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GRADIENT ELUTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. THEORETICAL BASIS FOR REVERSED-PHASE SYSTEMS

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CONTENTS

1. INTRODUCTION

At the present time it is probable that 60–80% of all separations by high-performance liquid chromatography (LC) are carried out in the reversed-phase mode with bonded-phase (*e.g.*, C_{18}) packings, generally using 5–10-μm totally porous particles. When gradient elution is added to this technique, which is increasingly the case,

the resulting separation system offers the same broad applicability for non-volatile samples that temperature-programmed gas chromatography (GC) provides for volatile mixtures. Gradient elution is a powerful separation procedure, but one that is at best difficult to understand. As a result, its optimal application in a given situation would be facilitated by a simple set of guidelines which relate various separation characteristics to the different operating parameters or experimental variables. That is the aim of the present and the following[1] papers.

Over the past few years, a general theory of gradient elution separation has been developed (*e.g.*, refs. 2–8), but many of the practical implications of this work seem to have gone largely unnoticed. More recently, the special case of reversed-phase gradient elution (RP-GE) has been examined theoretically[7,9,10]. Most of this work, however, has focused on detailed mathematical expressions for sample retention times and resolution. While these approaches in principle allow precise calculations of separation in a given RP-GE system for a particular sample (of known composition), they offer little help for the case of unknown samples. Further, such calculations require detailed studies of the elution of the sample in question under isocratic conditions, in order to specify the dependence of isocratic sample retention on mobile phase composition.

Our intention in this paper is to provide an approximate theoretical treatment for understanding and controlling separation in RP-GE for the general case, where the composition of the sample may not be known, and where no information is available on the variation of sample capacity factor (k') values with mobile phase composition. In Part II[1] we shall illustrate some of our theoretical conclusions with experimental examples, and offer a detailed set of rules for optimizing RP-GE separations. Our primary emphasis will be on resolution and detection sensitivity as a function of experimental conditions.

2. EXPERIMENTAL

This paper is almost entirely theoretical, and any new experimental data referred to are described in detail in Part II[1]. Most of the figures and some of the tables presented here are based on rigorous calculations, in most instances involving numerical integration of eqn. i-1 (Appendix I). Some assumptions are implicit not only in these calculations, but in much of the attendant discussion. These include the following:

(1) It is assumed that the sample is injected as a very small volume, relative to the larger volume of later eluting bands; *i.e.*, the sample volume does not affect the final band width.

(2) It is assumed that there is no time delay between the gradient generator and sample injector; *i.e.*, the gradient program begins at the column inlet at the moment of sample injection. This is generally not true of actual gradient elution systems, where a finite volume exists in the tubing connecting the gradient mixer and the column inlet.

(3) It is assumed that the mobile phase gradient entering the column is not affected by any sorption of mobile phase components on the column. In actual systems involving reversed-phase operation this is probably a reasonable approximation.

3. GRADIENT ELUTION

As far as possible, our approach here will be to explain gradient elution in terms of isocratic elution, and to transfer the various concepts to gradient elution which are applicable for isocratic separation. While it can be argued that some mathematical precision is sacrificed in this procedure, this is inevitable in terms of our goal of developing general guidelines for carrying out RP-GE separations. To follow more easily the discussion in the balance of this paper, it will prove useful to first look at what happens in gradient elution in qualitative terms. We shall also define several retention parameters that play a key role in any theory of gradient elution.

The following examples of isocratic and gradient elution are calculated as described under Experimental. The particular conditions assumed (*e.g.*, gradient shape and steepness) are for the most part both practical and reasonable. Fig. 1 shows an isocratic separation of an eight-component mixture, for the experimental conditions summarized in the figure caption. The k' values for bands 1, 2, 3, ..., 8 increase in geometric progression: 0.5, 1.0, 2.0, ..., 64. The bands are shown as triangles rather than Gaussian bands, with the base of each band equal to 4σ.

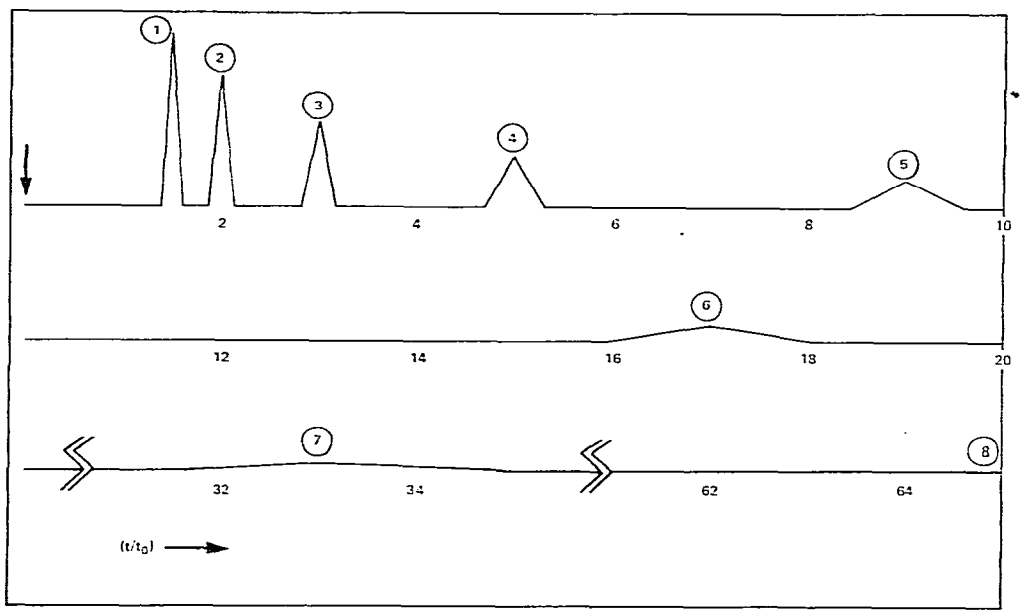

Fig. 1. Calculated isocratic separation of eight-component sample by reversed-phase LC. Mobile phase, 20% methanol–water; $N = 1000$; k' values for bands 1–8 are 0.5, 1.0, 2.0, . . ., 64.

Fig. 2 shows a proposed gradient or solvent program for the RP-GE separation of the same sample as in Fig. 1. A linear gradient from 20 to 80% methanol–water is assumed, and the light solid curve shows the change in mobile phase composition at the column inlet as a function of time t after sample injection (expressed in units of t/t_0, which we shall comment on shortly). The dashed curve in Fig. 2 (labeled "outlet")

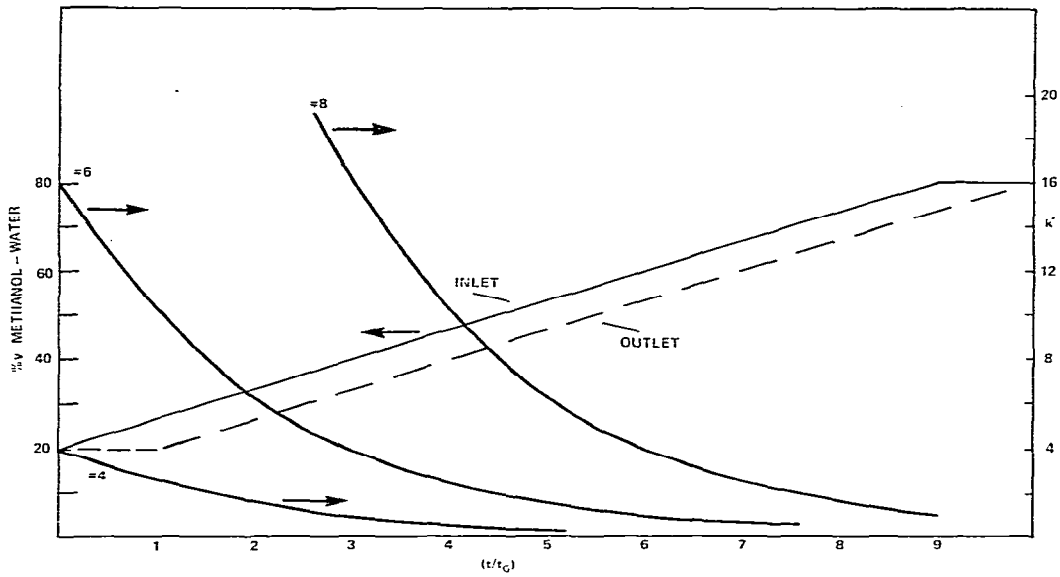

Fig. 2. Linear solvent program for separation by reversed-phase gradient elution. Program runs from 20 to 80% methanol–water; k_i values assume a 2-fold decrease for each 10% increase in methanol concentration.

shows the composition of the mobile phase as it leaves the column; this lags the inlet composition by one column volume or a time equal to t_0. Also superimposed on Fig. 2 are heavy curves for the k' values of bands 4, 6 and 8 as they would exist at the column inlet as a function of time (t/t_0). That is, these latter inlet k' values (k_i) refer to the isocratic k' for a mobile phase with the given inlet composition at time t/t_0. The actual bands will have migrated some distance across the column, and at any given time t/t_0 they will be moving in a somewhat weaker solvent. The instantaneous or actual k' value for the band (k_a) will therefore be slightly larger than k_i at any given time.

