12 chapter

Basic Principles of Chromatography

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12.1 INTRODUCTION

Chromatography has a great impact on all areas of analysis and, therefore, on the progress of science in general. Chromatography differs from other methods of separation in that a wide variety of materials, equipment, and techniques can be used. [Readers are referred to references [1–29] for general and specific information on chromatography.] This chapter will focus on the principles of chromatography, mainly **liquid chromatography** (LC). Detailed principles and applications of **gas chromatography** (GC) will be discussed in Chap. 14. In view of its widespread use and applications, high-performance liquid chromatography (HPLC) will be discussed in a separate chapter (Chap. 13). The general principles of extraction are first described as a basis for understanding chromatography

12.2 EXTRACTION

In its simplest form, extraction refers to the transfer of a solute from one liquid phase to another. Extraction in myriad forms is integral to food analysis – whether used for preliminary sample cleanup, concentration of the component of interest, or as the actual means of analysis. Extractions may be categorized as **batch**, **continuous**, or **countercurrent** processes. (Various extraction procedures are discussed in detail in other chapters: traditional solvent extraction in Chaps. 14, 17, and 33; accelerated solvent extraction in Chap. 33; solid-phase extraction in Chaps. 14 and 33; and solid-phase microextraction and microwave-assisted solvent extraction in Chap. 33).

12.2.1 Batch Extraction

In **batch extraction** the solute is extracted from one solvent by shaking it with a second immiscible solvent. The solute **partitions**, or distributes, itself between the two phases, and, when equilibrium has been reached, the **partition coefficient**, K, is a constant:

$$K = \frac{\text{Concentration of solute in phase 1}}{\text{Concentration of solute in phase 2}} \qquad (12.1)$$

After shaking, the phases are allowed to separate, and the layer containing the desired constituent is removed, for example, in a separatory funnel. In batch extraction, it is often difficult to obtain a clean separation of phases, owing to emulsion formation. Moreover, partition implies that a single batch extraction is usually incomplete.

12.2.2 Continuous Extraction

Continuous extraction requires special apparatus, but is more efficient than batch separation. One example is the use of a Soxhlet extractor (Chap. 17, Sect. 17.2.5) for extracting fat from solids using organic solvents. Solvent is recycled so that the solid is repeatedly extracted with fresh solvent. Other types of equipment have been designed for the continuous extraction of substances from liquids and/or solids, and different extractors are used for solvents that are heavier or lighter than water.

12.2.3 Countercurrent Extraction

Countercurrent distribution refers to a serial extraction process. It separates two or more solutes with different partition coefficients from each other by a series of partitions between two immiscible liquid phases. Liquid-liquid partition chromatography (Sect. 12.4.2), also known as countercurrent chromatography, is a direct extension of countercurrent extraction. Years ago the countercurrent extraction was done with a "Craig apparatus" consisting of a series of glass tubes designed such that the lighter liquid phase (**mobile phase**) was transferred from one tube to the next, while the heavy phase (**stationary phase**) remained in the first tube [5]. The liquid-liquid extractions took place simultaneously in all tubes of the apparatus, which was usually driven electromechanically. Each tube in which a complete equilibration took place corresponded to one theoretical plate of the chromatographic column (refer to Sect. 12.5.1.2.1). The greater the difference in the **partition coefficients** of various substances, the better was the separation. A much larger number of tubes were required to separate mixtures of substances with close partition coefficients, which made this type of countercurrent extraction very tedious. Modern **liquid-liquid partition chromatography** (Sect. 12.4.2) is much more efficient and convenient.

12.3 CHROMATOGRAPHY

12.3.1 Historical Perspective

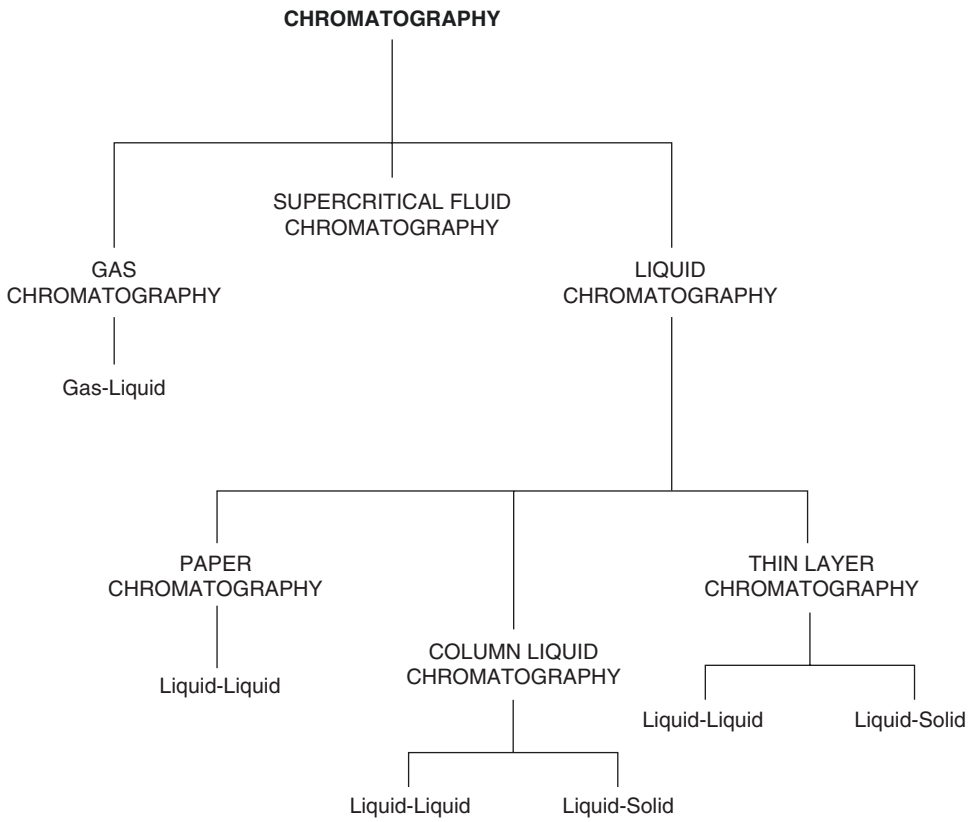
Modern chromatography originated in the late nineteenth and early twentieth centuries from independent work by David T. Day, a distinguished American geologist and mining engineer, and Mikhail Tsvet, a Russian botanist. Day developed procedures for fractionating crude petroleum by passing it through Fuller's earth, and Tsvet used a column packed with

chalk to separate leaf pigments into colored bands. Because Tsvet recognized and correctly interpreted the chromatographic processes and named the phenomenon **chromatography**, he is generally credited with its discovery.

After languishing in oblivion for years, chromatography began to evolve in the 1940s due to the development of column partition chromatography by Martin and Synge and the invention of paper chromatography. The first publication on GC appeared in 1952. By the late 1960s, GC, because of its importance to the petroleum industry, had developed into a sophisticated instrumental technique, which was the first instrumental chromatography to be available commercially. Since early applications in the mid-1960s, HPLC, profiting from the theoretical and instrumental advances of GC, has extended the area of LC into an equally sophisticated and useful method. Supercritical fluid chromatography (SFC), first demonstrated in 1962, has been gaining popularity in food analysis [7]. Efficient chromatographic techniques, including automated systems, continue to be developed for utilization in the characterization and quality control of food ingredients and products [4, 7–13].

12.3.2 General Terminology

Chromatography is a general term applied to a wide variety of separation techniques based on the partitioning or distribution of a sample (**solute**) between a moving or mobile phase and a fixed or stationary phase. Chromatography may be viewed as a series of equilibrations between the mobile and stationary phase. The relative interaction of a solute with these two phases is described by the **partition** (**K**) or **distribution** (**D**) **coefficient** (ratio of concentration of solute in stationary phase to concentration of solute in mobile phase). The mobile phase may be either a gas (for GC) or liquid (for LC) or a supercritical fluid (for SFC). The stationary phase may be a liquid or a solid. The field of chromatography can be subdivided according to the various techniques applied (Fig. 12.1) or according to the physicochemical principles involved in the separation. Table 12.1 summarizes some of the chromatographic procedures or methods that have been developed on the basis of different mobile-stationary phase combinations. Inasmuch as the nature of interactions between solute molecules and the mobile or stationary phases differ, these methods have the ability to separate different kinds of molecules. (The reader is urged to review Table 12.1 again after having read this chapter.)



12.1 figure A scheme for subdividing the field of chromatography, according to various applied techniques

12.1 table Characteristics of different chromatographic methods

Method	*Mobile/phase*	*Stationary phase*	*Retention varies with*
Gas-liquid chromatography	Gas	Liquid	Molecular size/polarity
Gas-solid chromatography	Gas	Solid	Molecular size/polarity
Supercritical fluid chromatography	Supercritical fluid	Solid	Molecular size/polarity
Reversed-phase chromatography	Polar liquid	Nonpolar liquid or solid	Molecular size/polarity
Normal-phase chromatography	Less polar liquid	More polar liquid or solid	Molecular size/polarity
Ion-exchange chromatography	Polar liquid-Ionic solid	Ionic solid	Molecular charge
Size-exclusion chromatography	Liquid	Solid	Molecular size
Hydrophobic interaction chromatography	Polar liquid	Nonpolar liquid or solid	Molecular size/polarity
Affinity chromatography	Water	Binding sites	Specific structure

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12.3.3 Gas Chromatography

Gas chromatography is a column chromatography technique, in which the mobile phase is gas and the stationary phase is mostly an immobilized liquid on an inert solid support in either a packed or capillary-type column. GC is used to separate thermally stable volatile components of a mixture. Gas chromatography, specifically gas-liquid chromatography, involves vaporizing a sample and injecting it onto the head of the column. Under a controlled temperature gradient, the sample is transported through the column by the flow of an inert, gaseous mobile phase. Volatiles are then separated based on several properties, including boiling point, molecular size, and polarity. Physiochemical principles of separation are covered in Sect. 12.4. However, details of the chromatographic theory of separation as it applies specifically to GC, as well as detection and instrumentation of GC, are detailed in Chap. 14.

12.3.4 Liquid Chromatography

There are several **liquid chromatography** techniques applied in food analysis, namely, **planar chromatography** (both paper and thin-layer chromatography) and column liquid chromatography, all of which involve a liquid mobile phase and either a solid or a liquid stationary phase. However, the physical form of the stationary phase is quite different in each case. Separation of the solutes is based on their physicochemical interactions with the two phases, which is discussed in Sect. 12.4.

12.3.4.1 *Planar Chromatography*

12.3.4.1.1 Paper Chromatography

Paper chromatography was introduced in 1944, and today it is mostly used as a teaching tool. In paper chromatography the stationary phase (water) and the mobile phase (organic solvent) are both liquid (**partition chromatography**, see Sect. 12.4.2), with paper (usually cellulose) serving as a support for the liquid stationary phase. The support also may be impregnated with a nonpolar organic solvent and developed with water or other polar solvents (reversed-phase paper chromatography). The dissolved sample is applied as a small spot or streak about 1.5 cm from the edge of a strip or square of the paper, which is then allowed to dry. The dry strip is suspended in a closed container in which the atmosphere is saturated with the **developing solvent** (mobile phase) and the paper chromatogram is **developed**. The end closer to the sample is placed in contact with the solvent, which then travels up or down the paper by capillary action (depending on whether **ascending** or **descending** development is used), separating the sample components in the process. When the solvent front has traveled the length of the paper, the strip is removed from the developing chamber, and the separated zones are detected by an appropriate method.

In the case of complex sample mixtures, a two-dimensional technique may be used. The sample is spotted in one corner of a square sheet of paper, and one solvent is used to develop the paper in one direction. The chromatogram is then dried, turned 90°, and developed again, using a second solvent of different polarity. Another means of improving resolution is the use of **ion-exchange** (Sect. 12.4.4) papers, i.e., paper that has been impregnated with ion-exchange resin or paper, with derivatized cellulose hydroxyl groups (with acidic or basic moieties).

In planar chromatography, components of a mixture are often characterized by their relative mobility (R_f) value, where:

$$R_f = \frac{\text{Distance moved by component}}{\text{Distance moved by solvent}} \qquad (12.2)$$

Unfortunately, R_f values are not always constant for a given solute/sorbent/solvent but depend on many

factors, such as the quality of the stationary phase, layer thickness, humidity, development distance, and temperature.

12.3.4.1.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC), first described in 1938, has largely replaced paper chromatography because it is faster, more sensitive, and more reproducible. The resolution in TLC is greater than in paper chromatography because the particles on the plate are smaller and more regular than paper fibers. Experimental conditions can be easily varied to achieve separation and can be scaled up for use in column chromatography, although thin-layer and column procedures are not necessarily interchangeable, due to differences such as the use of binders with TLC plates, vapor phase equilibria in a TLC tank, etc. There are several distinct advantages to TLC over paper chromatography and in some instances over column chromatography: high sample throughput, separations of complex mixtures, low cost, analysis of several samples and standards simultaneously, minimal sample preparation, and possibility to store the plate for later identification and quantification. Advances in TLC led to the development of **high-performance thin-layer chromatography** (HPTLC), which simply refers to TLC performed using plates coated with smaller, more uniform particles. This permits better separations in shorter times.

