

INTRODUCTION

Begin at the beginning ... and go on till you come to the end: then stop.

—Lewis Carroll, *Alice's Adventures in Wonderland*

1.1 THE “GENERAL ELUTION PROBLEM” AND THE NEED FOR GRADIENT ELUTION

Prior to the introduction of gradient elution, liquid chromatographic separation was carried out with mobile phases of fixed composition or eluent strength, that is, *isocratic* elution. Isocratic separation works well for many samples, and it represents the simplest and most convenient form of liquid chromatography. For some samples, however, no single mobile phase composition can provide a generally satisfactory separation, as illustrated by the reversed-phase liquid chromatography (RP-LC) examples of Figure 1.1(*a, b*) for the separation of a nine-component herbicide sample. We can use a weaker mobile phase such as 50 percent acetonitrile–water (50 percent B) or a stronger mobile phase such as 70 percent acetonitrile–water (70 percent B). With 50 percent acetonitrile (Fig. 1.1*a*), later peaks are very wide and have inconveniently long retention times. As a result, run time is excessive (140 min) and later peaks are less easily detected (in this example, peak 9 is only 3 percent as high as peak 1). The use of 70 percent acetonitrile (Fig. 1.1*b*) partly addresses the latter two difficulties, but at the same time it introduces another problem: the poor separation of peaks 1–3. This example illustrates the *general elution problem*: the inability of a single isocratic separation to provide adequate separation within reasonable time for samples with a wide range in retention (peaks with very different retention factors *k*).

Very early in the development of chromatography, Tswett introduced a practical solution to the general elution problem (cited in [1]; see also [2]). If separation is begun with a weaker mobile phase (e.g., 50 percent acetonitrile–water), a better separation of early peaks is possible within a reasonable time, following which the mobile phase can be changed (e.g., to 70 percent acetonitrile–water) for the faster elution of the remainder of the sample. This *stepwise* (or “step-gradient”) elution of the sample is illustrated in Figure 1.1(*c*) for the same sample, with other conditions

2 CHAPTER 1 INTRODUCTION

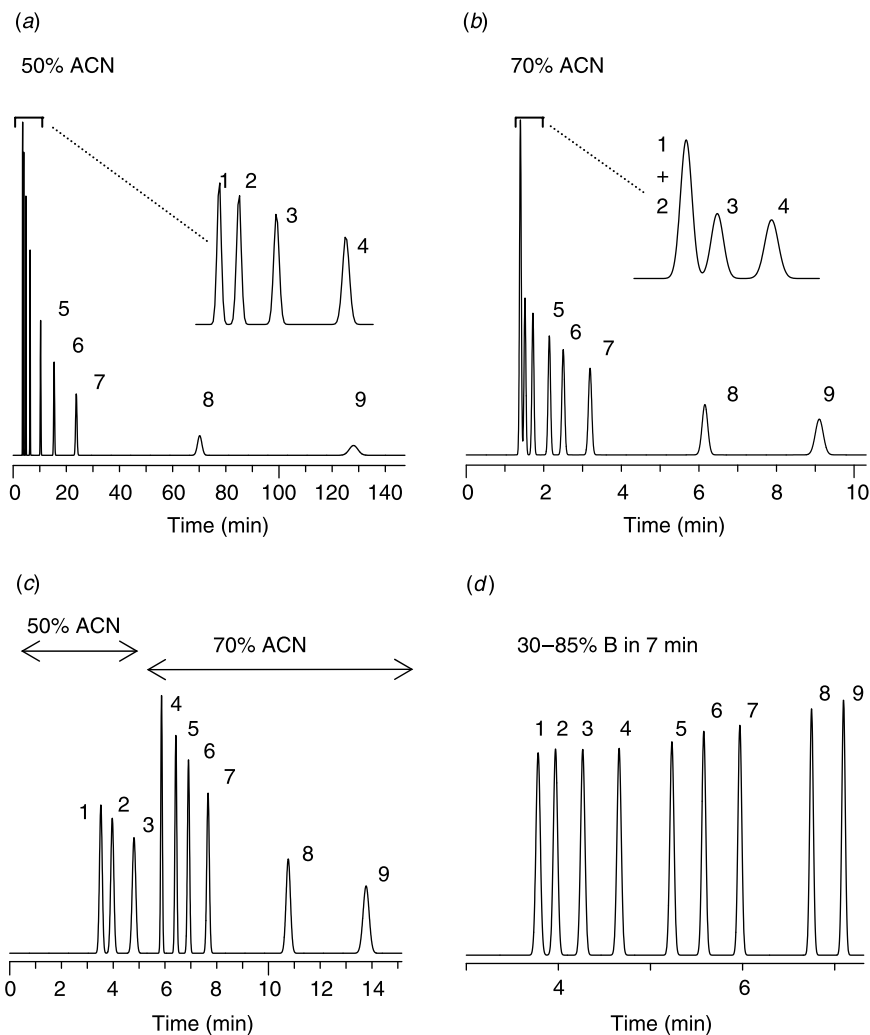


Figure 1.1 Illustration of the general elution problem and its solution. The sample is a mixture of herbicides described in Table 1.3 (equal areas for all peaks). (a) Isocratic elution using 50 percent acetonitrile (ACN)–water as mobile phase; 150×4.6 mm C_{18} column ($5 \mu\text{m}$ particles), 2.0 mL/min, ambient temperature; (b) same as (a), except 70 percent ACN–water; (c) same as (a), except stepwise elution with 50 percent ACN for 5 min, followed by 70 percent ACN for 10 min; (d) same as (a), except gradient elution: 30–85 percent ACN in 7 min. Computer simulations based on the experimental data of Table 1.3.

held constant. Now, all nine peaks are separated to baseline in a total run time of only 15 min.

For the sample of Figure 1.1, stepwise elution (c) is an obvious improvement over the isocratic separations of Figure 1.1(a, b), but it is not a perfect answer to the general elution problem. Significant differences in peak width and ease of detection

still persist in Figure 1.1(c), accompanied by sizable variations in peak spacing (representing wasted space within the chromatogram). For some samples, a two-step gradient as in Figure 1.1(c) would still suffer from the problems illustrated in Figure 1.1(a, b). Furthermore, step gradients are (a) more difficult to reproduce experimentally, and (b) a potential source of “peak splitting”: the appearance of two peaks for a single compound. *Gradient elution* refers to a *continuous* change in the mobile phase during separation, such that the retention of later peaks is continually reduced; that is, the mobile phase becomes steadily stronger as the separation proceeds. An illustration of the power of gradient elution is shown in Figure 1.1(d), where, all peaks are separated to baseline in a total run time of just 7 min, with approximately constant peak widths and comparable detection sensitivity for each peak.

In many cases, the advantage of gradient elution vs isocratic or stepwise elution can be even more pronounced than in the example of Figure 1.1. For several years after the introduction of gradient elution in the early 1950s, the relative merits of continuous vs stepwise elution were widely argued, with many workers expressing a preference for stepwise elution (p. 39 of [3]). For the above (and other) reasons, however, stepwise elution is much less used today, except for special applications, for example, the preparative isolation of a single compound, as described in Section 7.3.2.1 and illustrated in [4]. Software for the development of optimized multistep gradients has been described [5], although the applicability of such gradients appears somewhat limited.

