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DESIGN OF OPTIMIZED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC GRADIENTS FOR THE SEPARATION OF EITHER SMALL OR LARGE MOLECULES

II.* BACKGROUND AND THEORY

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SUMMARY

The effect of the gradient on high-performance liquid chromatographic separations has been examined from a theoretical standpoint, using computer simulations to visualize the effects of different variables. Samples to be separated by gradient elution can be classified according to their separation characteristics into three groups (referred to here as cases I, II and III). Each of these sample types responds differently to a change in gradient conditions.

Case III samples exhibit changes in band spacing when the gradient conditions are varied, and gradients composed of multiple linear segments are especially useful for controlling band spacing and resolution for such samples. The effect of different gradient conditions (starting %B, gradient steepness and gradient shape) on the separation of case III samples is examined in detail.

INTRODUCTION

In some respects, the design of an appropriate gradient for a given sample in high-performance liquid chromatography (HPLC) is not a difficult task. Conventional wisdom[1–3] suggests that gradient steepness can be decreased for adequate resolution, then the gradient range (change in %B during the gradient) can be narrowed to save time. This procedure is illustrated in Figs. 1 and 2 for the simulated separation by gradient elution of a ten-component sample. Fig 1 shows that a 100-min gradient provides adequate resolution (Fig. 1d), which is not improved by longer gradients. In Fig. 2, a similar separation as in Fig. 1d results by trimming the gradient from 5–100% B to 40–85% B (Fig. 2f) while holding the gradient steepness constant.

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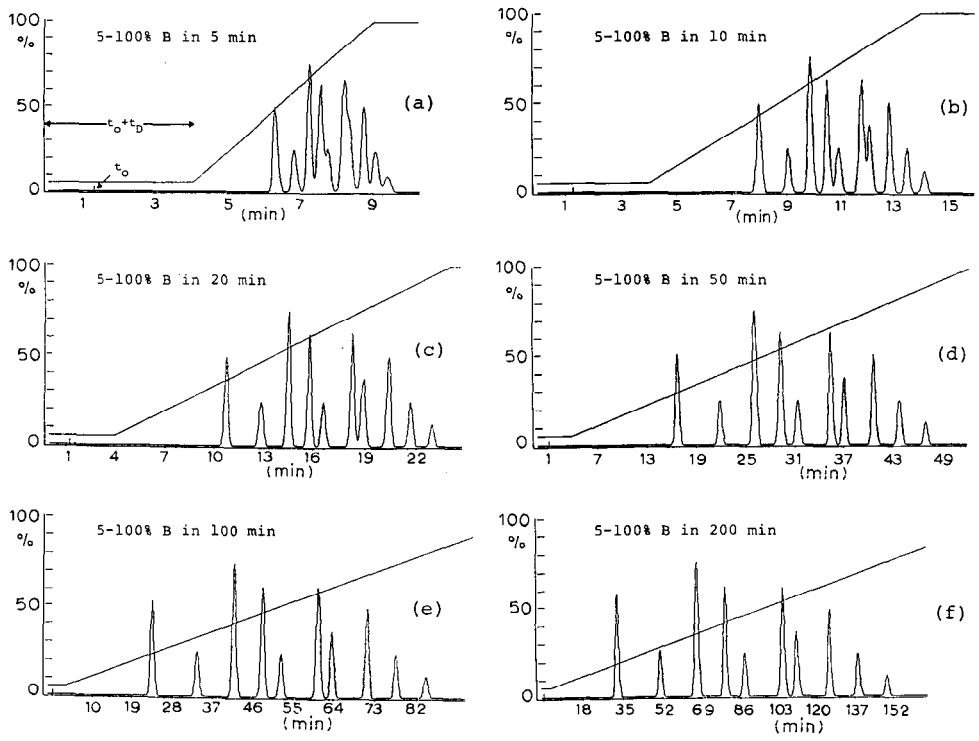

Fig. 1. Computer simulations (DryLab G) of the effect of gradient time on separation. Input conditions: V_D = 5.5 ml; 25 × 0.46 cm I.D. column; flow-rate, 2 ml/min; 5–100% B; t_G = 20 and 60 min; S = 5 for all compounds; log k_w = 2.0, 2.5, 2.9, 3.2, 3.4, 3.8, 3.95, 4.3, 4.6 and 4.9.

However, the latter separation requires only half as much time (47 min in Fig. 2f *vs.* 100 min in Fig. 1d).

