CHAPTER ONE

INTRODUCTION

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High-performance liquid chromatography (HPLC) is one of several chromatographic methods for the separation and analysis of chemical mixtures (Section 1.3). Compared to these other separation procedures, HPLC is exceptional in terms of the following characteristics:

- almost universal applicability; few samples are excluded from the possibility of HPLC separation
- remarkable assay precision (±0.5% or better in many cases)
- a wide range of equipment, columns, and other materials is commercially available, allowing the use of HPLC for almost every application
- most laboratories that deal with a need for analyzing chemical mixtures are equipped for HPLC; it is often the first choice of technique

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As a result, HPLC is today one of the most useful and widely applied analytical techniques. Mass spectrometry rivals and complements HPLC in many respects; the use of these two techniques in combination (LC-MS) is already substantial (Section 4.14), and will continue to grow in importance.

In the present chapter we will:

- examine some general features of HPLC
- summarize the history of HPLC
- very briefly consider some alternatives to HPLC, with their preferred use for certain applications
- list other sources of information about HPLC

1.1 BACKGROUND INFORMATION

1.1.1 What Is HPLC?

Liquid chromatography began in the early 1900s, in the form illustrated in Figure 1.1*a–e*, known as "classical column chromatography". A glass cylinder was packed with a finely divided powder such as chalk (Fig. 1.1*a*), a sample was applied to the top of the column (Fig. 1.1*b*), and a solvent was poured onto the column (Fig. 1.1*c*). As the solvent flows down the column by gravity (Fig. 1.1*d*), the components of the sample (A, B, and C in this example) begins to move through the column at different speeds and became separated. In its initial form, colored samples were investigated so that the separation within the column could be observed visually. Then portions of the solvent leaving the column were collected, the solvent was evaporated, and the separated compounds were recovered for quantitative analysis or other use (Fig. 1.1*e*). In those days a new column was required for each sample, and the entire process was carried out manually (no automation). Consequently the effort required for each separation could be tedious and time-consuming. Still, even at this stage of development, chromatography provided a unique capability compared to other methods for the analysis of chemical mixtures.

A simpler form of liquid chromatography was introduced in the 1940s, called *paper chromatography* (Fig. 1.1*f*). A strip of paper replaced the column of Figure 1.1*a*; after the sample was spotted near the bottom of the paper strip, the paper was placed in a container with solvent at the bottom. As the solvent migrated up the paper by capillary action, a similar separation as seen in Figure 1.1*d* took place, but in the opposite direction. This "open bed" form of chromatography was later modified by coating a thin layer of powdered silica onto a glass plate—as a replacement for the paper strip used in paper chromatography. The resulting procedure is referred to as *thin-layer chromatography* (TLC). The advantages of either paper or thin-layer chromatography included (1) greater convenience, (2) the ability to simultaneously separate several samples on the same paper strip or plate, and (3) easy detection of small amounts of separated compounds by the application of colorimetric reagents to the plate, after the separation was completed.

HPLC (Fig. 1.1*g*, *h*) represents the modern culmination of the development of liquid chromatography. The user begins by placing samples on a tray for automatic injection into the column (Fig. 1.1*g*). Solvent is continually pumped