The initial idea of gradient elution has been attributed to Arne Tiselius in the 1940s (cited in [1]), followed by its experimental implementation in 1950 by A.J.P. Martin [6]. Several independently conceived applications of gradient elution were reported in the early 1950s by different workers, as summarized in [3, 7]. Major credit for its subsequent rapid exploitation has been ascribed by Elberton [8] to R.J.P. Williams of the Tiselius group. Soon after the introduction of high-performance liquid chromatography (HPLC) in the late 1960s, commercial equipment became available for routine gradient elution. For further details on the early history of gradient elution, see [3, 7] and references therein. A conceptual understanding of how gradient elution works (as detailed in Chapters 2 and 9) has developed more slowly.

Temperature programming in gas chromatography (GC), which serves a similar purpose to gradient elution in liquid chromatography (LC), evolved about the same time (1952–1958) [9]. A theoretical description of these two separation procedures is remarkably similar, as can be seen from a comparison of [10] with the present book; the rate at which either the mobile phase composition (LC) or temperature (GC) is changed leads to fully analogous changes in the final separation [11, 12].

Apart from stepwise elution (Figure 1.1c), several other experimental procedures have been suggested as alternatives to gradient elution as a means of solving the general elution problem: flow programming [13–15], temperature programming [16, 17], and column switching [18]. However, for reasons summarized in Table 1.1, none of these alternative LC techniques is able to fully duplicate the advantages of gradient elution for the separation of wide-range samples. For a further discussion and comparison of these different programming techniques, see [18, 19].

TABLE 1.1 Alternatives to Gradient Elution

| Procedure | Basis | Comment |
|-------------------------------------|--|--|
| Flow programming [13–15] | Increase in flow rate during separation | Very limited ability to deal with wide-range samples Much reduced peak heights and areas for later peaks For most detectors, peak area varies with small changes in flow rate ^a Ability to use this approach is limited by the pressure tolerance of the system |
| Temperature programming [16, 17] | Increase in temperature during separation | Limited ability to deal with wide-range samples, because temperature has less effect on retention than a change in %B Possible sample reaction during separation of later peaks, due to their elution at higher temperature Many columns will not tolerate large changes in temperature |
| Column switching [18] | Transfer of sample fraction from a first column to a second column | Similar disadvantages as for stepwise elution More complicated method development and equipment Less reproducible method transfer |

^aAt constant flow, the analyte mass under the peak is proportional to the peak area multiplied by the flow rate. When flow rate is programmed, the flow rate during the time each peak is eluted becomes less controllable, as does peak area.

1.2 OTHER REASONS FOR THE USE OF GRADIENT ELUTION

Apart from the need for gradient elution in the case of wide-range samples like that of Figure 1.1, a number of other applications of this technique exist (Table 1.2). Large molecules, such as proteins or synthetic polymers, cannot be conveniently separated by isocratic elution, because their retention can be extremely sensitive to small changes in mobile phase composition (%B). For example, the retention factor k of a 200 kDa polystyrene can change by 25 percent as a result of a change in the mobile phase of only 0.1 percent B [21]. This behavior can

TABLE 1.2 Reasons for the Use of Gradient Elution

| Problem | Application |
|---|--|
| General elution problem | Samples with a wide retention range |
| Compounds whose retention changes markedly for small changes in mobile phase %B | Large biomolecules and synthetic polymers |
| Generic separation | A large number of samples of variable and/or unknown composition; the development of separate procedures for each sample would be economically prohibitive |
| Efficient method development | All samples; the final method can be either isocratic or gradient |
| Sample preparation needed | Samples that contain extraneous material that might interfere with HPLC separation |
| Tailing peaks | Especially for samples that are prone to exhibit tailing peaks, such as protonated bases |

make it extremely difficult to obtain reproducible separations of macromolecules from one laboratory to another, or even within the same laboratory. Furthermore, the isocratic separation of a mixture of macromolecules usually results in the immediate elution of some sample components (with no separation), and such slow elution of other components that it appears that they never leave the column. With gradient elution, on the other hand, there is a much smaller problem with irreproducible retention times for large molecules, and their resulting separation can be fast, effective, and convenient (Chapter 6).

In some applications of RP-LC, a single generic separation procedure is needed that can be used for samples composed of different components, for example, compounds *A*, *B*, and *C* in sample 1, compounds *D*, *E*, and *F* in sample 2. Typically, each sample will be separated just once within a fixed run time, with no further method development for each new sample. In this way, hundreds or thousands of unique samples can be processed in minimum time and with minimum cost. Generic separations by RP-LC (with fixed run times, for automated analysis) are only practical by means of gradient elution and are commonly used to assay combinatorial libraries [22] and other samples [23]. Generic separation is also often combined with mass spectrometric detection [24], which allows both the separation and identification of the components of samples of previously unknown composition (Section 8.1).

Efficient HPLC method development is best begun with one or more gradient experiments (Section 3.2). A single gradient run at the start of method development can replace several trial-and-error isocratic runs as a means for establishing the best solvent strength (value of %B) for isocratic separation. An initial gradient run can also establish whether isocratic or gradient elution is the best choice for a given sample.

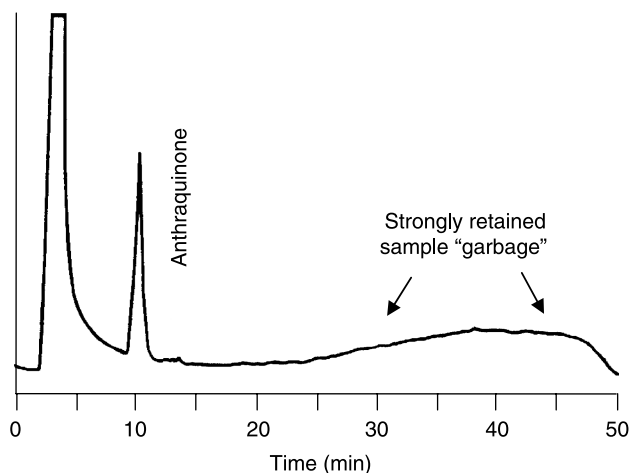


Figure 1.2 Illustration of a gradient separation that eliminates the need for sample pretreatment. The sample is a wood-pulp extract that contains anthraquinone. Conditions: 250×4.6 mm C_{18} column ($10 \mu\text{m}$ particles); A, solvent, water; B, solvent, methanol; 20–20–100–100 percent B in 0–15–20–25 min. Adapted from [26].

Many samples are unsuitable for direct injection followed by isocratic elution. Typically, some kind of sample preparation (pretreatment) is needed [25], in order to remove interfering peaks and prevent the buildup of strongly retained components on the column. In some cases, however, gradient elution can minimize (or even eliminate) the need for sample preparation. As an example, consider the HPLC analysis of wood-pulp extracts for anthraquinone with UV detection [26]. These samples can be separated isocratically with 20 percent by volume methanol–water as mobile phase. A sharp anthraquinone peak results, which is well separated from adjacent peaks in the chromatogram. However, the continued isocratic analysis of these samples results in a gradual deterioration of separation, due to a buildup on the column of strongly retained sample components that are of no interest to the analyst. A separate sample pretreatment *could* be used to remove these strongly retained sample constituents prior to analysis by RP-LC, and this is often the preferred option. However, when gradient elution is used for these samples (Fig. 1.2), any strongly retained material is washed from the column *during* each separation, so that column performance does not degrade rapidly over time. In this example, the use of gradient elution eliminates the need for sample pretreatment, while minimizing column deterioration.

