28

chapter

High-Performance Liquid Chromatography

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28.1 INTRODUCTION

High-performance liquid chromatography (HPLC) developed during the 1960s as a direct offshoot of classic column liquid chromatography through improvements in the technology of columns and instrumental components (pumps, injection valves, and detectors). Originally, HPLC was the acronym for *high-pressure liquid chromatography*, reflecting the high operating pressures generated by early columns. By the late 1970s, however, *high-performance liquid chromatography* had become the preferred term, emphasizing the effective separations achieved. In fact, newer columns and packing materials offer high performance at moderate pressure (although still high pressure relative to gravity-flow liquid chromatography). HPLC can be applied to the analysis of any compound with solubility in a liquid that can be used as the mobile phase. Although most frequently employed as an **analytical** technique, HPLC also may be used in the **preparative** mode. There are many *advantages* of HPLC over traditional low pressure column liquid chromatography:

1. Speed (many analyses can be accomplished in 30 min or less)
2. A wide variety of stationary phases
3. Improved resolution
4. Greater sensitivity (various detectors can be employed)
5. Easy sample recovery (less eluent volume to remove)

Application of HPLC to the analysis of food began in the late 1960s, and its use increased with the development of column packing materials that would separate sugars. Using HPLC to analyze sugars was justified economically as a result of sugar price increases in the mid 1970s, which motivated soft drink manufacturers to substitute high-fructose corn syrup for sugar. Monitoring sweetener content by HPLC assured a good quality product. Other early food applications included the analysis of pesticide residues in fruits and vegetables, organic acids, lipids, amino acids, toxins (such as aflatoxins in peanuts), and vitamins (1). HPLC continues to be applied to these, and many more, food-related analyses today (2–5).

28.2 COMPONENTS OF AN HPLC SYSTEM

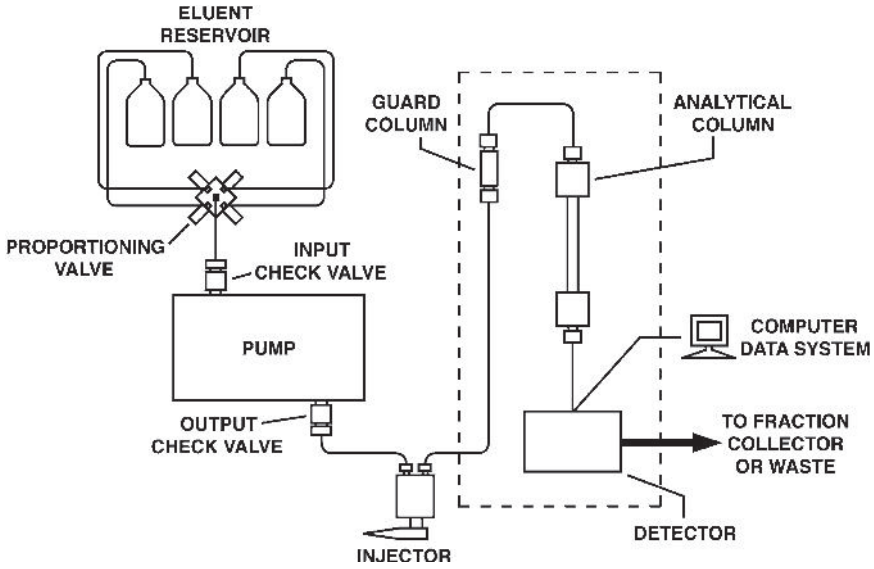
A schematic diagram of a basic HPLC system is shown in Fig. 28-1. The main components of this system – **pump, injector, column, detector**, and **data system** – are discussed briefly in the sections below. Also important are the mobile phase (**eluent**) reservoirs, and a fraction collector, which is used if further analysis of separated components is needed. Connecting tubing, tube fittings, and the materials out of which components are constructed also influence system performance and lifetime. References (1) and (6–10,15) include detailed discussions of HPLC equipment, with the book by Bidlingmeyer (1) especially appropriate for beginners. The unique organization of reference (8) is intended for those who may need to learn chromatography quickly in an industrial environment. Two useful books on HPLC troubleshooting are those written by Gertz (11) and Dolan and Snyder (12). In addition, much information on HPLC equipment, hardware, and troubleshooting hints may be found in publications such as *LC·MS, American Laboratory, Chemical & Engineering News*, and similar periodicals. Manufacturers are also a source of practical information on HPLC instrumentation and columns/stationary phase material.

28.2.1 Pump

The **HPLC pump** delivers the mobile phase through the system, typically at a flow rate of 0.4–1 ml/min, in a controlled, accurate, and precise manner. The majority of pumps currently used in HPLC (>90%) are reciprocating, piston-type pumps. The dual piston pump systems with ball check valves are the most efficient pumps available. One disadvantage of reciprocating pumps is that they produce a pulsating flow, requiring the addition of pulse dampers to suppress fluctuations. A mechanical **pulse damper** or **dampener** consists of a device (such as a deformable metal component or tubing filled with compressible liquid) that can change its volume in response to changes in pressure.

Gradient elution systems for HPLC are used to vary the mobile phase concentration during the run, by mixing mobile phase from two or more reservoirs. This is accomplished with low-pressure mixing, in which mobile phase components are mixed before entering the high-pressure pump, or high-pressure mixing, in which two or more independent, programmable pumps are used. For low-pressure gradient systems, a computer-controlled proportioning valve, followed by a mixing chamber at the inlet to the pumps is used, which results in extremely accurate and reproducible gradients. Gradient HPLC is extremely important for the effective elution of all components of a sample and for optimal resolution. It is routinely applied to all modes of HPLC except size-exclusion chromatography.