TLC, more so HPTLC, is applied in many fields, including environmental, clinical, forensic, pharmaceutical, food, flavors, and cosmetics. Within the food industry, TLC may be used for quality control. For example, corn and peanuts are tested for aflatoxins/mycotoxins prior to their processing into corn meal and peanut butter, respectively. Applications of TLC to the analysis of a variety of compounds, including lipids, carbohydrates, vitamins, amino acids, natural pigments, and sugar substitutes, are discussed in references [14, 17, 18].

1. **TLC General Procedures**. TLC utilizes a thin (ca. 250 μm thick) layer of **sorbent** or **stationary phase** bound to an **inert support**. The support is often a glass plate (traditionally, 20 × 20 cm), but plastic sheets and aluminum foil also are used. Pre-coated plates, of different layer thicknesses, are commercially available in a wide variety of sorbents, including chemically modified silica. Four frequently used TLC sorbents are silica gel, alumina, diatomaceous earth, and cellulose. Modified silica for TLC may contain polar or nonpolar groups, so both normal and reversed-phase (see Sect. 12.4.2.1) thin-layer separations may be carried out.
   If **adsorption** TLC is to be performed, the sorbent is first **activated** by drying for a specified time and temperature. As in paper chromatography, the sample (in carrier solvent) is applied as a spot or streak about 1.5 cm from one end of the plate. After evaporation of the carrier solvent, the TLC plate is placed in a closed **developing chamber**, solvent migrates up the plate (**ascending development**) by capillary action, and sample components are separated. After the TLC plate has been removed from the chamber and solvent allowed to evaporate, the separated bands are made visible or detected by other means. Specific **chemical reactions (derivatization)**, which may be carried out either before or after chromatography, often are used for this purpose. Two examples are reaction with sulfuric acid to produce a dark charred area (a **destructive chemical method**) and the use of iodine vapor to form a colored complex (a **nondestructive method** inasmuch as the colored complex is usually not permanent). Common **physical detection methods** include the measurement of absorbed or emitted electromagnetic radiation, such as measuring fluorescence when stained with 2,7-dichlorofluorescein, and measurement of β-radiation from radioactively labeled compounds. Different reagents that can react selectively to generate colored products also are used [17]. **Biological methods** or biochemical inhibition tests can be used to detect toxicologically active substances. An example is measuring the inhibition of cholinesterase activity by organophosphate pesticides.
   Quantitative evaluation of thin-layer chromatograms may be performed [17]: (1) in situ (directly on the layer) by using a **densitometer** [18], or (2) scraping a zone off the plate, eluting compound from the sorbent, and then analyzing the resultant solution (e.g., by liquid scintillation counting).
2. **Factors Affecting Thin-Layer Separations**. In both planar and column liquid chromatography, the nature of the compounds to be separated determines what type of stationary phase is used. Separation can occur by adsorption, partition, ion-exchange, size-exclusion, or multiple mechanisms (Sect. 12.4). Table 12.2 lists the separation mechanisms involved in some typical applications on common TLC sorbents.

Solvents for TLC separations are selected for specific chemical characteristics and **solvent strength** (a measure of interaction between solvent and sorbent; see Sect. 12.4.1). In simple adsorption TLC, the higher the solvent strength, the greater the R_f value of the solute. An R_f value of 0.3–0.7 is typical. Mobile phases have been developed for the separation of various compound classes on the different sorbents (see Table 7.1 in reference [19]).

In addition to the sorbent and solvent, several other factors must be considered when performing planar chromatography. These include the **type of developing chamber** used, **vapor phase conditions** (saturated vs. unsaturated), **development mode** (ascending, descending, horizontal, radial, etc.), and **development distance**. For additional reading refer to references [14–18].

12.3.4.2 *Column Liquid Chromatography*

Column liquid chromatography generally has enhanced resolution of solutes in a mixture and enables precise analysis compared to planar chromatography. Fractionation of solutes occurs as a result of differential migration through a closed tube of stationary phase, and analytes can be monitored while the separation is in progress. In column liquid chromatography, the mobile phase is liquid, and the stationary phase can be either solid or liquid supported by an inert solid. A system for **low-pressure** (i.e., performed at or near atmospheric pressure) column liquid chromatography is illustrated in Fig. 12.2.

Having selected a stationary and mobile phase suitable for the separation problem at hand, the analyst must first prepare the **stationary phase** (**resin**, **gel**, or **packing material**) for use according to the supplier's instructions (e.g., the stationary phase often must be **hydrated** or **preswelled** in the mobile phase). The prepared stationary phase then is **packed** into a column

12.2 table Thin-layer chromatography sorbents and mode of separation

Sorbent	*Chromatographic mechanism*	*Typical application*
Silica gel	Adsorption	Steroids, amino acids, alcohols, hydrocarbons, lipids, aflatoxins, bile acids, vitamins, alkaloids
Silica gel RP	Reversed phase	Fatty acids, vitamins, steroids, hormones, carotenoids
Cellulose, kieselguhr	Partition	Carbohydrates, sugars, alcohols, amino acids, carboxylic acids, fatty acids
Aluminum oxide	Adsorption	Amines, alcohols, steroids, lipids, aflatoxins, bile acids, vitamins, alkaloids
PEI cellulose[a]	Ion exchange	Nucleic acids, nucleotides, nucleosides, purines, pyrimidines
Magnesium silicate	Adsorption	Steroids, pesticides, lipids, alkaloids

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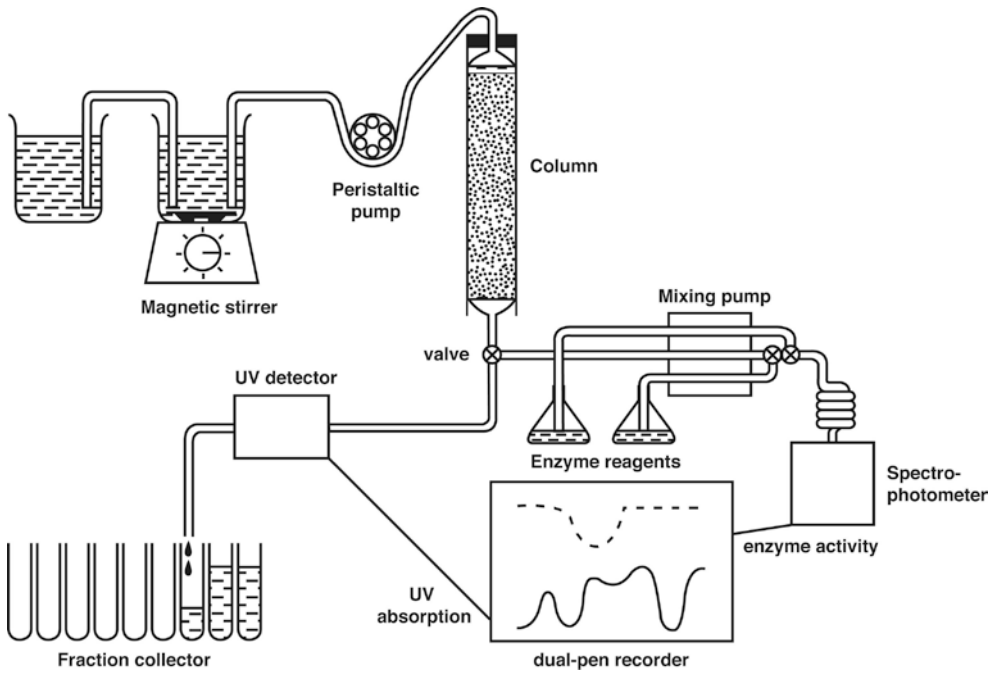
[a]PEI cellulose refers to cellulose derivatized with polyethyleneimine (PEI)



12.2 figure A system for low-pressure column liquid chromatography. In this diagram, the column effluent is being split between two detectors in order to monitor both enzyme activity (*at right*) and UV absorption (*at left*). The two tracings can be recorded simultaneously by using a dual-pen recorder (Adapted from Scopes [28], with permission)

(usually glass), the length and diameter of which are determined by the amount of sample to be loaded, the separation mode to be used, and the degree of resolution required. Longer and narrower columns usually enhance resolution and separation (Sect. 12.5.1). Adsorption columns may be either dry or wet packed; other types of columns are wet packed. The most common technique for wet packing involves making a slurry of the adsorbent with the solvent and pouring it into the column to the desired bed height. Pouring uniform columns is an art that is mastered with practice. If the packing solvent is different from the initial eluting solvent, the column must be thoroughly washed **(equilibrated** with 2–3 column volumes) with the starting mobile phase.

The sample to be fractionated is dissolved in a minimum volume of the starting mobile phase, injected through a sample injection port, and carried by the mobile phase onto the column. Low-pressure chromatography utilizes only **gravity** flow or a high-precision **peristaltic pump** to maintain a constant flow of **mobile phase** (**eluent** or **eluting solvent**) through the column. If eluent is fed to the column by a peristaltic pump (see Fig. 12.2), then the flow rate is determined by the pump speed. Depending on the dimensions of the column, the flow rate is adjusted not to exceed the max pressure sustained by the pump.

The process of passing the mobile phase through the column is called **elution**, and the portion that emerges from the outlet end of the column is called the **eluate** (or effluent). Elution may be **isocratic** (constant mobile phase composition) or a **gradient** (changing the mobile phase, e.g., increasing solvent strength or pH). Gradient elution enhances resolution and decreases analysis time (see also Sect. 12.5.1). As elution proceeds, components of the sample are selectively retarded by the stationary phase based on the strength of interaction with the stationary phase and thus are eluted at different times.

The column eluate may be directed through a detector and then into tubes, changed at intervals by a fraction collector. The detector response, in the form of an electrical signal, may be recorded (the **chromatogram**) using a computerized software. Signals are then integrated for either qualitative or quantitative analysis (Sects. 12.5.2 and 12.5.3). The fraction collector may be set to collect eluate at specified time intervals or after a certain volume or number of drops have been collected. Components of the sample that have been chromatographically separated and collected can be further analyzed as needed.

12.3.5 Supercritical Fluid Chromatography

Supercritical fluid chromatography is performed above the **critical pressure** (P_c) and **critical temperature** (T_c) of the mobile phase. A supercritical fluid (or compressed gas) is neither a liquid nor a typical gas. The combination of P_c and T_c is known as the **critical point**. A supercritical fluid can be formed from a conventional gas by increasing the pressure or from a conventional liquid by raising the temperature. Carbon dioxide frequently is used as a mobile phase for SFC; however, it is not a good solvent for polar and high-molecular-weight compounds. A small amount of a polar, organic solvent such as methanol can be added to a nonpolar supercritical fluid to enhance solute solubility, improve peak shape, and alter selectivity. Other supercritical fluids that have been used in food applications include nitrous oxide, trifluoromethane, sulfur hexafluoride, pentane, and ammonia.

Supercritical fluids confer chromatographic properties intermediate to LC and GC. The **high diffusivity** and **low viscosity** of supercritical fluids mean decreased analysis times and improved resolution compared to LC. An additional benefit of short analysis time is the reduced solvent consumption. SFC offers a wide range of **selectivity** (Sect. 12.5.2) adjustment, by changes in **pressure** and **temperature** as well as changes in **mobile phase composition** and the **stationary phase**. Compared to HPLC (Chap. 13), SFC is better for separating compounds with a broader range of polarities. In addition, SFC makes possible the separation of **nonvolatile**, **thermally labile compounds**, which cannot be analyzed by GC without derivatization. In fact, SFC today is mainly used for the analysis of nonvolatiles.

Either **packed** or **capillary** columns can be used in SFC**.** Packed column materials are similar to those used for HPLC. Small particle, porous, high surface area, hydrated silica, either bare or bonded silica, is commonly used as the column packing material (Sect. 12.4.2.3 and Chap. 13). Capillary columns are generally coated with a polysiloxane (-Si-O-Si) film containing different functional groups, which is then cross-linked to form a polymeric stationary phase that cannot be washed off by the mobile phase. Polysiloxanes containing different functional groups, such as methyl, phenyl, pyridine, or cyano, may be used to vary the polarity of this stationary phase.

The development of SFC benefited from advancement in the instrumentation for HPLC and GC. One major difference in instrumentation is the presence of a back pressure regulator to control the **outlet pressure** of the system. Without this device, the fluid would expand to a low-pressure, low-density gas. Detectors used in GC and HPLC also can be used with SFC, including coupling with mass spectrometry (MS) (Chap. 11).

