower, more concentrated band that can then be separated into its components as they travel through the running gel.

Detection in gel electrophoresis

There are several ways in which analytes can be detected in gel electrophoresis. This detection is performed after the analytes have been separated either by looking at their location on the gel or by transferring these substances to a different support for detection. In some cases, it is possible to perform direct detection of analytes on a gel. Direct detection can sometimes be conducted visually when dealing with intensely colored proteins, like hemoglobin, or by using absorbance measurements and a scanning device called a densitometer. However, direct detection requires that an analyte be present at a relatively high level in a sample or concentrated by a pretreatment step prior to the separation.

The most common approach for detection in gel electrophoresis is to treat the support with a stain or reagent that makes it easier to see analyte bands. Examples of stains used for proteins are Amido black, Coomassie Brilliant Blue, and Ponceau S. Silver nitrate, giving rise to a method known as "silver staining," is also used when looking at low concentration proteins. DNA bands are often detected by using ethidium bromide. When separating enzymes, the natural catalytic ability of these substances can be used for their detection (e.g., detecting production of the fluorescent compound NAD(P)H by enzymes that generate this substance in their reactions). Sometimes biological ligands are used to react with analytes on a gel for detection. This approach is used in rocket immunoelectrophoresis, where antibodies are used to form precipitation bands that help identify and measure specific proteins in a sample.

A third approach for detection in gel electrophoresis is to transfer a portion of the analyte bands to a second support (e.g., nitrocelluose), where they are reacted with a labeled agent. This approach is known as "blotting." There are several different types of blotting methods. A Southern blot is used to detect specific sequences of DNA by having these sequences bind to an added, known sequence of DNA containing a radioactive tag (^{32}P) or other easily detected label. A Northern blot is similar in format but is instead used to detect specific sequences of RNA by using a labeled DNA probe. A Western blot is used for detecting a given protein by reacting this protein with labeled antibodies that can bind to such analytes.

Special types of gel electrophoresis

Whenever there is a porous support in an electrophoretic system, it is possible that analytes may be separated by size as well as their electrophoretic mobilities. This effect

is used for proteins in a technique known as "sodium dodecyl sulfate polyacrylamide gel electrophoresis" (SDS-PAGE). In SDS-PAGE, the proteins in a sample are first denatured and their disulfide bonds are broken through the use of a reducing agent. This pretreatment converts the proteins into a set of single-stranded polypeptides. These polypeptides are then treated with sodium dodecyl sulfate (SDS). This is a surfactant with a nonpolar tail and a negatively charged sulfonic group at the other end. The nonpolar end of this surfactant coats the protein, forming a roughly linear rod with a layer of negative charges on the outside. The result for a mixture of proteins is a series of rods with different lengths but similar charge-to-mass ratios. Next, these protein rods are passed through a porous polyacryamide gel in the presence of an electric field. The negative charges on these rods from the SDS coating cause them all to move toward the positively charged electrode, while the pores of the gel cause small rods to travel more quickly than larger ones. At the end of the run, the positions of the protein bands from a sample are compared with those obtained in a protein ladder for a series of protein standards with known molecular masses and applied to the same gel, as illustrated in Fig. 8.5A. This comparison can be performed either qualitatively or by preparing a calibration curve by plotting the log of the molecular masses for the protein standards versus their migration distances.

Another type of electrophoresis that often makes use of supports is isoelectric focusing (IEF). This is a method used to separate zwitterions (i.e., substances with both acidic and basic groups) based on differences in their isoelectric points. This type of separation is accomplished by having the zwitterions migrate in an electric field across a pH gradient. In this pH gradient, each zwitterion will migrate until it reaches a region where the pH is equal to its isoelectric point (pI). Once this situation has occurred, the zwitterion will no longer have any net charge, and its electrophoretic mobility will become zero, causing the analyte to stop migrating. This results in a series of tight bands, where each band appears at the point where the pH is equal to the pI for a given analyte. IEF is useful in separating proteins with similar properties but slightly different amino acid compositions. A common example is its use in separating the isoforms of an enzyme. To obtain a separation in IEF, it is necessary to have a stable pH gradient. This pH gradient is produced by placing in the electric field a mixture of small reagent zwitterions known as "ampholytes." These are usually polyprotic amino carboxylic acids with a range of pK_a values. When these ampholytes are placed in an electric field, they will travel through the system and align in the order of their pK_a values. The result is the formation of a pH gradient. This gradient can be used directly or by first cross-linking the ampholytes to a support to keep them stationary in the system.