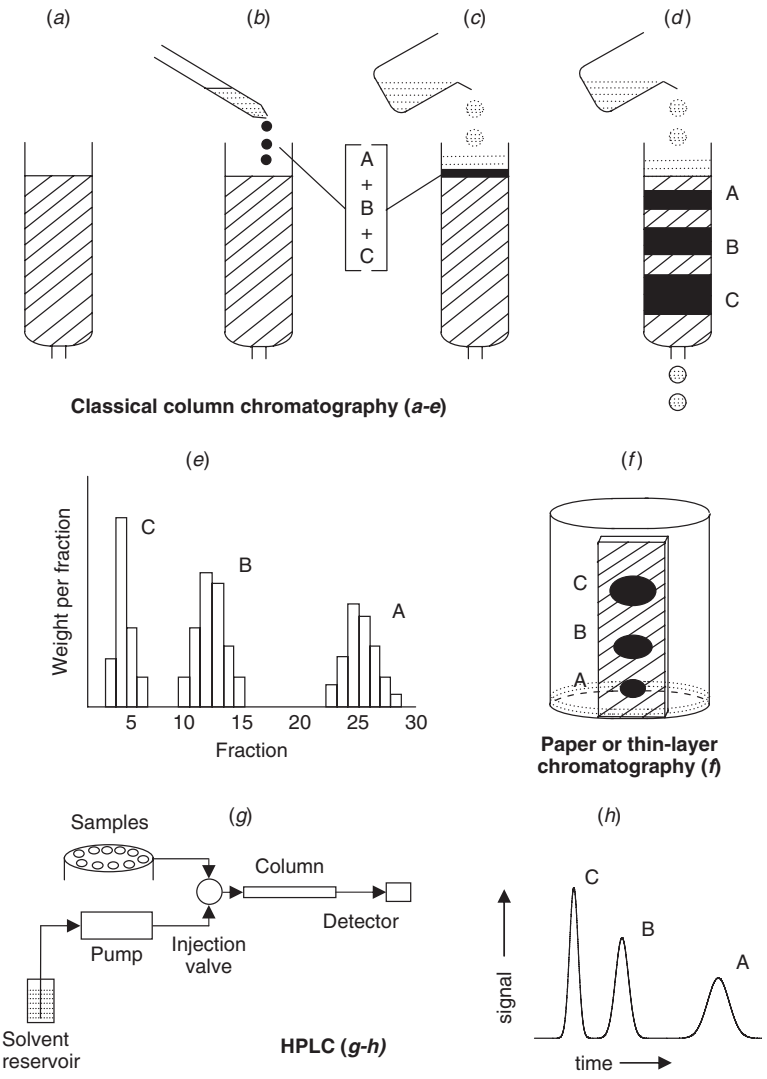

Figure 1.1 Different stages in the development of chromatography.

through the column, and the separated compounds are continuously sensed by a detector as they leave the column. The resulting detector signal plotted against time is the *chromatogram* of Figure 1.1*h*, which can be compared with the result of Figure 1.1*e*—provided that the sample A + B + C and experimental conditions are the same. A computer controls the entire operation, so the only manual intervention required is the placement of samples on the tray. The computer can also generate a final analysis report for the sample. Apart from this automation of the entire process, HPLC is characterized by the use of high-pressure pumps for faster separation, re-usable and more effective columns for enhanced separation, and a better control of the overall process for more precise and reproducible results. More discussion of the history of HPLC can be found in Section 1.2.

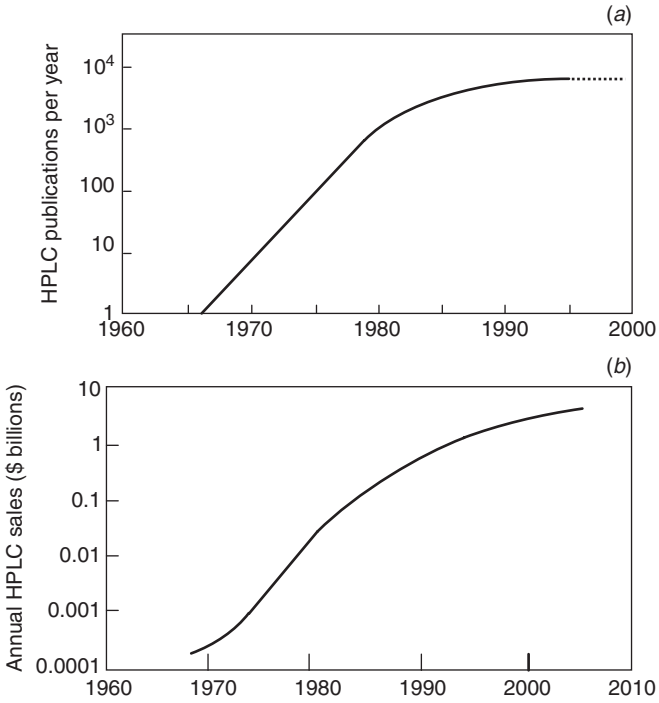

Figure 1.2 The expanding importance of HPLC research and application since 1966. (*a*) Number of HPLC-related publications per year [1]; (*b*) total sales of HPLC equipment and supplies per year (approximate data compiled from various sources).

The growth of HPLC, following its introduction in the late 1960s (Section 1.2), is illustrated in Figure 1.2. In (Fig. 1.2*a*) the annual number of HPLC publications is plotted against time. The first HPLC paper appeared in 1966 [2], and the number of publications grew each year exponentially, leveling off only after 1980. By 1990 the primary requirements of HPLC had largely been satisfied in terms of an understanding of the separation process, and the availability of suitable equipment and columns. At this time HPLC could be considered to have become a mature technique—one that is today practiced in every part of the world. While new, specialized applications of HPLC continued to emerge after 1990, and remaining gaps in our understanding receive ongoing attention, major future changes to our present understanding of HPLC seem unlikely.

As the pace of HPLC research reached a plateau by 1990, a comparable flattening of the HPLC economy took a bit longer—as suggested by the plot in Figure 1.2*b* of annual expenditures against time for all HPLC products (not adjusted for inflation). The money spent annually on HPLC at the present time exceeds that for any other analytical technique.