Many commercially available HPLC pumping systems and connecting lines are made of grade ANSI 316 stainless steel, which can withstand the pressures generated. Also it is resistant to corrosion by oxidizing agents, acids, bases, and organic solvents, although mineral acids and halide ions do attack stainless



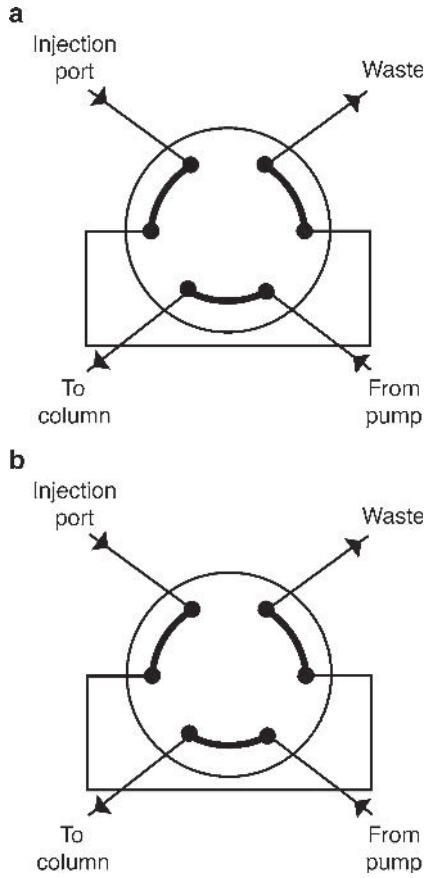
28-1 figure Schematic representation of a system for high-performance liquid chromatography (not drawn to scale). Column(s) and detector may be thermostatted, as indicated by the *dashed line*, for operation at elevated temperature.

steel. In other systems, all components that come into contact with the eluent are made of sturdy, inert polymers, and even employ sapphire pistons, which are resistant to extreme pH and high salt concentration. The latter systems can be used for all applications except normal phase, which uses organic solvents as the mobile phase. The polymer-based systems have facilitated a wider application of ion exchange HPLC.

All HPLC pumps contain moving parts such as check valves and pistons, and are quite sensitive to dust and particulate matter in the liquid being pumped. Therefore, it is advisable to filter the mobile phase using 0.45 or 0.22-µm filters prior to use. Degassing HPLC eluents, by the application of a vacuum or by sparging with helium, also is recommended to prevent the problems caused by air bubbles in a pump or detector.

28.2.2 Injector

The role of the injector is to place the sample into the flowing mobile phase for introduction onto the column. Virtually all HPLC systems use **valve injectors**, which separate sample introduction from the high-pressure eluent system. With the injection valve in the LOAD position (Fig. 28-2a), the sample is loaded into an **external, fixed-volume loop** using a syringe. Eluent, meanwhile, flows directly from the pump to the column at high pressure. When the valve is rotated to the INJECT position (Fig. 28-2b), the loop becomes part of the eluent flow stream and sample is carried onto the column. Such injectors are generally trouble free and afford good precision.



28-2 figure Valve-type injector. The valve allows the sample loop to be (**a**) isolated from the pump eluent stream (LOAD position) or (**b**) positioned in it (INJECT position). [from (9), used with permission.]

Changing the loop allows different volumes to be injected. Although injection volumes of 10–100 µl are typical, both larger (e.g., 1–10 ml) and smaller (e.g., ≤2 µl) sample volumes can be loaded by utilizing special hardware. An important advantage of the loop valve design is that it is readily adapted to automatic operation. Thus, automated sample injectors, or **autosamplers**, may be used to store and inject large numbers of samples. Samples are placed in uniform-size vials, sealed with a septum, and held in a (possibly refrigerated) tray. A computer actuated needle penetrates the septum to withdraw solution from the vial, and a mechanically or pneumatically operated valve introduces it onto the column. Autosamplers can reduce the tedium and labor costs associated with routine HPLC analyses and improve assay precision. However, because samples may remain unattended for 12–24 h prior to automatic injection, sample stability is a limiting factor for using this accessory.

28.2.3 Column

28.2.3.1 Column Hardware

An **HPLC column** is usually constructed of stainless steel tubing with terminators that allow it to be connected between the injector and detector of the system (Fig. 28-1). Columns also are made from glass, fused silica, titanium, and polyether ether ketone (PEEK) resin; the PEEK columns are essential for the high pH, high salt concentrations necessary for the powerful ion exchange HPLC systems. Many types and sizes of columns are commercially available, ranging from 5 cm × 50 cm (or larger) preparative columns down to wall-coated capillary columns.

28.2.3.1.1 Precolumns Auxiliary columns that precede the analytical HPLC column are termed **precolumns**. Short (≤5 cm) expendable columns, called **guard columns**, often are used to protect the analytical column from strongly adsorbed sample components. A guard column (or cartridge) is installed between the injector and analytical column via short lengths of capillary tubing (or a cartridge holder). They may be filled with either **pellicular** media (see Sect. 28.2.3.2.2) of the same bonded phase as the analytical column, or with **microparticulate** (≤10 µm) packing material identical to that of the analytical column. Microparticulate guard columns are usually purchased as prepacked, disposable inserts for use in a special holder, and cost much less than replacing an analytical column. A guard column (or cartridge) should be repacked or replaced before its binding capacity is exceeded and contaminants pollute the analytical column.

28.2.3.1.2 Analytical Columns The most commonly used **analytical** HPLC columns are 10, 15, or 25-cm long with an internal diameter of 4.6 or 5 mm (9). Short (3 cm) columns, packed with ≤3 µm particles, are gaining popularity for fast separations; for example, in method development or process monitoring. In recent years, the use of columns with smaller internal diameters (<0.5–2.0 mm), including wall-coated capillary columns, has increased. The advantages of using smaller diameter columns include a decreased consumption of mobile phase, an increased peak concentration, increased resolution, and the ability to couple HPLC with mass spectrometry (MS) (13).

Various names have been used for the reduced-volume columns. Dorsey et al. (14) refer to columns with internal diameters of 0.5–2.0 mm as **microbore**, while packed or open tubular columns having internal diameters of <0.5 mm are termed **microcolumns** or **capillary columns** (a capillary column is a narrow-bore open tubular column, in which the inner surface is coated with a thin layer of stationary phase). In the case of the packed columns, the microbore or microcolumns contain very small particle size packing material. Because of the extremely high operating pressures of these systems, they are often referred to as **ultra-HPLC** (UHPLC). To achieve good performance from microcolumns, it is essential to have an HPLC system with very low dead-volume, so that peak broadening outside the column does not destroy resolution achieved within the column. Pumps, injectors, and other hardware designed specifically for use with these columns are available from commercial suppliers.

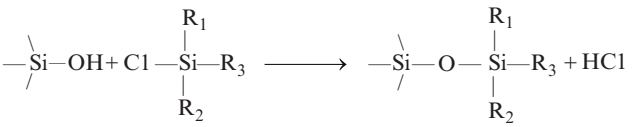
28.2.3.2 HPLC Column Packing Materials

The development of a wide variety of column packing materials has contributed substantially to the success and widespread use of HPLC.