Gradient shape is less often manipulated, although for some samples it is obvious that step gradients or gradient holds will be beneficial. As the sample complexity increases, however, there are greater rewards to be reaped from optimizing the gradient shape, *i.e.*, precisely tailoring the gradient to meet the needs of a given sample. Samples also often differ with respect to how relative retention changes with the gradient conditions; such differences can further favor the use of gradients having special shapes

The relationship between changes in gradient conditions and resulting changes in the chromatogram are widely, if somewhat vaguely, understood. These rough perceptions are often inadequate when dealing with very complex samples (such as many mixtures of peptides or proteins). This immediately becomes clear when using computer simulation as a means of developing a gradient method[4]. With this approach, many different gradients can be tried within a few minutes, and any limitations on the accuracy of our intuitional predictions quickly become apparent.

In this paper, we have tried to develop a better understanding of how chromatographic separation responds to various changes in gradient shape. Our

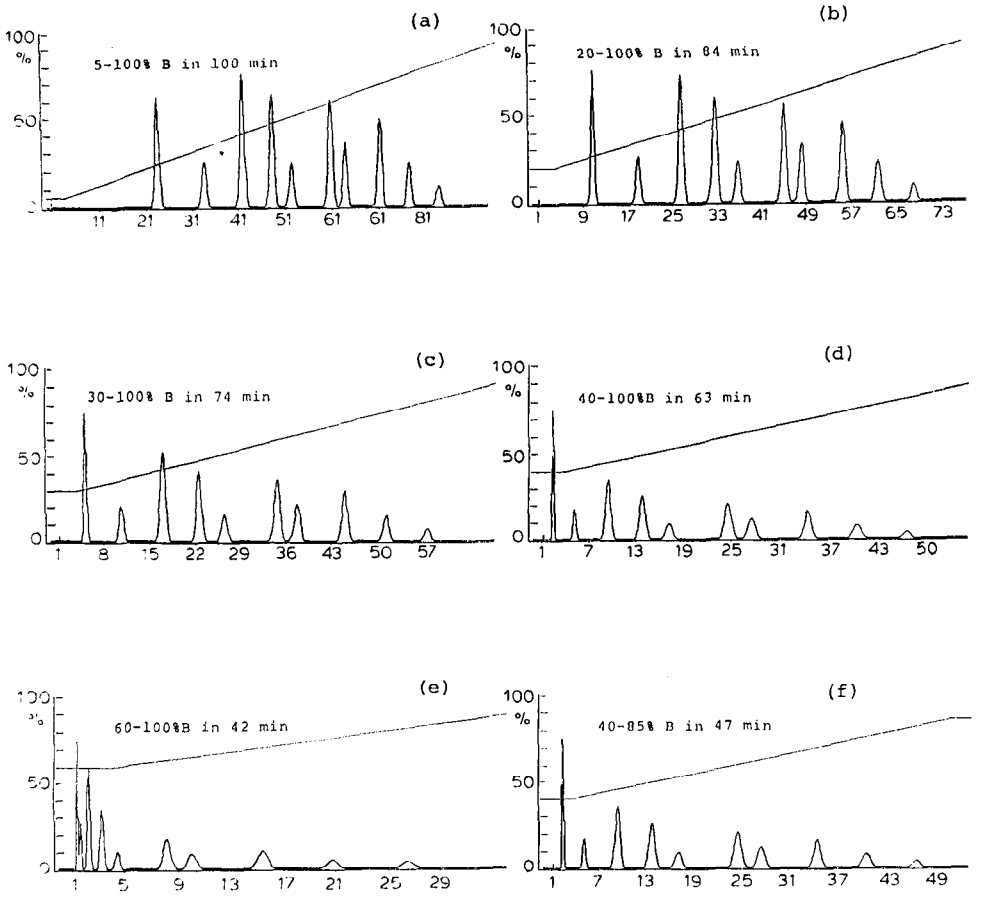

Fig. 2. Computer simulations (DryLab G) of the effect of gradient range on separation. Gradient steepness held constant. Conditions as in Fig. 1, unless indicated otherwise.

approach has been to use computer simulations (DryLab G software), where the results of change in the gradient are modeled according to theory without any of the (many) complications that can occur in experimental gradient separations. We have shown elsewhere[4–8] that (with suitable precautions) gradient simulation accurately predicts the results obtained from actual experimental runs, thus validating the approach that follows.

In the following paper[8], we apply the principles presented here for the design of optimized gradients for various samples.