The resulting gradient elution separation of the sample of Fig. 1, using the solvent program of Fig. 2, is shown in Fig. 3a. Here, it is assumed again that the various sample bands are of equal area, corresponding, *e.g.*, to the case of equal concentrations and equal detector sensitivities. Comparison of Figs. 1 and 3a shows that the first-eluted bands (1–3) are eluted in a similar fashion by either isocratic or gradient elution; the retention times are similar, the band widths are comparable and the peak heights are about the same. The reason is that in gradient elution the bands elute within the first 3 column volumes, before the mobile phase composition leaving the column has changed much (*cf.*, Fig. 2), and before the k_i values for these bands have decreased greatly from the starting isocratic values (k_0) as in Fig. 1. Note that the mobile phase composition for Fig. 1 is the same (20% methanol–water) as for the start of the gradient elution separation in Fig. 3a (see Fig. 2).

The appearance of the last eluted bands (5–8) differs greatly in gradient (Fig. 3a) from isocratic (Fig. 1) elution. The reason is that the initial k' or k_0 values for these bands are fairly large, and as a result the bands elute under essentially

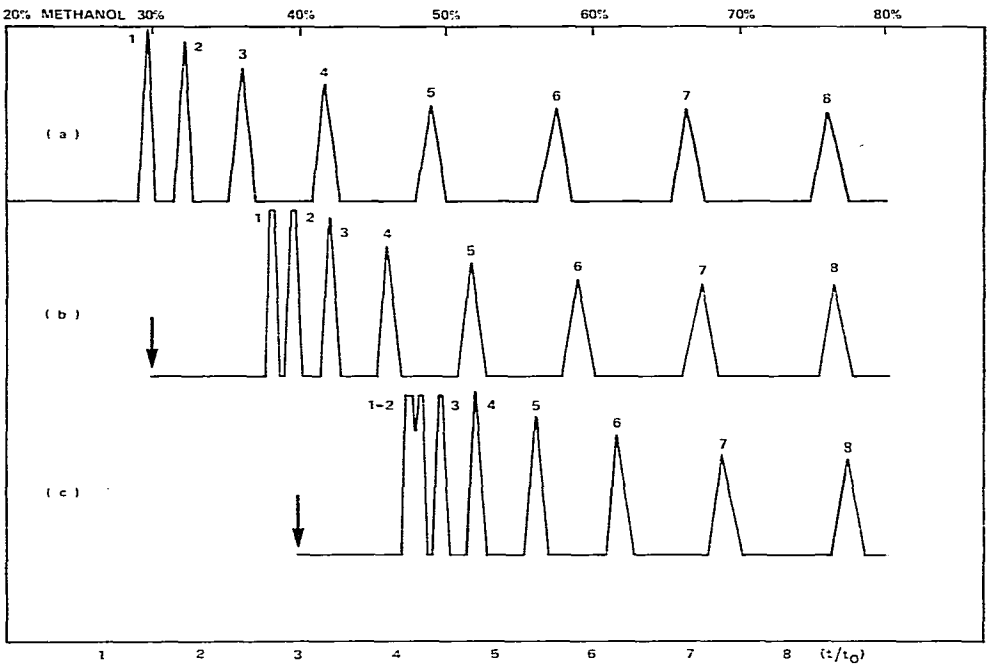

Fig. 3. Calculated gradient elution separation for sample and column of Fig. 1. (a) Using solvent program of Fig. 2 ($b = 0.2$); (b) same, except program starts at 30% methanol–water; (c) same, except program starts at 40% methanol–water.

gradient conditions, rather than near-isocratically as for bands 1–3. As a result, these later eluted bands in gradient elution have approximately equal band widths (and similar detection sensitivities), and are more closely spaced in the chromatogram. When the sample k_0 values are in geometric progression as in this example, equal spacing of the bands results. Let us look more closely at the elution of these late bands in RP-GE, as in Fig. 4a. Here, the fractional movement x of band 8 across the column is shown as a function of time t/t_0. It is seen that the movement of band 8 along the column is negligible ($x \leqslant 0.1$) prior to $t/t_0 \approx 3$. At this point, band 8 begins to move across the column increasingly rapidly, until its elution at $t/t_0 = 8.4$. The instantaneous k' value for band 8 (k_a) during this RP-GE separation is also shown in Fig. 4a. The value of k_a remains large up to $t/t_0 \approx 3$, then drops rapidly as band 8 begins to migrate. The average or effective value of k_a during elution of the band determines the resolution of that band, just as k' determines the resolution of a band in isocratic elution. If we define this average k_a as $\bar{k}$, equal roughly to the value of k_a at $x = 0.5$ ($\bar{k} = 4.2$ in Fig. 4a), then the resolution, R_s, for the band in gradient elution will be given as

$$R_s = \tfrac{1}{4}(\alpha - 1)\, N^{\frac{1}{2}} [\bar{k}/(1 + \bar{k})] \qquad (1)$$

which is exactly analogous to the corresponding relationship for isocratic elution (*e.g.*, ref. 11). Here, α is the separation factor for two adjacent bands and N is the column

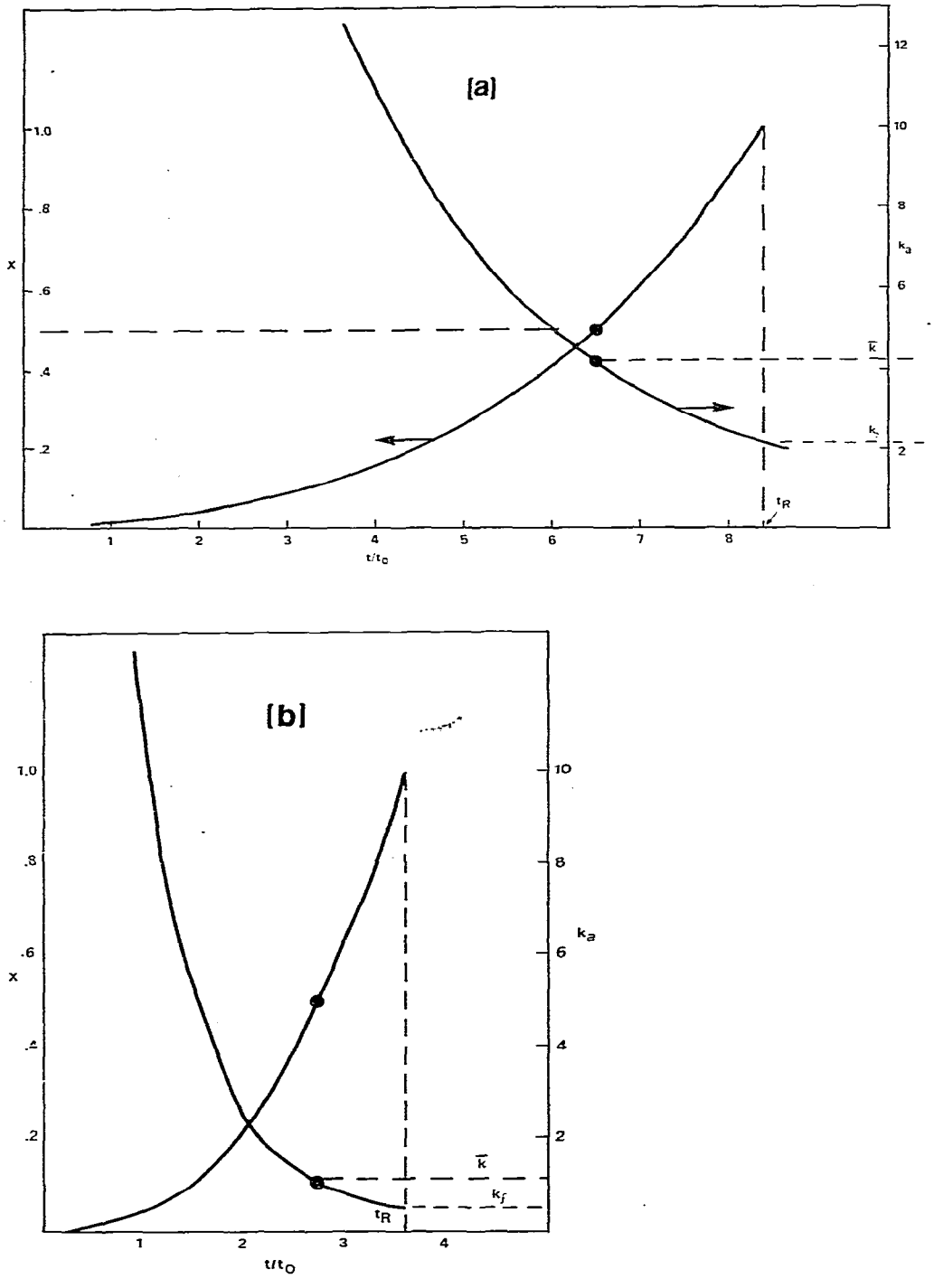

Fig. 4. Migration of band 8 along column in separation of Fig. 3. (a) Conditions as in Figs. 2 and 3; (b) same, except steeper gradient ($b = 0.8$).

plate number. Note in Fig. 4a that the value of $\bar{k}$, comparable to k' in isocratic elution, is in the optimal range of 2–5 (*cf.*, discussion in ref. 11).