28.2.3.2.1 General Requirements A **packing material** serves, first of all, to form the chromatographic bed; however, in most modes of chromatography the column packing material serves as both **support** and the **stationary phase**. Requirements for HPLC column packing materials are good chemical stability, sufficient mechanical strength to withstand pressure generated during use, and the availability of a well-defined particle size, with a narrow particle size distribution (10). Two materials that meet the above criteria are porous silica and synthetic organic resins (see Sects. 28.2.3.2.2 and 28.2.3.2.3, respectively).

28.2.3.2.2 Silica-Based Column Packings **Porous silica** meets the above criteria quite well and can be prepared in a wide range of particle and pore sizes, with a narrow particle size distribution. Both **particle size** and **pore diameter** are important: Small particles reduce the distance a solute must travel between stationary and mobile phases, which facilitates equilibration and results in good column efficiencies (Chap. 27, Sect. 27.5.2.2.2). However, small particles also yield greater flow resistance and higher pressure at equivalent flow rates. Spherical particles of 3, 5, or 10-μm diameter are utilized in analytical columns. One-half or more of the volume of porous silica consists of the **pores** (10). Use of the smallest possible pore diameter will maximize **surface area** and **sample capacity**, which is the amount of sample that can be separated on a given column. Packing materials with a pore diameter of 50–100 Å and surface area of 200–400 m^2/g are used for low-molecular-weight (<500 Da) solutes. For increasingly larger molecules, such as proteins and polysaccharides, it is necessary to use wider pore materials (pore diameter ≥300 Å), so that internal surface is accessible to the solute (10).

Bonded phases (Fig. 28-3a) are made by covalently bonding hydrocarbon moieties to −OH groups (silanols) on the surface of silica particles (10, 16). Often, the silica is reacted with an organochlorosilane:



[1]

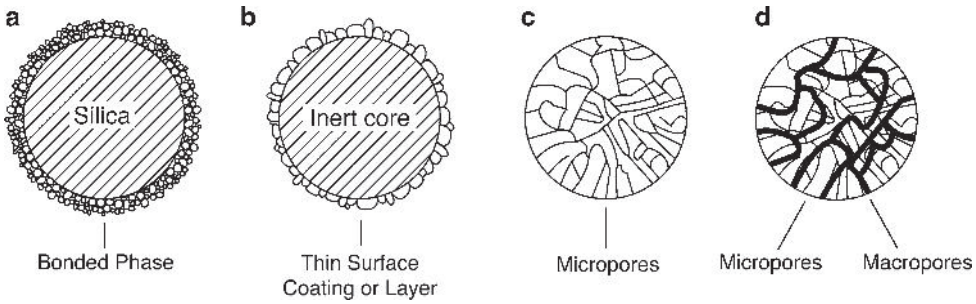
Substituents R_1 and R_2 may be halides or methyl groups. The nature of R_3 determines whether the resulting bonded phase will exhibit normal-phase, reversed-phase, or ion-exchange chromatographic behavior. The main disadvantage of silica and silica-based bonded-phase column packings is that the silica skeleton slowly dissolves in aqueous solutions, and the rate of this process becomes prohibitive at pH<2 and >8.

A **pellicular packing material** (Fig. 28-3b) is made by depositing a thin layer or coating onto the surface of an inert, usually nonporous, microparticulate **core**. Core material may be either inorganic, such as silica, or organic, such as poly(styrene-divinylbenzene) or latex. Functional groups, such as ion-exchange sites, are then present at the surface only. The rigid core ensures good physical strength, whereas the thin stationary phase provides for rapid mass transfer and favorable column efficiency.

28.2.3.2.3 Porous Polymeric Column Packings Synthetic organic resins offer the advantages of good chemical stability and the possibility to vary interactive properties through direct chemical modification. Two major categories of porous polymeric packing materials exist.

Microporous or gel-type resins (Fig. 28-3c) are comprised of crosslinked copolymers in which the apparent porosity, evident only when the gel is in its swollen state, is determined by the degree of crosslinking. These gel-type packings undergo swelling and contraction with changes in the chromatographic mobile phase. Microporous polymers of less than ca. 8% crosslinking are not sufficiently rigid for HPLC use.

Macroporous resins are highly crosslinked (e.g., ≥50%) and consist of a network of microspheric gel beads joined together to form a larger bead (Fig. 28-3d). Large, permanent pores, ranging from 100 to 4000 Å or more in diameter, and large surface areas (≥100 m^2/g) are the result of interstitial spaces between the microbeads (16). Rigid microparticulate poly(styrene-divinylbenzene) packing materials of the macroporous type are popular for HPLC use. They are stable from pH 1 to 14 and are available in a variety of particle and pore sizes. These resins can be used in unmodified form for reversed-phase chromatography or functionalized for use in other HPLC modes.



28-3 figure Some types of packing materials utilized in HPLC. (**a**) Bonded-phase silica; (**b**) pellicular packing; (**c**) microporous polymeric resin; (**d**) macroporous polymeric resin. [Adapted from (16), p. 621, by courtesy of Marcel Dekker, Inc.]

28.2.4 Detector

A **detector** translates sample concentration changes in the HPLC column effluent into electrical signals. Spectrochemical, electrochemical, or other properties of solutes may be measured by a variety of instruments, each of which has advantages and disadvantages. The choice of which to use depends on solute type and concentration, and on detector sensitivity, linear range, and compatibility with the solvent and elution mode to be used. Cost also may influence detector selection. One common feature of most HPLC detectors is the presence of a flow cell, through which the eluent flows as it is analyzed by the detector system. These flow cells are often delicate and easily polluted or damaged, so care must be taken when handling them.

The most widely used HPLC detectors are based on ultraviolet-visible (UV-Vis) and fluorescence spectrophotometry, refractive index determination, and electrochemical analysis (see Chap. 22 for detailed discussion of UV-Vis and fluorescence spectrophotometry). Many other methods, such as light scattering or mass spectrometry, also can be applied to the detection of analytes in HPLC eluents. More than one type of HPLC detector may be used in series, to provide increased **specificity** and **sensitivity** for multiple types of analytes. In one food-related application, a multidetector HPLC system equipped with a diode array absorption detector coupled to fluorescence and electrochemical detectors was used to monitor a wide variety of Maillard reaction products (2).

28.2.4.1 UV-Vis Absorption Detectors

Many HPLC analyses are carried out using a **UV-Vis absorption** detector, which can measure the absorption of radiation by chromophore-containing compounds. The three main types of UV-Vis absorption detectors are **fixed wavelength, variable-wavelength**, and **diode array spectrophotometers** (9). As its name implies, the simplest design operates at a single, **fixed wavelength**. A filter is used to isolate a single emission line (e.g., at 254 nm) from a source such as a mercury lamp. This type of detector is easy to operate and inexpensive, but of limited utility.