SFC has been used for the analysis of a wide range of compounds. Fats, oils, and other lipids are compounds to which SFC is increasingly applied. For example, the noncaloric fat substitute, Olestra®, was characterized by SFC-MS. Other researchers have used SFC to detect pesticide residues, study thermally labile

compounds from members of the *Allium* genus, fractionate citrus essential oils, and characterize compounds extracted from microwave packaging [20]. Bernal et al. [11] highlighted the use of packed column and capillary SFC for the analysis of food and natural products, namely, lipids and their derivatives, carotenoids, fat-soluble vitamins, polyphenols, carbohydrates, and food adulterants such as Sudan dyes.

12.4 PHYSICOCHEMICAL PRINCIPLES OF CHROMATOGRAPHIC SEPARATION

Several physicochemical principles (illustrated in Fig. 12.3) are involved in chromatography mechanisms employed to separate or fractionate various compounds of interest, regardless of the specific techniques applied (discussed in Sect. 12.3). The mechanisms described below apply mainly to liquid chromatography; GC mechanisms will be detailed in Chap. 14. Although it is more convenient to describe each of these phenomena separately, it must be emphasized that more than one mechanism may be involved in a given fractionation. For example, many cases of partition chromatography also involve adsorption. Table 12.3 summarizes the different separation modes and associated stationary phases, mobile phases, and types of interactions.

12.4.1 Adsorption (Liquid-Solid) Chromatography

Adsorption chromatography is the oldest form of chromatography and originated with Tsvet in 1903 in the experiments that spawned modern chromatography. In this chromatographic mode, the stationary phase is a finely divided solid to maximize the surface area. The stationary phase (**adsorbent**) is chosen to permit differential interaction with the components of the sample to be resolved. The intermolecular forces thought to be primarily responsible for chromatographic adsorption include the following:

- Van der Waals forces
- Electrostatic forces
- Hydrogen bonds
- Hydrophobic interactions

Sites available for interaction with any given substance are heterogeneous. Binding sites with greater affinities, the most active sites, tend to be populated first, so that additional solutes are less firmly bound. The net result is that adsorption is a concentration-dependent process, and the **adsorption coefficient** is *not* a constant (in contrast to the **partition coefficient**). Sample loads exceeding the adsorptive capacity of the stationary phase will result in relatively poor separation (Sect. 12.5.1).

Classic adsorption chromatography utilizes mostly **silica** (slightly acidic), **alumina** (slightly basic), or charcoal (nonpolar). Both silica and alumina are polar adsorbents, possessing surface hydroxyl groups, and Lewis acid-type interactions determine their adsorption characteristics. The elution order of compounds from these adsorptive stationary phases can often be predicted on the basis of their relative **polarities** (Table 12.4). Compounds with the most polar functional groups are retained most strongly on polar adsorbents and, therefore, are eluted last. Nonpolar solutes are eluted first.

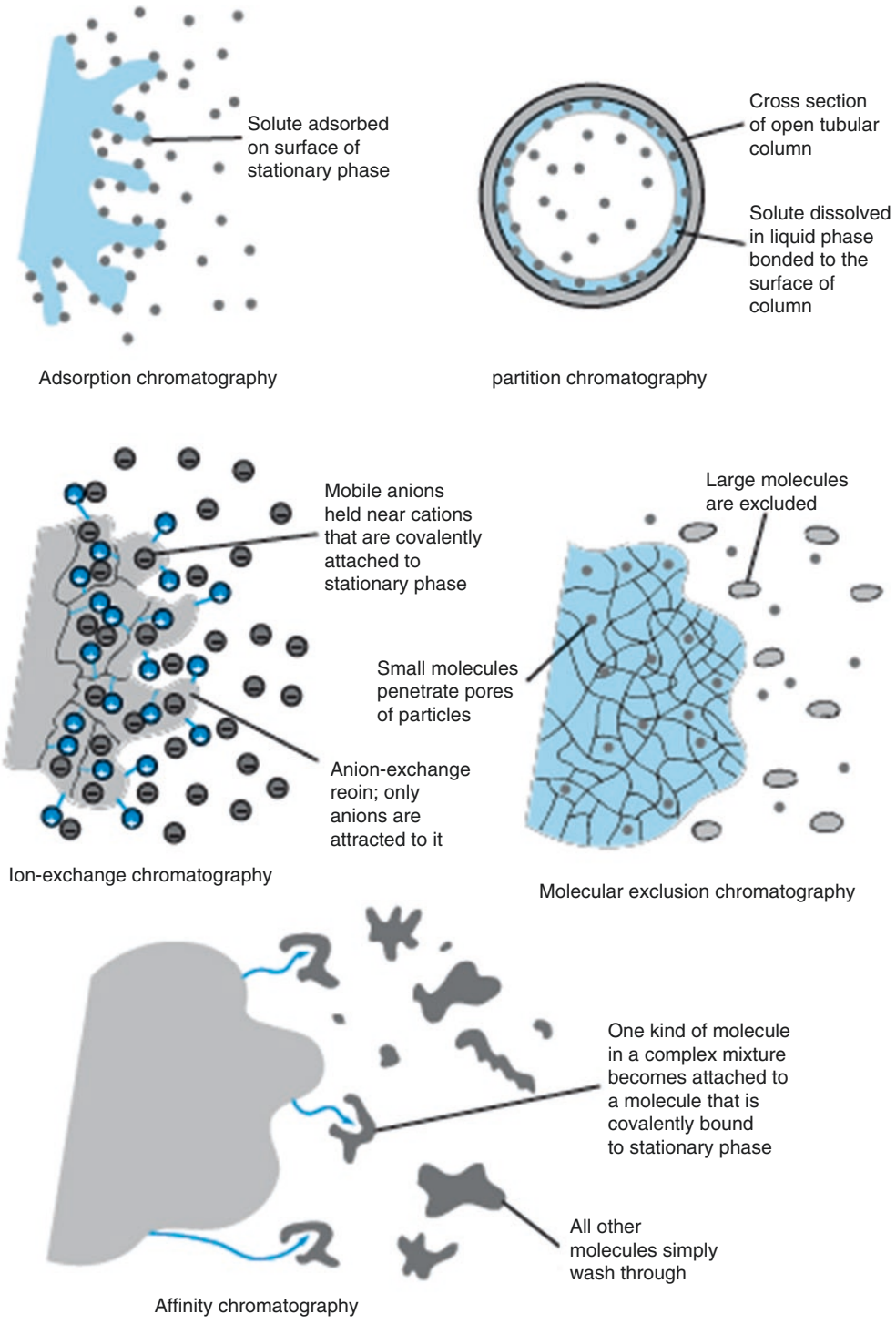
One model proposed to explain the mechanism of liquid-solid chromatography is that **solute** and **solvent** molecules are competing for active sites on the adsorbent. Thus, as relative adsorption of the mobile phase increases, adsorption of the solute must decrease. Solvents can be rated in order of their strength of adsorption on a particular adsorbent, such as silica. Such a **solvent strength** (or solvent polarity in this case) **scale** is called an **eluotropic series**. An eluotropic series for alumina is listed in Table 12.5. Silica has a similar rank ordering. Once an adsorbent has been chosen, solvents can be selected from the eluotropic series for that adsorbent. Mobile phase strength (polarity in this case) can be increased (often by admixture of more polar solvents) until elution of the compound(s) of interest has been achieved.

Adsorption chromatography can be used to separate aromatic or aliphatic nonpolar compounds, based primarily on the type and number of functional groups present. The labile, fat-soluble chlorophyll and carotenoid pigments from plants have been studied extensively by adsorption column chromatography. Adsorption chromatography also has been used for the analysis of fat-solu is also another ble vitamins. Frequently, it is used as a batch procedure for the removal of impurities from samples prior to other analyses. For example, disposable solid-phase extraction cartridges (see Chap. 14) containing silica have been used for analyses of lipids in soybean oil, carotenoids in citrus fruit, and vitamin E in grain. Adsorption chromatography is also applied in specialized forms for the analysis of a wide range of compounds. Several of the chromatographic separation techniques described in the following sections are forms of specialized adsorption chromatography.

12.4.2 Partition (Liquid-Liquid) Chromatography

12.4.2.1 *Introduction*

In 1941, Martin and Synge undertook an investigation of the amino acid composition of wool, using a countercurrent extractor (Sect. 12.2.3) consisting of 40 tubes



12.3 figure Physicochemical principles of chromatography (From Harris [2] *Quantitative Chemical Analysis* 9e, by Daniel C. Harris, copyright 2016 by W.H. Freeman and Company. Used with permission of publisher)

12.3 table

Summary of different chromatographic separation modes

Separation mode	*Stationary phase*	*Mobile phase*	*Increasing mobile phase strength*	*Compounds eluting first/ eluting last*	*Type of interactions between solutes and stationary phase*
Normal phase (can be in the form of adsorption or partition chromatography)	Polar sorbent (e.g., silica, alumina, water)	Nonpolar solvent (e.g., aqueous methanol, acetonitrile)	Decreasing concentration of organic solvent (i.e., increasing polarity, making the mobile phase more like the stationary phase)	Least polar/most polar	H-bonding mostly
Reversed-phase (can be in the form of adsorption or partition chromatography)	Nonpolar sorbent (e.g., bonded silica, C8 or C18)	Polar solvent (e.g., water)	Increasing concentration of organic solvent (i.e., decreasing polarity, making the mobile phase more like the stationary phase)	Most polar/least polar	H-bonding; Van der Waals, hydrophobic interactions
Hydrophobic interaction	Nonpolar sorbent (e.g., butyl-sepharose and phenyl-sepharose)	Salt solution/buffer (e.g., 1 *M* ammonium sulfate; Phosphate buffer)	Decreasing concentration of salt (result in reduced interaction of solute with the sorbent)	Least hydrophobic surface/most hydrophobic surface	Hydrophobic interactions
Cation exchange	Negatively charged functional groups (e.g., $RSO3^-$, $RCO2^-$)	Buffers of specific pH and ionic strength	Increasing the pH (in case of weak cation exchanger) or increasing salt concentration (e.g., increasing the pH will result in deprotonation, i.e., loss of positive charge of the solute so it no longer interacts with the functional group of the stationary phase, and increasing the salt concentration will provide counter ions that will displace the solute on the functional groups of the stationary phase)	Solutes with the least positive charge density/most positive charge density	Electrostatic
Anion exchange	Positively charged functional groups (e.g., RNR′3+, R-NHR′2+)	Buffers of specific pH and ionic strength	Decreasing the pH (in case of weak anion exchanger) or increasing salt concentration (e.g., decreasing the pH will result in protonation, i.e., loss of negative charge of the solute so it no longer interacts with the functional group of the stationary phase, and increasing the salt concentration will provide counter ions that will displace the solute on the functional groups of the stationary phase)	Solutes with the least negative charge density/most negative charge density	Electrostatic
Affinity	Highly specific ligand bound to inert surface (e.g., antibodies, enzyme inhibitors, lectins)	Buffer	Changing the pH or ionic strength, or adding a ligand similar to the bound ligand of the stationary phase	Solutes with least affinity to bound ligands/most affinity to bound	H-bonding; Van der Waals, hydrophobic interactions, electrostatic
Size exclusion	Porous inert material (e.g., Sephadex, a cross-linked dextran)	Mostly water or buffer	Not applicable	Largest in size/smallest in size	None

with chloroform and water flowing in opposite directions. The efficiency of the extraction process was improved enormously when a column of finely divided inert support material was used to hold one liquid phase (stationary phase) immobile, while the second liquid, an immiscible solvent (mobile phase), flowed over it, thus providing intimate contact between the two phases. Solutes partitioned between the two liquid phases according to their partition coefficients, hence the name **partition chromatography**.

In partition chromatography, depending on the characteristics of the compounds to be separated, the nature of the two liquid phases can be varied, usually by combination of solvents or pH adjustment of buffers. Often, the more polar of the two liquids is held stationary on the inert support, and the less polar solvent is used to elute the sample components (**normal-phase chromatography**). Reversal of this arrangement, using a **nonpolar stationary phase** and a **polar mobile phase**, has come to be known as **reversed-phase chromatography** (see Sect. 12.4.2.3).

Polar **hydrophilic** substances, such as amino acids, carbohydrates, and water-soluble plant pigments, are often separable by **normal-phase** partition chromatography. **Lipophilic** compounds, such as lipids, fat-soluble pigments, and **polyphenols**, may be resolved better with **reversed-phase** systems. Liquid-liquid partition chromatography has been invaluable to carbohydrate chemistry. Column liquid chromatography on finely divided cellulose has been used extensively in preparative chromatography of sugars and their derivatives.

12.4.2.2 *Coated Supports*

In its simplest form, the stationary phase for partition chromatography consists of a liquid coating on a solid matrix. The solid support should be as inert as possible and have a large surface area in order to maximize the amount of liquid held. Some examples of solid supports that have been used are silica, starch, cellulose powder, and glass beads. All are capable of holding a thin film of water, which serves as the stationary phase. It is important to note that materials prepared for adsorption chromatography must be **activated** by drying them to remove surface water. Conversely, some of these materials, such as silica gel, may be used for partition chromatography if they are deactivated by impregnation with water or the desired stationary phase. One disadvantage of liquid-liquid chromatographic systems is that the liquid stationary phase is often stripped off. This problem can be overcome by chemically bonding the stationary phase to the support material.