1.1.2 What Can HPLC Do?

When the second edition of this book appeared in 1979, some examples of HPLC capability were presented, two of which are reproduced in Figure 1.3. Figure 1.3*a*

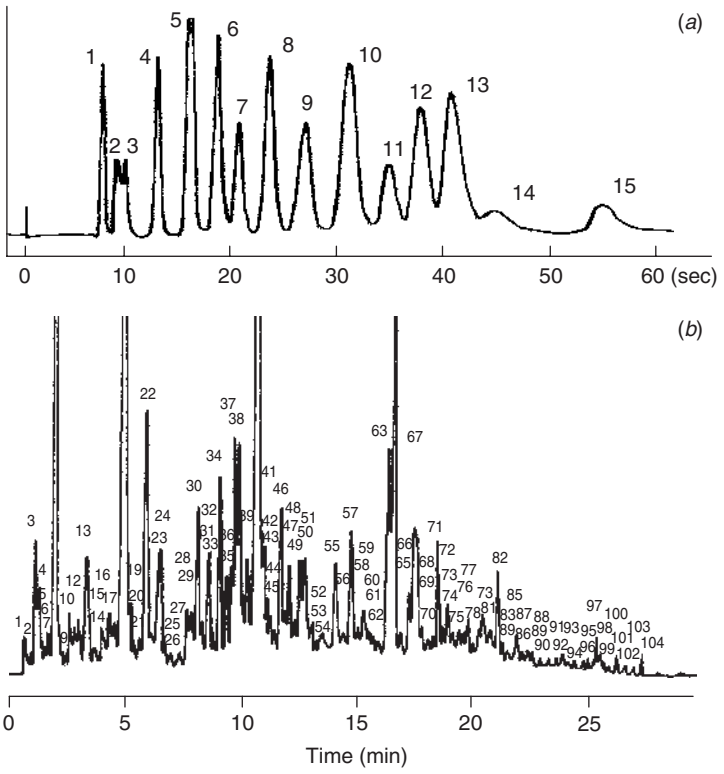

Figure 1.3 Examples of HPLC capability during the mid-1970s. (*a*) Fast separation of a mixture of small molecules [3]; (*b*) high-resolution separation of a urine sample [4]. (*a*) is adapted from [3], and (*b*) is adapted from [4].

shows a fast HPLC separation where 15 compounds are separated in just one minute. Figure 1.3*b* shows the separation power of HPLC by the partial separation of more than 100 recognizable peaks in just 30 minutes. In Figure 1.4 are illustrated comparable separations that were carried out 25 years later. Notice that in Figure 1.4*a*, six proteins are separated in 7 seconds, while in Figure 1.4*b*, *c*, about 1000 peptides plus proteins are separated in a total time of 1.5 hours. The improvement in Figure 1.4*a* compared with Figure 1.3*a* can be ascribed to several factors, some of which are discussed in Section 1.2. The separation of 1000 compounds in Figure 1.4*b*, *c* is the result of so-called two-dimensional separation (Section 9.3.10): a first column (Fig. 1.4*b*) provides fractions for further separation by a second column (Fig. 1.4*c*). In this example 4-minute fractions were collected from the first column and further separated with the second column; Figure 1.4*c* shows the separation of fraction 7. The total number of (recognizable) peaks in the sample is then obtained by adding the unique peaks present in each of the fractions. The enormous progress made in HPLC performance (Fig. 1.4 vs. Fig. 1.3) suggests that comparable major improvements in speed or separation power in the coming years are not so likely.

Some other improvements in HPLC since 1979 have been equally significant. Beginning in the 1980s, the introduction of suitable columns for the separation of proteins and other large biomolecules [7, 8] has opened up an entirely new