The most popular general purpose HPLC detector today is the **variable-wavelength** detector in which deuterium and tungsten lamps serve as sources of ultraviolet and visible radiation, respectively. Wavelength selection is provided by a **monochromator**, a device that acts somewhat like a prism to deflect light. An exit slit in the monochromator allows light from a limited range of wavelengths to pass through, and rotating the monochromator allows one to change the operating wavelength.

Diode array spectrophotometric detectors can provide much more information about sample composition than is possible with monochromatic detection. In this instrument, all the light from a deuterium lamp is spread out into a spectrum that falls across an array of photodiodes mounted on a silicon chip. These are read almost simultaneously by a microprocessor to provide the full absorption spectrum from 200 to 700 nm every 0.1 s, which may enable the components of a mixture to be identified. Although considerably more expensive than variable-wavelength detectors, they are useful in method development and in routine analyses in which additional evidence of peak identity, without further analysis, is needed.

28.2.4.2 Fluorescence Detectors

Some organic compounds can re-emit a portion of absorbed UV-Vis radiation at a longer wavelength (lower energy). This is known as **fluorescence**, and measurement of the **emitted light** provides another useful detection method. Fluorescence detection is both selective and very sensitive, providing up to 1000-fold lower detection limits than for the same compound in absorbance spectrophotometry (10). Although relatively few compounds are inherently fluorescent, analytes often are converted into fluorescent derivatives (see Sect. 28.2.4.7). Ideal for trace analysis, fluorescence detection has been used for the determination of various vitamins in foods and supplements, monitoring aflatoxins in stored cereal products, and the detection of aromatic hydrocarbons in wastewater.

28.2.4.3 Refractive Index Detectors

Refractive index (RI) detectors measure change in the **RI** of the mobile phase due to dissolved analytes, which provides a nearly universal method of detection. However, because a bulk property of the eluent is being measured, RI detectors are less sensitive than other types. Another disadvantage is that they cannot be used with gradient elution, as any change in eluent composition will alter its RI, thereby changing the baseline signal. RI detectors are widely used for analytes that do not contain UV-absorbing chromophores, such as carbohydrates and lipids, when the analytes are present at relatively high concentration.

28.2.4.4 Electrochemical Detectors

Electroanalytical methods used for HPLC detection are based either on electrochemical oxidation–reduction of the analyte or on changes in conductivity of the eluent. **Amperometric detectors** measure the change in current as the analyte is oxidized or reduced

by the application of voltage across electrodes of the flow cell. This method is highly selective (nonreactive compounds give no response) and very sensitive. A major application of electrochemical detection has been for the routine determination of catecholamines, which are phenolic compounds of clinical importance that are present in blood and tissues at very low levels. The development of a triple-pulsed amperometric detector, which overcame the problem of electrode poisoning (accumulation of oxidized product on the electrode surface), has allowed electrochemical detection to be applied to the analysis of carbohydrates (see Sect. 28.3.3.2.2). Pulsed electrochemical detection also has excellent sensitivity for the quantification of flavor-active alcohols, particularly terpenols (2).

Analytes that are ionized and carry a charge can be detected by measuring the change in eluent **conductivity** between two electrodes. **Conductivity detection** has been used mainly to detect inorganic anions and cations and organic acids upon elution from weak ion-exchange columns. Its principal application has been the basis of **ion chromatography** (Sect. 28.3.3.2.1). An excellent overview of electrochemical detection is provided by Swedesh (8).

28.2.4.5 Other HPLC Detectors

Unfortunately, there is no *truly universal* HPLC detector with *high sensitivity*. Thus, there have been many attempts to find new principles that could lead to improved instrumentation. One interesting concept is the **evaporative light scattering detector**. The mobile phase is sprayed into a heated air stream, evaporating volatile solvents and leaving nonvolatile analytes as aerosols. These droplets or particles can be detected because they will scatter a beam of light (8). HPLC with light scattering detection has been applied to the analysis of wheat flour lipids. Also, light scattering detectors are quite useful for the characterization of polymers by size-exclusion chromatography. Improvements in laser applications brought about the development of **low-angle laser light scattering** (LALLS) and **multi-angle laser light scattering** (MALLS) detectors. With these detectors, there is no need to evaporate the mobile phase, as the laser beam is directed at the flow cell, and scattered laser light is then monitored by photo detectors set at specific angles to the cell. In MALLS there may be as many as 18 different photo detectors at discrete angles, each continuously collecting and analyzing the scattered light; from this data, the computer can determine the molecular weight of the eluting sample.

Radioactive detectors are widely used for pharmacokinetic and metabolism studies with radiolabeled drugs. Decay of a radioactive nucleus leads to excitation of a scintillator, which subsequently loses its excess energy by photon emission. Photons are counted by a photomultiplier tube and the number of counts per second is proportional to radiolabeled analyte (9).

A **chemiluminescent nitrogen detector** (CLND) allows nitrogen-containing compounds, such as amino acids, to be detected without using chemical derivatization (Sect. 28.2.4.7). This nitrogen-specific detection system has been used to quantify caffeine in coffee and soft drink beverages, and to analyze capsaicin in hot peppers (2).

28.2.4.6 Coupled Analytical Techniques

To obtain more information about the analyte(s), eluent from an HPLC system can be passed on to a second analytical instrument, such as infrared (IR), nuclear magnetic resonance (NMR), or MS [see Chaps. 23, 25, and 26, respectively, or reference (6)]. The coupling of spectrometers with liquid chromatography (LC) was initially slow to gain application, due to many practical problems. For example, in the case of **HPLC with mass spectrometric detection** (LC–MS), the liquid mobile phase affected the vacuum in the MS. This problem was addressed by the development of commercial interfaces that allow solvent to be evaporated so that only analyte is carried to the spectrometer. Two commonly used interface techniques are discussed in detail by Harris (6). The use of microbore or capillary HPLC columns with a low flow volume also facilitates direct coupling of the two instruments (13). LC–MS systems continue to improve, and the applications are expanding to nearly every class of relatively low molecular weight compounds, including bioactives and contaminants.