12.4.2.3 *Bonded Supports*

The liquid stationary phase may be covalently attached to a support by a chemical reaction. These **bonded phases** have become very popular for HPLC use, and a wide variety of both polar and nonpolar stationary phases is now available. It is important to note that mechanisms other than partition may be involved in the separation using bonded supports. Reversed-phase HPLC (see Chap. 13), with a nonpolar bonded stationary phase (e.g., silica bonded with C8 or C18 groups) and a polar solvent (e.g., water-acetonitrile), is widely used. Bonded-phase HPLC columns have greatly facilitated the analysis of vitamins in foods and feeds, as discussed in Chap. 3 of reference [21]. Additionally, bonded-phase HPLC is widely used for the separation and identification of polyphenols such as phenolic acids (e.g., *p*-coumaric, caffeic, ferulic, and sinapic acids) and flavonoids (e.g., flavonols, flavones, isoflavones, anthocyanidins, catechins, and proanthocyanidins).

12.4.3 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) has gained popularity over recent years for the purification of compounds on a preparative and semi-analytical scale. In HIC biomolecules adsorb to a weak hydrophobic surface at high salt concentration. Elution of adsorbed molecules is achieved by decreasing the salt concentration of the mobile phase over time. This technique takes advantage of hydrophobic moieties on the surface of a compound. Accordingly, HIC is very commonly used for the purification of food proteins, enzymes, and antibodies while offering high resolution. The high salt concentration allows biomolecules with high surface charge to adsorb to the hydrophobic **ligands** by shielding the charges. Salt precipitation of proteins, in particular, onto the column does not cause denaturation, since the interaction with the hydrophobic ligands is weak.

The stationary phase in HIC consists of hydrophilic support bonded to hydrophobic ligands. Several polymeric materials can be used as support, including cellulose, agarose, chitosan, polymethacrylate, or silica. The support must be hydrophilic so as not to contribute additional hydrophobicity and hence strong interactions that may cause denaturation of the protein. Often the polymer used has a high degree of cross-linking to provide rigidity and high surface area. Most commonly used ligands, chemically bonded to the support polymer, are linear chain alkanes. The size of the alkyl chains used depends on the surface hydrophobicity of the biomolecules to be separated, with the length of the chain being higher for more hydrophobic biomolecules. Sometimes phenyl and other aromatic groups are also used. Often times though, ligands with intermediate hydrophobicity are used to avoid strong interactions. Butyl-Sepharose® and phenyl-Sepharose® are among the most commonly used stationary phases.

12.4 table Compounds class polarity scale

Fluorocarbons
Saturated hydrocarbons
Olefins
Aromatics
Halogenated compounds
Ethers
Nitro compounds
Esters ≈ ketones ≈ aldehydes
Alcohols ≈ amines
Amides
Carboxylic acids

From Johnson and Stevenson [5], used with permission
Listed in order of increasing polarity

12.5 table Eluotropic series for alumina

Solvent
1-Pentane
Isooctane
Cyclohexane
Carbon tetrachloride
Xylene
Toluene
Benzene
Ethyl ether
Chloroform
Methylene chloride
Tetrahydrofuran
Acetone
Ethyl acetate
Aniline
Acetonitrile
2-Propanol
Ethanol
Methanol
Acetic acid

From Johnson and Stevenson [5], used with permission
Listed in order of increasing polarity

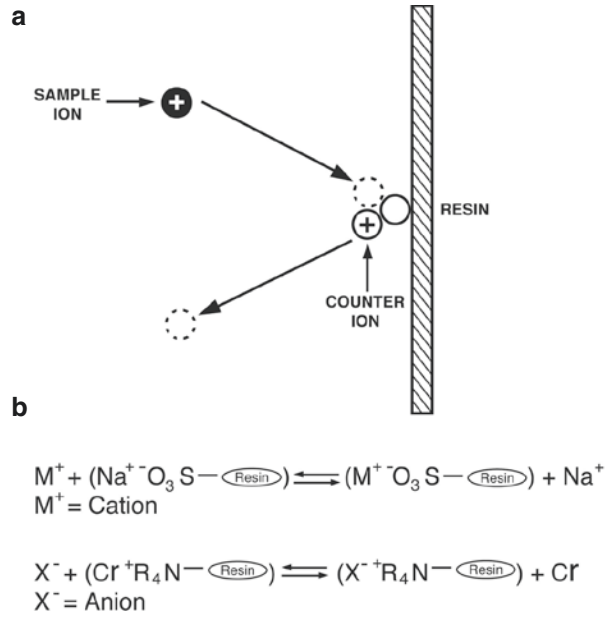
Different salts are used in HIC depending on their effects on protein precipitation. The most commonly used salt is ammonium sulfate. Concentration of the salt is also a determining factor for the precipitation of the protein on the column. Often 1 *M* ammonium sulfate is used for initial screening. It is important to prepare the sample in the same salt solution/buffer as the mobile phase. This, however, necessitates care in loading the sample to avoid precipitation of the protein prior to reaching the column. A wash step generally precedes loading the sample to allow for washing out impurities. Depending on the sample characteristics, elution can be performed gradually by decreasing salt concentration over time, which may allow for isolation of different proteins in the sample in less time. Changing of salt concentration over time requires optimization for best resolution and shortest analysis time. Switching directly to water after the salt wash also may be performed to elute all bound protein with minimal separation. This is dependent on the level of fractionation and purification required. If a compound resists elution even after reducing salt concentration to zero, other HIC ligands should be tried. Cleaning and regeneration of the column are required after several runs. Often 0.1–1 *M* NaOH is used to prevent fouling of the column, and sometimes detergents and alcohol washes are used.

The pH of the mobile phase also can influence retention on the column and elution. However, oftentimes buffers with pH 7 are used. Additives, such as water-miscible alcohols and detergents, are sometimes used to help elute the protein faster. The hydrophobic parts of these additives will compete with the protein for binding to the ligand, causing desorption of the protein. Finally, temperature may play a role in HIC. As temperature increases, hydrophobic interaction increases allowing for better retention on the column; lowering the temperature aids in elution. Control of temperature during a run may enhance resolution and reduce analysis time (see also Sect. 2.5.1). For further details on HIC and applications, readers are directed to reference [22].

12.4.4 Ion-Exchange Chromatography

Separation by ion exchange can be categorized into three types: (1) ionic from nonionic, (2) cationic from anionic, and (3) mixtures of similarly charged species. In the first two cases, one substance binds to the ion-exchange medium, whereas the other substance does not. Batch extraction methods can be used for these two separations; however, chromatography is needed for the third category.

Ion-exchange chromatography is viewed as a specialized type of adsorption chromatography in which interactions between solute and stationary phase are primarily **electrostatic** in nature. The stationary phase (ion exchanger) contains fixed functional groups that are either negatively or positively charged (Fig. 12.4a). Exchangeable counterions preserve charge neutrality. A sample ion (or charged sites on large molecules) can exchange with the counterion for the interaction with the charged functional group. Ionic equilibrium is established as depicted in Fig. 12.4b. The functional group of the stationary phase determines whether cations or anions are



12.4 figure

The basis of ion-exchange chromatography. (**a**) Schematic diagram of the ion-exchange process; (**b**) ionic equilibria for cation- and anion-exchange processes (From Johnson and Stevenson [5], used with permission)

exchanged. **Cation exchangers** possess covalently bound negatively charged functional groups, whereas **anion exchangers** possess bound positively charged groups. The chemical nature of these acidic or basic residues determines how stationary phase ionization is affected by the mobile phase pH.

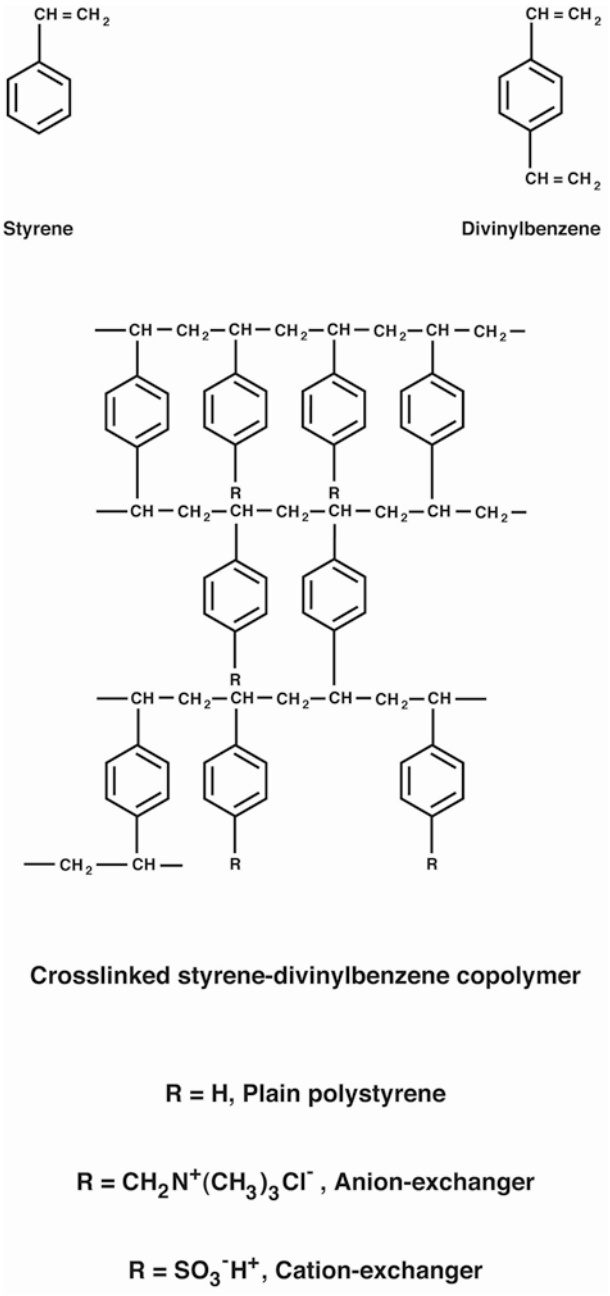
The strongly acidic sulfonic acid moieties (RSO3-) of "strong" cation exchangers are completely ionized at all pH values above 2. Strongly basic quaternary amine groups (RNR′3+) on "strong" anion exchangers are ionized at all pH values below 10. Since maximum negative or positive charge is maintained over a broad pH range, the exchange or binding capacity of these stationary phases is essentially constant, regardless of mobile phase pH. "Weak" cation exchangers contain weakly acidic carboxylic acid functional groups (RCO2−); consequently, their exchange capacity varies considerably between ca. pH 4 and 10. Weakly basic anion exchangers possess primary, secondary, or tertiary amine residues (R-NHR′2+) that are deprotonated in moderately basic solution, thereby losing their positive charge and the ability to bind anions. Thus, one way of eluting solutes bound to weak ion-exchange medium is to change the mobile phase pH. Changing of the pH in the case of weak ion exchangers allows for enhanced separation and better selectivity compared to strong ion exchangers when separating compounds with very similar charge densities. While strong ion exchangers are generally used for initial screening and optimization of the separation, oftentimes, weak ion exchangers are used to separate compounds that have similar adsorption coefficient, making use of a pH gradient. Changing the pH, however, may present a challenge when separating proteins. One must avoid the isoelectric point of the proteins during chromatographic separation; otherwise they will fall out of solution. A second way to elute bound solutes from either strong or weak ion exchangers is to increase the ionic strength (e.g., use NaCl) of the mobile phase, to weaken the electrostatic interactions.

Chromatographic separations by ion exchange are based upon differences in affinity of the exchangers for the ions (or charged species) to be separated. The factors that govern **selectivity** of an exchanger for a particular ion include: (1) ionic valence, radius, and concentration, (2) nature of the exchanger (including its displaceable counterion), and (3) composition and pH of the mobile phase. To be useful as an ion exchanger, a material must be both ionic in nature and highly permeable. Synthetic ion exchangers are thus cross-linked polyelectrolytes, and they may be inorganic (e.g., aluminosilicates) or, more commonly, organic compounds. Two commonly used types of organic compound-based synthetic ion exchangers are polystyrene and polysaccharide.

Polystyrene, made by cross-linking styrene with divinylbenzene (DVB), may be modified to produce either anion- or cation-exchange resins (Fig. 12.5). Polymeric resins such as these are commercially available in a wide range of particle sizes and with different degrees of cross-linking (expressed as weight percent of DVB in the mixture). The extent of cross-linking controls the rigidity and porosity of the resin, which, in turn, determines its optimal use. Lightly cross-linked resins permit rapid equilibration of solute, but particles swell in water, thereby decreasing charge density and selectivity (relative affinity) of the resin for different ions. More highly cross-linked resins exhibit less swelling, higher exchange capacity, and selectivity, but longer equilibration times. The small pore size, high charge density, and inherent hydrophobicity of the older ion-exchange resins have limited their use to small molecules [molecular weight (MW) <500].