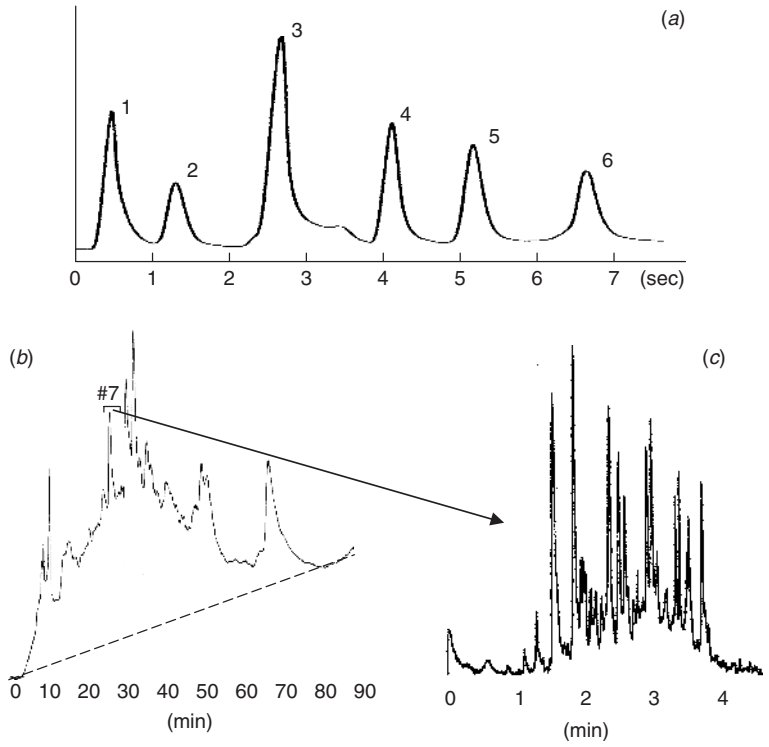

Figure 1.4 Recent examples of HPLC capability. (*a*) Fast separation of six proteins, using gradient elution with a 150 × 4.6-mm column packed with 1.5-μm-diameter pellicular particles [5]; (*b*) initial separation of peptides and proteins from human fetal fibroblast cell by gradient cation-exchange chromatography; (*c*) further separation of fraction 7 (collected between 24–28 min) on a second column by gradient reversed-phase chromatography [6]. Figures adapted from original publications [5, 6].

field of application and facilitated major advances in biochemistry. Similarly the development of chiral columns for the separation of enantiomeric mixtures by Pirkle [9] and others enabled comparable advances in the areas of pharmaceuticals and related life sciences. The use of HPLC for large-scale purification is also increasing, as a result of the availability of appropriate equipment, an increase in our understanding of how such separations should best be carried out, and regulatory pressures for higher purity pharmaceutical products.

1.2 A SHORT HISTORY OF HPLC

We have noted the development of liquid chromatography prior to the advent of HPLC (Section 1.1). For a more complete account of this pre-1965 period, several review articles have been written by Leslie Ettre, our "historian of chromatography":

- precursors to chromatography; developments prior to 1900 [10, 11]
- invention of chromatography by M. S. Tswett in the early 1900s [12]

- rediscovery of chromatography in the early 1930s [13]
- A. J. P. Martin's invention of partition and paper chromatography in the early 1940s [14]
- development of the amino-acid analyzer by S. Moore and W. S. Stein in the late 1950s [15]
- development of the gel-permeation chromatograph by Waters Associates in the early 1960s [16]

Carl Runge, a German dye-chemist born in 1856, first reported crude dye separations by means of a technique similar to paper chromatography [10], but neither he nor others pursued the practical possibilities of this work. In the late 1890s David Day at the US Geological survey carried out separations of petroleum by a technique that resembles classical column chromatography [11]; however, his goal was not the development of a separation technique, but rather the demonstration that petroleum deposits of different quality result from their separation during migration through the ground. As in the case of Runge's work, Day's investigations did not proceed further. In the early 1900s, Mikhail Tswett invented classical column chromatography and demonstrated its ability to separate different plant extracts [12]. This was certainly the beginning of chromatography, but the value of his work was not appreciated for another two decades. In the early 1930s, Tswett's work was rediscovered [13], leading to an explosive subsequent growth of chromatography. The invention of paper chromatography by A.J.P. Martin followed in 1943 [14], accompanied by the development of thin-layer chromatography between the late 1930s and the mid-1950s [17]. This short summary necessarily omits numerous other contributions to the development of chromatography before 1955.

The amino-acid analyzer, introduced in the late 1950s [15], was an important precursor to HPLC; it was an automated means for analyzing mixtures of amino acids by use of ion-exchange chromatography (Section 7.5). This was followed by the invention of gel permeation chromatography (Section 13.7) by Moore [18] and the introduction in the early 1960s of a gel-permeation chromatograph by Waters Associates [16]. Each of these latter techniques was close in concept to what later became HPLC, differing little from the schematic of Figure 1.1*g*. In each case the solvent was pumped at high pressure through a reusable, small-particle column, the column effluent was continuously monitored by a detector, and the output of the device was a chromatogram as in Figure 1.1*h*. What each of these two systems lacked, however, was an ability to separate and analyze other kinds of samples. The amino-acid analyzer was restricted to the analysis of mixtures of amino acids, while the gel-permeation chromatograph was used exclusively for determining the molecular weight distribution of synthetic polymers. In neither case were these devices readily adaptable for the separation of other samples.