28.2.4.7 Chemical Reactions

Detection sensitivity or specificity may sometimes be enhanced by converting the analyte to a **chemical derivative** with different or additional characteristics. An appropriate reagent can be added to the sample prior to injection (i.e., **precolumn derivatization**) or combined with column effluent before it enters the detector (i.e., **postcolumn derivatization**). Automated amino acid analyzers utilize postcolumn derivatization, usually with ninhydrin, for reliable and reproducible analysis of amino acids. Precolumn derivatization of amino acids with *o*-phthalaldehyde or similar reagents permits highly sensitive HPLC determination of amino acids using fluorescence detection (Chap. 15, Sect. 15.3.1.2). In addition, fractions may be collected after passing through the detector and aliquots of each

fraction analyzed by various means, including chemical/colorimetric assays, such as the Lowry protein assay (Chap. 9, Sect. 9.2.5) or a total carbohydrate assay (Chap. 10, Sect. 10.3.2). The results can then be plotted and overlaid with the detector plot, yielding very important information about the compounds eluting in various peaks.

28.2.5 Data Station Systems

A detector provides an electronic signal related to the composition of the HPLC column effluent. It is the job of the last element in the chain of HPLC instrumentation to display the chromatogram, and integrate the peak areas. **Data stations** and **software packages** are nearly ubiquitous with modern HPLC, and all come with very powerful tools for sample identification and quantitation. As an HPLC analysis progresses, the data from the HPLC detector(s) are digitized and saved to the hard drive of a dedicated computer. The operator can then manipulate the data, by assigning and integrating the peaks, for example, and then print out plots and tables for further assessment. Importantly, the software programs can be set up prior to the analysis to perform nearly all these functions, without further input from the operator. For example, retention times can be calculated relative to an internal standard in pesticide residue analysis, and the results compared to a stored database of standards that the software automatically accesses when the analysis is complete. The software will then assign and integrate the peaks, and construct a complete report that will be displayed when the file is opened.

The data stations are now more than simply for interpreting results: the software packages also include all the parameters needed to run the HPLC, including start and stop, injection of the sample, and developing the gradient via control of the proportioning pump systems. When combined with an autosampler, the data station can carry out the entire operation, on hundreds of samples, in the absence of an operator, and, with networking, deliver the analysis file/report to an office computer.

28.3 APPLICATIONS IN HPLC

The basic physicochemical principles underlying all liquid chromatographic separations – adsorption, partition, ion exchange, size exclusion, and affinity – are discussed in Chap. 27, and details will not be repeated here. The number of separation modes utilized in HPLC, however, is greater than that available in classic chromatography. Examples of HPLC applications in food analysis are given in Table 28-1. This is attributable to the success of bonded phases, initially developed to facilitate liquid–liquid partition chromatography (Chap. 27, Sect. 27.4.2). In fact, reversed-phase chromatography is the most widely used separation mode in modern HPLC.

28.3.1 Normal Phase

28.3.1.1 Stationary and Mobile Phases

In **normal-phase** HPLC, the **stationary phase** is a **polar adsorbant**, such as bare silica or silica to which polar nonionic functional groups – hydroxyl, nitro, cyano (nitrile), or amino – have been chemically attached. These bonded phases are moderately polar and the surface is more uniform, resulting in better peak shapes. The **mobile phase** for this mode consists of a **nonpolar solvent**, such as hexane, to which is added a more polar modifier, such as methylene chloride, to control solvent strength and selectivity. Solvent

28-1 table **Example Applications of HPLC in the Analysis of Various Food Constituents**

Analyte	*Separation Mode*	*Method of Detection*	*Chapter*	*Section*
Mono- and oligosaccharides	Ion exchange; normal- or reversed phase	Electrochemical; refractive index; postcolumn analysis	10	10.3.4.1
Vitamin E	Normal or reversed phase	Fluorescence; electrochemical; UV	11	11.2.5.2
Amino acids	Ion exchange; reversed-phase	Post or precolumn derivatization	15	15.3.1
Pesticides	Normal or reversed phase	UV; fluorescence; mass spectrometry	18	18.3.3.2.3; 18.3.3.3.2
Mycotoxins	Reversed phase; immunoaffinity	UV; fluorescence	18	18.4.3.2.1
Antibiotics	Reversed phase	UV	18	18.5.2.2
Melamine	Reversed phase, ion exchange	UV; mass spectrometry	18	18.8.3
Sulfites	Ion exchange	UV; electrochemical	18	18.8.2

strength refers to the way a solvent affects the migration rate of the sample. **Weak solvents** increase retention (large k' values) and **strong solvents** decrease retention (small k' values).

28.3.1.2 Applications of Normal-Phase HPLC

In the past, normal-phase HPLC was used for the analysis of fat-soluble vitamins, although reverse phase is currently applied more frequently for these analyses (see Table 28-1). Normal phase is currently used for the analyses of biologically active polyphenols from natural plant sources, such as grape and cocoa. It is also used for the analysis of relatively polar vitamins, such as vitamins A, D, E, and K (see Chap. 11), and also natural carotenoid pigments, which impart both color and health benefits to foods. Highly hydrophilic species, such as carbohydrates (see Chap. 10, Sect. 10.3.4.1), also may be resolved by normal-phase chromatography, using amino bonded-phase HPLC columns (10).