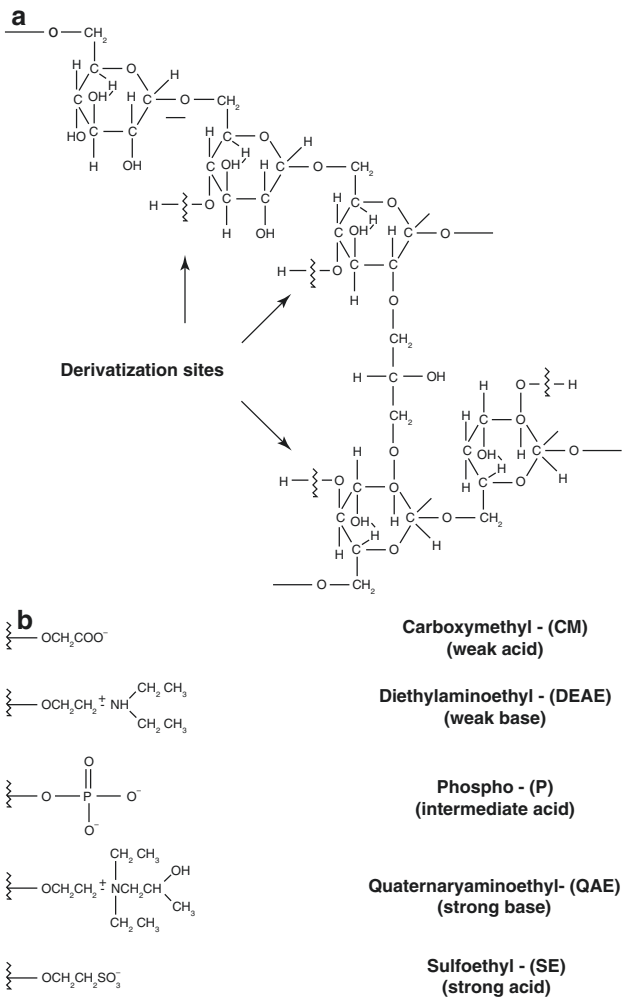
Ion exchangers based on **polysaccharides**, such as cellulose, dextran, or agarose, have proven very useful for the separation and purification of large molecules, such as proteins and nucleic acids. These materials, called **gels**, are much softer than polystyrene resins, thus may be derivatized with strong or with weak acidic or basic groups via OH moieties on the polysaccharide backbone (Fig. 12.6). They have much larger pore sizes and lower charge densities than the older synthetic resins.

Food-related applications of ion-exchange chromatography include the separation of amino acids, sugars, alkaloids, and proteins. Fractionation of amino



12.5 figure Chemical structure of polystyrene-based ion-exchange resins

acids in protein hydrolysates was initially carried out by ion-exchange chromatography; automation of this process led to the development of commercially produced amino acid analyzers (see Chap. 24). Many drugs, fatty acids, and organic acids, being ionizable compounds, may be chromatographed in the ion-exchange mode. For additional details on the principles and applications of ion chromatography, please refer to reference [23].



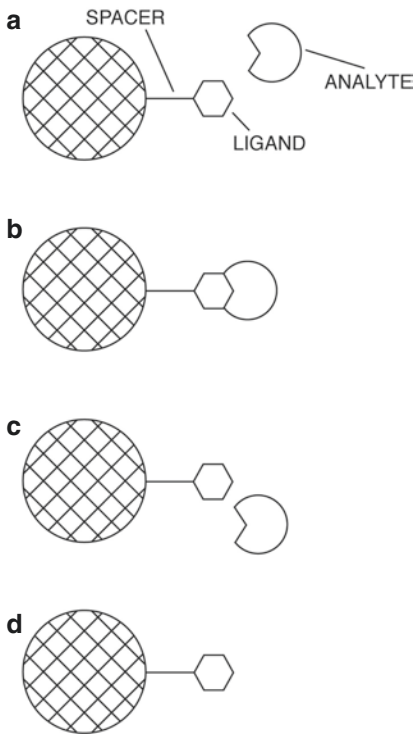
12.6 figure Chemical structure of one polysaccharide-based ion-exchange resin. (**a**) Matrix of cross-linked dextran ("Sephadex," Pharmacia Biotech Inc, Piscataway NJ); (**b**) functional groups that may be used to impart ion-exchange properties to the matrix

12.4.5 Affinity Chromatography

Affinity chromatography is also another specialized form of adsorption chromatography, with the separation being based on very specific, reversible interaction between a solute molecule and a ligand immobilized on the chromatographic stationary phase (types of interactions are listed in Sect. 12.4.1). Affinity chromatography involves immobilized biological ligands as the stationary phase. These ligands can be antibodies, enzyme inhibitors, lectins, or other molecules that selectively and reversibly bind to complementary analyte molecules in the sample. Although both ligands and the species to be isolated are usually biological macromolecules, the term affinity chromatography also encompasses other systems, such as separation of small molecules containing *cis*-diol

groups via phenylboronic acid moieties on the stationary phase.

The principles of affinity chromatography are illustrated in Fig. 12.7. A ligand, chosen based on its specificity/selectivity and strength of interaction with the analyte, is immobilized on a suitable support material. As the sample is passed through the column, analytes that are complementary to the bound ligand are adsorbed, while other sample components are eluted. Bound analytes are subsequently eluted via a change in the mobile phase composition as will be discussed below. After reequilibration with the initial mobile phase, the stationary phase is ready to be used again. The ideal support for affinity chromatography should be a porous, stable, high-surface-area material that does not adsorb anything itself. Thus, polymers such as agarose, cellulose, dextran, and polyacrylamide are used, as well as controlled pore glass.



12.7 figure

Principles of bioselective affinity chromatography. (**a**) The support presents the immobilized ligand to the analyte to be isolated. (**b**) The analyte makes contact with the ligand and attaches itself. (**c**) The analyte is recovered by the introduction of an eluent, which dissociates the complex holding the analyte to the ligand. (**d**) The support is regenerated, ready for the next isolation (Reprinted from Heftmann [1], p. A311, with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands)

Affinity ligands are usually attached to the support or matrix by covalent bond formation, and optimum reaction conditions often must be found empirically. Immobilization generally consists of two steps: **activation** and **coupling**. During the activation step, a reagent reacts with functional groups on the support, such as hydroxyl moieties, to produce an activated matrix. After removal of excess reagent, the ligand is coupled to the activated matrix. (Preactivated supports are commercially available, and their availability has greatly increased the use of affinity chromatography.) The coupling reaction most often involves free amino groups on the ligand, although other functional groups can be used. When small molecules such as phenylboronic acid are immobilized, a **spacer arm** (containing at least four to six methylene groups) is used to hold the ligand away from the support surface, enabling it to reach into the binding site of the analyte.

Ligands for affinity chromatography may be either **specific** or **general** (i.e., group specific). Specific ligands, such as antibodies, bind only one particular solute. General ligands, such as nucleotide analogs and lectins, bind to certain classes of solutes. For example, the lectin concanavalin A binds to all molecules that contain terminal glucosyl and mannosyl residues. Bound solutes then can be separated as a group or individually, depending upon the elution technique used. Some of the more common general ligands are listed in Table 12.6. Although less selective, general ligands provide greater convenience.

Elution methods for affinity chromatography may be divided into **nonspecific** and **(bio)specific** methods. Nonspecific elution involves disrupting ligand analyte binding by changing the mobile phase pH, ionic strength, dielectric constant, or temperature. If additional selectivity in elution is desired, for example, in the case of immobilized general ligands, a biospecific elution technique is used. Free ligand, either identical to or different from the matrix-bound ligand, is added to the mobile phase. This free ligand competes for binding sites on the analyte. For example, glycoproteins bound to a concanavalin A (lectin) column can be eluted by using buffer containing an excess of lectin. In general, the eluent ligand should display greater affinity for the analyte of interest than the immobilized ligand.

In addition to protein purification, affinity chromatography may be used to separate supramolecular structures such as cells, organelles, and viruses; concentrate dilute protein solutions; investigate binding mechanisms; and determine equilibrium constants. Affinity chromatography has been useful especially in the separation and purification of enzymes and glycoproteins. In the case of the latter, carbohydrate-derivatized adsorbents are used to isolate specific lectins, such as concanavalin A, and lentil

12.6 table General affinity ligands and their specificities

Ligand	*Specificity*
Cibacron Blue F3G-A dye, derivatives of AMP, NADH, and NADPH	Certain dehydrogenases via binding at the nucleotide binding site
Concanavalin A, lentil lectin, wheat germ lectin	Polysaccharides, glycoproteins, glycolipids, and membrane proteins containing sugar residues of certain configurations
Soybean trypsin inhibitor, methyl esters of various amino acids, D-amino acids	Various proteases
Phenylboronic acid	Glycosylated hemoglobins, sugars, nucleic acids, and other *cis*-diol-containing substances
Protein A	Many immunoglobulin classes and subclasses via binding to the F_c region
DNA, RNA, nucleosides, nucleotides	Nucleases, polymerases, nucleic acids

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or wheat germ lectin. The lectin then agarose may be coupled to agarose, such as concanavalin A-agarose or lentil lectin-agarose, to provide a stationary phase for the purification of specific glycoproteins, glycolipids, or polysaccharides. Other applications of affinity chromatography include purification and quantification of mycotoxins [24] and pesticide residues/metabolites [25] in food/crops. For additional details on affinity chromatography, refer to references [26, 27].

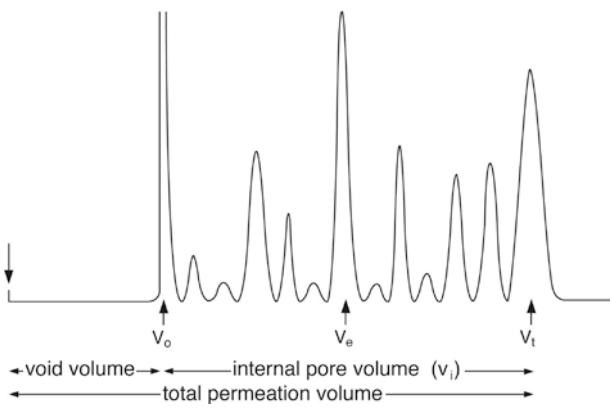
12.4.6 Size-Exclusion Chromatography

Size-exclusion chromatography (SEC), also known as molecular exclusion, gel permeation (GPC), and gel filtration chromatography (GFC), is probably the easiest mode of chromatography to perform and to understand. It is widely used in the biological sciences for the resolution of macromolecules, such as proteins and carbohydrates, and also is used for the fractionation and characterization of synthetic polymers. Unfortunately, nomenclature associated with this separation mode developed independently in the literature of the life sciences and in the field of polymer chemistry, resulting in inconsistencies.

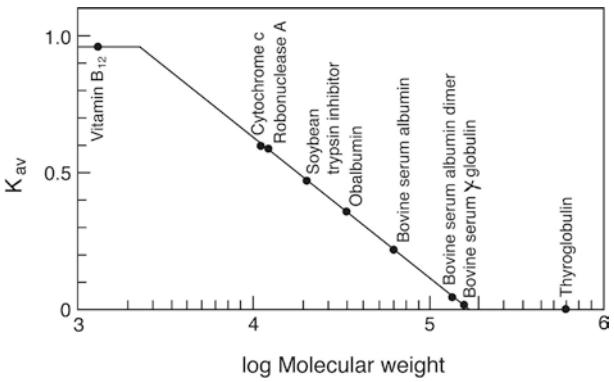
In the ideal SEC system, molecules are separated solely on the basis of their size; no interaction occurs between solutes and the stationary phase. In the event that solute/support interactions do occur, the separation mode is termed nonideal SEC. The stationary phase in SEC consists of a column packing material that contains pores comparable in size to the molecules to be fractionated. Solutes too large to enter the pores travel with the mobile phase in the interstitial space (between particles) outside the pores. Thus, the largest molecules are eluted first from an SEC column. The volume of the mobile phase in the column, termed the column **void volume**, Vo, can be measured by chromatographing a very large (totally excluded) species, such as Blue Dextran, a dye of molecular weight (MW) two million.

As solute dimensions decrease, approaching those of the packing pores, molecules begin to diffuse into the packing particles and, consequently, are slowed down. Solutes of low MW (e.g., glycyltyrosine) that have free access to all the available pore volume are eluted in the volume referred to as Vt, the **total permeation volume** of the column. The Vt is equal to the column void volume, Vo, plus the volume of liquid inside the sorbent pores (**internal pore volume**), Vi (Vt = Vo + Vi). These relationships are illustrated in Fig. 12.8. Solutes are ideally eluted between the void volume and the total permeation volume of the column. Because this volume is limited, only a relatively small number of solutes (ca. 10), of a particular size range, can be completely resolved by SEC under ordinary conditions.