Another way gel electrophoresis can be utilized is in a method known as "two-dimensional electrophoresis." This is a high-resolution technique used to look at complex protein mixtures. In this method, two types of electrophoresis are performed on a single sample. The first of these separations is usually based on IEF, and the second is based on SDS-PAGE. The fact that two different characteristics of each protein are used in this separation (pI values and size) makes it possible to resolve a much larger number of proteins than is possible by either IEF or SDS alone. This method is popular in the analysis of large protein libraries, as is used for finding new disease biomarkers or in studying disease pathology.

Capillary electrophoresis

General terms and concepts

Another type of electrophoresis is the method of CE. This is a technique that separates analytes by electrophoresis that is carried out in a capillary. This method is typically conducted in capillaries with inner diameters of 20−100 μm and lengths of 20−100 cm. The use of these narrow bore capillaries allows efficient removal of Joule heating, which helps decrease band-broadening and provides much more efficient and faster separations than gel electrophoresis.

If there is no appreciable Joule heating present and no interactions between the analytes and the capillary, the main source of band-broadening in CE will be longitudinal diffusion. Under these conditions, an increase in applied voltage results in less band-broadening, because shorter times are allowed for analytes to undergo diffusion along the length of the capillary. Besides providing efficient separations, the ability to use high electric fields also reduces the time needed for a separation. This combination of good efficiency and speed has made CE of interest for the analysis of complex biological samples like urine and blood.

An example of a CE system is shown in Fig. 8.6. In addition to the capillary, this system includes a power supply and electrodes for applying the electric field, two buffer containers that supply a contact between these electrodes and the solution within the capillary, an on-line detector, and a means for injecting samples onto the capillary. Most CE instruments can supply voltages up to 25−30 kV. To prevent injury to laboratory workers, these instruments have safety features that isolate the region of high voltage from the user and that turn off this voltage when the system is opened for maintenance or for the insertion of samples and reagents.

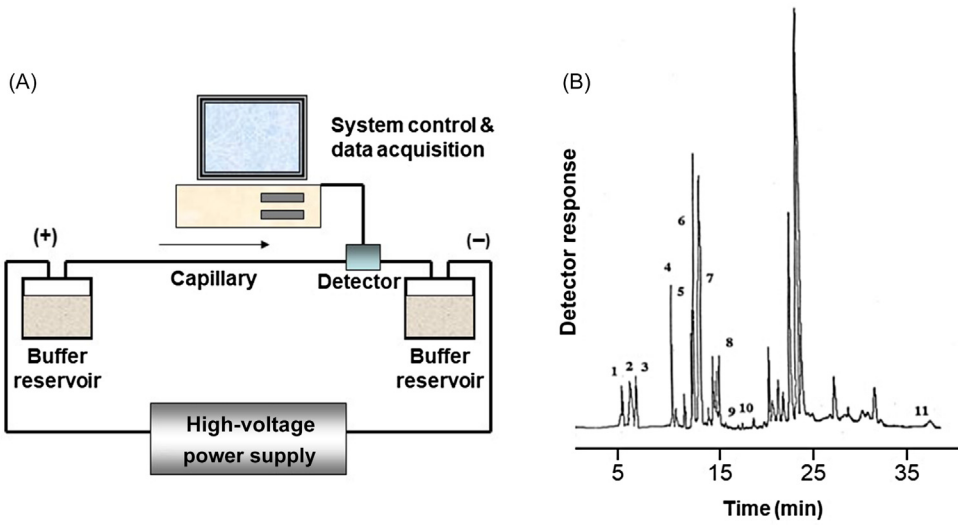

FIGURE 8.6 (A) A system for performing capillary electrophoresis and (B) a typical separation performed by capillary electrophoresis, using the analysis of biogenic amines (peaks 1–11) in urine. *The electropherogram in (B) is reproduced with permission from H. Siren, U. Karjalainen, Study of catecholamines in patient urine samples by capillary electrophoresis, J. Chromatogr. A 853 (1999) 527 [12]; courtesy Elsevier.*

Supports and sample application in capillary electrophoresis

The capillary in a CE system is usually fused silica. A fused silica capillary is often used directly, but it can also be modified to place various coatings on its interior surface. With a fused silica capillary, flow of the buffer due to electroosmosis can have a significant impact on the overall mobility of analytes. This electroosmosis is created by the negative charge present on surface of silica at a neutral or basic pH. The presence of these negatively charged groups creates an electrical double layer that causes movement of the running buffer toward the negatively charged electrode.

One useful feature of electroosmosis is it causes most analytes, regardless of their charge, to travel in the same direction through the CE capillary. This effect means that a sample containing many types of ions can often be injected at one end of the capillary (at the positively charged electrode), with electroosmosis then carrying these through to the other end (to the negatively charged electrode) and past a detector. This format is referred to as the "normal polarity mode" of CE. It is important to remember in this situation that a separation of ions will still occur but that the observed mobility will now be the sum of the inherent electrophoretic mobility for an analyte plus the mobility due to electroosmosis. The observed migration time for the analyte and the efficiency and resolution obtained for the separation are also affected.