28.3.2 Reversed Phase

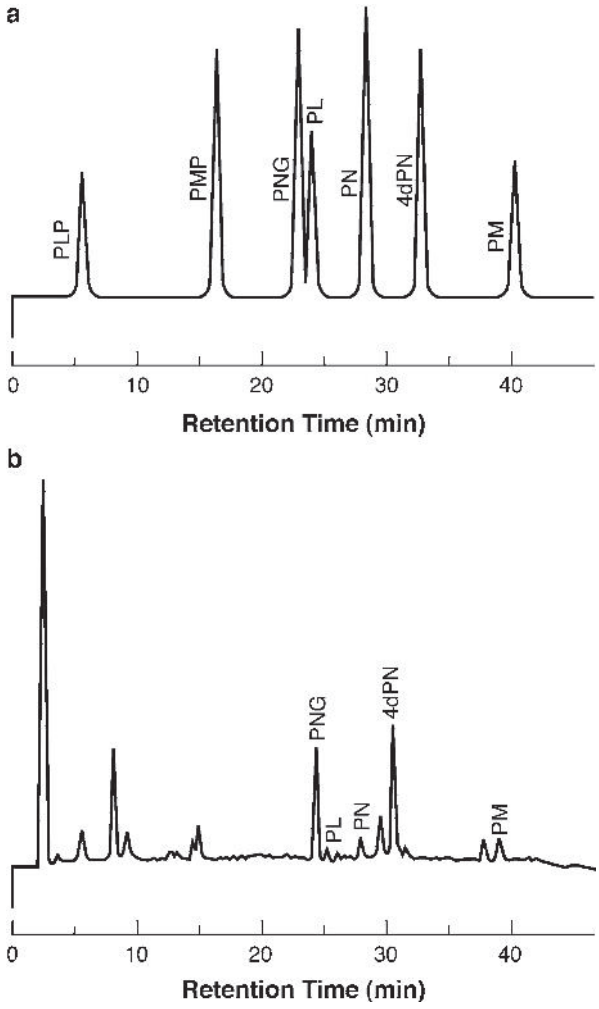
28.3.2.1 Stationary and Mobile Phases

More than 70% of all HPLC separations are carried out in the reversed-phase mode, which utilizes a **nonpolar stationary phase** and a **polar mobile phase**. **Octadecylsilyl** (ODS) **bonded phases**, with an octadecyl (C_{18}) chain [$-(CH_2)_{17}CH_3$], are the most popular reversed-phase packing materials, although shorter chain hydrocarbons [e.g., octyl (C_8) or butyl (C_4)] or phenyl groups are also used. Many silica-based, reversed-phase columns are commercially available. Differences in their chromatographic behavior result from variation in the type of organic group bonded to the silica matrix or the chain length of organic moiety.

Reversed-phase HPLC utilizes **polar mobile phases**, usually water mixed with methanol, acetonitrile, or tetrahydrofuran. Solutes are retained due to **hydrophobic interactions** with the **nonpolar stationary phase** and are eluted in order of increasing hydrophobicity (decreasing polarity). Increasing the polar (aqueous) component of the mobile phase increases solute retention (larger k' values) (see Chap. 27, Sect. 27.5.2.2.4), whereas increasing the organic solvent content of the mobile phase decreases retention (smaller k' values). Various additives can serve additional functions. For example, although ionic compounds often can be resolved without them, **ion-pair reagents** may be used to facilitate chromatography of ionic species on reversed-phase columns. These reagents are ionic surfactants, such as octanesulfonic acid, which can neutralize charged solutes and make them more lipophilic. This type of chromatography is referred to as **ion-pair reversed-phase**.

28.3.2.2 Applications of Reversed-Phase HPLC

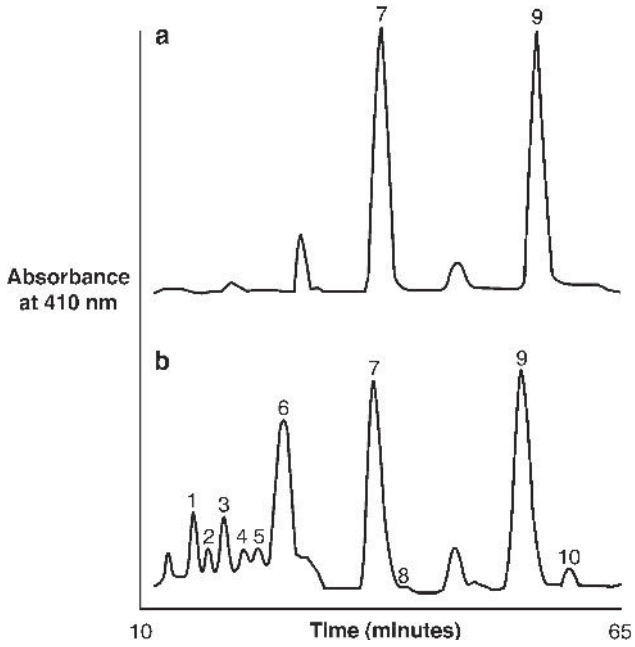
Reversed-phase has been the HPLC mode most used for analysis of plant proteins. Cereal proteins, among the most difficult of these proteins to isolate and characterize, are now routinely analyzed by this method (10). Both water- and fat-soluble vitamins (Chap. 11) can be analyzed by reversed-phase HPLC (2–5), and the availability of fluorescence detectors has enabled researchers to quantitate very small amounts of the different forms of vitamin B_6 (vitamers) in foods and



28-4 figure Analysis of vitamin B_6 compounds by reversed-phase HPLC with fluorescence detection. Some of the standard compounds (**a**) are present in a sample of rice bran extract (**b**). Sample preparation and analytical procedures are described in reference (17). *PL*, pyridoxal; *PLP*, pyridoxal phosphate; *PM*, pyridoxamine; *PMP*, pyridoxamine phosphate; *PN*, pyridoxine; *PNG*, pyridoxine β-D-glucoside. [Reprinted in part with permission from (17). Copyright 1991 American Chemical Society.]

biological samples. Figure 28-4 shows the separation of several of these vitamers in a rice bran extract achieved by reversed-phase ion-pair HPLC (17).

Reversed-phase ion-pair HPLC can be used to resolve carbohydrates on C_{18} bonded-phase columns (10), and the constituents of soft drinks (caffeine, aspartame, etc.) can be rapidly separated. Reversed-phase HPLC using a variety of detection methods, including RI, UV, and light scattering, has been applied to the analysis of lipids (2–5,10). Antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can be extracted from dry foods and analyzed with simultaneous UV and fluorescence detection (3). Phenolic flavor compounds (such as vanillin) and pigments (such as chlorophylls, carotenoids, and anthocyanins) are also easily analyzed (2–5,10). A typical chromatogram of carotenoids present in a carrot extract is shown in Fig. 28-5. Reversed-phase ion-pair chromatography also is used for the separation of synthetic food colors (e.g., FD&C Red No. 40 and FD&C Blue No. 1) (5).



28-5 figure Reversed-phase HPLC separation of α-carotene (AC) and β-carotene (BC) isomers in (**a**) fresh and (**b**) canned carrots using a 5 µm C_{30} stationary phase. Peak 1, 13-*cis* AC; 2, unidentified *cis* AC; 3,13′-*cis* AC; 4, 15-*cis* BC; 5, Unidentified *cis* AC; 6, 13-*cis* BC; 7, all-*trans* AC; 8, 9-*cis* AC; 9, all-*trans* BC; 10, 9-*cis* BC. [Reprinted with permission from (18). Copyright 1997 American Chemical Society.]