The behavior of a molecule in a size-exclusion column may be characterized in several different ways. Each solute exhibits an **elution volume**, Ve, as illustrated in Fig. 12.9. However, Ve depends on



12.8 figure Schematic elution profile illustrating some of the terms used in size-exclusion chromatography (Adapted from Heftmann [1], p. A271, with kind permission from Elsevier Science – NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands)



12.9 figure

Relationship between K_{av} and log (molecular weight) for globular proteins chromatographed on a column of Sephadex G-150 Superfine (Reproduced by permission of Pharmacia Biotech, Inc., Piscataway, NJ)

column dimensions, the way in which the column was packed, and the sorbent pore size. The **available partition coefficient**, Kav, is used to define solute behavior independent of these variables:

$$\text{Kav} = (\text{Ve} - \text{Vo}) / (\text{Vt} - \text{Vo}) \qquad (12.3)$$

where:

Kav = available partition coefficient
Ve = elution volume of solute
Vo = column void volume
Vt = total permeation volume of column

The value of Kav calculated from experimental data for a solute chromatographed on a given SEC column defines the proportion of pores that can be occupied by that molecule. For a large, totally excluded species, such as Blue Dextran or DNA, Ve = Vo and Kav = 0. For a small molecule with complete access to the internal pore volume, such as glycyltyrosine, Ve = Vt and Kav = 1.

For each size-exclusion packing material, a plot of Kav vs. the logarithm of the MW for a series of solutes, similar in molecular shape and density, will give an S-shaped curve (Fig. 12.9). In the case of proteins, Kav is actually better related to the Stokes radius, the average radius of the protein in solution. The central, linear portion of this curve describes the **fractionation range** of the matrix, wherein maximum separation among solutes of similar MW is achieved. This correlation between solute elution behavior and MW (or size) forms the basis for a widely used method for characterizing large molecules such as proteins and polysaccharides. A size-exclusion column is calibrated with a series of solutes of known MW (or Stokes radius) to obtain a curve similar to that shown in Fig. 12.9. The value of Kav for the unknown is then determined, and an estimate of MW (or size) of the unknown is made by interpolation of the calibration curve.

Column packing materials for SEC can be divided into two groups: semirigid, hydrophobic media and soft, hydrophilic gels. The former are usually derived from polystyrene and are used with organic mobile phases (GPC or nonaqueous SEC) for the separation of polymers, such as rubbers and plastics. Soft gels, polysaccharide-based packings, are typified by Sephadex, a cross-linked dextran (see Fig. 12.6a). These materials are available in a wide range of pore sizes and are useful for the separation of water-soluble substances in the MW range $1–2.5 \times 10^7$ Da. In selecting an SEC column packing, both the purpose of the experiment and size of the molecules to be separated must be considered. If the purpose of the experiment is group separation, for which molecules of widely different molecular sizes need to be separated, a matrix is chosen such that the larger molecules, e.g., proteins, are eluted in the void volume of the column, whereas small molecules are retained in the total volume. When SEC is used for separation of macromolecules of different sizes, the molecular sizes of all the components must fall within the fractionation range of the gel.

As discussed previously, SEC can be used, directly, to fractionate mixtures, to purify, or, indirectly, to obtain MW/size information about a dissolved species. In addition to MW estimations, SEC is used to determine the MW distribution of natural and synthetic polymers, such as dextrans and gelatin preparations. Fractionation of biopolymer mixtures is probably the most widespread use of SEC, since the mild elution conditions employed rarely cause denaturation or degradation. Often times SEC is used as an early chromatographic separation step in a multidimensional chromatographic approach toward purification. It is also a fast, efficient alternative to dialysis for desalting solutions of macromolecules, such as proteins.

12.5 ANALYSIS OF CHROMATOGRAPHIC PEAKS

Once the chromatographic technique (Sect. 12.3) and chromatographic mechanism (Sect. 12.4) have been chosen, the analyst has to ensure adequate separation of constituents of interests from a mixture, in a reasonable amount of time. After separation is achieved and chromatographic peaks are obtained, qualitative as well as quantitative analysis can be carried out. Basic principles of separation and resolution will be discussed in the subsequent sections. Understanding these principles allows the analyst to optimize separation and perform qualitative and quantitative analysis.

12.5.1 Separation and Resolution

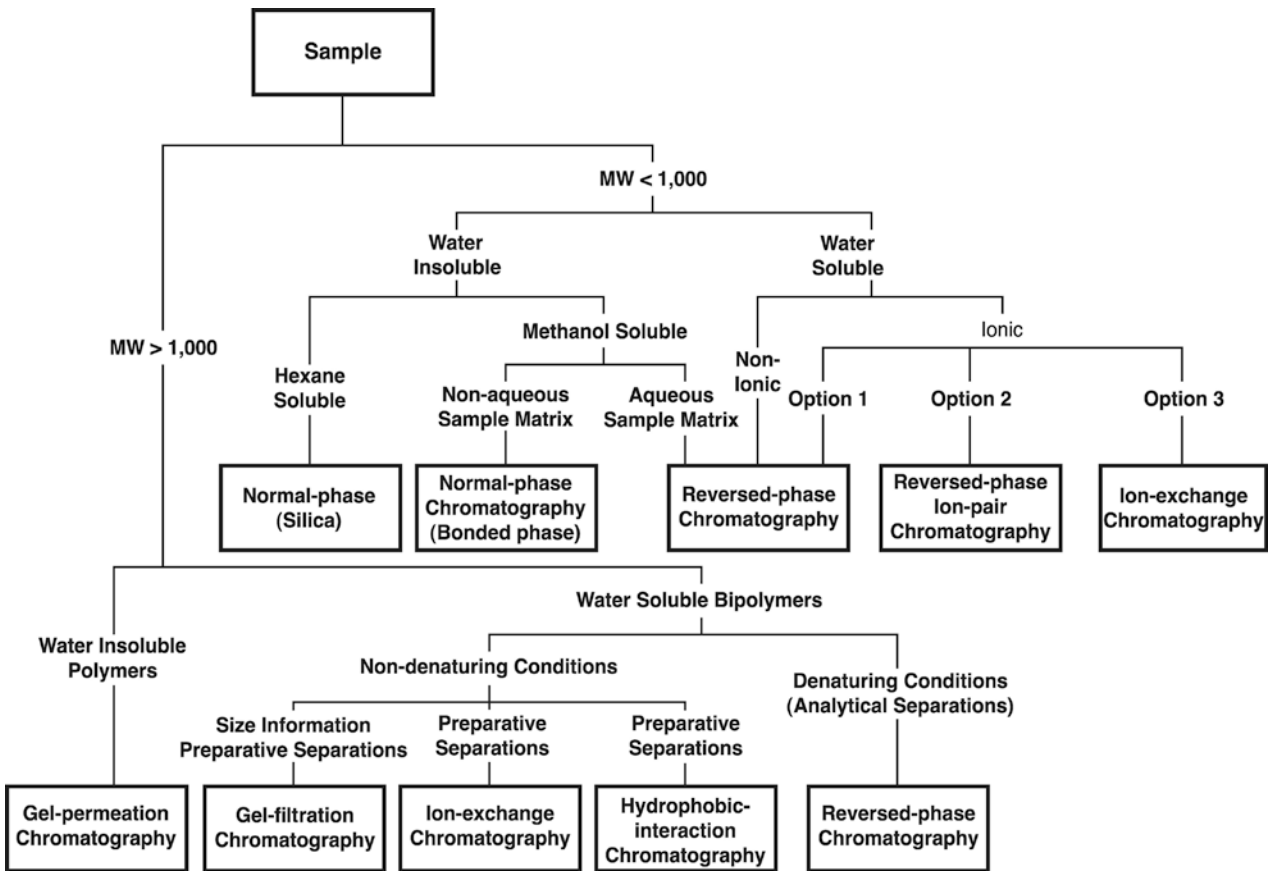
This section will discuss separation and resolution as it pertains mainly to LC; separation and resolution optimization as it pertains specifically to GC will be discussed in Chap. 14.

12.5.1.1 *Developing a Separation*

There may be numerous ways to accomplish a chromatographic separation for a particular compound. In many cases, the analyst will follow a standard laboratory procedure or published methods. In the case of a sample that has not been previously analyzed, the analyst begins by evaluating what is known about the sample and defines the goals of the separation. How many components need to be resolved? What degree of resolution is needed? Is qualitative or quantitative information needed? Molecular weight (or MW range)/size, polarity, and ionic character of the sample will guide the choice of chromatographic separation mechanism (**separation mode**). Figure 12.10 shows that more than one correct choice may be possible. For example, small ionic compounds may be separated by ion-exchange, ion-pair reversed-phase (see Sect. 13.3.2.1), or reversed-phase LC. In this case, the analyst's choice may be based on convenience, experience, and personal preference.

Having chosen a separation mode for the sample at hand, one must select an appropriate stationary phase, elution conditions, and a detection method. Trial experimental conditions may be based on the results of a literature search, the analyst's previous experience with similar samples, or the general recommendations from chromatography experts.

To achieve separation of sample components by all modes except SEC, one may utilize either isocratic or gradient elution. **Isocratic elution** is the simplest technique, in which solvent composition and flow rate are held constant throughout the run. **Gradient elution** involves reproducibly varying mobile phase composition or flow rate (flow programming) during the LC analysis. Gradient elution is used when sample components vary a lot in inherent characteristics such as polarity and/or charge density, so that an isocratic mobile phase does not elute all components within a reasonable time. The change may be continuous or stepwise. Gradients of increasing ionic strength, or



12.10 figure A schematic diagram for choosing a chromatographic separation mode based on sample molecular weight and solubility (From Lough and Wainer [29], used with permission)

changing pH, are extremely valuable in ion-exchange chromatography (see Sect. 12.4.4), whereas gradients of increasing or decreasing polarity are valuable in normal or reversed-phase chromatography, respectively (Sect. 12.4.2). Increasing the "strength" of the mobile phase (Sect. 12.4.1), either gradually (continuously) or in a stepwise fashion, shortens the analysis time.

Method development may begin with an isocratic mobile phase, possibly of intermediate solvent strength; however, using gradient elution for the initial separation may ensure that some level of separation is achieved within a reasonable time period and nothing is likely to remain on the column. Data from this initial run allows one to determine if isocratic or gradient elution is needed, and to estimate optimal isocratic mobile phase composition or gradient range.

Once an initial separation has been achieved, the analyst can proceed to optimize resolution (separation of closely related analytes). This generally involves changing mobile phase variables, including the following: (1) nature and percentage of organic solvents (and type of organic solvents), (2) pH, (3) ionic strength, (4) nature and concentration of additives (such as ion-pairing agents), (5) flow rate, and (6) temperature. In the case of gradient elution, gradient steepness (slope, i.e., rate of change) is another variable to be optimized. However, the analyst must be aware of the principles of chromatographic resolution as will be discussed in the following section.

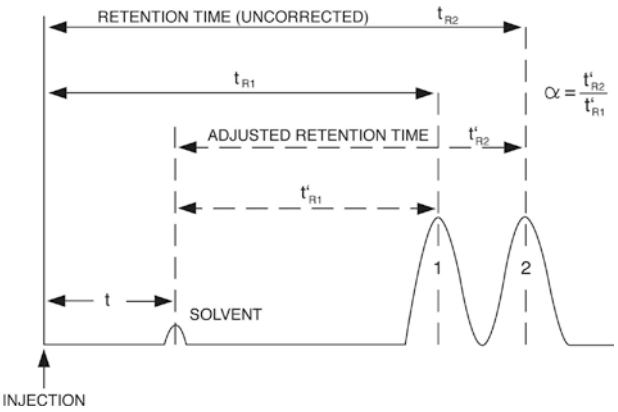
12.5.1.2 *Chromatographic Resolution*

12.5.1.2.1 Introduction

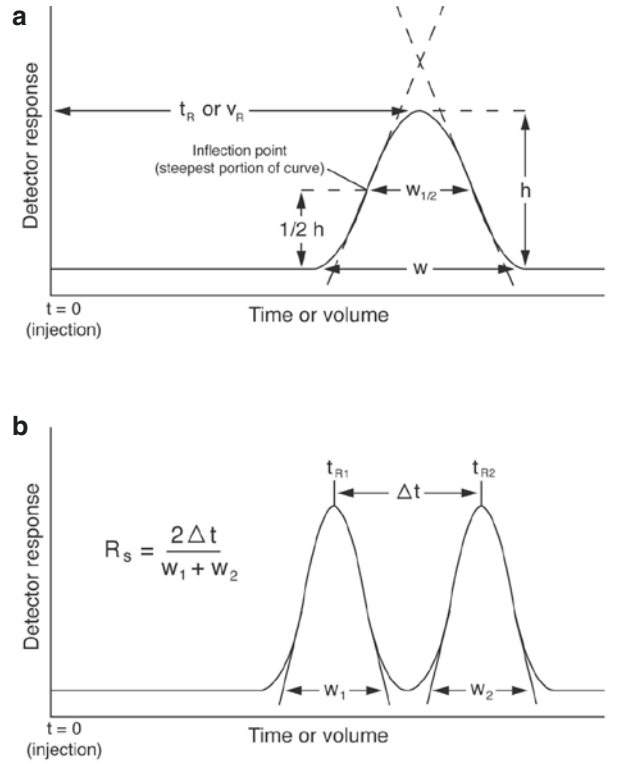
The main goal of chromatography is to segregate components of a sample into separate bands or peaks as they migrate through the column. A **chromatographic peak** is defined by several parameters including **retention time** (Fig. 12.11), **peak width**, and **peak height** (Fig. 12.12). The volume of the mobile phase required to elute a compound from an LC column is called the **retention volume**, V_R. The associated time is the **retention time**, t_R. Shifts in retention time and changes in peak width greatly influence **chromatographic resolution**.