Band width and detection sensitivity in gradient elution are determined by the value of N and by the k_a value of the band at the time of elution (k_f), just as in isocratic elution. In Fig. 4a we see that k_f for band 8 is 2.1, which is small enough to yield a fairly narrow, easily detected band. In contrast, band 8 in the isocratic separation in Fig. 1 has $k' = 64$, yielding a barely detectable band which is about 32-fold wider than in Fig. 3a.

For later eluting bands in RP-GE, the progression of the band along the column proceeds very much as for band 8 in Fig. 4a. The only difference is either an earlier or later beginning of migration; *i.e.*, at t/t_0 values less than or greater than 3. Because the shape of the x or k_a *versus* t/t_0 plots for later eluting bands are essentially similar to that for band 8 in Fig. 4a (for a properly designed solvent program, as in Fig. 2), similar values of $\bar{k}$ and k_f result for every band. This means that resolution for all later eluted bands is comparable when their isocratic α values are similar, and band widths for all later eluting compounds will be essentially constant.

A. Linear solvent strength separations

The most basic and important experimental variable in gradient elution separation is the solvent program: the varying composition of the mobile phase during the elution of sample from the column (as in Fig. 2). The optimal solvent program in turn depends on the relationship of sample k' values (isocratic separation) to the composition of the mobile phase. In a later section we shall consider this latter relationship in more detail. For the moment we shall concentrate instead on gradient programs which are fundamentally optimal from the standpoint of separation; as discussed elsewhere[2–5,12] such solvent programs are of the so-called linear solvent strength (LSS) form. With an LSS solvent program, the inlet k_i values for individual sample compounds decrease during gradient elution according to

$$\log k_i = \log k_0 - b(t/t_0) \tag{2}$$

As previously, k_0 refers to the k' value for the band in question at the beginning of gradient elution; *i.e.*, for isocratic elution with the mobile phase composition corresponding to the beginning of the solvent program (20% methanol–water in Figs. 2 and 3a). The parameter b should remain constant throughout the solvent program, and ideally b will have the same value for all compounds in the sample (in principle, this is never exactly possible; see later discussion).

Among the advantages of properly designed LSS programs in gradient elution separations are the following:

(1) approximately constant band widths for all bands in the chromatogram;

(2) comparable resolution or effective plate number NQ^2 (see ref. 4) for both early- and late-eluting bands (*i.e.*, equal values of NQ^2 for all bands in the chromatogram);

(3) a regular spacing of bands throughout the chromatogram, without bunching of peaks at the beginning or end (if values of $\alpha - 1$ for all adjacent bands are reasonably large);

(4) a conceptual simplicity which makes it possible to understand easily how separation varies as different experimental variables are changed.

These features of LSS separation will be further illustrated and discussed below.

It has been argued that "custom" (non-LSS) gradients are more appropriate for some samples. With such solvent programs it is possible to tailor the separation at individual points within the chromatogram to provide maximal resolution for difficult to separate band pairs. However, few samples require this approach in practice.

B. Retention time, band width and resolution in LSS gradient elution

Previous papers[2,5,6] have derived relationships for these separation parameters in LSS systems, with particular emphasis on liquid–solid (adsorption) LC. Here we shall generalize this treatment for all forms of LC, and in a following section focus on the special case of RP-GE.

(a) Retention time

As derived in Appendix I, the retention volume, V_g (ml), of a given band in an LSS separation is

$$V_g = (V_m/b) \log(2.3\, k_0 b + 1) + V_m \tag{3}$$

where V_m is the total volume of mobile phase contained within the column. Eqn. 3 has been verified experimentally for the special case of liquid–solid LC[13]. There is no reason to doubt its validity for other forms of LC, including reversed-phase separations.

The retention time, t_g (sec), is then given as V_g/F, where F is the flow-rate (ml/sec) of mobile phase through the column:

$$t_g = (t_0/b) \log(2.3\, k_0 b + 1) + t_0 \tag{3a}$$

Here, t_0 is the column "dead-time", equal to V_m/F.

It can be seen in eqn. 3a that the retention time, t_g, decreases as the gradient steepness, b, increases. This is similar to the case of isocratic elution, where retention times, t_R, decrease with increase in solvent strength. This analogy between b in gradient elution and solvent strength in isocratic elution becomes more quantitative if we rearrange eqn. 3a as follows:

$$(t_g - t_0)/t_0 = (1/b) \log(2.3\, k_0 b + 1)$$

For the case of later eluting bands (large k_0), we then have

$$(t_g - t_0)/t_0 \approx (1/b)\,[\log 2.3 + \log k_0 + \log b] \approx (\log k_0)/b \tag{3b}$$

The term on the left is analogous to k' in isocratic elution, and it is seen to be approximately proportional to $1/b$, for a given band (*i.e.* given value of k_0). The time

of separation, t_s, is equal to t_g for the last eluted compound, or to the time required to complete the gradient. In either case, as $t_s \approx (t_g - t_0)$, the separation time is proportional to $1/b$. Note that in isocratic elution the time of separation is proportional to $1 + k'$ for the last-eluted compound in the sample (*e.g.*, ref. 11), or approximately to k'. Thus, so far as the separation time, t_s, is concerned, $1/b$ in gradient elution corresponds to k' in isocratic elution.

Consider next the effect of beginning the gradient with a mobile phase of higher strength. This is illustrated in Fig. 3b and c, where the starting mobile phase consists of 30% and 40% methanol–water, respectively (compared with 20% in Fig. 3a). We have noted that the initial bands in a gradient elution separation are eluted more or less isocratically, so that bands 1–3 in these examples elute in a stronger solvent in going from Fig. 3a to 3c. The resulting changes in the separation of these bands reflect this increase in solvent strength, the retention times becoming shorter, bands narrower and taller, and resolution poorer. The elution of later bands, however, is less sensitive to the starting composition of the solvent program. While the retention times for these bands (6–8 in Figs. 3) are decreased by the time saved in starting the solvent program at a later point, these later eluting bands still elute at about the same mobile phase composition; see top scale of Fig. 3, *i.e.*:

Starting mobile phase (methanol–water) composition (% methanol)	*Inlet mobile phase (methanol–water) composition at t_g (% methanol)*		
	Band 6	*Band 7*	*Band 8*
20	58	67	76
30	59	68	77
40	62	69	77

The widths, detectability and resolution of these later bands are also essentially constant in Fig. 3a–c.

(b) Band width

The band width in gradient elution separation is the result of three more-or-less independent processes:

(1) the normal broadening of sample bands as they move through a column[11];

(2) a "band compression" phenomenon, which arises from the faster migration of the tail of bands in gradient elution, *versus* the equal migration of all parts of the band in isocratic elution[4];

(3) the instantaneous k' value of the band (k_f) as it leaves the column[14].

If we consider the width, σ_x, of sample bands on the column bed, at the time each band leaves the column, where σ_x is the standard deviation of the Gaussian distribution in length units (cm), then the plate number N of the column can be expressed as[15]

$$N = (L/\sigma_x)^2 \tag{4}$$

where L is the length of the column. The value of N in isocratic elution is generally assumed to be independent of k' (see discussion in refs. 4 and 5), which means that

σ_x will be constant for different bands, and for different mobile phases of similar viscosity. This in turn implies that σ_x will be constant for different bands in a given gradient elution separation, which is indeed the case. The value of σ_x is then given by eqn. 4.

Band compression in gradient elution has been discussed earlier[4]. The band width, σ_x, that would be obtained in isocratic elution is reduced in gradient elution by the factor G, where G can be calculated by numerical integration[4]. For LSS gradients, G is solely a function of the gradient parameter b, as shown in Fig. 5. Note that for intermediate values of b ($0.2 < b < 0.5$), G is roughly constant and equal to 0.8, which means that bands in gradient elution are compressed by about 20% in the usual case. The possibility of further compression of gradient elution bands at higher values of b, as for increasing detection sensitivity in trace analysis, is considered in a following section.

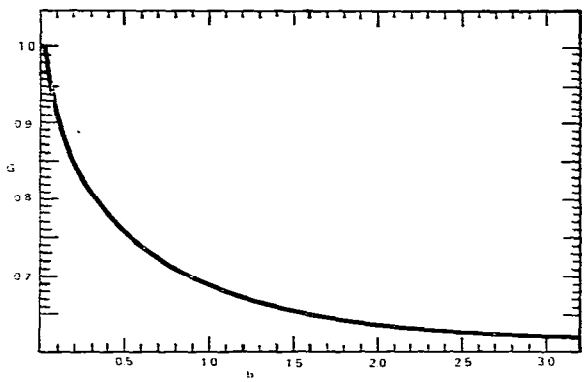

Fig. 5. Band compression factor, G, as a function of gradient steepness, b. Calculated by numerical integration of eqn. i-1.