Although many analytes will travel in the same direction as electroosmotic flow through a CE system, it is possible for some to have migration rates faster and in the opposite direction from electroosmosis. The analysis of these ions in a silica capillary is performed by injecting them by the negatively charged electrode and allowing them to migrate toward the positively charged electrode against electroosmotic flow. This format is referred to as the "reversed polarity mode" of CE. In addition, electroosmotic flow can be altered by changing the pH, which changes the charge on silica, or by placing a coating on the support surface. In this latter case, a neutral coating helps to reduce or eliminate electroosmosis while a positively charged coating will reverse the direction of this flow toward the positively charged electrode.

There are two features of CE that place special demands on how samples can be injected in this method. First, the small volume of this device must be considered. For instance, a typical 50-μm I.D. × 25-cm-long capillary for CE will contain only 0.5 μL of running buffer. This type of capillary requires the use of much smaller injection volumes to allow samples to be separated into their individual components. Another factor to consider is the high resolution of CE, which further restricts the sample sizes that can be employed. In practice, this factor generally requires the use of samples that are $<2\%$ of the capillary's internal volume, or <10 nL for a 0.5-μL volume capillary.

Sample volumes of nanoliters or less cannot be easily delivered by the types of injection valves used for LC. Thus alternative means for injection must be employed. One is hydrodynamic injection, which uses a difference in pressure to deliver a sample to the capillary. Hydrodynamic injection can be performed by placing one end of the capillary into a sample in an enclosed chamber and applying a pressure to this chamber for a fixed period of time. The amount of injected sample will depend on the size of the pressure difference and the amount of time this pressure is applied to the capillary. A similar effect occurs if a vacuum is pulled at the other end of the capillary. Once the sample has entered the capillary, the capillary is placed back into contact with the running buffer and electrodes, and the separation is begun.

Another technique frequently employed in CE is electrokinetic injection. This method again begins by placing the capillary into the sample; however, an electrode is also now placed into the sample. When an electric field is placed across the capillary, electroosmotic flow causes analytes to enter the capillary. The amount of each analyte that is injected will depend on the rate of electroosmotic flow, the analyte's inherent electrophoretic mobility, the applied electric field, and the time over which this field is applied. The capillary is then placed back into a container of the running buffer, and another electric field is applied to separate the sample components. One disadvantage to this approach is it favors the injection of analytes with electrophoretic mobilities that move them into the capillary over those that are applied only through the presence of electroosmotic flow.

Like traditional gel electrophoresis, there are various methods for concentrating samples and providing narrow analyte bands in CE. Sample stacking is one approach for accomplishing this goal and occurs when the ionic strength (and therefore the conductivity) of the sample is less than that of the running buffer. When an electric field is applied, analytes will migrate quickly through the sample matrix until they come to the boundary between the sample and the running buffer. Because the running buffer has a higher ionic strength, this situation causes the rate of migration to slow down and the analytes to concentrate as they enter the running buffer.

Detection in capillary electrophoresis

Many of the same detection techniques used for LC are also used in CE. However, one important difference between detection in LC and CE is the need in CE for methods that can work with very small sample sizes. This difference is a result of the small injection volumes that are required in CE to avoid adding excessive band-broadening. Selective detection methods that work particularly well for this purpose are electrochemical and fluorescence detection. However, UV−vis absorbance, conductance, and mass spectrometry detection are also employed.

Special types of capillary electrophoresis

One useful feature of gel electrophoresis is the ability of some supports in this method to separate analytes based on size, as occurs for proteins in SDS-PAGE. The same effect can be obtained in CE by including an agent in the CE system that sieves the analytes, or separates them based on size. There are several ways of doing this. The first way is to place a porous gel in the capillary, like the polyacrylamide gels employed in SDS-PAGE. This approach is called "capillary gel electrophoresis." However, these gels are not always stable in the high electric fields used in CE and must frequently be replaced. A second approach is to add a large polymer to the running buffer that is not immobilized but that can entangle with analytes and alter their rate of migration. This technique provides a system with better reproducibility and stability than those using gels, because the polymer is continuously renewed as the running buffer passes through the capillary.