An early goal of gradient elution was the reduction of peak tailing during isocratic separation [27]. Because of the increase in mobile phase strength during the time a peak is eluted in gradient elution, the tail of the peak moves faster than the peak front, with a resulting reduction in peak tailing and peak width. This peak compression effect is illustrated in Figure 1.3 for (a) isocratic and (b) gradient separation of the same sample by means of anion-exchange chromatography. Note the pronounced tailing in the isocratic run (a) of peaks 12 and 13 (asymmetry factor, ASF = 2–4), but their more symmetrical shape (ASF = 1.2) in the gradient run (b).

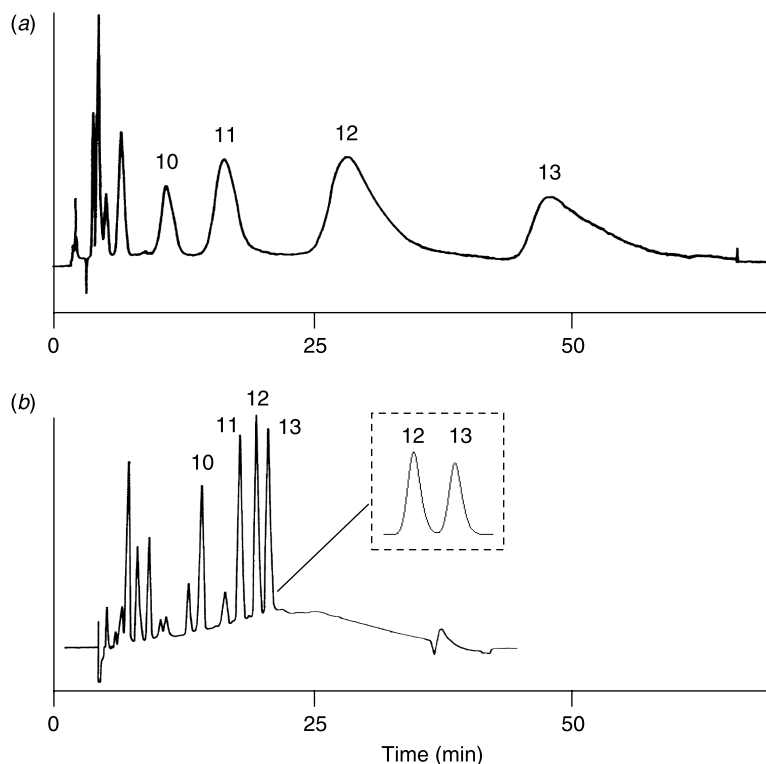


Figure 1.3 Illustration of reduced peak tailing in (b) gradient elution vs (a) isocratic elution. Separations of a mixture of aromatic carboxylic acids by anion-exchange chromatography. Conditions: (a) 0.055 M NaNO_3 in water; (b) gradient from 0.01 to 0.10 M NaNO_3 in 20 min. Adapted from [28].

1.3 GRADIENT SHAPE

By “gradient shape,” we mean the way in which mobile phase composition (%B \equiv percentage by volume organic in RP-LC) changes with time during a gradient run. Gradient elution can be carried out with different gradient shapes, as illustrated in Figure 1.4(a–f). Most gradient separations use linear gradients (a), which are strongly recommended during the initial stages of method development. Curved gradients (b, c) have been used in the past for certain kinds of samples, but today such gradients have been largely replaced by segmented gradients (d). Segmented gradients can provide all the advantages of curved gradients, and also furnish a greater control over separation (as well as freedom from the need for specialized gradient formers). The use of segmented gradients as a means of enhancing separation is examined in Section 3.3.4. Gradient delay, or “isocratic hold” (e), and a step gradient (f) are also illustrated in Figure 1.4.

A linear gradient can be described (Fig. 1.4g) by the initial and final mobile phase compositions, and gradient time (the time during which the mobile phase is changing). We can define the initial and final mobile phase compositions in terms

of %B, or we can use the volume-fraction ϕ of solvent B in the mobile phase (equal to 0.01 percent B), that is, values ϕ_0 and ϕ_f , respectively, for the beginning and end of the gradient. The change in %B or ϕ during the gradient is defined as the *gradient range* and designated by $\Delta\phi = \phi_f - \phi_0$. In the present book, values of %B and ϕ

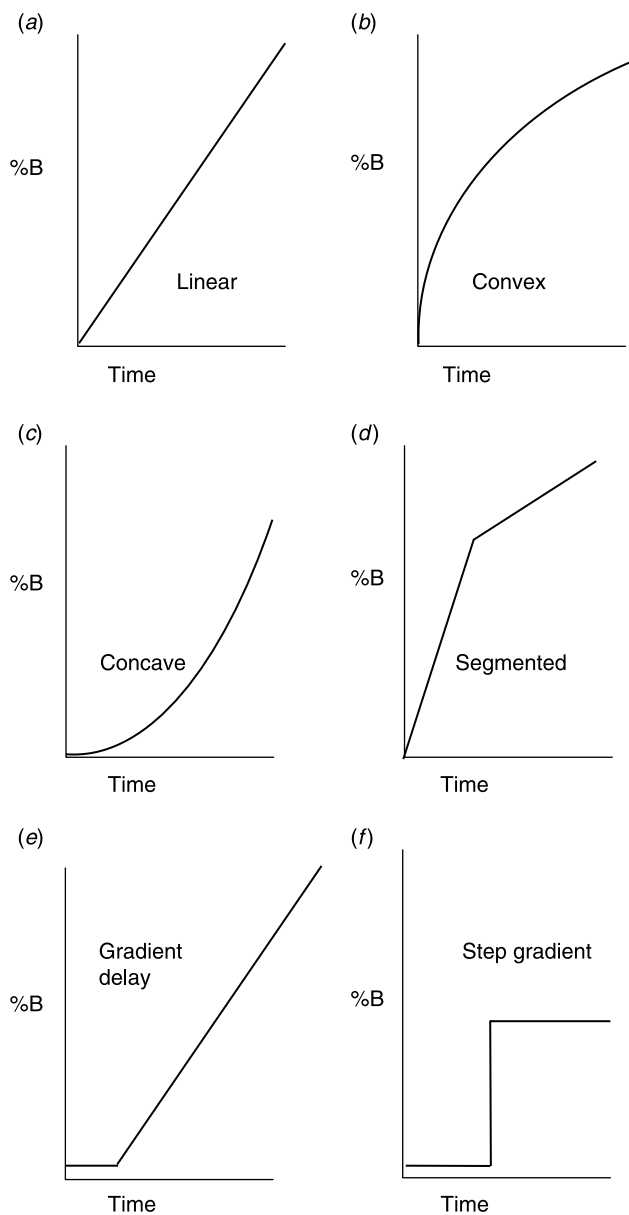


Figure 1.4 Illustration of different gradient shapes (plots of %B at the column inlet vs time). See text for details.