During the early 1960s, two different groups embarked on the development of a general-purpose HPLC system, under the leadership of Csaba Horváth in the United States and Josef Huber in Europe. Each of these two men have described their early work on HPLC in a collection of personal recollections [19], and Ettre has provided additional detail on early work in Horváth's laboratory [20]. The immediate results of these two groups, plus related work by others that was carried out a few years later, are described in publications that appeared in 1966 to 1968 [2, 21–24]. The introduction of commercial equipment for HPLC followed in the

late 1960s, with systems from Waters Associates and DuPont initially dominating the market. Other companies soon offered competing equipment, and research on HPLC began to accelerate (as seen from Fig. 1.2*a*). By 1971, the first HPLC book had been published [25], and an HPLC short course was offered by the American Chemical Society (Modern Liquid Chromatography), with J. J. Kirkland and L. R. Snyder as course instructors).

Progressive improvements in HPLC from 1960 to 2010 are illustrated by the representative separations of Figure 1.5*a–f*, which show separation times decreasing by several orders of magnitude during this 50-year interval. Figure 1.5*g* shows how this reduction in separation time (◦,—) was related to increases in the pressure drop across the column (- - -) and a reduction in the size of particles (•) that were used to pack the column. In the early days of HPLC the technique was sometimes referred to as "high-pressure liquid chromatography" or "high-speed liquid chromatography," for reasons suggested by Figure 1.5*g*. Figure 1.5*h* shows corresponding changes in column length (•) and flow rate (◦) for the separations of Figure 1.5*a–e*.

A theoretical foundation for the eventual development of HPLC was established well before the 1960s. In 1941, Martin reported [27] that "the most efficient columns . . . should be obtainable by using very small particles and high-pressure differences across the length of the column;" this summarized the requirements for HPLC separation in a nutshell (as demonstrated by Fig. 1.5*g*). In the early 1950s, the related technique of gas chromatography was invented by Martin [28]; its rapid acceptance by the world [29] led to a number of theoretical studies that would prove relevant to the later development of HPLC. Giddings summarized and extended this work for specific application to HPLC in the early 1960s [30], work that was later to prove important for both column design and the selection of preferred experimental conditions.

For a further background on the early days of HPLC, see [19, 31–33]. Additional historical details on the progress of HPLC after 1980 are provided by the collected biographies of several HPLC practitioners [34].

1.3 SOME ALTERNATIVES TO HPLC

Two, still-important techniques, each of which can substitute for HPLC in certain applications, existed prior to 1965: gas chromatography (GC) and thin-layer chromatography (TLC). Countercurrent chromatography (CCC) is another pre-1965 technique that, in principle, might compete with HPLC in many applications but falls considerably short of the speed and separation power of HPLC. Several additional, potentially competitive, techniques were introduced after HPLC: supercritical fluid chromatography (SFC) in the 1970s, capillary electrophoresis (CE) in the 1980s, and capillary electrochromatography (CEC) in the 1990s.

1.3.1 Gas Chromatography (GC)

Because GC [35] is limited to samples that are volatile below 300°C, this technique is not applicable for very-high-boiling or nonvolatile materials. Thus about 75% of all known compounds cannot be separated by GC. On the other hand, GC is considerably more efficient than HPLC (higher values of the plate number N),