THEORY

As discussed in the preceding paper[7], isocratic retention in reversed-phase systems can be approximated by

$$\log k' = \log k_w - S\varphi \tag{1}$$

For the definition of these symbols and others introduced in Part I[7], see the Glossary of Symbols in that paper. There is generally some relationship between values of S and k_w for the components of a given sample, and this relationship determines how the gradient conditions will affect separation.

It is useful to recognize three possible relationships between S and k_w (or sample retention):

Case I: S values are approximately constant for all sample bands (independent of k_w).

Case II: S values tend to increase with increasing values of k_w (and increasing solute retention).

Case III: S values vary randomly with k_w and band retention.

Other cases (of limited practical interest) can also be imagined.

Cases I and II (no band-spacing changes)

For many samples comprising mixtures of closely related compounds, values of S for each compound are approximately equal, leading to parallel plots of isocratic retention *vs.* mobile-phase composition φ as in Fig. 3a. The dashed horizontal line in Fig. 3a corresponds to conditions of constant k' for every compound in the sample, or to values of k' at elution (k_f) in gradient elution (which are also constant for all bands). If a linear gradient is used, the quantity k_f is given by

$$k_f = 0.42\, t_G\, F/V_m\, \Delta\varphi\, S \qquad (2)$$

In a given separation, values of t_G, F, V_m and $\Delta\varphi$ are constant. This means that if the values of S are equal for all sample components, the values of k_f will also be equal; this is equivalent to values of k_f falling on the dashed horizontal line in Fig. 3a for a given run.

Retention in gradient elution (linear gradients) will parallel the intersection of these (solid) plots with horizontal (dashed) lines, as in Fig. 3a. Hence, values of k_f determine corresponding values of φ at elution (φ_e), and values of φ are proportional to time t during the gradient (the scale for the chromatogram at the bottom of Fig. 3a).

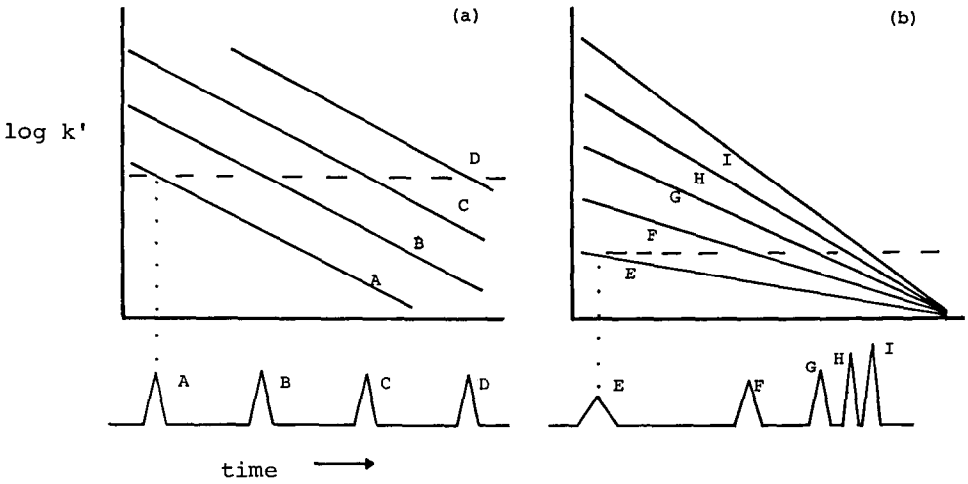

Fig. 3. Illustration of samples having a regular dependence of S on k_w. (a) Case I; (b) case II.

For constant S values (Fig. 3a) it is seen that the spacing of bands within the chromatogram tends to be regular and does not change with the gradient conditions (a change in t_G, F, V_m or $\Delta\varphi$ results in a change in k_f and a vertical shifting of the dashed line in Fig. 3a to higher or lower k_f values).

For many samples, there is a general tendency for values of S and log k_w to be linearly related[9,10]; in these cases S and sample retention usually increase continuously with increasing k_w. This gives rise to plots of log k' *vs.* φ as in Fig. 3b. It is seen that this results in a tendency for later bands to bunch together, with a resulting decrease in resolution; that is, a more or less regular spacing of bands is observed for Fig. 3a, but not for Fig. 3b* (linear gradients in each instance). However, the band sequence does not change with change in gradient conditions (just as for case I in Fig. 3a).