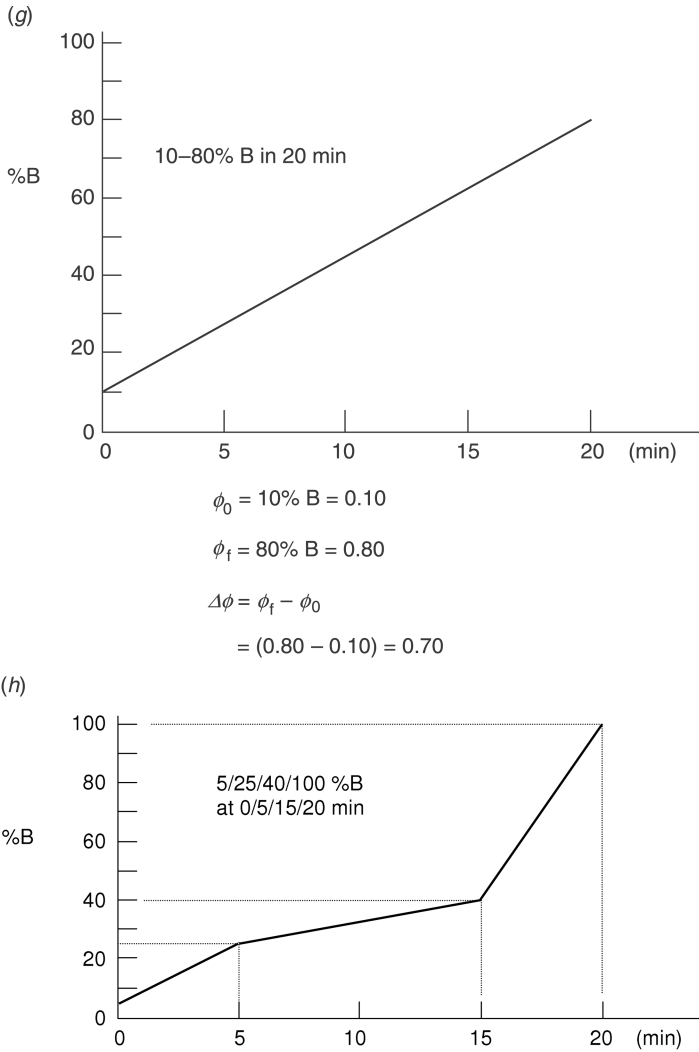


Figure 1.4 (Continued)

will be used interchangeably; that is, ϕ always equals 0.01 percent B, and 100 percent B means pure organic solvent ($\phi = \phi_f = 1.00$). For reasons discussed in Chapter 5, the A- and/or B-reservoirs may contain *mixtures* of the A- and B-solvents, rather than pure water and organic, respectively, for example, 5 percent acetonitrile–water in the A-reservoir and 95 percent acetonitrile–water in the B-reservoir. For the latter example, a 0–100 percent B gradient would correspond to 5–95 percent acetonitrile.

By a *gradient program*, we refer to the description of how mobile phase composition changes with time. Linear gradients represent the simplest example, for example, a gradient from 10 to 80 percent B in 20 min (Fig. 1.4g), which can also be described as 10–80 percent B in 0–20 min (10 percent B at time 0 to

80 percent B at 20 min). Segmented programs are usually represented by values of %B and time for each linear segment in the gradient, for example, 5–25–40–100 percent B at 0–5–15–20 min (Fig. 1.4*h*).

1.4 SIMILARITY OF ISOCRATIC AND GRADIENT ELUTION

A major premise of the present book is that isocratic and gradient separations are fundamentally similar, so that well-established concepts for developing isocratic methods can be used in virtually the same way to develop gradient methods [25]. This similarity of isocratic and gradient elution is hinted at in the examples of Figure 1.1. Thus, the stepwise gradient in Figure 1.1(c) is seen to represent a combination of the two isocratic separations of Figure 1.1(a, b). As the number of isocratic steps is increased from two (as in Fig. 1.1c) to a larger number, the separation eventually approaches that of a continuous gradient (as in Fig. 1.1d).

1.4.1 Gradient and Isocratic Elution Compared

The movement of a band through the column as a function of time proceeds in similar fashion for both isocratic and gradient elution (Figs 1.5 and 1.6, respectively). First consider Figure 1.5 for an isocratic separation, where the position of the

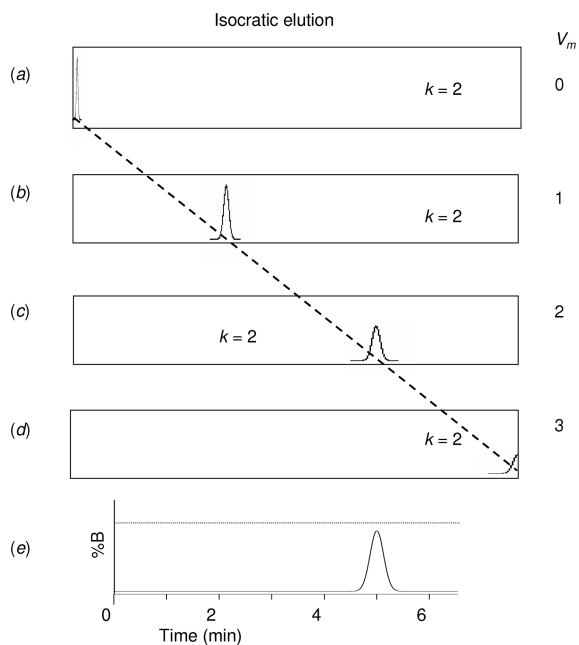


Figure 1.5 Illustration of band migration within the column during isocratic elution. See text for details.

band within the column is noted during its migration from column inlet to outlet. In (a), the solute band is shown at the column inlet just after sample injection. In (b), one column-volume (V_m) of mobile phase has moved through the column, and the band has broadened while migrating one-third of the way through the column [note that the fractional migration R_F is equal to $1/(1+k)$; in Figure 1.5, $k=2$ is assumed]. Here k refers to the retention factor of the solute (Section 2.1.1). In (c), a second column volume has entered the column, and the band has now migrated two-thirds of the way through the column with further broadening. After the passage of a third column volume in (d), the band has arrived at the column outlet and is ready to leave the column and appear as a peak in the final chromatogram (e); %B [dotted line in (e)] does not change with time (isocratic elution). Note that the band moves at constant speed through the column in isocratic elution, as indicated by the dashed, straight line of Figure 1.5 through the band centers at each stage of peak migration.

Figure 1.6 shows the similar separation of a band during gradient elution. Most sample-compounds in a gradient separation are initially “frozen” at the column inlet, because of their strong retention in the starting (relatively weak) mobile phase. However, as the separation proceeds, the mobile phase becomes progressively stronger, and the value of k for the band continually decreases. The example of Figure 1.6 begins (a) after five column volumes have passed through the column; because of the strong initial retention of the band, only a limited migration has occurred at this point in the separation. When the sixth column

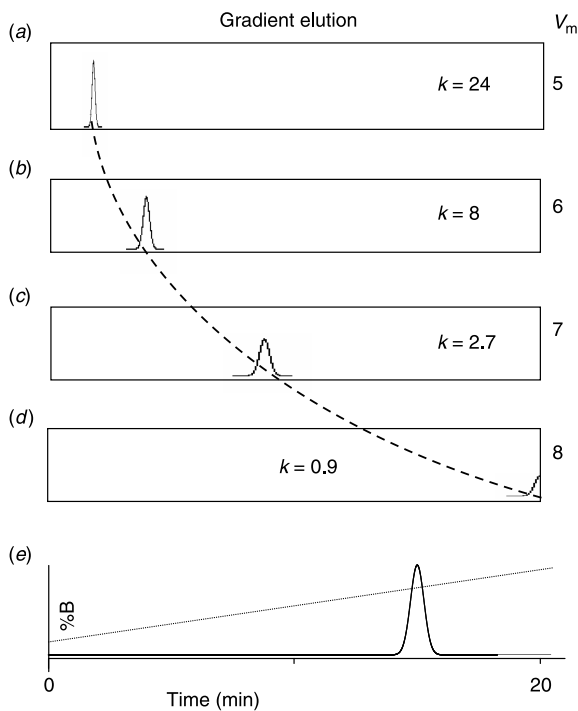


Figure 1.6 Illustration of band migration within the column during gradient elution. See text for details.

volume passes through the column (*b*), with an average value of $k = 8$, the band now moves appreciably further through the column (with additional broadening). For the next column volume (*c*), with an average $k = 2.7$, the extent of band migration and broadening is considerably greater, because of the stronger mobile phase. Finally, after the eighth column volume (*d*), the band reaches the end of the column and appears as a peak in the chromatogram of (*e*); note that %B (dotted line) increases with time (gradient elution). The dashed curve in Figure 1.6 marks the continued acceleration of the peak as it moves through the column in gradient elution, in contrast to its constant migration rate in isocratic elution (Fig. 1.5).