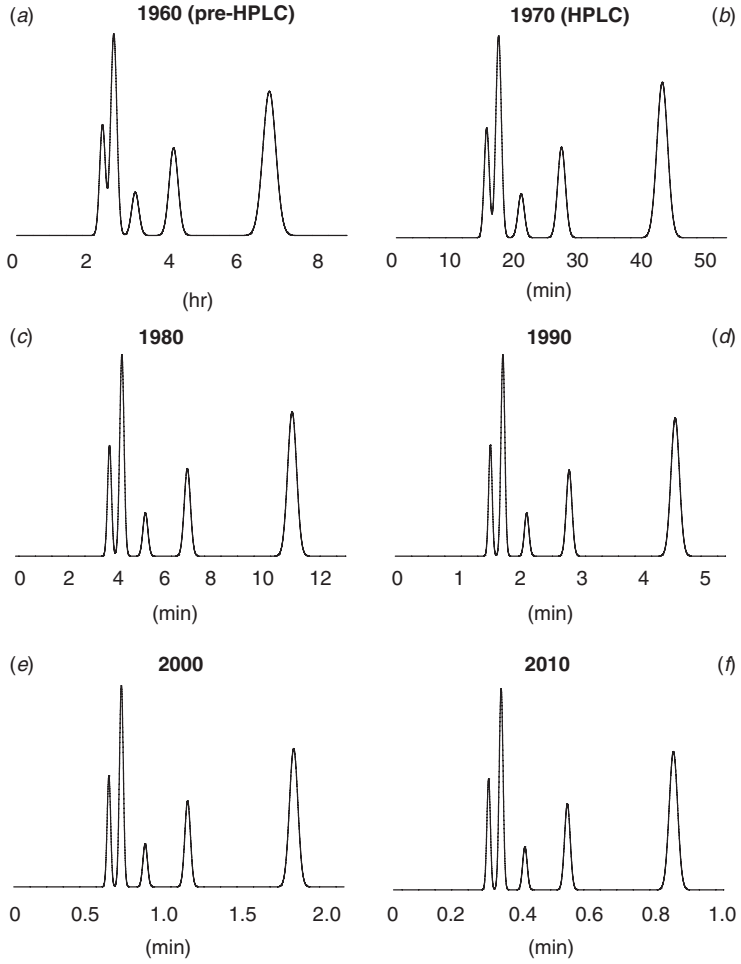

Figure 1.5 Representative chromatograms that illustrate the improvement in HPLC performance over time. Sample: five herbicides. Conditions: 50% methanol-water, ambient temperature. Chromatograms *a–f* are DryLab^R computer simulations (Section 10.2), based on data of [26]; *g* and *h* provide details for the separations of *a–f*. Column-packings of identical selectivity and 4.6-mm-diameter columns are assumed.

which means faster and/or better separations are possible. GC is therefore preferred to HPLC for gases, most low-boiling samples, and many higher boiling samples that are thermally stable under the conditions of separation. GC also has available several very sensitive and/or element-specific detectors that permit considerably lower detection limits.

1.3.2 Thin-Layer Chromatography (TLC)

The strong points of TLC [36] are its ability to separate several samples simultaneously on a single plate, combined with the fact that every component in the sample is visible on the final plate (strongly retained compounds may be missed in

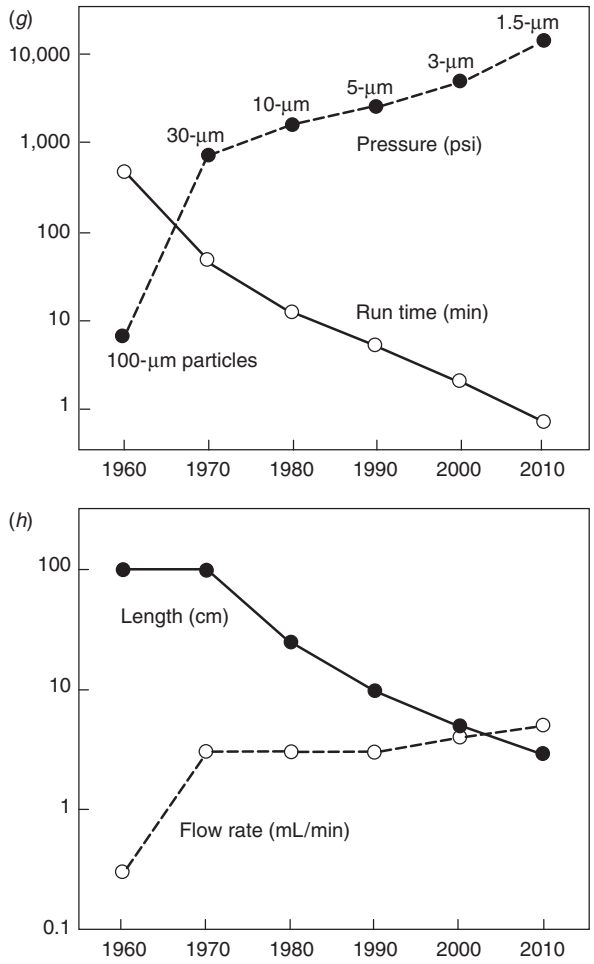

Figure 1.5 (*Continued*)

HPLC). With the advent of specialized equipment for the pressurized flow of solvent across the plate, so-called high-performance TLC (HP-TLC) has become possible. Regardless of how it is carried out, however, TLC lacks the separation efficiency of HPLC (as measured by values of N), and quantitation is less convenient and less precise. At the time of publication of the present book, TLC was used relatively infrequently in the United States for quantitative analysis, although it is a convenient means for semi-quantitative analysis and for the detection of sample impurities. It is widely used for screening large numbers of samples, with little need for sample cleanup (e.g., plasma drug screening). In Europe HP-TLC is more popular than in the United States but much less popular than HPLC.