It is possible to make the spacing of bands as in Fig. 3b more even by using a convex gradient (*e.g.*, Fig. 16.9 in ref. 1 and Fig. 21 in ref. 2). However, such curved gradients come in a variety of forms, and the optimization of gradient curvature for a given sample is not straightforward. In the following paper[8], it will be seen that curved gradients are seldom required for samples as in Fig. 3b.

Case III (band-spacing changes)

The general pattern of Fig. 3b is often observed for samples such as homologues, benzologues, oligonucleotides and other oligomers[11–13]. For most samples containing peptides or proteins, however, the dependence of S on k_w is weak, leading to plots of log k' *vs.* φ as in Fig. 4a. Here, it is seen that the retention plots for each compound (A–G) are not parallel or congruent as in Fig. 3, but instead frequently intersect as φ is varied. For this example, four different gradient runs are indicated (dashed lines, t_G = 20, 40, 80 and 160 min). Note that gradient steepness can be defined as[1,2]

$$b = V_m \Delta\varphi \, S/t_G \, F = 1/(2.3 \, k_f) \tag{3}$$

If values of S for the sample are similar (as is often the case for non-oligomeric samples), then the value of k_f for each compound in a given gradient run (with V_m, $\Delta\varphi$, t_G and F fixed) will be roughly constant, corresponding to the horizontal dashed lines in Fig. 4a (as in Fig. 3a for case I samples).

The corresponding chromatograms for the 20-, 40- 80- and 160-min runs in Fig. 4a are shown in Fig. 4b. Because of the intersection of the different log k' *vs.* φ plots in Fig. 4 as φ is varied, the band spacing in these four runs varies:

20 min: A < B < E < D = C < F < G
40 min: A < B < C < D = E < F < G
80 min: A < B = C < D < E < F < G
160 min: A < B = C < D < E < F < G

If we compare Fig. 4a with the chromatograms in Fig. 4b, it is seen that the retention

* Note also that values of S increase with increasing solute retention, leading to smaller values of k_f and band narrowing (eqn. 2). This decrease in k_f also means that the dashed line in Fig. 3b (corresponding to band retention with a linear gradient) should be tilted (slightly) downward to the left.