Figure 1.7 extends the example of Figure 1.6 for two different compounds (*i* and *j*) that are separated during gradient elution. Consider first the results for the initially eluted compound *i* in (*a*). The solid curve [$x(i)$] marks the fractional migration x of the band through the column as a function of time (note that $x = 1$ on the y-axis corresponds to elution of the band from the column; $x = 0$ corresponds to the band position at the start of the separation). This behavior is similar to that shown by the dashed curve in Figure 1.6, that is, accelerating migration with time, or an upward-curved plot of x vs t . Also plotted in Figure 1.7(*a*) is the instantaneous value of k for band *i* [dashed curve, " $k(i)$ "] as it migrates through the column. The quantity k_i is the value of k if the compound were run under the isocratic

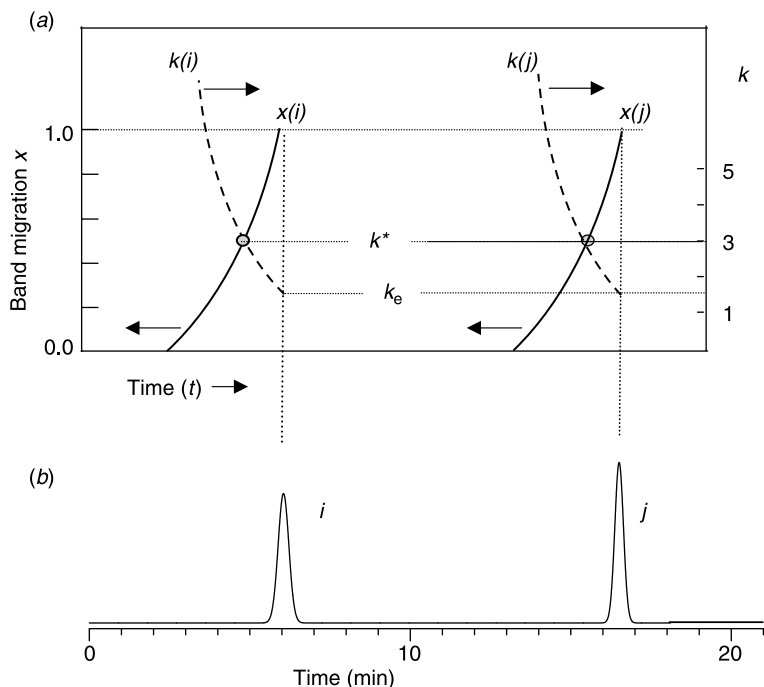


Figure 1.7 Peak migration during gradient elution; (*a*) band-migration plots showing average (k^*) and final values of k at elution (k_e); (*b*) resulting chromatogram. See text for details.

conditions present at that instant in time, that is, using a mobile phase whose composition (%B) is the same as the mobile phase in contact with the band at a given time during band migration. As we will see in Chapter 2, peak retention and resolution in gradient elution depend on the median value of k (i.e., the instantaneous value of k when the band has migrated halfway through the column, defined as the *gradient retention factor* k^*), while peak width is determined by the value of k when the peak is eluted or leaves the column (defined as k_e).

A comparison in Figure 1.7(a) of the two compounds i and j shows a generally similar behavior for each band as it migrates through the column, apart from a greater delay in the migration of band j . Specifically, values of k^* and k_e for both early and late peaks in the chromatogram are about the same for i and j , suggesting that resolution and peak spacing will not decrease for earlier peaks; compare the gradient separation of Figure 1.1(d) with the isocratic separation of Figure 1.1(b). Small values of k (or k^*) generally result in poorer separation (e.g., the early portion of Fig. 1.1b), whereas larger values give better separation (e.g., later peaks in Fig. 1.1b). Constant (larger) values of k^* in gradient separation should improve separation throughout the chromatogram. Values of k_e are also usually similar for early and late peaks in gradient elution, meaning that peak width will be similar for both early and late peaks in the chromatogram (as also observed in the gradient separation of Fig. 1.1d). *The relative constancy of values of k^* and k_e for a given gradient separation contributes to the pronounced advantage of gradient over isocratic elution for the separation of many samples.*

1.4.2 The Linear-Solvent-Strength Model

The linear-solvent-strength (LSS) model for gradient elution is based on an approximation for isocratic retention in RP-LC as a function of *solvent strength*. In terms of the retention factor k and the percentage-volume of organic solvent in the water-organic mobile phase (%B),

$$\log k = a - b(\%B) \quad (1.1)$$

Here, a and b are usually positive constants for a given compound, with only %B varying. Equation (1.1) is an empirical relationship that was cited in almost a dozen separate reports in the mid-1970s [25], not to mention its earlier recognition in analogous thin-layer chromatography separations [29].

Equation (1.1) is illustrated in Figure 1.8(a) for nine different solutes (1–9); for examples of plots with individual data points, see Figure 6.1. Equation (1.1) is more often represented by

$$\log k = \log k_w - S\phi \quad (1.2)$$

where ϕ is the volume-fraction of organic solvent in an RP-LC mobile phase (or %B expressed in decimal form; $\phi = 0.01$ percent B), S is a constant for a given compound and fixed experimental conditions (other than ϕ), and k_w is the (extrapolated) value of k for $\phi = 0$ (i.e., water as mobile phase). Values of $\log k_w$ and S for the compounds of Figure 1.8 are listed in Table 1.3 (“regular” sample).