1.3.3 Supercritical Fluid Chromatography (SFC)

SFC [37] is carried out with equipment and columns that are similar to HPLC. The solvent is, by definition, a supercritical fluid, usually a gas such as CO_2,

under conditions of elevated pressure and temperature. SFC can be regarded as an extension of GC, in that supercritical fluids can dissolve and separate samples that are normally considered to be nonvolatile. SFC may be considered as a hybrid of GC and HPLC, as it is characterized by greater separation efficiency than for HPLC (higher N) but lower efficiency than GC. Similarly the solvent in SFC plays a greater role in determining separation than in GC, but less so than in HPLC. Detection sensitivity is also intermediate between what is possible with HPLC compared to GC. A major application of SFC is for the analysis of natural or synthetic polymeric mixtures, for example, the separation of polyphenols as described in [38]. Whereas HPLC may be unable to resolve individual polymeric species with molecular weights above some maximum value, SFC can usually extend this upper molecular-weight limit considerably. SFC has also been used for separating enantiomers, whose very similar retention may require greater separation efficiency (larger value of N).

1.3.4 Capillary Electrophoresis (CE)

CE [1, 39] is not a form of chromatography, but it competes effectively with HPLC for the separation of certain classes of compounds. The principle of separation is the differential migration of sample compounds in a capillary, under the influence of an electric field, with the result that compounds are separated on the basis of their mass-to-charge ratio (m/z); compounds with smaller m/z migrate faster. Consequently compounds that are to be separated by CE must carry an ionic charge. CE is characterized by a greater separation efficiency than for HPLC (higher value of N), especially for the separation of compounds of high molecular weight. However, detection sensitivity is usually much poorer than for HPLC. CE is heavily used for the genomic analysis of various species, based on the fractionation of DNA fragments. CE has also proved popular for analytical separations of enantiomeric samples, where its performance may exceed that of HPLC for two reasons. First, these separations are often difficult and therefore are facilitated by the larger values of N available from CE. Second, HPLC separations of enantiomers usually rely on *chiral columns*. The separation of a particular enantiomeric sample may require the trial-and-error testing of several different (and expensive) columns before a successful separation is achieved. CE allows the use of small amounts of different chiral complexing agents—instead of different columns, allowing for a faster, cheaper, and more versatile alternative to HPLC. The required flow rates for HPLC compared with CE (e.g., mL/min vs. μL/min) make the use of costly chiral complexing reagents impractical for HPLC. Several variations of CE exist, which allow its extension to other sample types; for example, non-ionized compounds can be separated by micellar electrokinetic chromatography [40].

1.3.5 Countercurrent Chromatography

CCC [41, 42] is an older form of liquid–liquid partition chromatography that was later improved in various ways. HPLC with a liquid stationary phase was since replaced by bonded-phase HPLC, the use of CCC as an alternative to HPLC has become relatively less frequent. An often-cited feature of CCC is its freedom from problems caused by irreversible attachment of the sample to the large internal surface present in HPLC columns. However, the improved HPLC columns used today are largely free from this problem. CCC may possess certain advantages for

the preparative separation of enantiomers [43]; otherwise, the technique is used mainly for the isolation of labile natural products.

1.3.6 Special Forms of HPLC

The five separation techniques mentioned above (Sections 1.3.1–l.3.5) differ in essential ways from HPLC. Four other procedures, which will not be discussed in this book, can be regarded as HPLC variants. However, much of the information in following chapters can be adapted for use with the following procedures.

Capillary electrochromatography [44, 45] (CEC) is generally similar to HPLC, except that the flow of solvent is achieved by means of an electrical potential across the column (endoosmotic flow), rather than by use of a pump. Because solvent flow is not affected by the size of particles within the column (and column efficiency can be greater for small particles), much larger values of N are, in principle, possible by means of CEC. Higher values of N also result from endoosmotic flow per se. Because of these potentially greater values of N in CEC than in HPLC, considerable effort has been invested since 1995 into making this technique practical. However, major technical problems remain to be solved, and CEC had not become a routine alternative to HPLC at the time this book went to press.

HPLC on a chip [46] is a recently introduced technology for the convenient separation of very small samples. A micro-column (e.g., 43 × 0.06 mm) forms part of the chip, which can be interfaced between a micro pump and a mass spectrometer. The principles of separation are the same as for HPLC with conventional columns and equipment, but a chip offers advantages in terms of separation power and convenience for very small samples.

Ion chromatography [47, 48] is widely used for the analysis of mixtures that contain inorganic anions and cations; for example, Cl^- and Na^+, respectively. While the principles of separation are the same as for ion-exchange HPLC (Section 7.5), ion chromatography involves special equipment and is used mainly for inorganic analysis.

Micellar liquid chromatography is a variant of reversed-phase chromatography in which the usual aqueous-organic solvent is replaced by an aqueous surfactant solution [49]. It is little used at present because of the lower efficiency of these separations.