The approach using polymers to entangle and separate analytes based on their size is often used when performing DNA sequencing by CE. The process of DNA sequencing by CE typically makes use of the Sanger method, in which the DNA template sequence to be examined is mixed with a smaller DNA primer that binds to part of this sequence. This mixture is then combined with: (1) nucleotides that contain the bases cytosine (C), guanine (G), adenine (A), or thymine (T); (2) the enzymes that are needed to add these nucleotides to the primer and extend it to match the template; and (3) small amounts of labeled nucleotides (also containing C, G, A, or T) that will stop this extension. The DNA strands that have been formed are then separated by CE based on their size, or strand length. The type of labeled nucleotide that ends each sequence is then determined and used to provide the sequence of the original template.

Ordinary CE works well for separating cations and anions, but it cannot be used to separate neutral substances, which migrate as a single peak that travels with the electroosmotic flow. However, CE can be extended to such compounds by placing in the running buffer a charged agent that can interact with these substances. One way of accomplishing this is to employ micelles as additives, giving a method known as "micellar electrokinetic chromatography." A micelle is a particle formed by the aggregation of a large number of surfactant molecules, such as SDS. As indicated earlier, SDS has a long nonpolar tail attached to a negatively charged sulfate group. When the concentration of a surfactant like SDS reaches a certain threshold level (i.e., the critical micelle concentration), some of the surfactant molecules come together to form micelles. If this situation occurs in a polar solvent like water, the nonpolar tails of the surfactant will be on the inside of the aggregate (giving a nonpolar interior), while the charged groups at the other end will be on the outside by the solvent.

When micelles based on SDS are placed into the running buffer of a CE system, they will be attracted toward the positively charged electrode. If a sample with several neutral compounds is now injected, some of these neutral substances may enter the micelles and interact with their nonpolar interior. Although these neutral compounds normally travel with electroosmotic flow through the capillary, while they are in the micelles they travel with the

micelles in the opposite direction. This effect gives a separation of neutral compounds based on the degree to which they enter the micelles. Micelles can also alter the migration times for charged substances through a combination of partitioning and charge interactions between the analytes and the micelles.

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Self-assessment questions

1. Which part of a chromatographic system interacts with the components of a sample and delays the rate at which they travel through the system?
   a. the mobile phase
   b. the stationary phase
   c. the support material
   d. the detector
2. Which of the following statements is false?
   a. The retention factor is related to the retention time of a compound.
   b. The van Deemter equation shows how the retention of a compound changes with linear velocity.
   c. The resolution between two peaks will increase with their degree of retention.
   d. The retention factor is affected by the amount of stationary phase in the column.
3. Which of the following items is NOT desired for a good separation in chromatography?
   a. a large value for the number of theoretical plates, N
   b. a large value for the peak resolution, R_s
   c. a large value for the plate height, H
   d. a large value for the separation factor, α
4. What is the most common type of column used in clinical applications of GC?
   a. a packed column with a bonded stationary phase
   b. a packed column with a liquid stationary phase
   c. an open-tubular column with a bonded stationary phase
   d. an open-tubular column with a liquid stationary phase
5. A GC detector that is selective for compounds with electronegative groups is _____.
   a. a thermal conductivity detector
   b. a flame ionization detector
   c. a nitrogen−phosphorus detector
   d. an electron capture detector
6. Liquid chromatography is different from gas chromatography in that _____.
   a. it has less band-broadening
   b. it has a liquid mobile phase that affects analyte retention
   c. it requires volatile analytes
   d. it can use a gas or liquid as the mobile phase
7. Which type of liquid chromatography uses a nonpolar stationary phase?
   a. normal-phase chromatography
   b. reversed-phase chromatography
   c. ion-exchange chromatography
   d. size-exclusion chromatography
8. Which type of liquid chromatography uses a support with fixed charges to separate chemicals?
   a. reversed-phase chromatography
   b. ion-exchange chromatography
   c. size-exclusion chromatography
   d. affinity chromatography
9. Which of the following detectors might be used in LC to record an absorbance spectrum?
   a. photodiode array detector
   b. evaporative light scattering detector
   c. conductivity detector
   d. electrochemical detector
10. Separations in electrophoresis are based mainly on _____.
    a. interactions of analytes with a stationary phase
    b. the different volatilities of analytes
    c. the migration rates of analytes in an electric field
    d. the use of radioactive labels for detection
11. Which term is used to describe the pH at which a zwitterion has no net charge?
    a. electrophoretic mobility
    b. size or mass
    c. isoelectric point
    d. amino acid composition
12. Which of the following is NOT an advantage of capillary electrophoresis?
    a. It has low band-broadening due to effective removal of Joule heating.
    b. It can provide faster separations than gel electrophoresis.
    c. It can be used with large sample volumes.
    d. It can be used with an on-line detector.

Answers

1. b
2. b
3. c
4. c
5. d
6. b
7. b
8. b
9. a
10. c
11. c
12. c