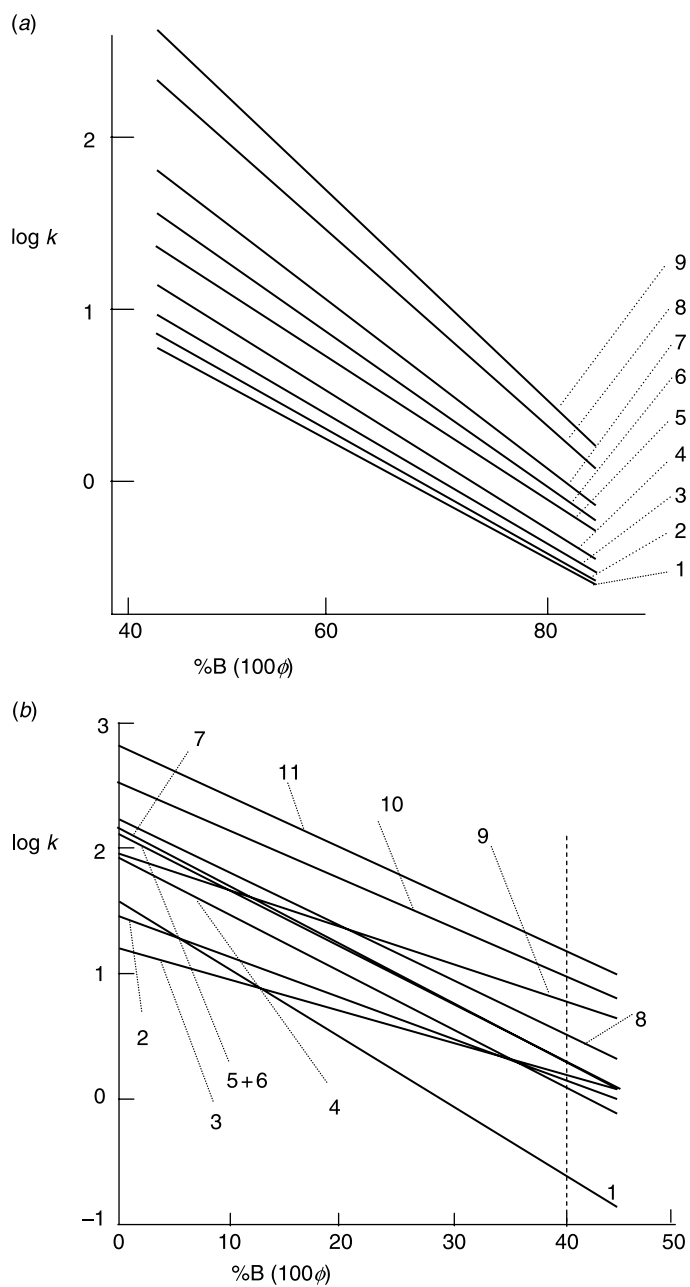


Figure 1.8 Plots of $\log k$ vs $\%B$ for (a) the "regular" and (b) "irregular" samples of Table 1.3. Separation conditions defined in the footnotes of Table 1.3.

TABLE 1.3 Values of $\log k_w$ and S for the Representative “Regular” and “Irregular” Samples Used in this Book

| “Regular” sample ^a | | | “Irregular” sample ^b | | |
|-------------------------------|------------|-------|---------------------------------|------------|------|
| Compound | $\log k_w$ | S | Compound | $\log k_w$ | S |
| 1. Simazine | 2.267 | 3.41 | 1. Phthalic acid | 1.58 | 5.46 |
| 2. Monolinuron | 2.453 | 3.65 | 2. 2-Nitrobenzoic acid | 1.47 | 3.34 |
| 3. Metobromuron | 2.603 | 3.746 | 3. 4-Chloroaniline | 1.23 | 2.50 |
| 4. Diuron | 2.816 | 3.891 | 4. 2-Fluorobenzoic acid | 1.90 | 4.46 |
| 5. Propazine | 3.211 | 4.222 | 5. 3-Nitrobenzoic acid | 2.12 | 4.55 |
| 6. Chloroxuron | 3.602 | 4.636 | 6. 2-Chlorobenzoic acid | 2.12 | 4.55 |
| 7. Neburon | 3.920 | 4.882 | 7. 3-Fluorobenzoic acid | 2.17 | 4.61 |
| 8. Prometryn | 4.731 | 5.546 | 8. 2,6-Dimethylbenzoic acid | 2.22 | 4.29 |
| 9. Terbutryn | 5.178 | 5.914 | 9. 2-Chloroaniline | 1.95 | 2.90 |
| | | | 10. 3,4-Dichloroaniline | 2.52 | 3.80 |
| | | | 11. 3,5-Dichloroaniline | 2.81 | 4.02 |

^aData of [20] for methanol–water mixtures as mobile phase and a 5 μm C₁₈ column; ambient temperature.

^bData of [38] for acetonitrile–buffer mixtures as mobile phase and a 5 μm C₁₈ column; the buffer was 25 mM citrate (pH 2.6); 32.1°C.

If a linear gradient is used (Fig. 1.4a or g), %B is related to time t as

$$\%B = c + dt \quad (1.3)$$

where c and d are also constants. The combination of Equations (1.1) and (1.3) then gives

$$\log k = a - bc - bdt \equiv (\text{constant}) - (\text{constant})t \quad (1.4)$$

Here, k refers to the value the solute would have at the column inlet, for an isocratic mobile phase having a composition ϕ at time t (this ignores the migration of the solute during the gradient). A gradient in which retention is described by Equation (1.4) is referred to as a *linear-solvent-strength* gradient. Equation (1.4) predicts a linear decrease in $\log k$ during the gradient with either time or the volume of mobile phase that has left (or entered) the column. Equations (1.2) and (1.4) are never exact relationships, especially for large changes in k or ϕ , but Equation (1.4) nevertheless allows accurate predictions of separation in gradient elution for “practical” experimental conditions. The advantages of a linear gradient and Equation (1.4) include (a) the easy determination of constants a – d , followed by quantitative predictions of separation, and (b) the ability of Equation (1.4) to relate gradient separations to corresponding isocratic separations. As a result, chromatographers who have experience in the development and use of isocratic RP-LC methods can apply this knowledge directly to the development and troubleshooting of corresponding gradient methods. The extension of the LSS model to non-reversed-phase HPLC methods is possible, with a slight decrease in reliability or some increase in mathematical complexity (Sections 8.2 and 8.3).

When Equation (1.4) is combined with a fundamental equation of gradient elution [Equation (9.2) in Chapter 9], it becomes possible to accurately predict retention time, peak width, and resolution as functions of experimental conditions, and to express these results in terms that are equivalent to those used in isocratic elution. We will next present a qualitative picture of an important relationship: the dependence of values of the gradient retention factor k^* (Fig. 1.7) on gradient conditions. In Chapter 2, we will see that Equation (1.5) below can be used to conveniently compare gradient and isocratic separation. *The discussion of the following three paragraphs [ending with the paragraph that contains Equation (1.7) below] can be skipped if the reader is primarily interested in the practical application of gradient elution, rather than insight into its fundamental basis.*

In Figure 1.6, the value of k when the band reaches the column mid-point (k^*) is determined by how fast k changes during the migration of the band through the column. This can be seen by comparing Figure 1.6 with Figure 1.9. In Figure 1.6, values of k are reduced 3-fold after the passage of each successive column volume. As a result, the

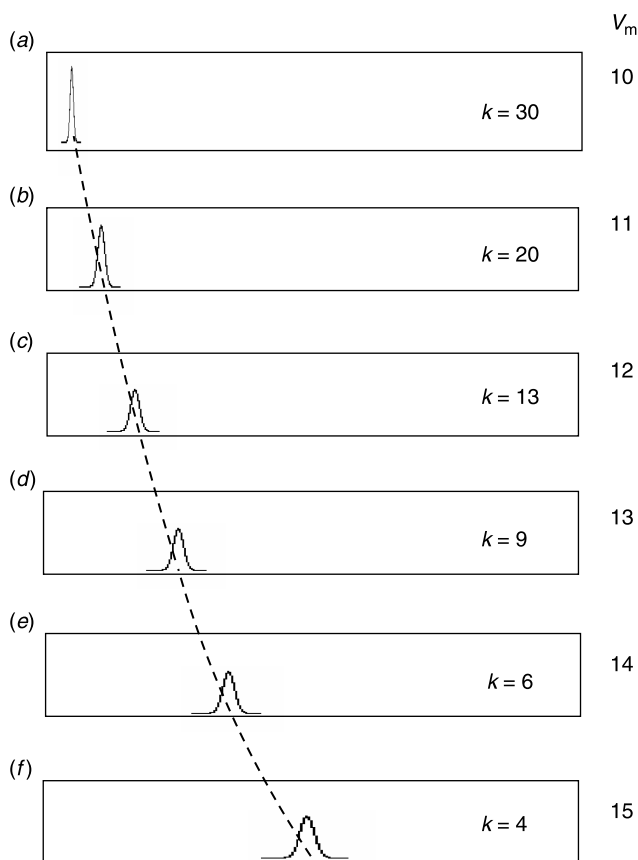


Figure 1.9 Illustration of band migration within the column during gradient elution (shallower gradient and slower peak migration than in Fig. 1.6). See text for details.