28.3.3 Ion Exchange

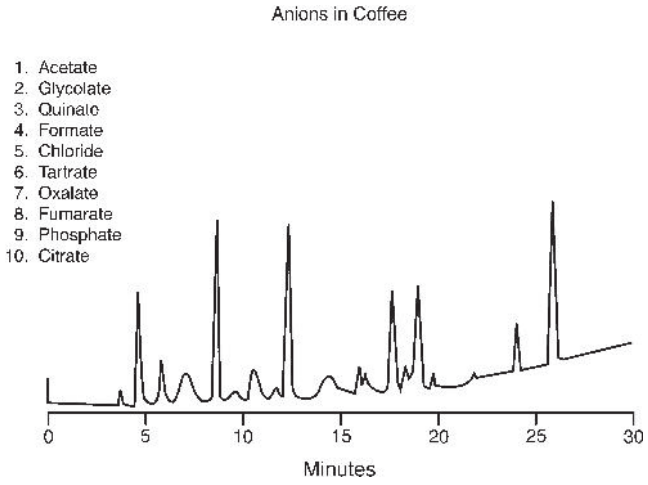
28.3.3.1 Stationary and Mobile Phases

Packing materials for **ion-exchange HPLC** are usually **functionalized organic resins**, such as sulfonated or aminated poly(styrene-divinylbenzene) (Chap. 27, Sect. 27.4.3). **Macroporous resins** are most effective for HPLC columns due to their rigidity and permanent pore structure. **Pellicular packings** also are utilized, particularly in the CarboPac™ (Dionex) series, in which the nonporous, latex resin beads are coated with functionalized microbeads. The **mobile phase** in ion-exchange HPLC is usually an **aqueous buffer**, and solute retention is controlled by changing mobile phase ionic strength and/or pH. **Gradient elution** (gradually increasing ionic strength) is frequently employed.

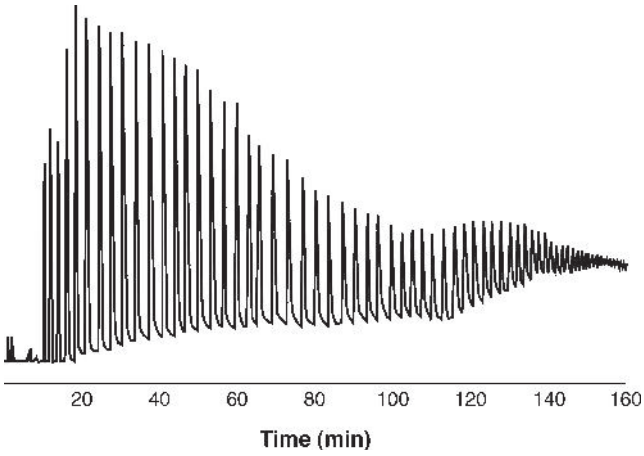
28.3.3.2 Applications of Ion-Exchange HPLC

Ion-exchange HPLC has many applications, ranging from the detection of simple inorganic ions, to analysis of carbohydrates and amino acids, to the preparative purification of proteins oligosaccharides.

28.3.3.2.1 Ion Chromatography **Ion chromatography** is simply high-performance ion-exchange chromatography using a relatively **low-capacity stationary phase** (either anion- or cation-exchange) and, usually, a **conductivity detector**. All ions conduct an electric current; thus, measurement of electrical conductivity is an obvious way to detect ionic species. Because the mobile phase also contains ions, however, background conductivity can be relatively high. One step toward solving this problem is to use much lower capacity ion-exchange packing materials, so that more dilute eluents may be employed. In **nonsuppressed** or **single-column ion chromatography**, the detector cell is placed directly after the column outlet and eluents are carefully chosen to maximize changes in conductivity as sample components elute from the column. **Suppressed ion chromatography** utilizes an eluent that can be selectively removed by the use of ion-exchange membranes (10). Suppressed ion chromatography permits the use of more concentrated mobile phases and gradient elution. Ion chromatography can be used to determine inorganic anions and cations, transition metals, organic acids, amines, phenols, surfactants, and sugars. Some specific examples of ion chromatography applied to food matrices include the determination of organic and inorganic ions in milk; organic acids in coffee extract and wine; chlorine in



28-6 figure Ion-chromatographic analysis of organic acids and inorganic anions in coffee. Ten anions (listed) were resolved on an IonPac AS5A column (Dionex) using a sodium hydroxide gradient and suppressed conductivity detection. (Courtesy of Dionex Corp., Sunnyvale, CA.)



28-7 figure Anion exchange analysis of iso-amylase-treated waxy corn starch. The enzyme debranches the amylopectin, and the chromatogram represents the branch chain-length distribution, from four sugars in length and up. The analysis was performed with anion-exchange HPLC (Dionex™), with a pulsed amperometric detector.

infant formula; and trace metals, phosphates, and sulfites in foods. Figure 28-6 illustrates the simultaneous determination of organic acids and inorganic anions in coffee by ion chromatography.

28.3.3.2.2 Ion Exchange Chromatography of Carbohydrates and Proteins Both cation- and anion-exchange stationary phases have been applied to HPLC of carbohydrates. The advantage of separating carbohydrates by **anion exchange** is that retention and selectivity may be altered by changes in eluent composition. Carbohydrate analysis has benefited greatly by the development of a system that involves **anion-exchange HPLC** at high pH ($\geq$12), and detection by a **pulsed amperometric detector** (PAD). Pellicular column packings (see Sect. 28.2.3.2.2), consisting of nonporous latex beads coated with a thin film of strong anion exchanger, provide the necessary fast exchange, high efficiency, and resistance to strong alkali. These systems may be used in a variety of applications, from routine quality control to basic research. One common application is the determination of oligosaccharide distributions in corn syrups and other starch hydrolysates (Fig. 28-7).

Amino acids have been resolved on polymeric ion exchangers for more than 40 years (see Chap. 15, Sect. 15.3.1.2). Ion exchange is one of the most effective modes for HPLC of proteins and, recently, has been recognized as valuable for the fractionation of peptides.

28.3.4 Size Exclusion

Size-exclusion chromatography (SEC) fractionates solutes solely on the basis of size, with larger molecules eluting first. Due to the limited separation volume available in this chromatographic mode, as explained in Chap. 27 (Sect. 27.4.4), the peak capacity of a size-exclusion column is relatively small. Thus, the "high-performance" aspect of HPLC is not really applicable in the case of size exclusion. The main advantage gained from use of small particle packing materials is speed. Relatively small amounts of sample can be analyzed or separated and collected in $\leq$60 min, compared to $\leq$24 h separations using classic low pressure systems (10). A second advantage is that the sample concentration is higher and the relative volume is lower, so there is much less eluent to remove.