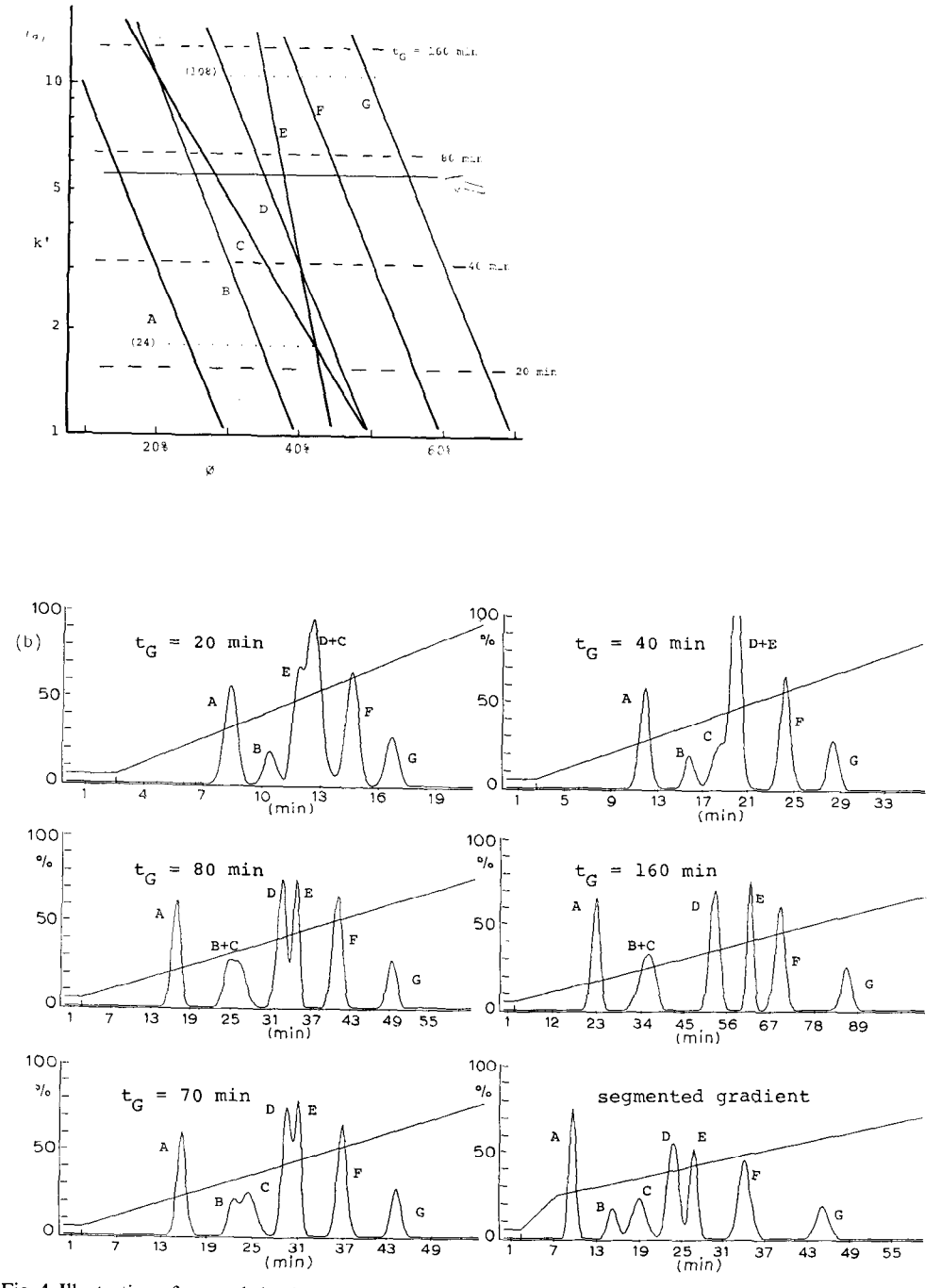

Fig. 4. Illustration of a sample having an irregular dependence of S on k_w (case III). (a) isocratic plots of log k' *vs.* φ (heavy diagonal lines); (b) gradient runs with t_G varying (5–100%B gradients). DryLab G simulations; arrow in (a) indicates the gradient time for an optimum overall band spacing (and resolution).

order for different gradient times is accurately predicted by the isocratic plots in Fig. 4a*.

For this particular sample, no single gradient time (20–160 min) provides a good spacing of the bands within the chromatogram, although the optimal time [t_G = 70 min; see Fig. 4a (arrow) and b] give a marginal resolution of all bands. However, we see that t_G = 20 min provides a good separation of bands A–C, and gradient times of 80–160 min provide good resolution for bands C–G. This suggests beginning with a steep gradient (similar to the 20-min gradient) for the elution and good resolution of bands A–C, followed by a decrease in gradient steepness (80 < t_G < 160 min) for the successful resolution of the remainder of the sample. This separation is shown in Fig. 4b (last chromatogram, "segmented gradient"). Now all seven bands are much better resolved.

The design of the optimal segmented gradient in Fig. 4b can be inferred from the log k' *vs.* φ plots in Fig. 4a. Thus in Fig. 4a it is seen that a gradient time of 24 min (4.8%/min) provides an optimal spacing of bands A–C (lower dotted horizontal line), whereas a gradient time of 108 min (0.9%/min) provides an optimal spacing of bands C–G (upper dotted horizontal line). The segmented gradient in Fig. 4b begins with a gradient steepness of 4.8% B/min, then changes to 0.9% B/min just prior to elution of band A. However, choosing the point in the gradient (φ*) for a change in gradient steepness is not so obvious, as discussed below.

Segmented gradients: effects of slope changes on retention

Segmented gradients (as in the foregoing example) have been successfully used for the improved resolution of complex samples[5,6]. However, this approach is less simple than suggested by the above discussion. The reason is that retention and band spacing depend not only on the steepness of the gradient during the time two bands are eluted, but also on the steepness (and duration) of the preceding gradient segment.

Consider first the migration of a band through the column during gradient elution, as pictured in Fig. 5. During the beginning of gradient elution (time < 10 min), the k' value of the band is usually large and the band remains at the column inlet. At some time during the gradient, the value of φ becomes large enough to decrease k' for the band to *ca.* 20, and the band begins to move through the column (at about 12 min in Fig. 5). Eventually the band leaves the column (at 24.5 min in the example in Fig. 5). Changes in the gradient prior to the time when $k' \approx 20$ will have little effect on the separation of a band from adjacent bands in the sample, because during this time ($k' > 20$) there has been little movement (< 10%) of the band(s) from the column inlet; that is, the history of the separation up to the point where $k' < 20$ is irrelevant to the subsequent migration and separation of these sample bands.