peak reaches the column mid-point in Figure 1.6 with a value of $k^* \approx 2$. In Figure 1.9, the change in k is slower (i.e., a flatter gradient), with only a 1.5-fold decrease in k^* for each successive column volume; the resulting value of $k^* \approx 4$. That is, for a slower change in k during gradient elution, the value of k when the peak reaches the column mid-point (k^*) will be larger than for a steeper gradient. The reason for a larger value of k^* , when k changes more slowly, is that (for a given change in %B or ϕ) a larger volume of mobile phase passes through the column, carrying the band further along the column. The faster migration of the band (in terms of the change in ϕ) therefore results in an earlier arrival of the band at the column midpoint, with a smaller value of ϕ for the adjacent mobile phase, and a larger value of $k = k^*$ [i.e., Equation (1.2)]. In the limit, for the flattest possible gradient or no decrease in k during band migration (isocratic elution), the value of k^* is equal to k at the start of elution (the largest possible value of k^* in gradient elution).

Conversely, for a faster change of k during gradient elution, the value of k^* will be smaller. Thus, the value of k^* is determined by the rate of change in k during gradient elution, or by *gradient steepness*. The steeper the gradient, the smaller is k^* . This picture of gradient elution suggests a fundamental definition of gradient steepness, namely the rate of change in $\log k$ during the gradient per volume of mobile phase passing through the column. We will define the latter quantity as the *intrinsic gradient steepness* b . The quantity b is therefore determined by the total change in $\log k$ during the gradient divided by the number of column-volumes of mobile phase that have passed through the column during the gradient (t_G/t_0). The change in ϕ during the gradient (final value of ϕ minus the initial value) will be defined as $\Delta\phi$, so that the change in $\log k$ during the gradient is $\Delta\phi S$ [difference in values of $\log k$ for initial vs final values of ϕ in the gradient; Equation (1.2)]. Therefore, b is given by $(\Delta\phi S)/(t_G/t_0)$, or

$$b = t_0 \Delta\phi S / t_G \quad (1.5)$$

Because column dead-time $t_0 = V_m/F$, where F is flow rate, Equation (1.5) can also be written as

$$b = V_m \Delta\phi S / t_G F \quad (1.6)$$

For all but early-eluting peaks in gradient elution, we can show (Section 9.1.1) that $k^* = 1/1.15b$, and values of k^* determine peak width and resolution in gradient elution.

Note that b in Equation (1.6) is determined by gradient conditions ($\Delta\phi$, t_G), column dead-volume V_m , flow rate F , and the parameter S from Equation (1.2). Values of S for a given sample and separation conditions can be obtained either from two isocratic measurements with ϕ varied [Equation (1.2)] or from two gradient experiments where gradient time is varied (Section 9.3.3). This in turn allows the calculation of values of b and k^* for each solute in each of the two gradient runs, which can be used to predict gradient separation as a function of experimental conditions. Later chapters will show that the intrinsic gradient steepness b is of fundamental importance in understanding gradient elution.

Values of S in RP-LC can be approximated (Section 6.1.1) by

$$S \approx 0.25(\text{molecular weight})^{1/2} \quad (1.7)$$

so that typical small molecules with molecular weights around 200 have $S \approx 4$, while larger molecules have larger values of S ; for example, $S \approx 11$ for a solute with a molecular weight of 2000. Macromolecules such as proteins, DNA, or synthetic polymers (with molecular weights $> 10^4$) can have very large values of S , with important consequences for their gradient separation (Chapter 6).

Some potential advantages of LSS gradients were first recognized in 1964 [30], and the LSS model presented here was then developed over the next 35 years [31–34]. For further information and a detailed, current review of this concept and its application to gradient elution, see Chapters 2 and 9.

1.5 COMPUTER SIMULATION

The LSS model allows the reliable calculation of separation in gradient elution as a function of experimental conditions (Chapters 2 and 9). Thus, there is a predictable effect on separation of (a) gradient steepness (%B/min), (b) initial and final values of %B in the gradient, (c) gradient shape, (d) flow rate, and (e) column dimensions; other conditions such as temperature, mobile phase pH, etc. are assumed to be held constant while the latter conditions are changed. Calculations of gradient separation in this manner require values of k_w and S for each compound in the sample [Equation (1.2)], which can be obtained from two gradient runs for a given sample, where only gradient time t_G is varied (Section 9.3.3). Computer simulation makes use of the foregoing calculations as a result of two or more initial gradient separations (experimental “calibration runs”), in order to then predict isocratic or gradient separation as a function of different experimental conditions. Computer simulation begins with the entry of (a) experimental data from the calibration runs, plus (b) separation conditions. The computer program then determines values of k_w and S for each compound in the sample, following which separation can be predicted as a function of the above (and other) experimental conditions. In this way, the process of developing a gradient RP-LC method can be made more efficient, with resulting methods that are better, as well as less costly to develop; see Section 3.4 for further details, as well as some examples of computer simulation.

A good way to demonstrate the various principles of gradient elution is also provided by computer simulation, which we will use extensively in the present book (DryLab[®] software, Rheodyne LLC, Rohnert Park, CA, USA [35–37]). By selecting representative samples, it is possible to generate simulated chromatograms which show what happens when gradient conditions are changed. Because predicted separations from computer simulation are usually quite reliable (Section 9.3), they can be considered equivalent to corresponding experimental runs. Computer simulation can be assumed for chromatograms shown in the present book, unless a literature reference (for a “real” separation) is given.

1.6 SAMPLE CLASSIFICATION

As we will see, samples can differ in two important respects: sample type (“regular” vs “irregular”), and sample molecular weight (greater or less than 1000 Da). The nature of the sample (defined in this way) plays an important role in determining gradient separation as a function of experimental conditions. The present book deals mainly with samples of “low” molecular weight (<1000 Da), while Chapter 6 can be consulted for the separation of high-molecular-weight samples. A definition of what we mean by sample type, and an examination of some of its consequences for gradient elution, will be considered next.

1.6.1 Sample Compounds of Related Structure (“Regular Samples”)

For compounds of highly related structure (e.g., homologs), plots of $\log k$ vs %B vary in a regular fashion, as illustrated by the sample of Figure 1.8(a). We will refer to samples of this kind as “regular,” in contrast to “irregular” samples as in Figure 1.8(b) (note that this definition of “regular” samples differs from that used in [25]). The relative retention of a “regular” sample does not change when %B is varied in isocratic elution; thus, regardless of %B, the order of peak elution for the sample of Figure 1.8(a) is always $1 < 2 < 3 < \dots < 9$. As we will see in Chapter 2, there is also no change in relative retention when gradient conditions are changed for the separation of “regular” samples (e.g., Fig. 2.5). In this book, we have chosen a particular sample that is representative of “regular” samples: a mixture of nine herbicides (phenylureas and triazines) summarized in Table 1.3, whose retention as a function of %B is illustrated in Figure 1.8(a) (this “regular” sample was also used for the examples of Fig. 1.1). Separations of this sample as a function of both isocratic and gradient conditions will be illustrated in later chapters.