The final width of a gradient elution band in time units, σ_t, is determined by the value of σ_x (the width of the band on the bed, just prior to elution), and the instantaneous k' value of the band as it leaves the column (k_f) (see the discussion in ref. 14). This final value of σ_t is then given[4,14] as

$$\sigma_t = (1 + k_f)\,\sigma_x\,G(t_0/L) \tag{5}$$

Similarly, as shown in Appendix I for LSS separations,

$$k_f = 1/(2.3b + 1/k_0) \tag{6}$$

As k_0 for most bands is large, eqn. 6 can be approximated by

$$k_f = 1/2.3b \tag{6a}$$

Finally, eqns. 4 and 5 can be combined and re-stated in time units:

$$N = G(1 + k_f)t_0/\sigma_t^2 \tag{7}$$

Combination of eqns. 6a and 7 then yields

$$\sigma_t = (2.3b + 1)Gt_0/2.3bN^{\frac{1}{2}} \tag{7a}$$

Experimental verification of eqn. 7a is afforded by one study involving liquid–solid gradient elution LC[4], as summarized in Table 1. The agreement found between calculated and experimental σ_t values is adequate, considering the various approximations that enter into eqn. 6a, and uncertainty in the values of b that can be estimated in ref. 4.

TABLE 1

COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF AVERAGE BAND WIDTH IN GRADIENT ELUTION LIQUID-SOLID CHROMATOGRAPHY (WITH LSS PROGRAMS)

b*	N	G	σ_t/t_0	
			*Experimental***	*Calculated****
0.15	1090	0.93	0.10	0.11
0.27	920	0.90	0.09	0.08
0.52	1600	0.86	0.06	0.04

* Re-calculation of b (on basis of average $A_s = 10$ for the compounds studied; see ref. 4) gave 30% lower values than those reported in ref. 4.

** Data from ref. 4.

*** Eqn. 7a.

According to eqn. 7a, for LSS gradient programs the widths of eluted bands are predicted to be constant throughout the chromatogram. Using the more exact eqn. 6 as opposed to eqn. 6a, early eluting (small k_0) bands are predicted to have slightly reduced band widths compared with later bands. This pattern is apparent in the various calculated chromatograms in Fig. 3. For wider gradients (*e.g.*, 0–100% methanol–water) than shown in Fig. 3, most of the bands in the chromatogram would appear to have equal band widths.

In isocratic elution, band widths increase approximately in proportion to k' (more exactly, $1 + k'$), or in inverse proportion to solvent strength. In gradient elution (eqn. 7a), band width varies inversely as b. Thus, again we see an analogy between gradient steepness b in gradient elution and solvent strength or $1/k'$ in isocratic elution.

(c) Resolution

Resolution in LSS gradient elution has already been discussed in some detail[4,5] and only a practical summary will be repeated here, plus some updating for more recent developments in column technology. The analogies we have already drawn between b in gradient elution and $1/k'$ in isocratic elution will be found to apply to various aspects of resolution. Resolution, R_s, in gradient elution can be defined in much the same way as for isocratic elution (*e.g.*, ref. 11):

$$R_s = (t_2 - t_1)/2(\sigma_1 + \sigma_2) \tag{8}$$

Here, t_1 and t_2 refer to retention times, t_g, in gradient elution for adjacent bands 1 and 2, respectively; σ_1 and σ_2 are the corresponding band widths (σ_t values).

Resolution in gradient elution can also be described in terms of eqn. 1, which is analogous to the corresponding expression for isocratic elution (*e.g.*, ref. 11):

$$R_s = \tfrac{1}{4}(\alpha - 1)\, N^{\frac{1}{2}}\, [k'/(1 + k')]^2 = \tfrac{1}{4}(\alpha - 1)\, (NQ^2)^{\frac{1}{2}} \qquad (9)$$

The quantity NQ^2 in eqn. 9 is referred to as the effective plate number of the column, and a similar definition is applicable for gradient elution; *i.e.*, in eqn. 1, $NQ^2 = N[\bar{k}/(1 + \bar{k})]^2$. Larger values of NQ^2 provide generally better resolution, other factors (*e.g.*, α) equal. The value of Q (and NQ^2) in LSS gradient elution tends to a constant, limiting value for later eluting bands[4], and this limiting value of Q is determined by the value of b for the solvent program. Q^2 as a function of b is summarized in Table 2, as calculated by numerical integration in ref. 4 of the fundamental gradient elution equation (eqn. i-1 in Appendix I).

TABLE 2

EFFECTIVE PLATES AND DETECTION SENSITIVITY IN LSS GRADIENT ELUTION AS A FUNCTION OF b

b	$\bar{k} = 1/1.3b$	Q^{2*}	$[\bar{k}/(1+\bar{k})]^{2**}$	s_g^{***}	$s_k^{\S}$
0.1	7.7	0.79	0.78	0.2	0.1
0.2	3.8	0.63	0.63	0.4	0.2
0.4	1.9	0.45	0.43	0.6	0.3
0.6	1.3	0.33	0.32	0.8	0.4
1.0	0.8	0.20	0.19	1.0	0.6
1.5	0.5	0.12	0.12	1.2	0.7
2.5	0.3	0.06	0.06	1.4	0.8

* Calculated from ref. 4 by numerical integration of eqn. i-1.
** Calculated from the value of b and eqn. 10.
*** Eqns. 11a and 6a.
§ Eqn. iii-6.

If the quantity Q^2 from Table 2 is compared with $[\bar{k}/(1 + \bar{k})]^2$ (*cf.*, eqn. 9 and third column in Table 2), it is found that these two functions are approximately related if we assume

$$\bar{k} = 1/1.3b \qquad (10)$$

That is, substitution of $\bar{k}$ from eqn. 10 for various values of b leads to values of $[\bar{k}/(1 + \bar{k})]^2$ in Table 2 which are almost identical with gradient elution Q^2 values for the same value of b. Thus, so far as resolution is concerned, we can regard the term $1/1.3b = \bar{k}$ as an average or effective value of k' for gradient elution.

As in isocratic elution there exists an optimal value of k', so in LSS gradient elution there exists an optimal value of $\bar{k}$ and b. In isocratic elution three experimental cases can be defined, for each of which the optimal k' value is different[11]:

(1) constant mobile phase velocity u, with column length L and pressure drop P varying; k' (optimal) = 2;

(2) constant L, with u and P variable; k' (optimal) = 3–6;

(3) constant P, with L and u variable; k' (optimal) = 2.5–3.

For cases (2) and (3) above, the optimal value of k' varies mainly with the particle size, d_p, of the column packing and the resulting value of the column parameter n ($0.3 \leqslant n \leqslant 0.6$, see ref. 11).

Case (1) is of limited interest in gradient elution. Case (2) is the commonest situation, and is discussed in detail in Appendix II. We can summarize by saying that there is an optimal value of b in gradient elution for this case, equal variously to 0.1–0.3 depending on the experimental conditions. An average, optimal value of b in gradient elution of 0.2 can generally be assumed. Note that this range in b values (0.1–0.3) is the same as can be calculated from eqn. 10 and the isocratic (optimal) k' values: $3 < k' < 6$. Again, we see a rather precise analogy between b in gradient elution and k' in isocratic separation. Extending the analogy, although we have not pursued this theoretically, the optimal value of b for case (3) should be 0.25–0.30.

Effective plates (NQ^2) and resolution in gradient elution can always be increased by a decrease in b, as shown in Table 2 and illustrated in Fig. 3a ($b = 0.2$) *vs.* Fig. 6 ($b = 0.8$). However, this results in an increase in separation time, which must be weighed against the alternative of simply slowing the flow-rate through the column while holding b constant (*i.e.*, increasing N rather than Q). The significance of the above optimal values of b is that for an increase in separation time t_s, it is preferable to hold b constant at about 0.2, and to decrease the flow-rate for an increase in N. This approach will generally yield maximum resolution.

(d) Detection sensitivity

Because the bands in gradient elution are generally narrower than those in isocratic elution, gradient elution offers a means of increasing the detection sensitivity

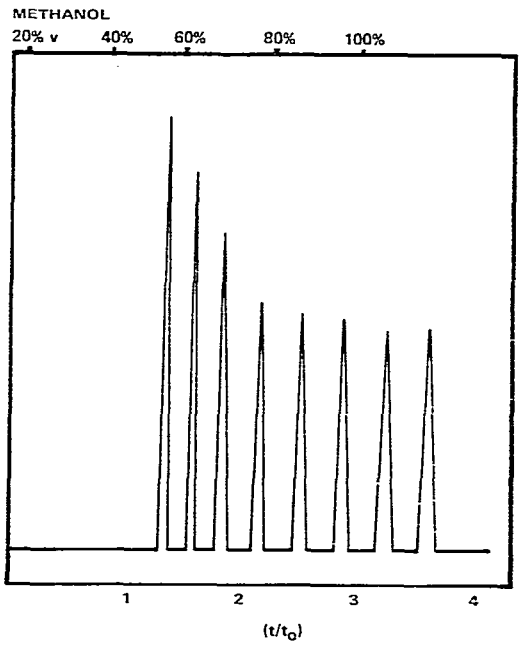

Fig. 6. Calculated gradient elution separation for sample and column of Fig. 1. Same conditions as for Fig. 3a, except $b = 0.8$. Chromatogram attenuated by a factor of 2×.

in applications such as trace analysis. In isocratic elution, the band width decreases and the detection sensitivity increases as k' is decreased (see discussion in ref. 16). We can define a sensitivity function s_k for isocratic elution, equal to the height of some band with $k' \neq 0$, relative to the height of a band eluted at t_0. As discussed in Appendix III,

$$s_k = 1/(1 + k') \tag{11}$$

Similarly, a sensitivity function s_g can be defined, equal to the height of a band in gradient elution, relative to the height of a band eluted isocratically from the same column at t_0. A comparison of s_g with s_k values then permits the increased detection sensitivity in gradient elution to be assessed.