On the other hand, a change in gradient steepness after significant band migration has occurred (segmented gradient as in Fig. 4b) means that the band *will* have

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* The horizontal lines in Fig. 4a correspond to k' values for each solute when that solute has migrated 40% of the way through the column, corresponding to $k' = 1.25\,\bar{k}$. This reflects the fact that separation is influenced more by initial elution ($k' > \bar{k}$) than by later elution, because resolution is greater for larger values of k', other factors being equal.

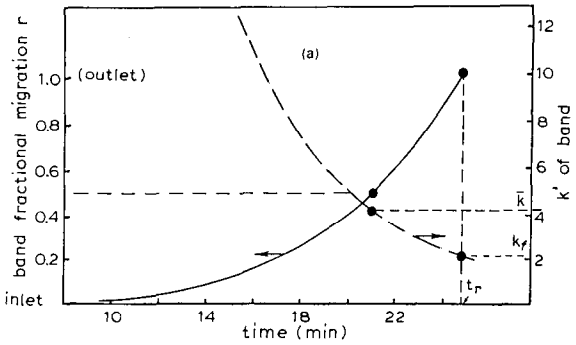

Fig. 5. Illustration of migration of a sample band along the column during gradient elution (solid curve); corresponding dependence of solute k' value on time is indicated by the dashed line. See text for details.

been influenced by both gradient segments. In this instance, we cannot assume that the separation will be independent of the preceding gradient segment. In the use of segmented gradients, it is conceptually convenient to assume that the final separation is determined by the segment during which bands of interest are eluted. This then raises the question of how much time must have elapsed since a change in gradient steepness in order for this to be true; or, if the change in gradient steepness occurs at some value of $\varphi(\varphi^*)$, what value of $\varphi_e - \varphi^*$ is required for there to be no effect on separation by the preceding gradient segment.

The condition for 10% (or less) migration of a band during gradient elution is readily derived. The retention time of a band during gradient elution is given by eqn. 2 in ref. 7 (assumes that k_0 is large):

$$t_g = (t_0/b)\,[\log(2.3\,k_0\,b)] + t_0 + t_D \tag{4}$$

Similarly, the time required for migration of the band 10% along the column (from the inlet) is[2]

$$(t_g)_{10\%} = (t_0/b)\,[\log(0.23\,k_0\,b)] + 0.1\,t_0 + t_D \tag{5}$$

The last two terms in eqns. 4 and 5 can be ignored as far as band spacing is concerned, as they comprise a constant term for each of two adjacent bands. The difference in retention times (corresponding to band migration from 10 to 100% of the column length) is then

$$t_g - (t_g)_{10\%} = (t_0/b)[\log(10)] \tag{6}$$

which (with eqn. 3) gives

$$t_g - (t_g)_{10\%} = t_G/(\Delta\varphi\,S) \tag{7}$$

or

$$t_g(\Delta\varphi/t_G) - (t_g)_{10\%}\,(\Delta\varphi/t_G) = 1/S \tag{7a}$$

The two left-hand-side terms correspond to values of φ at elution (φ_e) and after 10% migration through the column (φ^*), so that

$$\varphi_e - \varphi^* = 1/S \tag{8}$$

Eqn. 8 indicates the condition for a prior gradient segment *not* affecting separation in a following segment. For example, if a band is eluted at $\varphi_e = 0.6$, and $S = 20$ for the compound, then a change in gradient steepness (new gradient segment) should start at $\varphi^* = 0.60 - (1/20) = 0.55$ or earlier, if the separation of the band from adjacent bands is not to be influenced by the preceding gradient segment (however, smaller values of $\varphi_e - \varphi^*$ may still yield acceptable resolution for a given sample, despite a significant change in resolution as a function of φ^*).

APPLICATION

Case III: principles of separation

Samples corresponding to case III are the most amenable to gradient optimization; that is, fine tuning the gradient can make a more significant difference in the final separation than for case I or II samples. Mixtures of peptides and proteins generally fall into case III, as do many other samples of both high and low molecular weight. Case III samples usually show an average improvement in resolution as gradient time is increased, similarly to the case I example in Fig. 1. For samples corresponding to case III, however, one or more band pairs will show maximum resolution for intermediate values of t_G. This is clearly different from the examples in Fig. 1 (case I) and Fig. 1 of the following paper[8] (case II).