1.6.2 Sample Compounds of Unrelated Structure (“Irregular” Samples)

Samples which contain compounds which are structurally diverse often exhibit “irregular” retention vs %B, in contrast to the “regular” sample of Figure 1.8(a). This is illustrated in Figure 1.8(b) for the “irregular” sample of Table 1.3, where it is seen that relative retention changes as %B is varied. This differing behavior of the “regular” and “irregular” sample is better illustrated in Figure 1.10, for the separation of compounds 1–7 of the “regular” sample in (a) and (b), and the separation of compounds 1–5 of the “irregular” sample in (c) and (d). For a change in mobile phase from 40 to 60 percent B for the “regular” sample, peaks are more bunched together in (b), but there is no change in retention sequence: peak $1 < 2 < 3 < 4 < 5 < 6 < 7$. For the separation of the “irregular” sample, there is a considerable change in retention order for a change of mobile phase from 20 to 40 percent B: (20 percent B) peak $1 < 3 < 2 < 4 < 5$; (40 percent B) peak $1 < 2 = 4 < 3 < 5$. Thus, by changing %B it is possible to change the order of peak elution for an “irregular” sample (but not a “regular” sample), and (more important) improve resolution (cf. Fig. 1.10c vs d). As will be seen in Chapter 2, a change in

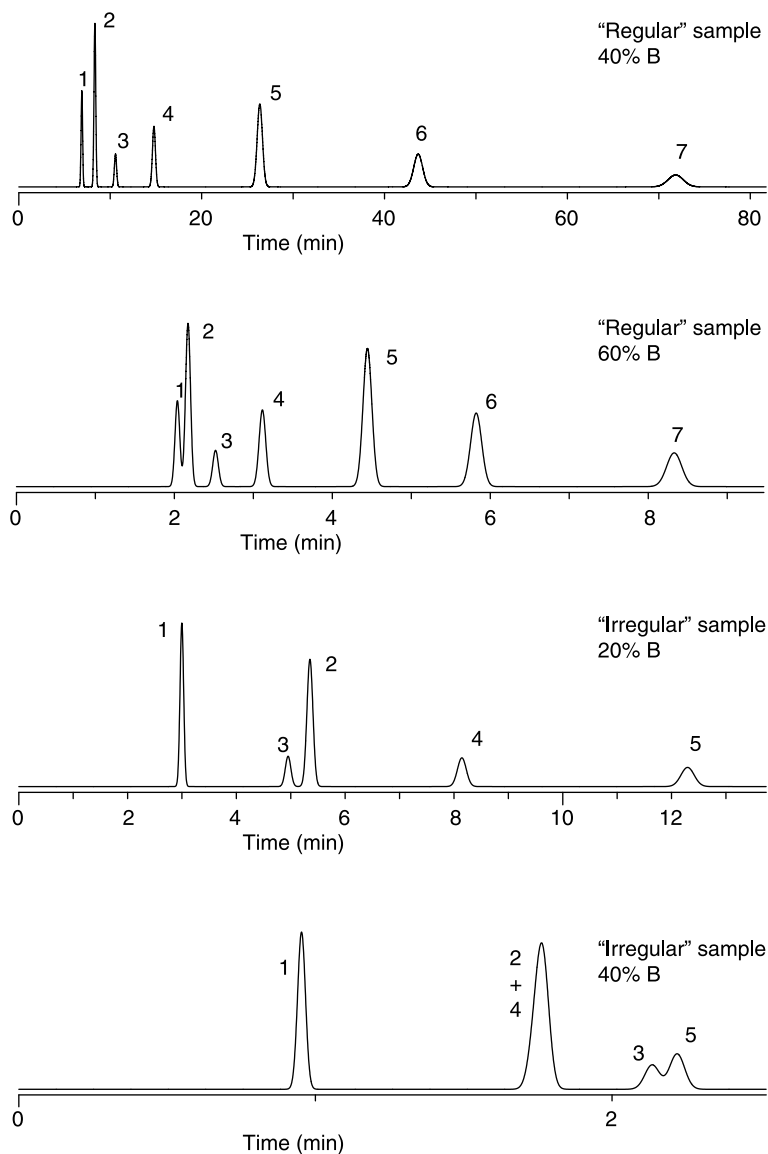


Figure 1.10 Separation of "regular" and "irregular" samples of Table 1.3 as a function of %B. Conditions: 150×4.6 mm C_{18} columns; 2.0 mL/min; other conditions in Table 1.3 or in figure.

gradient conditions can also result in dramatic changes in separation order for "irregular" samples (Figs 2.10 and 2.16), similar to the effects of a change in isocratic %B as in Figure 1.10(c, d). In later chapters, we will contrast the gradient separation of "regular" vs "irregular" samples when changing different experimental conditions (e.g., column length, flow rate, gradient time and range). *It is important to note that*

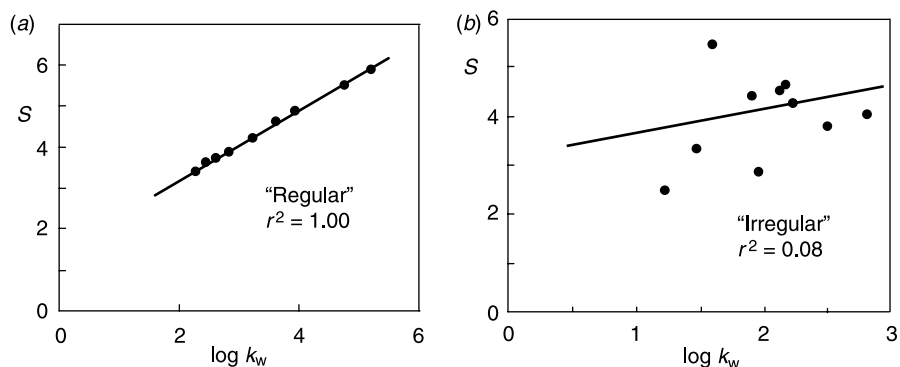


Figure 1.11 Plots of S vs $\log k_w$ for (a) “regular” sample and (b) “irregular” sample. Data of Table 1.3.

the “structural similarity” of the components of a sample, as it relates to sample “irregularity,” is usually not obvious. As illustrated in the following chapters, sample “regularity” or “irregularity” is best identified on the basis of observed chromatographic behavior, rather than molecular structure; if there are noticeable changes in relative retention when only gradient time is changed, then the sample should be regarded as “irregular.”

Plots of $\log k$ vs %B for individual compounds in a “regular” sample (as in Fig. 1.8a) exhibit slopes [values of $S = d(\log k)/d\phi$] that increase regularly for more retained compounds (larger values of k_w), while this is not the case for the “irregular” sample of Figure 1.8(b). This is illustrated in Figure 1.11 for the “regular” (a) and “irregular” (b) samples of Table 1.3. Values of S vs $\log k_w$ for the “regular” sample are highly correlated ($r^2 = 1.00$), while a similar plot for the “irregular” sample exhibits considerable scatter ($r^2 = 0.08$).

The specific “regular” and “irregular” samples of Table 1.3 will be used extensively in following chapters as representative examples of each sample type (all separations of these samples shown in this book are created by computer simulation). Most samples share the characteristics of both “regular” and “irregular” samples, resulting in an intermediate behavior as %B is changed in isocratic elution, or gradient conditions are changed for gradient separation. As a result, changes in isocratic %B or gradient time t_G often result in potentially useful changes in compound separation order or selectivity.

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