As derived in Appendix III, s_g in gradient elution is given as

$$s_g = 1/G\,(1 + k_f) \tag{11a}$$

By comparing eqns. 11a and 11, we again see the analogy between k' values in isocratic elution and b values in gradient elution, as $k_f = 1/2.3b$ (eqn. 6a). A further comparison of s_g and s_k values is shown in Table 2, where s_g as a function of b is compared with s_k as a function of $b = 1/2.3\ k_f$. Thus the s_g values are for $0.3 < k_f < 8$. Because, for a given value of b in Table 2, s_g is generally larger than s_k, this means that the detection sensitivity in gradient elution is always better than the corresponding isocratic case, when the resolution or NQ^2 is the same ($k' = 1/1.3b$) for both separations.

In view of the band compression effect, it might be assumed that gradient elution bands can be "squeezed" down to any width desired, using a sufficiently steep gradient (large enough value of b). In practice, this is not the case, as seen in Table 2. Because G (Fig. 1) decreases slowly with b*, impractically large b values are required for significant reduction in band widths, relative to bands eluted isocratically at t_0. Thus, in Table 2, it is seen that a value $b = 2.5$ results in only a 40% increase in band height *versus* a band eluted at t_0 (*i.e.*, $s_g = 1.4$), while the entire gradient is compressed into a time 0.7 t_0 (*i.e.*, 1.7–1).

Gradients this steep (or steeper) are not practical with presently available equipment, and it should also be noted that resolution suffers greatly for b values greater than 1 ($Q^2 < 0.2$). Nevertheless, from Table 2 it appears that b values as large as 1.0 allow about a 3-fold increase in detection sensitivity in gradient elution, *versus* the case of $b = 0.2$ for optimal resolution. However, an increase of b to 1.0 also results in a 3-fold reduction in column efficiency, NQ^2.

Much larger increases in detection sensitivity can be achieved by gradient elution[16] in other ways. For example, very large sample volumes can be charged if the k_0 values of compounds of interest are fairly large. In this case, the bands of interest will be held initially at the column inlet, and the large sample volume will not appreciably widen the bands for these compounds when they are eventually eluted.

* *E.g.*, for $b = 3.0$, $G = 0.6$ (only a 40% "squeezing" of the band).

(e) Separation selectivity

By separation selectivity we mean differences in the retention times of two compounds in gradient elution, which in turn implies an average value of α in eqn. 1 which is different from 1. By reference to eqn. 3a, it is apparent that differences in retention time can result from differences in k_0 or b for the two compounds in question. As discussed in the following section, b values for different compounds are usually of similar magnitude in a given gradient elution system, which means that we rely mainly on differences in k_0 to achieve separation selectivity. Differences in k_0 for two compounds can in turn only be achieved by changing the nature of the organic solvent in the RP-GE system, and this is the commonest approach where $\alpha - 1$ must be increased. Several studies have shown (*e.g.*, refs. 17–19 and especially 20) that significant changes in α can be achieved in this fashion. One example is shown below, for a change in α for isocratic RP separation[18] of various glycosides.

Band pair	α	
	37% acetonitrile–water	*45% dioxane–water*
Gitaloxigenin, gitoxin	1.07	2.7
Lanatoside B, lanatoside E	1.00	1.04
Gitaloxin, lanatoside A	1.02	1.25

Alternatively, it is possible to change α by changing the gradient steepness b or the rate of change of the volume fraction of the organic solvent, when the b values for two compounds in a given RP-GE system differ. This is discussed in greater detail in Appendix IV, which is based on the further discussion of mobile phase composition in RP-GE in the following section.

4. SOLVENT STRENGTH *VERSUS* COMPOSITION IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

Several studies have been reported on the variation of sample k' values in isocratic, reversed-phase LC, as a function of mobile phase composition (*e.g.*, refs. 10 and 17–34). For mobile phases consisting usually of water–methanol or water–acetonitrile mixtures, and a wide range in sample compounds, it is usually observed for a given system (a specific column and organic component of the binary solvent, *e.g.*, methanol) that sample k' values are related to the volume fraction, Φ_b, of organic solvent B in the mobile phase as

$$\log k' = \log k_w - S\Phi_b \tag{12}$$

Here k_w refers to the isocratic k' value for pure water as mobile phase, and is usually an extrapolated value. Several studies (*e.g.*, refs. 21–25 and 27–32) have shown that eqn. 12 is valid within experimental error over wide limits in both k' and Φ_b. The coefficient S in eqn. 12 is seen to be related to the strength of pure solvent B as mobile phase, as larger values of S lead to a faster decrease in k' with increase in Φ_b. For a given reversed-phase system, *e.g.*, different mixtures of methanol–water as mobile

phase, and a given column, the parameter S is often observed to be roughly constant, even for solutes of varying molecular size and structure. The value of S when B is either methanol or acetonitrile is usually about 3, but varies (in different studies) from 2 to 4 for reasons that are not yet clear. As discussed below, S varies further as the solvent B is changed for less polar solvents such as ethanol or tetrahydrofuran, with S increasing as the polarity of B decreases.

A. Optimal gradients for reversed-phase LC

It will next be shown that LSS gradients in reversed-phase systems correspond to linear solvent composition gradients; *i.e.*, where the volume fraction Φ_b of the organic solvent B increases linearly with time:

$$\Phi_b = \Phi_0 + \Phi' t \tag{13}$$

Here, Φ_0 is the value of Φ_b at the beginning of the separation, and $\Phi' = \mathrm{d}\Phi_b/\mathrm{d}t$ is the rate of change of Φ_b with time (and is constant for a given separation). If eqns. 12 and 13 are combined, we obtain

$$\log k' = (\log k_w - S\Phi_0) - S\Phi' t \tag{13a}$$

This is of the same form as eqn. 2 (LSS gradient), provided that $\log k_w - S\Phi_0$ is set equal to $\log k_0$, and the factor $S\Phi'$ is equated to b/t_0. As we have argued that LSS gradients are generally optimal, it follows that linear gradients as in eqn. 13 are likewise optimal for reversed-phase gradient elution in LC.

(a) Exceptions to eqns. 12 and 13

Schoenmakers *et al.*[10] have noted that eqn. 12 is not strictly obeyed in reversed-phase systems studied by them, but instead plots of $\log k'$ *versus* Φ_b are slightly concave. Other studies[33,34] show a similar relationship between k' and Φ_b, and in extreme cases these plots actually pass through a minimum in the region of $\Phi_b \approx 0.9$. A more careful analysis of these effects and their impact on our preceding analysis is presented in Appendix V and also Part II[1]. To summarize that discussion, it appears that these deviations of experimental data from eqn. 12 have essentially no effect on the conclusions so far presented in this paper.

The data of ref. 31 clearly show that S increases regularly with molecular size in a homologous series of solutes. A similar increase in S with increasing size of the solute molecule is suggested for oligomeric series of polymers (*e.g.*, ref. 35). For homologous or oligomeric samples, use of a linear gradient (eqn. 13) is expected to provide poorer resolution of later bands, together with progressive narrowing of these bands. This can be corrected by maintaining an LSS gradient. As b and $S\Phi'$ must remain constant throughout an LSS gradient, if S increases (for polymeric samples), Φ' must decrease with time; *i.e.* a convex gradient. Poile[9] has argued that this is also true for the elution of polycyclic aromatic hydrocarbons, as their S and t_g values increase regularly with molecular size.

B. The strength, S, of other solvents B in reversed-phase LC

A number of studies have been reported which allow values of S to be derived for methanol as the organic solvent B in water–organic mixtures as the mobile phase. While S is usually fairly constant for a given study (and column), its values vary from one study to another by ± 1 unit, *i.e.*, $2 < S < 4$. It thus appears that S is not a constant which is characteristic of a given solvent B, but varies somewhat with other separation parameters. A few studies have compared k' values in reversed-phase systems for more than one solvent B, which in turn allows estimates of the variation of S as a function of the solvent B. Data from several such studies are summarized in Table 3. The resulting S values are in rough agreement for different solvents, and are averaged at the bottom of Table 3. Because of the as yet unexplained variability of S, for a given solvent B (as in Table 3), these "best" values of S in Table 3 must be considered to be approximate at most. There is a definite need for a definitive experimental study of S as a function of all probable variables that might affect it such as sample molecular structure, the solvent B, variations in the column packing (coverage, alkyl chain length, etc.) and separation temperature.

TABLE 3

SUMMARY OF SOLVENT STRENGTH (S) VALUES FOR DIFFERENT ORGANIC SOLVENTS B IN REVERSED-PHASE LC (25°)

Reference	*Solvent B*						
	Methanol	*Acetonitrile*	*Ethanol*	*Acetone*	*Dioxane*	*Isopropanol*	*Tetrahydrofuran*
18–29	2–4						
1	3.5	2.9					4.2
10	2.7		3.4			4.1	
17	(3)*	4.1					4.7
36		(2.5)*		3.4	3.5	4.2	4.4
"Best"	3.0	3.1	3.6	3.4	3.5	4.2	4.4

* Assumed value for calculation of S for other solvents (k' *versus* Φ_b data not provided).