28.3.4.1 Column Packings and Mobile Phases

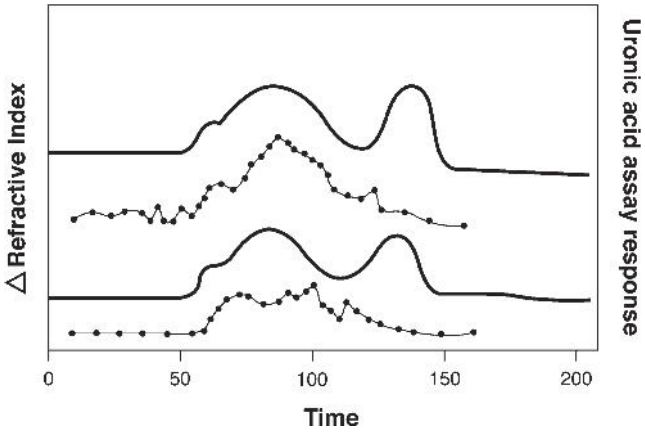
Size-exclusion packing materials or columns are selected so that matrix pore size matches the molecular weight range of the species to be resolved. Prepacked columns of microparticulate media are available in a wide variety of pore sizes. **Hydrophilic packings**, for use with water-soluble samples and aqueous mobile phases, may be surface-modified silica or methacrylate resins. **Poly(styrene-divinylbenzene) resins** are useful for nonaqueous size-exclusion chromatography of synthetic polymers.

The mobile phase in this mode is chosen for sample solubility, column compatibility, and minimal solute–stationary phase interaction. Otherwise, it has little effect on the separation. Aqueous buffers are used for biopolymers, such as proteins and polysaccharides, both to preserve biological activity and to prevent adsorptive interactions. Tetrahydrofuran or dimethylformamide is generally used for size-exclusion chromatography of other polymer samples, to ensure sample solubility.

28.3.4.2 Applications of High Performance SEC

Hydrophilic polymeric size-exclusion packings are used for the rapid determination of **average molecular weight** and **molecular weight range** of polysaccharides, including amylose, amylopectin, and other soluble gums such as xanthan, pullulan, guar, and water-soluble cellulose derivatives. **Molecular weight distribution** can be determined directly from high performance size-exclusion chromatography, if LALLS or MALLS is used for detection (8, 10). The application of aqueous size-exclusion chromatography to two commercially important polysaccharides, xanthan and carboxymethyl cellulose, is discussed in detail in reference (8).

SEC analysis has been useful to better understand numerous food components and systems. SEC analysis of tomato cell-wall pectin from hot-and cold-break tomato preparations (Fig. 28-8) showed that the cell-wall pectin was not differentially degraded by the different processing procedures. Size-exclusion HPLC has been shown to be a rapid, one-step method for assessing soybean cultivars on the basis of protein content (proteins in the extracts of nondefatted flours from five soybean cultivars were separated into six common peaks, and cultivars could be identified by the percent total area of the fifth peak). A size-exclusion liquid chromatographic method also has been applied to the determination of polymerized triacylglycerols in oils and fats (2).



28-8 figure Analysis of tomato cell-wall pectin from hot- and cold-break tomato preparations by size-exclusion chromatography. The *solid lines* are from a refractive index detector response. The lines with markers result from a postchromatography analysis. Aliquots of collected fractions were analyzed by a colorimetric chemical assay that is specific for pectic sugars.

28.3.5 Affinity

Affinity chromatography is based on the principle that the molecules to be purified can form a selective but reversible interaction with another molecular species that has been immobilized on a chromatographic support. Although almost any material can be immobilized on a suitably activated support, the major ligands are proteins, including lectins (Chap. 15, Sect. 15.2.3.2.2), nucleic acids, and dyes (Chap. 27, Sect. 27.4.5). Affinity chromatography is used to purify many glycoproteins. Affinity chromatography using immobilized folate-binding protein is an effective tool in purifying sample extracts for HPLC analysis of folates in foods [e.g., reference (19)].

28.4 SUMMARY

HPLC is a chromatographic technique of great versatility and analytical power. A basic HPLC system consists of a pump, injector, column, detector, and data system. The pump delivers mobile phase through the system. An injector allows sample to be placed into the flowing mobile phase for introduction onto the column. The HPLC column consists of stainless steel or polymer hardware filled with a separation packing material. Various auxiliary columns, particularly guard columns, may be used prior to the analytical column. Detectors used in HPLC include UV-Vis absorption, fluorescence, RI, electrochemical, and light scattering, as well as coupled analytical systems, such as a mass spectrometer. Detection sensitivity or specificity sometimes can be enhanced by chemical derivatization of the analyte. Computer-controlled data station systems offer data collection and processing capabilities, and can run the instrument when an automated system is needed. A broad variety of column packing materials have contributed greatly to the widespread use of HPLC. These column packing materials may be categorized as silica-based (porous silica, bonded phases, pellicular packings) or polymeric (microporous, macroporous, or pellicular/nonporous). The success of silica-based bonded phases has expanded the applications of normal-phase and reversed-phase

modes of separation in HPLC. Separations also are achieved with ion-exchange, size-exclusion, and affinity chromatography. HPLC is widely used for the analysis of small molecules and ions, such as sugars, vitamins, and amino acids, and is applied to the separation and purification of macromolecules, such as proteins and polysaccharides.

28.5 STUDY QUESTIONS

1. Why might you choose to use HPLC rather than traditional low-pressure column chromatography?
2. What is a guard column and why is it used?
3. Give three general requirements for HPLC column packing materials. Describe and distinguish among porous silica, bonded phases, pellicular, and polymeric column packings, including the advantages and disadvantages of each type.
4. What is the primary function of an HPLC detector (regardless of type)? What factors would you consider in choosing an HPLC detector? Describe three different types of detectors and explain the principles of operation for each.
5. You are performing HPLC 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?
6. Why are external standards commonly used for HPLC (unlike in GC, for which internal standards are more commonly used)?
7. Ion chromatography has recently become a widely promoted chromatographic technique in food analysis. Describe ion chromatography and give at least two examples of its use.
8. Describe one application each for ion-exchange and size-exclusion HPLC.

28.6 ACKNOWLEDGMENTS

Mary Ann Rounds (deceased) has been included as an author in this chapter, in recognition of the key role she played in writing the chapter on HPLC for the 1st–3rd editions of this textbook. Much of her writing was included in this chapter for the 4th edition. Dr. Reuhs, the primary author of this chapter, also wishes to acknowledge the involvement of Dr. Jesse F. Gregory, III, with this chapter for previous editions of the book. Dr. Baraem Ismail is acknowledged for her thoughtful suggestions on reorganizing the chromatography chapters, and her preparation of Table 28-1.

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