1.4 OTHER SOURCES OF HPLC INFORMATION

A wide variety of resources is available that can be consulted to supplement the use of the present book. These include various other publications (Sections 1.4.1–1.4.3), short courses (Section 1.4.4), and the Internet (Section 1.4.5).

1.4.1 Books

Literally hundreds of books on chromatography have now been published, as reference to Amazon.com and other internet sources can readily verify. Books on HPLC can be divided into two groups: (1) specialized texts that address the HPLC separation of a certain kind of sample (e.g., proteins, carbohydrates, enantiomers),

or by means of special detection (e.g., mass spectrometer, chemical derivatization), and (2) more general books, such as the present book, that cover all aspects of HPLC. Specialized HPLC books are referenced in later chapters that address different HPLC topics. Table 1.1 provides a partial listing of more general HPLC books published after 1995 that might serve as useful supplements to the present book.

1.4.2 Journals

Technical articles that involve HPLC can appear in most journals that deal with the chemical or biochemical sciences. However, the journals below are of special value to those readers wishing to keep abreast of new developments in the field.

- *Analytical Chemistry*, American Chemical Society
- *Chromatographia*, Springer
- *Journal of Chromatographic Science*, Preston
- *Journal of Chromatography A*, Elsevier
- *Journal of Chromatography B*, Elsevier
- *Journal of Liquid Chromatography*, Wiley
- *Journal of Separation Science*, Wiley
- *LCGC*, Advanstar (separate issues for North America and Europe)

1.4.3 Reviews

Review articles that deal with HPLC can be found in the journals listed above and in other journals. Additionally there are series of publications that are devoted in part to HPLC, either as collections of review articles

- *Advances in Chromatography*, Dekker
- *High-Performance Liquid Chromatography. Advances and Perspectives*, Academic Press (published only between 1980 and 1986)

or as individual books:

- *Journal of Chromatography Library*, Elsevier

1.4.4 Short Courses

There are numerous short courses offered either "live" or on the Internet (see Section 1.4.5). For a current listing of short courses, see the back pages of *LCGC* magazine or search the Internet for "HPLC training."

1.4.5 The Internet

The dynamic nature of the Internet ensures that any listing in a book will soon be obsolete. A number of sites are links to other sites and, as such, presumably will be continuously updated:

http://www.lcresources.com

http://matematicas.udea.edu.co/~carlopez/index7.html

Table 1.1

Some HPLC Books of General Interest Published since 1995

Title	Author(s)	Publication Date	Publisher
General texts			
Handbook of HPLC	E. Katz, R. Eksteen, P. Schoenmakers, and N. Miller, eds.	1998	Dekker
High Performance Liquid Chromatography	S. Lindsay	2000	Wiley
High Performance Liquid Chromatography	E. Prichard	2003	Royal Society of Chemistry
HPLC, 2nd ed.	M.C. McMaster	2006	Wiley-Interscience
Modern HPLC for Practicing Scientists	M. W. Dong	2006	Wiley-Interscience
Practical High-Performance Liquid Chromatography, 4th ed.	V. R. Meyer	2006	Wiley-Interscience
Method development			
Practical HPLC Method Development, 2nd ed.	L. R. Snyder, J. L. Glajch, and J. J. Kirkland	1997	Wiley-Interscience
HPLC Made to Measure: A Practical Handbook for Optimization	S. Kromidas	2006	Wiley
Troubleshooting			
LC Troubleshooting	J.W. Dolan	1983–present	Monthly column in *LCGC Magazine*; past columns available at www.chromatographyonline.com
Troubleshooting HPLC Systems: A Bench Manual	P. C. Sadek	1999	Wiley
More Practical Problem Solving in HPLC	S. Kromidas	2005	Wiley
Pitfalls and Errors of HPLC in Pictures 2nd ed.	V. R. Meyer	2006	Wiley

Table 1.1

(*Continued*)

Title	Author(s)	Publication Date	Publisher
Preparative HPLC			
Practical Handbook of Preparative HPLC	D. A. Wellings	2006	Elsevier
HPLC columns			
HPLC Columns: Theory, Technology and Practice	U. D. Neue	1997	Wiley-VCH
HPLC solvents			
The HPLC Solvent Guide	P. C. Sadek	1996	Wiley
Gradient elution			
High-Performance Gradient Elution	L. R. Snyder and J. W. Dolan	2007	Wiley

http://lchromatography.com/hplcfind/index.html
thttp://tech.groups.yahoo.com/group/chrom-L/links
http://userpages.umbc.edu/~dfrey1/Freylink
http://www.infochembio.ethz.ch/links/en/analytchem_chromat.html
http://www.chromatographyonline.com

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