(a) Optimal value of Φ' in reversed-phase LC systems

We have argued in an earlier section that the optimal value of b in reversed-phase LC should generally be about 0.2. In terms of eqn. 13a, we have seen that

$$\Phi' = b/S\,t_0 \tag{14}$$

Values of $b = 0.2$ and S from Table 3 (for various solvents B) can now be inserted into eqn. 14 for calculation of the optimal gradient steepness (value of Φ') for maximum resolution (or NQ^2) per unit separation time. For example, with methanol–water solutions as mobile phase ($S = 3$), and a value of $t_0 = 1$ min, the optimal value of Φ' is 0.067, or a 6.7% increase in methanol concentration per minute for the gradient. Optimal values of Φ' for various values of t_0 and different solvents B are summarized in Table 4.

TABLE 4

OPTIMAL GRADIENT STEEPNESS (FOR MAXIMAL RESOLUTION) IN REVERSED-PHASE LC ($b = 0.2$)

Solvent B	*Φ′ (%v/min change in B)*			
	t_0 = *10 sec*	t_0 = *30 sec*	t_0 = *1 min*	t_0 = *2 min*
Methanol	40	13	6.7	3.3
Acetonitrile	39	13	6.5	3.2
Acetone	35	12	5.9	3.0
Dioxane	34	11	5.7	2.8
Ethanol	33	11	5.6	2.8
Isopropanol	29	10	4.8	2.4
Tetrahydrofuran	27	9	4.5	2.3

5. MISCELLANEOUS OTHER CONSIDERATIONS

A. Design of isocratic separations on the basis of initial gradient elution separation

In some cases gradient elution is used as a "scouting" technique for unknown samples. An initial separation by reversed-phase gradient elution provides an immediate picture of the sample, with often adequate separation in the first attempt. However, it may then be desired to repeat the separation isocratically, for any of several reasons. In this case, it is useful to be able to estimate the correct solvent strength for the isocratic separation from the results of the initial gradient elution separation.

For isocratic elution, we require appropriate k' values for bands of interest. In this connection, eqn. 6a is useful, as it defines the k' value (k_f) of a band as it leaves the column (in the mobile phase leaving the column at the same time). For the optimal b value of 0.2, $k' = k_f$ in an isocratic separation is then $1/2.3 \cdot 0.2 = 2.2$. For a column of fixed length, an optimal value of k' in an isocratic separation is about 4 (see preceding section), so that a somewhat weaker solvent is required in isocratic elution than actually elutes a band of interest in gradient elution. In fact, we require k' (isocratic elution) to be increased about 2-fold (over gradient elution). From eqn. 2, for $b = 0.2$, this corresponds to mobile phase leaving the column at $1.5\ t_0$ prior to the elution of the band of interest (gradient elution). As it is generally more convenient to consider the composition of mobile phase entering the column, the isocratic separation will require a solvent corresponding to that entering the gradient column at a time $t_g - 2.5\ t_0$, where t_g is the retention time of the band of interest in gradient elution.

B. Calculation of column plate number in gradient elution

It is apparent to most workers that the column plate number, N, cannot be determined from a gradient separation by means of the usual relationship for isocratic elution:

$$N = (t_R/\sigma_t)^2 \tag{15}$$

Application of eqn. 15 to a gradient chromatogram grossly overestimates N in most cases, because of the lower value of k_f at the time of elution of each band. Nevertheless, eqn. 15 is periodically used in the literature (*e.g.*, refs. 37–40) for this purpose. In many of these cases, the shortcomings of the resulting N values are acknowledged, but then conclusions based on these "apparent" N values are drawn. The use of such N values (eqn. 15) in gradient elution is not recommended, as even relative N values can vary with t_g by large factors. An alternative is to use the correct expression for N for gradient elution, which is derivable from eqn. 7a:

$$N = \left[\frac{(2.3\,b + 1)\,G\,t_0}{2.3\,b\,\sigma_t}\right]^2 \tag{15a}$$

Eqn. 15a allows the calculation of the plate number from a gradient elution separation. The gradient steepness parameter, b, must be known, but it can be calculated from eqn. 14. The compression factor, G, can in turn be estimated from Fig. 5, where G is plotted as a function of b. Finally, the experimental quantities t_0 and σ_t are determined from the chromatogram.

6. CONCLUSIONS

The reciprocal of the gradient steepness parameter, b, increases for shallower gradients (smaller values of Φ'). The quantity $1/b$ plays an almost identical role in gradient elution as the parameter k' does in isocratic separation. Thus separation in gradient elution can be understood and controlled very much as in isocratic elution. Part II[1] summarizes a number of specific rules in this connection, and provides experimental illustration and verification of various conclusions presented in preceding sections of this paper. The various similarities that exist between isocratic and gradient elution when we substitute $1/b$ from the latter for k' in the former are summarized in Table 5.

For reversed-phase gradient elution, the parameter b is defined by the experimental variables Φ' (%/min change in concentration of B in the mobile phase), the solvent strength S of the pure solvent B and the column dead-time t_0 (min) as

$$b = 100\,\Phi'\,S\,t_0 \tag{16}$$

TABLE 5

ANALOGIES BETWEEN ISOCRATIC AND GRADIENT ELUTION WHEN k' (ISOCRATIC) IS SUBSTITUTED FOR $1/b$ (GRADIENT)

Variable	*Depends on*		*Equation*
	isocratic elution	*gradient elution*	
Calculated capacity factor	$(t_R - t_0)/t_0 = k'$	$(t_g - t_0)/t_0 \approx (\log k_0)/b$	Eqn. 3b
Separation time, t_s	$1 + k'$	$(\log k_0)/b$	Eqn. 3b
Resolution, R_s	$k/(1 + k')$	$\bar{k}/(1 + \bar{k})$, where $\bar{k} = 1/1.3b$	Eqn. 10
Detection sensitivity	$1/(1 + k')$	$1/G(1 + k_f)$, where $k_f = 1/2.3b$	Eqn. 6a
Maximal resolution	Optimal k' varies with column, but is usually $3 < k' < 6$	Optimal b varies with column, but is usually given by $3 < \bar{k} < 6$, where $\bar{k} = 1/1.3b$	Appendix II

Values of S for various organic solvents are summarized in Table 3. Resolution increases as b decreases, but so does the separation time. For a given separation time, there is an optimal value of b which is generally close to 0.2, but which can vary by ± 0.1 with little effect on resolution. Resolution can be increased, while holding b constant, by either decreasing F or increasing L (see Part II[1] for a detailed discussion). Table 4 summarizes optimal gradient rates, Φ', for varying t_0 and different organic solvents B that are used in the water–B gradient. The parameter Φ' should be held constant during a gradient elution run, which means that the solvent gradient in reversed-phase gradient elution should be linear.

The detection sensitivity increases as b is increased. For a 5-fold increase in b (from the normally optimal value of 0.2–1.0), the detection sensitivity will increase 3-fold, but with a 3-fold loss in resolution (see Table 2).

7. SYMBOLS

A, B refers to solvents A (water) and B (organic) used in the gradient program.

b coefficient in eqn. 2; larger values of b correspond to steeper gradients (eqn. 14).

F flow-rate of mobile phase through column (ml/sec).

G band compression factor[4]; corresponds to fractional reduction in width of band as a result of compression; see Fig. 5 for G as a function of b.

k' capacity factor[11].

k_a actual value of k' for a band in gradient elution at some time t during elution; determined by the composition of the mobile phase at the same point in the column where the band is located.

k_f value of k' for a band at the moment it leaves the column in gradient elution; equal to k_a at time $t = t_g$.

k_i value of k' for a given compound if injected at the column inlet at any time t after a gradient elution separation begins; equal to k' in an isocratic separation, using mobile phase of the same composition as that entering the column at time t in gradient elution.

k_0 value of k' for a compound at the beginning of gradient elution ($k_0 = k_a$ or k_i at $t = 0$); equal to k' in an isocratic separation with same mobile phase used to begin gradient elution.

k_w a value of k' for a given compound, with water as mobile phase; an extrapolated value based on eqn. 12.

$\bar{k}$ a (roughly) average value of k_a during gradient elution (Fig. 3); $\bar{k}$ determines R_s as a function of b.

L column length (cm).

LSS linear solvent strength.

n column parameter as defined by eqn. ii-3 (Appendix II).

N column plate number, defined by eqn. 15 for isocratic elution or eqn. 15a for gradient elution.

P pressure drop across column (p.s.i.).

Q column efficiency factor, equal to $k'/(1 + k')$ for isocratic elution and $\bar{k}/(1 + \bar{k})$ for gradient elution.

RP-GE reversed-phase gradient elution.