Fig. 4 shows the advantage of using either an intermediate gradient time (70 min) or a multi-segment gradient for case III samples. Examples of this for both low- and high-molecular-weight samples have been reported[5,6]. Often the overall problem of designing an optimal gradient can be broken down into a series of "critical" band groups (*e.g.*, B/C and D/E in Fig. 4). By varying the gradient steepness for a critical group of adjacent bands, we can position the bands so as to achieve equal resolution for each band pair in the group (and thereby maximize the resolution of the poorest resolved band pair in the sample). In order to design an optimal gradient for case III samples, we need to understand how various changes in the gradient affect band spacing and resolution. The following discussion will attempt to provide this understanding, by using model three-component samples as examples.

Effects of gradient time and starting %B. Fig. 6 describes the isocratic retention of a hypothetical three-component sample as a function of mobile-phase composition (%B or φ). In this example, compound 2 has a much smaller value of S (3) than the other two compounds ($S = 5$). This will magnify the effects of gradient time and starting %B on band spacing. The separation of this sample with a 5–100% B gradient and different gradient times t_G is shown in Fig. 7a–c ("real" samples will normally exhibit less extreme changes in band spacing for similar changes in gradient conditions, because of smaller differences in S for adjacent bands). Band 2 is seen to change its position in the chromatogram as t_G (and gradient steepness) changes. Similarly, if the gradient steepness is held constant (runs in Fig. 7c–f), a change in the starting %B also

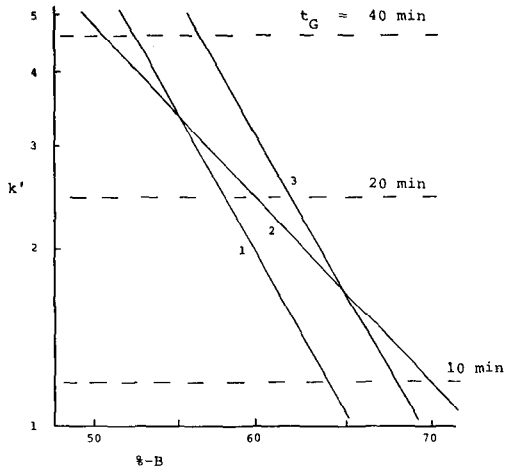

Fig. 6. Isocratic retention of a hypothetical three-component sample, showing band reversals with change in φ (isocratic) or t_G (gradient). Values of S: (1) 5.11; (2) 3.04; (3) 5.11. Values of log k_w: (1) 3.337; (2) 2.204; (3) 3.542.

leads to changes in band spacing. These variations in band spacing can be understood in terms of the retention diagram in Fig. 6. Thus, an increase in gradient time (10–40 min) corresponds to an increase in $k' = \bar{k}$ (dashed lines in Fig. 6), leading to the same change in band spacing as seen in Fig. 7a–c (relative retention of band 2 decreases). Likewise, an increase in %B (isocratic) leads to an increase in the relative retention of band 2 (Fig. 6); a similar increase in the relative retention of band 2 is seen in Fig. 7c–f for gradient elution when φ_0 is increased (as a result of the isocratic pre-elution of the sample by the starting mobile phase).

We have carried out simulations for other hypothetical samples, where both S and k_w for the bands were varied over wide limits. These results are plotted in Fig. 8 (circles) as values of $\varphi_e - \varphi_0$ *vs.* S for a 10% change in resolution. Also included (squares) are values for several critical band pairs found in the 30S ribosomal protein sample described in ref. 6. These data lie reasonably close to the solid curve in Fig. 8; the dashed curve is that predicted by eqn. 7. Differences between the two curves reflect (a) our arbitrary definition of a "sigificant" change in resolution (5% change in R_s), (b) a minor dependence of experimental values of $\varphi_e - \varphi_0$ on the differences in S values for the bands within the triplet and (c) other factors.

The main significance of Fig 8 is that it provides an approximate measure of when φ_0 (or φ^*) is likely to affect the separation of a band triplet. Note in Fig. 8 (top scale) that an estimate of $\varphi_e - \varphi_0$ (or $\varphi_e - \varphi^*$) can be obtained from the molecular weight of the sample; a value of S need not be known. Values of $\varphi_e - \varphi_0$ or $\varphi_e - \varphi^*$ estimated from Fig. 8 are conservative. Often larger values of φ_0 or φ^* can be used before an unacceptable loss of resolution occurs.