Differences in column dimensions, loading, temperature, mobile phase flow rate, system dead volume, and detector geometry may lead to discrepancies in retention time. By subtracting the time required for the mobile phase or a non-retained solute (t_m or t_o) to travel through the column to the detector, one obtains an **adjusted retention time**, t'_R (or volume), as depicted in Fig. 12.11. The adjusted retention time (or volume) corrects for differences in system dead volume and signifies the time the analyte spends adsorbed on the stationary phase.

The **resolution** of two peaks from each other is related to the **separation factor**, α. Values for α (Fig. 12.11)



12.11 figure Measurement of chromatographic retention (Adapted from Johnson and Stevenson [5], with permission)



12.12 figure Measurement of peak width and its contribution to resolution. (**a**) Idealized Gaussian chromatogram, illustrating the measurement of w and $w_{½}$; (**b**) the resolution of two bands is a function of both their relative retentions and peak widths (Adapted from Johnson and Stevenson [5], with permission)

depend on temperature, flow rate, stationary phase, and mobile phase used. Resolution is defined as follows:

$$R_s = \frac{2\Delta t}{w_2 + w_1} \qquad (12.4)$$

where:

R_s = resolution
Δt = difference between retention times of peaks 1 and 2
w_2 = width of peak 2 at baseline
w_1 = width of peak 1 at baseline

Figure 12.12 illustrates the measurement of peak width [part (a)] and the values necessary for calculating resolution [part (b)]. (Retention and peak or band width must be expressed in the same units, i.e., time or volume).

Chromatographic resolution is a function of column **efficiency**, **selectivity**, and the **capacity factor**. Mathematically, this relationship is expressed as follows:

$$R_s = \underbrace{\tfrac{1}{4}\sqrt{N}}_{a}\underbrace{\left(\frac{\alpha-1}{\alpha}\right)}_{b}\underbrace{\left(\frac{k'}{k'+1}\right)}_{c} \qquad (12.5)$$

where:

a = column **efficiency** term
b = column **selectivity** term
c = **capacity** term

These terms, and factors that contribute to them, will be discussed in the following sections.

12.5.1.2.2 Column Efficiency

If faced with the problem of improving resolution, a chromatographer should first examine the **efficiency** of the column. An efficient column keeps the solutes from spreading widely on the column and hence gives narrow peaks. Column efficiency can be calculated by:

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16\left(\frac{t_R}{w}\right)^2 = 5.5\left(\frac{t_R}{w_{1/2}}\right)^2 \qquad (12.6)$$

where:

N = number of theoretical plates
t_R = retention time
σ = standard deviation for a Gaussian peak
w = peak width at baseline ($w = 4\sigma$)
$w_{½}$ = peak width at half height

The measurement of t_R, w, and $w_{½}$ is illustrated in Fig. 12.12. (Retention volume may be used instead of t_R; in this case, peak width is also measured in units of volume.) Although some peaks are not actually Gaussian in shape, normal practice is to treat them as if they were. In the case of peaks that are incompletely resolved or slightly asymmetric, peak width at half height is more accurate than peak width at baseline.

The value N calculated from the above equation is called the number of **theoretical plates**. The theoretical plate concept, borrowed from distillation theory, can best be understood by viewing chromatography as a series of equilibrations of solutes between mobile and stationary phases, analogous to countercurrent distribution. Thus, a column would consist of N segments (theoretical plates) with one equilibration occurring in each. As a first approximation, N is independent of retention time and is therefore a useful measure of column performance. One method of monitoring column performance over time is to chromatograph a standard compound periodically, under constant conditions, and to compare the values of N obtained. It is important to note that columns often behave as if they have a different number of plates for different solutes in a mixture. Different solutes have different partition coefficient and thus have distinctive series of equilibrations between mobile and stationary phases. Peak broadening due to column deterioration will result in a decrease of N for a particular solute. Peak broadening is a result of an extended time for a solute to reach equilibrium between mobile and stationary phases (causing them to spread over a wider area on the column).

The number of theoretical plates is generally proportional to column length. The longer the column, the higher the number of theoretical plates, and hence the better is the resolution. Because columns are available in various lengths, it is useful to have a measure of column efficiency that is independent of column length. This may be expressed as:

$$\text{HETP} = \frac{L}{N} \qquad (12.7)$$

where:

HETP = height equivalent to a theoretical plate
L = column length
N = number of theoretical plates

The so-called **HETP, height equivalent to a theoretical plate**, is sometimes more simply described as **plate height** (H). If a column consisted of discrete segments, HETP would be the height of each imaginary segment. Small plate height values (a large number of plates) indicate good efficiency of separation (minimal spread of solute within the column, resulting in sharp peaks). Conversely, reduced number of plates results in poor separation due to the extended equilibrium time (spread of solutes on the column, resulting in wide peaks) in a deteriorating column.

In reality, columns are not divided into discrete segments and equilibration is not infinitely fast. The plate theory is used to simplify the equilibration concept. The movement of solutes through a chromatography column takes into account the finite rate at which a solute can equilibrate itself between stationary and mobile phases. Thus, peak shape depends on the rate of elution and is affected by solute diffusion. Any mechanism that causes a peak of solute to broaden

will increase HETP and decrease column efficiency. The various factors that contribute to plate height are expressed by the **Van Deemter equation**:

$$\text{HETP} = \text{A} + \frac{\text{B}}{u} + Cu \qquad (12.8)$$

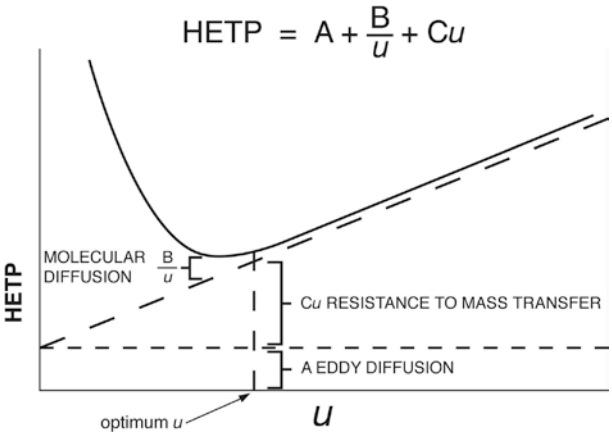
where:

HETP = height equivalent to a theoretical plate
A, B, C = constants
u = mobile phase rate

The constants A, B, and C are characteristic for a given column, mobile phase, and temperature. The A term represents the **eddy diffusion** or multiple flow-paths. Eddy diffusion refers to the different microscopic flowstreams that the mobile phase can take between particles in the column (analogous to eddy streams around rocks in a brook). Sample molecules can thus take different paths as well, depending on which flowstreams they follow. As a result, solute molecules spread from an initially narrow band to a broader area within the column. The larger is the particle size of the packing material, the more paths a solute might take. Eddy diffusion may be minimized by good column packing techniques and the use of small diameter particles of narrow particle size distribution.

The B term of the Van Deemter equation, sometimes called the **longitudinal diffusion** term, exists because all solutes diffuse from an area of high concentration (the center of a chromatographic peak) to one of low concentration (the leading or trailing edge of a chromatographic peak). In LC, the contribution of this term to HETP is small except at low flow rate of the mobile phase. With slow flow rates there will be more time for a solute to spend on the column; thus, its diffusion will be greater.

The C (mass transfer) term arises from the finite time required for solute to equilibrate between the mobile and stationary phases. **Mass transfer** is practically the partitioning of the solute into the stationary phase, which does not occur instantaneously and depends on the solute's partition and diffusion coefficients. If the stationary phase consists of porous particles (see Chap. 13, Sect. 13.2.3.2, Fig. 13.3), a sample molecule entering a pore ceases to be transported by the solvent flow and moves by diffusion only. Subsequently, this solute molecule may diffuse back to the mobile phase flow or it may interact with the stationary phase. In either case, solute molecules inside the pores are slowed down relative to those outside the pores, and peak broadening occurs. Contributions to HETP from the C term can be minimized by using porous particles of small diameter or pellicular packing materials (Chap. 13, Sect. 13.2.3.2.2). Also, using a narrower column with a smaller inner diameter reduces the C value, given



12.13 figure Van Deemter plot of column efficiency (HETP) vs. mobile phase rate (u). Optimum u is noted (Courtesy of Hewlett-Packard Co., Analytical Customer Training, Atlanta, GA)

that equilibrium time will be reduced since there is less packing material.

As expressed by the Van Deemter equation, **mobile phase flow rate**, u, contributes to plate height in opposing ways – increasing the flow rate increases the equilibration point (Cu), but decreases longitudinal diffusion of the solute particles (B/u). A Van Deemter plot (Fig. 12.13) may be used to determine the mobile phase flow rate at which plate height is minimized and column efficiency is maximized. Flow rates above the optimum may be used to decrease analysis time if adequate resolution is still obtained. However, at very high flow rates, there will be less time to approach equilibrium, which will lead to a shorter retention time and co-elution of closely related solutes.

In addition to flow rate, temperature can affect the longitudinal diffusion and the mass transfer. Increasing the temperature causes enhanced movement of the solute between the mobile phase and the stationary phase and within the column, thus leading to faster elution and narrower peaks.

12.5.1.2.3 Column Selectivity

Chromatographic resolution depends on column selectivity as well as efficiency. **Column selectivity** refers to the distance, or relative separation, between two peaks and is given by:

$$\alpha = \frac{t_{R2} - t_o}{t_{R1} - t_o} = \frac{t'_{R2}}{t'_{R1}} = \frac{K_2}{K_1} \qquad (12.9)$$

where:

α = separation factor
t_{R1} and t_{R2} = retention times of components l and 2, respectively
t_o (or t_m) = retention time of unretained components (solvent front)

t'_{Rl} and t'_{R2} = adjusted retention times of components l and 2, respectively
K_l and K_2 = distribution coefficients of components l and 2, respectively

Retention times (or volumes) are measured as shown in Fig. 12.11. The time, *t*o, can be measured by chromatographing a solute that is not retained under the separation conditions (i.e., travels with the solvent front). When this parameter is expressed in units of volume, V_o or V_m, it is known as the **dead volume** of the system. Selectivity is a function of the stationary and/or mobile phase. For example, selectivity in ion-exchange chromatography is influenced by the nature and number of ionic groups on the matrix but also can be affected by pH and ionic strength of the mobile phase. Changing the pH or the ionic strength of the mobile phase will impact the extent of interaction of the solutes with the column. Selectivity can be either static as in isocratic elution (the choice of the mobile phase from the beginning impact selectivity) or dynamic as in gradient elution (selectivity changing over time). Good selectivity is probably more important to a given separation than high efficiency (Fig. 12.14), since resolution is directly related to selectivity but is quadratically related to efficiency; thus, a fourfold increase in *N* is needed to double *R*s (Eq. 12.5).

12.5.1.2.4 Column Capacity Factor

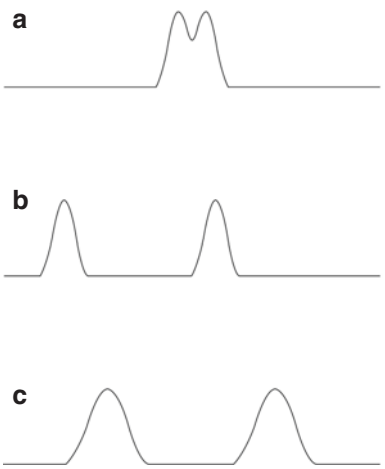
The **capacity** or **retention factor**, k', is a measure of the amount of time a chromatographed species (solute) spends in/on the stationary phase relative to the mobile phase. The relationship between capacity factors and chromatographic retention (which may be expressed in units of either volume or time) is shown below:

$$k' = \frac{KV_s}{V_m} = \frac{V_R - V_m}{V_m} = \frac{t_R - t_o}{t_o} \qquad (12.10)$$

where:

k' = capacity factor
K = distribution coefficient of the solute
V_s = volume of stationary phase in column
V_m = volume of mobile phase
V_R = retention volume of solute
t_R = retention time of solute
t_o = retention time of unretained components (solvent front)

Small values of k' indicate little retention, and components will be eluted close to the solvent front, resulting in poor separations. Overuse or misuse of the column may lead to the loss of some functional groups, thus resulting in small k' values. Large values of k' result in improved separation but also can lead to broad peaks and long analysis times. On a practical basis, k' values within the range of 1–15 are generally used. (In the equation for R_s, k' is actually the average of k_1' and k_2' for the two components separated.)