R_s resolution factor; eqns. 1, 8 and 9 for gradient elution. (see also ref. 11 for analogous expression in isocratic elution).
S solvent strength parameter; see eqn. 12.
s_g, s_k sensitivity parameters, equal to peak height in gradient (g) or isocratic (k) elution relative to height of t_0 band in isocratic elution (see eqns. iii-6 and iii-7, Appendix III.
t time after sample injection and start of gradient (sec).
t_g retention time in gradient elution; time (sec) from sample injection to elution of band maximum from column.
t_0 column dead time[11] (sec).
t_1, t_2 values of t_g for adjacent bands 1 and 2 (eqn. 8).
t_R retention time (sec) in isocratic elution[11].
t_s time to complete separation after sample injection; equal variously to time from beginning to end of gradient program, or time to elute last sample band (sec).
u velocity (cm/sec) of mobile phase in column.
V volume of mobile phase eluted from column after sample injection and start of gradient (ml).
V_g retention volume of band in gradient elution (ml); analogous to retention volume in isocratic separation[11].
V_m total volume of mobile phase contained within column (ml).
V_t instantaneous, corrected retention volume for a band at some time during gradient elution; equal to $k' V_m$ (ml).
x fractional distance a band has migrated along column at some time t (see Fig. 4).
α separation factor for two adjacent bands; defined for isocratic elution as in ref. 11.
σ_0 σ_t value in isocratic elution for $k' = 0$ (sec).
σ_t width of eluted band in either isocratic or gradient elution; standard deviation of Gaussian band (sec).
σ_1, σ_2 σ_t values for adjacent bands (1) and (2).
σ_x width of band on column, just prior to elution (cm).
Φ_b volume fraction of organic solvent B in water–organic mixture.
Φ' rate of change of Φ_b with time: $d\Phi_b/dt$ (sec^{-1}) (can also be expressed as %/min).
Φ_0 initial value of Φ at $t = 0$.
Φ_f final value of Φ at end of gradient.

8. APPENDIX I

Derivation of retention time, t_g, and capacity factor at time of elution, k_f, in LSS gradient elution

From the fundamental equation of gradient elution (*e.g.*, ref. 2), we have for the retention volume, V_g

$$\int_0^{V_g} (dV/V_a) = 1 \qquad \text{(i-1)}$$

Here dV refers to the passage of a differential volume of mobile phase through the band center, and V_a is the instantaneous, corrected retention volume (corresponding to k_a):

$$V_a = k_a V_m \tag{i-2}$$

The quantity V_m refers to the dead-volume of the column. Eqn. 2 can be re-stated as

$$\log k_i = \log k_0 - b(V/V_m) \tag{i-3}$$

and k_i and k_a then become equivalent in terms of eqn. i-1. Substitution of k_i from eqn. i-3 for k_a in eqn. i-2, followed by substitution of V_a from eqn. i-2 into eqn. i-1, then gives

$$\int_0^{V_g} \frac{10^{bV/V_m}\, dV}{k_0 V_m} = 1 \tag{i-4}$$

Integration of eqn. i-4 then gives eqn. 3.

The value of k' for a band at the time of elution (k_f) is obtained by substituting the corrected retention volume ($V_g - V_m$) from eqn. 2 into eqn. 1a:

$$\log k_f = \log k_0 - \log (2.3k_0 b \div 1) \tag{i-5}$$

Eqn. i-5 can then be rearranged into eqn. 6.

9. APPENDIX II

Optimal value of b in gradient elution for column length L, fixed and variable separation time

We desire to maximize the effective plate number, NQ^2, for a column of fixed length L, for various separation times t_s (and corresponding variation in mobile phase flow-rate, F, and velocity, u) by optimizing the gradient parameter b. For a given value of t_s, the k_i value of the last eluted band (k_z) is given from eqn. 2 as

$$\log k_z = \log k_0 - b(t_s/t_0)$$

which rearranges to

$$b = (\log k_0/k_z)\,(t_0/t_s)$$

As $u = L/t_0$,

$$b = [(\log k_0/k_z)\, L/t_s]u \tag{ii-1}$$

As the bracketed factor on the right is constant for a given separation, b is seen to vary inversely as u, *i.e.*,

$$b = C/u \tag{ii-2}$$

Now we can approximate the plate height H in most cases[11,16,17] by

$$H = D\,u^n \tag{ii-3}$$

Combining eqns. ii-2 and ii-3, we obtain

$$H = (DC^n)/b^n \tag{ii-4}$$

As the effective plate number of the column can be represented as

$$NQ^2 = (L/H)\,[\bar{k}/(1 + \bar{k})]^2$$

H from eqn. ii-4 and $\bar{k}$ from eqn. 10 can be substituted into the latter relationship to yield

$$NQ^2 = (L/DC^n)\,b^n\,[1/(1.3b + 1)]^2 \tag{ii-5}$$

The optimal value of b, for maximal NQ^2 in eqn. ii-5, is obtained in the usual fashion by differentiation to give

$$b\ (\text{optimal}) = n/(2.6 - 1.3n) \tag{ii-6}$$

The column parameter n for pellicular packings is 0.4, which yields an optimal value of $b = 0.24$. For large-particle separations with porous packings, n is usually 0.4–0.6, corresponding to $b = 0.24$–0.33. For small-particle (*e.g.*, $d_p \leqslant 10\ \mu$m) separations, n is usually smaller, as discussed in ref. 17. However, eqn. ii-3 is also less reliable, so a different approach to discussing the optimal value of b is indicated.

For the case of small particles, we can describe $H = hd_p$ as function of the reduced plate height, h and reduced velocity, $v = u\,d_p/D_m$ (see discussion in ref. 17), where D_m is the sample diffusion coefficient in the mobile phase. h is then given as a function of v for well packed columns of porous particles[17]:

$$h = 2/v + v^{0.33} + 0.05\,v \tag{ii-7}$$

Finally, NQ^2 can be calculated as above, substituting the latter expression for H for eqn. ii-3:

$$NQ^2 = (Lh/d_p)\,[\bar{k}/(1 + \bar{k})]^2 \tag{ii-8}$$

As an example, consider the case discussed in Part II[1] of a 25-cm column of 5-μm particles, a 5–95% gradient of methanol–water and a separation time of 20 min. Assume the calculation for the case of $b = 0.1$. Let the initial value of Φ_b (0.05) be given as Φ_0, and the final value ($\Phi_b = 0.95$) be Φ_f. Combination of eqns. 2 and 12 then yields

$$b = S\,(\Phi_f - \Phi_0)/(t_s/t_0) \tag{ii-9}$$

Inserting the above experimental conditions, we have

$$0.1 = 3.0\,(0.95 - 0.05)/(20 \cdot 60/t_0)$$

from which $t_0 = 44$ sec. The velocity, u, of the mobile phase is then $L/t_0 = 25/44 = 0.57$ cm/sec. We can calculate the reduced velocity, ν, as

$$\nu = u\, d_p/D_m \qquad \text{(ii-10)}$$

assuming that the solute diffusion coefficient is $3 \cdot 10^{-5}$ (see ref. 17): $\nu = 0.57 \cdot 0.0005/0.00003 \doteq 9.5$. From eqn. ii-7, this yields a value of $h = 2.78$. Similarly, from eqn. 10, $\bar{k} = 1/1.3 \cdot 0.1 = 7.7$. Inserting the latter values into eqn. ii-8, we obtain

$$NQ^2 = (25 \cdot 2.78/0.0005)\,(7.7/8.7)^2 = 10{,}900$$

It is found for a particular set of conditions that characteristic plots of NQ^2 *versus* ν result, as illustrated for a 10-μm particle, reversed-phase separation at room temperature, shown in Fig. 7 for a 25-cm column. In this case, as the separation time increases the optimal value of b shifts from a value of about 0.2 to lower values, *e.g.*, 0.08 for $t_s = 75$ min.

The curves in Fig. 7 do not change as L is varied, other than to give a proportionate increase in NQ^2 and t_s for an increase in L. For smaller particles, *e.g.*, 5-μm diameter packings, the same basic curves of Fig. 7 apply, except that the NQ^2 values are proportionately greater and t_s proportionately smaller, *e.g.*, 3.7, 11.2 and 37.5 min in Fig. 7.

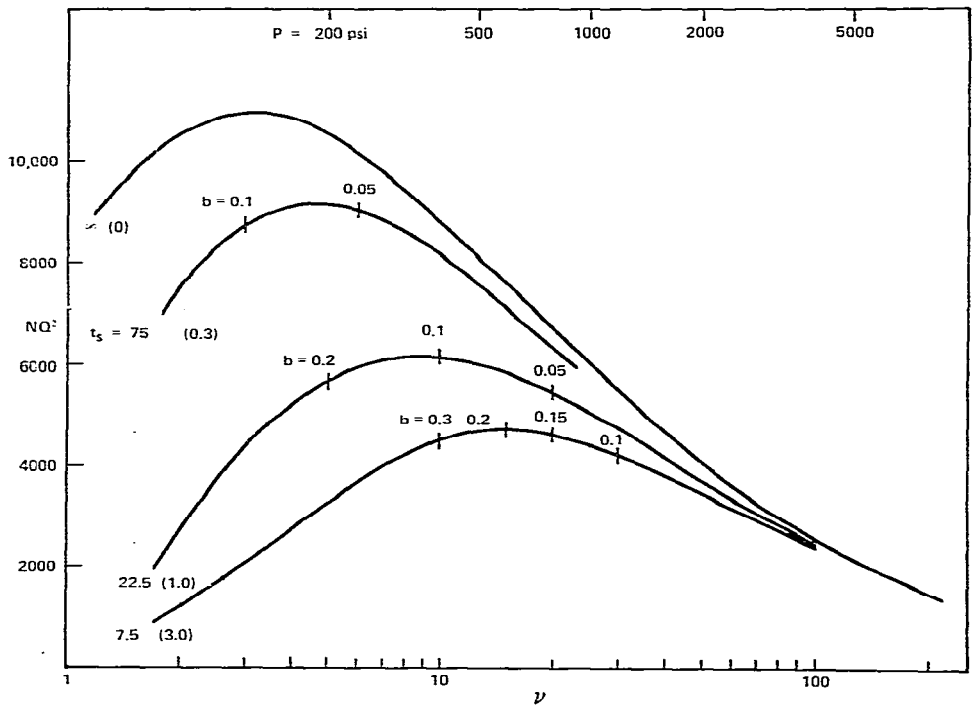

Fig. 7. Variation of NQ^2 with reduced velocity, ν, and b in gradient elution. Assumes 0–100% methanol–water gradient, 25-cm column of 10-μm porous particles for reversed-phase separation of molecular weight 300 sample at toom temperature. Values in parentheses refer to the product $b\nu$.

10. APPENDIX III

Derivations of sensitivity equations for isocratic and gradient elution

The column plate number, N, can be defined in terms of retention time, t_R, and band width, σ_t (standard deviation of Gaussian curve, in time units):

$$N = (t_R/\sigma_t)^2 \qquad \text{(iii-1)}$$

The retention time is in turn given as

$$t_R = (1 + k')\, t_0 \qquad \text{(iii-2)}$$

From eqns. iii-1 and iii-2, we obtain

$$\sigma_t = (1 + k')\, t_0/N^{\frac{1}{2}} \qquad \text{(iii-3)}$$

and the band width, σ_0, of a non-retained band ($k' = 0$) is then

$$\sigma_0 = t_0/N^{\frac{1}{2}} \qquad \text{(iii-4)}$$

We can define a sensitivity function, s_k:

$$s_k = \sigma_0/\sigma_t \qquad \text{(iii-5)}$$

As peak height and sensitivity are inversely proportional to band width, s_k represents the relative height (and sensitivity) of a band eluted with some value of k', relative to a band eluted at t_0. From eqns. iii-3–iii-5, we have

$$s_k = 1/(1 + k') \qquad \text{(iii-6)}$$

In a similar manner, we can define a sensitivity function, s_g, for gradient elution bands from eqn. 7a:

$$\begin{aligned} s_g &= \sigma_0/\sigma_{t,t} \\ &= 2.3\, b/(2.3\, b + 1)G \\ &= 1/G(1 + k_f) \end{aligned} \qquad \text{(iii-7)}$$

11. APPENDIX IV

Changes in separation selectivity with change in Φ' (reversed-phase LC)

Workers who have used gradient elution have often observed that for a given mobile phase A–B, a change in the gradient steepness, Φ', can lead to changes in band position within the final chromatogram. We shall show that this can occur for any two bands whose b or S values are different for that LC system.

Assume two compounds i and j with k_0 values of 100 (i) and 465 (j), S values of 3 (i) and 4.5 (j), and $t_0 = 1$ min. The parameter b is defined by eqn. 14 ($= \Phi'\, S\, t_0$),

and can be calculated for various values of Φ'. If we assume Φ' varies as below, we can then calculate t_g for each band (i and j) from eqn. 3a:

Band	t_g *(min)*		
	$\Phi' = 0.033$	$\Phi' = 0.067$	$\Phi' = 0.133$
i	14.8	9.4	5.9
j	15.7	9.4	5.7

It can be seen that the two bands have equal t_g values (no separation) for $\Phi' = 0.067$, whereas the bands are separable at either higher or lower values of Φ' (steeper or shallower gradients). Or, as the gradient steepness is increased from $\Phi' = 0.033$, band i is eluted first, but then band j overtakes band i for $\Phi' > 0.067$, and the band positions are reversed.

12. APPENDIX V

Deviations from eqn. 12 and their effect on separations in reversed-phase gradient elution

Schoenmakers *et al.*[10] carried out a detailed study of the variation of k' with Φ_b for 16 solutes and three different solvents B (methanol, ethanol and propanol). On the basis of these data they suggest that eqn. 12 is generally invalid, and k' as a function of Φ_b is instead given by a fitting function of the form

$$\log k' = A\,\Phi_b^2 + B\,\Phi_b + C \qquad \text{(v-1)}$$

Examples of resulting plots of $\log k'$ *versus* Φ_b for two such solutes from ref. 10 are redrawn in Fig. 8, and the curvature of these data is readily apparent. While the actual experimental data obtained by Schoenmakers *et al.*[10] are not reported for verification of these curves, other data[1,32–34] show similar non-linearity of these $\log k'$ *versus* Φ_b plots. However, the effect of this curvature of the plots in Fig. 8 (and elsewhere) on the resulting gradient elution separation is less pronounced than might be expected. The reason is that for an optimal gradient (LSS program with $b \approx 0.2$), migration of a band along the column occurs mainly when $2 < k_a < 8$ (*e.g.*, Fig. 4a, for $x \geqslant 0.2$). If we compare plots such as those in Fig. 8 with corresponding "best fit" linear curves over this range[2–8] in k' values, we find resulting deviations of experimental k' values from the linear curve of no more than about 3%. We can show this better by taking the average A, B and C values from ref. 10 for methanol–water as mobile phase and the 16 solutes studied. This average k' *versus* Φ_b plot is then given as

$$\log k' = 1.88\,\Phi_b^2 - 5.24\,\Phi_b + 3.06 \qquad \text{(v-2)}$$

The best linear fit to this curve can be calculated from the tangent at $x \approx 0.5$, or $k' \approx 4$. This yields a value of $S = 3.02$ ($\Phi_b = 0.59$). We can now calculate the various separation parameters for a model case, first assuming that eqn. 12 is correct ($S = 3.02$), then repeating the calculation using the true curve (eqn. v-2). The latter cal-

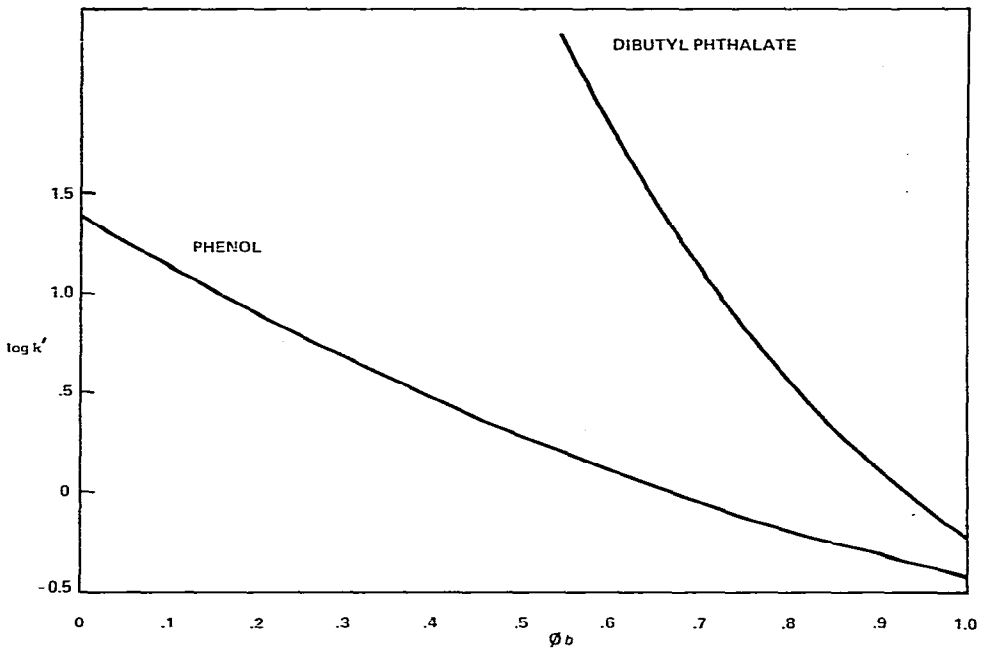

Fig. 8. Calculated plots of log k' *versus* Φ_b for phenol and dibutyl phthalate from data in ref. 10. Mobile phase, methanol–water.

culation requires numerical integration of eqn. i-1, while the former calculations are summarized in the text (eqns. 3a, 6a and 10). Calculated data for these two cases are as follows [assuming $b = 0.2$ at $k' = 4$, $k_0 = 64$ (eqn. 12) or 135 (eqn. v-2)]:

Parameter	*Value*	
	Eqn. 12	*Eqn. v-2*
$\frac{t_g - t_0}{t_0}$	7.4	7.5
k_f	2.1	2.0
$\bar{k}$	3.7	3.4

These deviations in absolute separation parameters due to the failure of eqn. 12 are even less significant in practical applications of the theory described in the main text. There we are concerned with relative, rather than absolute, changes as a function of separation parameters. Considerable cancellation of errors introduced by using eqn. 12 then results.

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14. SUMMARY

A general theory of separation is presented for gradient elution with reversed-phase systems. Expressions for retention, resolution, band width and other separation parameters are presented as a function of experimental variables. So-called "linear solvent strength" gradients are assumed.

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