12.14 figure Chromatographic resolution: efficiency vs. selectivity. (**a**) Poor resolution; (**b**) good resolution due to high column efficiency; (**c**) good resolution due to column selectivity (From Johnson and Stevenson [5], used with permission)

12.5.2 Qualitative Analysis

Once separation and resolution have been optimized, identification of the detected compounds can be achieved. (Various detection methods are outlined in Chaps. 13 and 14). Comparing V_R or t_R to that of standards chromatographed under identical conditions often enables one to identify an unknown compound. When it is necessary to compare chromatograms obtained from two different systems or slightly different columns, it is better to compare **adjusted retention time**, t'_R (see Sect. 12.5.1.2). Oftentimes comparison across different systems and columns is not possible. Different compounds may have identical retention times using the same system. In other words, even if the retention time of an unknown and a standard are equivalent, the two compounds might not be identical. Therefore, other techniques are needed to confirm peak identity. For example:

1. Spike the unknown sample with a known compound and compare chromatograms of the original and spiked samples to see which peak has increased. Only the height of the peak of interest should increase, with no change in retention time, peak width, or shape.
2. A diode array detector can provide absorption spectra of designated peaks (see Sects. 13.2.6 and 13.2.4.1). Although identical spectra do not prove identity, a spectral difference confirms

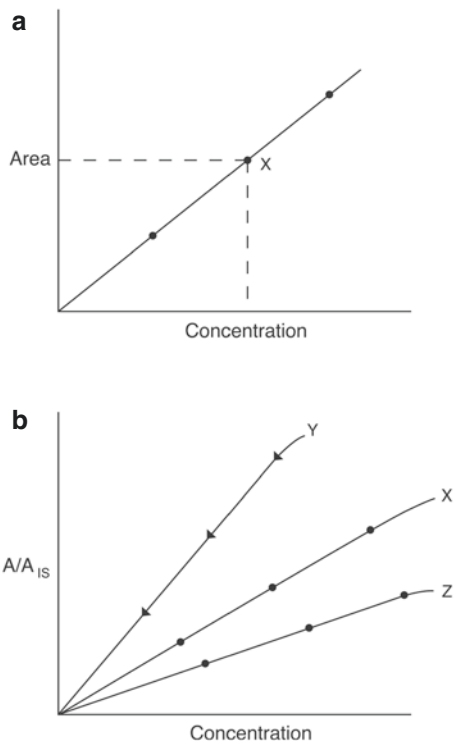
that sample and standard peaks are different compounds.
3. In the absence of spectral scanning capability, other detectors, such as absorption or fluorescence, may be used in a ratioing procedure. Chromatograms of sample and standard are monitored at each of two different wavelengths. The ratio of peak areas at these wavelengths should be the same if sample and standard are identical.
4. Peaks of interest can be collected and subjected to additional chromatographic separation using a different separation mode.
5. Collect the peak(s) of interest and establish their identity by another analytical method (e.g., mass spectrometry, which can give a mass spectrum that is characteristic of a particular compound; see Chap. 11).

12.5.3 Quantitative Analysis

Assuming that good chromatographic resolution and identification of sample components have been achieved, quantification involves mostly measuring peak area and comparing the data with those for standards of known concentration. Nowadays all chromatography systems use data analysis software that recognizes the start, maximum, and end of each chromatographic peak, even when not fully resolved from other peaks. These values then are used to determine retention times, peak height, and peak areas. At the end of each run, a report is generated that lists these data and post-run calculations, such as relative peak areas, areas as percentages of the total area, and relative retention times. If the system has been standardized, data from external or internal standards can be used to calculate analyte concentrations (discussed below). In low-pressure, preparative chromatography, post-chromatography analysis of collected fractions is sometimes used to identify samples eluted. Examples of post-chromatography analysis include the BCA (bicinchoninic acid) protein assay (Chap. 18, Sect. 18.4.2.3) and the phenol-sulfuric acid assay for carbohydrate (Chap. 19, Sect. 19.3). After obtaining the absorbance reading on a spectrophotometer for such assays, the results are plotted as fraction number on the *x*-axis and absorbance on the *y*-axis to determine which fractions contain protein and/or carbohydrate.

Having determined peak areas, one must compare these data with appropriate standards of known concentration to determine sample concentrations. Comparisons may be by means of **external** or **internal** standards. Comparison of peak area of unknown samples with standards injected separately (i.e., **external standards**) is common practice. Standard solutions covering the desired concentration range (preferably diluted from one stock solution) are chromatographed, and the peak area is plotted versus concentration to obtain a standard curve. An identical volume of sample is then chromatographed, and an area of the sample peak is used to determine sample concentration via the standard curve (Fig. 12.15a). This absolute calibration method requires precise analytical technique and requires that detector sensitivity be constant from day to day if the calibration curve is to remain valid.

The use of an **internal standard** can minimize errors due to losses incurred during sample preparation, apparatus noise (inherent apparatus error), and systematic errors in the operator's technique (including the volume of sample injected). In other words, the internal standard, upon addition to the sample, will be subjected to the same sequence of events that may introduce changes to the actual content of the compounds of interest and the standard. In this technique, compared to the compounds of interest in the sample, the internal standard compound must: (1) be chemically/structurally related to the compounds of interest, (2) elute at a different time, and (3) not be naturally present in the sample. Basically, the amount of each component in the sample is determined by comparing the area of that component peak to the area of the internal standard peak. However, variation in detector response between compounds of different chemical



12.15 figure

Calibration curves for quantification of a sample component, *x*. (**a**) External standard technique; (**b**) internal standard technique (Adapted from Johnson and Stevenson [5], with permission)

structure must be taken into account. One way to do this is by first preparing a set of standard solutions containing varying concentrations of the compound(s) of interest. Each of these solutions is made to contain a known and constant amount of the internal standard. These standard solutions are chromatographed, and peak area is measured. Ratios of peak area (compound of interest/internal standard) are calculated and plotted against concentration to obtain calibration curves such as those shown in Fig. 12.15b. A separate response curve must be plotted for each sample component to be quantified. Next, a known amount of internal standard is added to the unknown sample, and the sample is chromatographed. Peak area ratios (compound of interest/internal standard) are calculated and used to calculate the concentration of each relevant component using the appropriate calibration curve. The advantages of using internal standards are that injection volumes need not be accurately measured and the detector response need not remain constant since any change will not alter ratios.

Standards should be included during each analytical session, since detector response may vary from day to day. Analyte recovery should be checked periodically. This involves addition of a known quantity of standard to a sample (usually before extraction) and determination of how much is recovered during subsequent analysis. During routine analyses, it is highly desirable to include a control or check sample, a material of known composition. This material is analyzed parallel to unknown samples. When the concentration of analyte measured in the control falls outside an acceptable range, data from other samples analyzed during the same period should be considered suspect. Carefully analyzed food samples and other substances are available from the National Institute of Standards and Technology (formerly the National Bureau of Standards) for use in this manner.

12.6 SUMMARY

Chromatography is a separation method based on the partitioning of a solute between a mobile phase and a stationary phase. The mobile phase may be liquid, gas, or a supercritical fluid. The stationary phase may be an immobilized liquid or a solid, in either a planar or column form. Based on the physicochemical characteristics of the analyte, and the availability of instrumentation, a chromatographic system is chosen to separate, identify, and quantify the analyte. Chromatographic modes include adsorption, partition, hydrophobic interaction, ion exchange, affinity, and size-exclusion chromatography. Factors to be considered when developing a separation include mobile phase variables (polarity, ionic strength, pH, temperature, and/or flow rate) and column efficiency, selectivity, and capacity. Following detection, a chromatogram provides both qualitative and quantitative information via retention time and peak area data.

For an introduction to the techniques of HPLC and GC, the reader is referred again to Chaps. 13 and 14 in this text or to the excellent 5th edition of *Quantitative Chemical Analysis* by D.C. Harris [2]. The book by R.M. Smith [3] also contains information on basic concepts of chromatography and chapters devoted to TLC, LC, and HPLC, as well as an extensive discussion of GC. References [14–16] contain a wealth of information on TLC, and references [8] and [9] cover SFC. *Chromatography* [1], the standard work edited by E. Heftmann (2004 and earlier editions), is an excellent source of information on both fundamentals (Part A) and applications (Part B) of chromatography. Part B includes chapters on the chromatographic analysis of amino acids, proteins, lipids, carbohydrates, and phenolic compounds. In addition, *Fundamental and Applications Reviews* published (in alternating years) by the journal *Analytical Chemistry* relate new developments in all branches of chromatography, as well as their application to specific areas, such as food. Recent books and general review papers are referenced, along with research articles published during the specified review period.

12.7 STUDY QUESTIONS

1. Explain the principle of countercurrent extraction and how it developed into partition chromatography.
2. For each set of two (or three) terms used in chromatography, give a brief explanation as indicated to distinguish between the terms:

   (a) Adsorption vs. partition chromatography

	Adsorption	*Partition*
Nature of stationary phase		
Nature of mobile phase		
How solute interacts with the phases		

   (b) Normal-phase vs. reversed-phase chromatography

	Normal-phase chromatography	*Reversed-phase chromatography*
Nature of stationary phase		
Nature of mobile phase		
What elutes last		

(c) Cation vs. anion exchangers

	Cation exchanger	*Anion exchanger*
Charge on column		
Nature of compounds bound		

(d) Internal standards vs. external standards

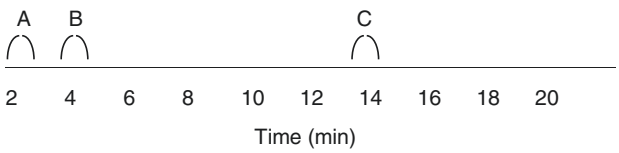
	Nature of stds.	*How stdsq. are handled in relation to samples*	*What is plotted on std. curve*
Internal standard			
External standard			

(e) TLC vs. column liquid chromatography

	Thin-layer chromatography	*Column liquid chromatography*
Nature and location of stationary phase		
Nature and location of mobile phase		
How sample is applied		
Identification of solutes separated		

(f) HETP vs. N vs. L (from the equation HETP = *L*/*N*)

3. State the advantages of TLC as compared to paper chromatography.
4. State the advantages of column liquid chromatography as compared to planar chromatography.
5. Explain how SFC differs from LC and GC, including the advantages of SFC.
6. What is the advantage of bonded supports over coated supports for partition chromatography?
7. You are performing LC using a stationary phase that contains a polar nonionic functional group. What type of chromatography is this, and what could you do to increase the retention time of an analyte?
8. You applied a mixture of proteins, in a buffer at pH 8.0, to an anion-exchange column. On the basis of some assays you performed, you know that the protein of interest adsorbed to the column:
   (a) Does the anion-exchange stationary phase have a positive or negative charge?
   (b) What is the overall charge of the protein of interest that adsorbed to the stationary phase?
   (c) Is the isoelectric point of the protein of interest (adsorbed to the column) higher or lower than pH 8.0?
   (d) What are the two most common methods you could use to elute the protein of interest from the anion-exchange column? Explain how each method works. (See also Chap. 24).
9. Would you use a polystyrene- or a polysaccharide-based stationary phase for work with proteins? Explain your answer.
10. Explain the principle of affinity chromatography, why a spacer arm is used, and how the solute can be eluted.
11. Explain how you would use SEC to estimate the molecular weight of a protein molecule. Include an explanation of what information must be collected and how it is used.
12. What is gradient elution from a column, and why is it often advantageous over isocratic elution?
13. A sample containing compounds A, B, and C is analyzed via LC using a column packed with a silica-based C_{18} bonded phase. A 1:5 solution of ethanol and H_2O was used as the mobile phase. The following chromatogram was obtained:



Assuming that the separation of compounds is based on their polarity:
   (a) Is this normal- or reversed-phase chromatography? Explain your answer.
   (b) Which compound is the most polar?
   (c) How would you change the mobile phase so compound C would elute sooner, without changing the relative positions of compounds A and B? Explain why this would work.
   (d) What could possibly happen if you maintained an isocratic elution mode at low solvent strength?
14. Using the Van Deemter equation, HETP, and N, as appropriate, explain why the following changes may increase the efficiency of separation in column chromatography:

(a) Changing the flow rate of the mobile phase
(b) Increasing the length of the column
(c) Reducing the inner diameter of the column
(d) Decreasing the particle size of the packing material

15. State the factors and conditions that lead to poor resolution of two peaks.
16. How can chromatographic data be used to quantify sample components?
17. Why would you choose to use an internal standard rather than an external standard? Describe how you would select an internal standard for use.
18. To describe how using internal standards works, answer the following questions:

    (a) What specifically will you do with the standards?
    (b) What do you actually measure and plot?
    (c) How do you use the plot?

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