Segmented gradients: pre-elution. The simplest segmented gradient results from the use of a linear (unsegmented) gradient under normal conditions. When there is an appreciable dwell volume V_D in the system (as in all the present examples, *e.g.*, Fig. 7a), the initial elution of the sample is effected by a mobile phase with $\varphi = \varphi_0$ (isocratic

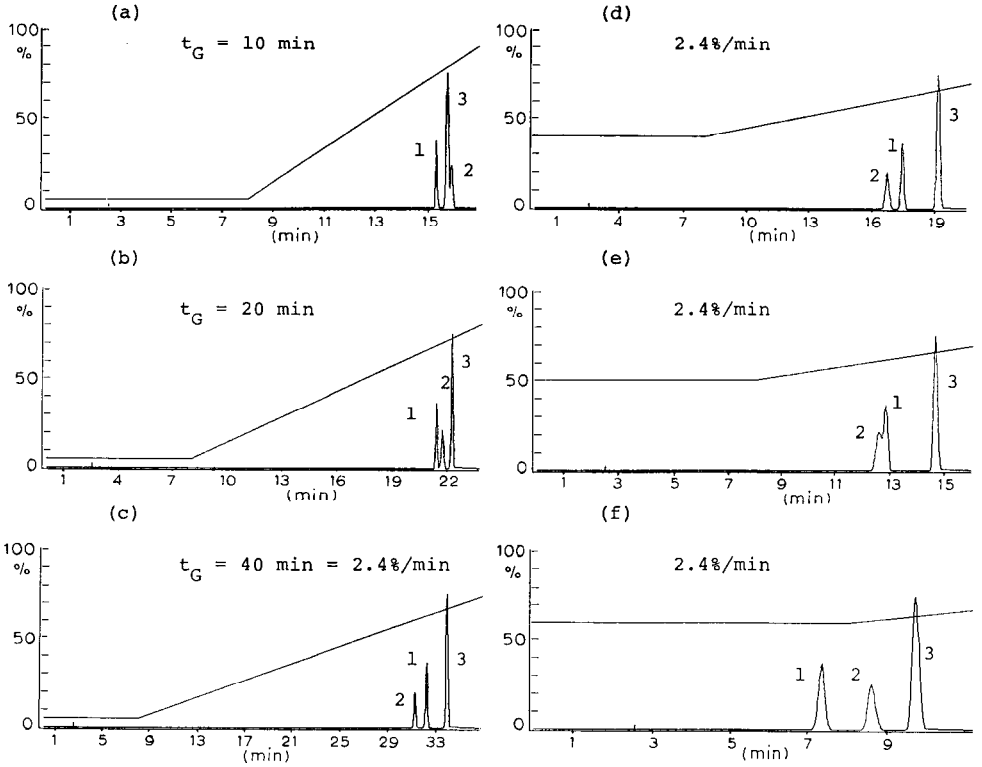

Fig. 7. Changes in band spacing for the sample in Fig. 6 when the gradient time t_G or the initial mobile-phase composition φ_0 is changed. Conditions: V_m = 2.5 ml; V_D = 5.5 ml; flow-rate, 1.0 ml/min; 5–100% B unless indicated otherwise.

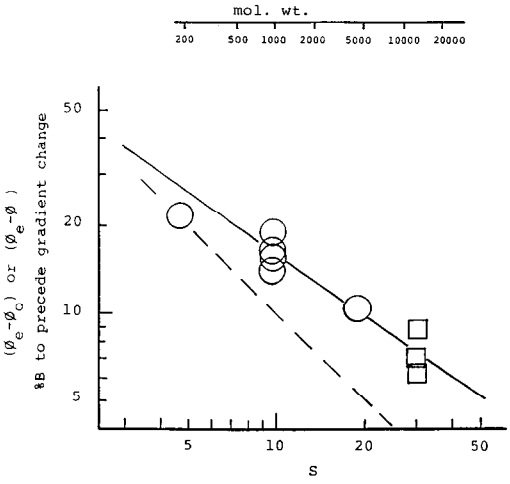

Fig. 8. Necessary length of gradient segment prior to elution of a critical band pair without loss of resolution. See text.

elution). Hence an initial segment equivalent to a very flat gradient precedes the gradient that is entered into the gradient controller. This particular case is of interest in its own right; it also provides a basis for understanding what happens when multi-segment gradients are intentionally used.

Fig. 9 shows further simulations for another hypothetical sample (involving smaller, more realistic, differences in S). Because the initial elution of the sample occurs under isocratic conditions (during passage through the column of a volume V_D of mobile phase having $\varphi = \varphi_0$), it is helpful to examine band elution under isocratic conditions. Fig. 9a shows the effect of a change in φ (isocratic elution) on the relative

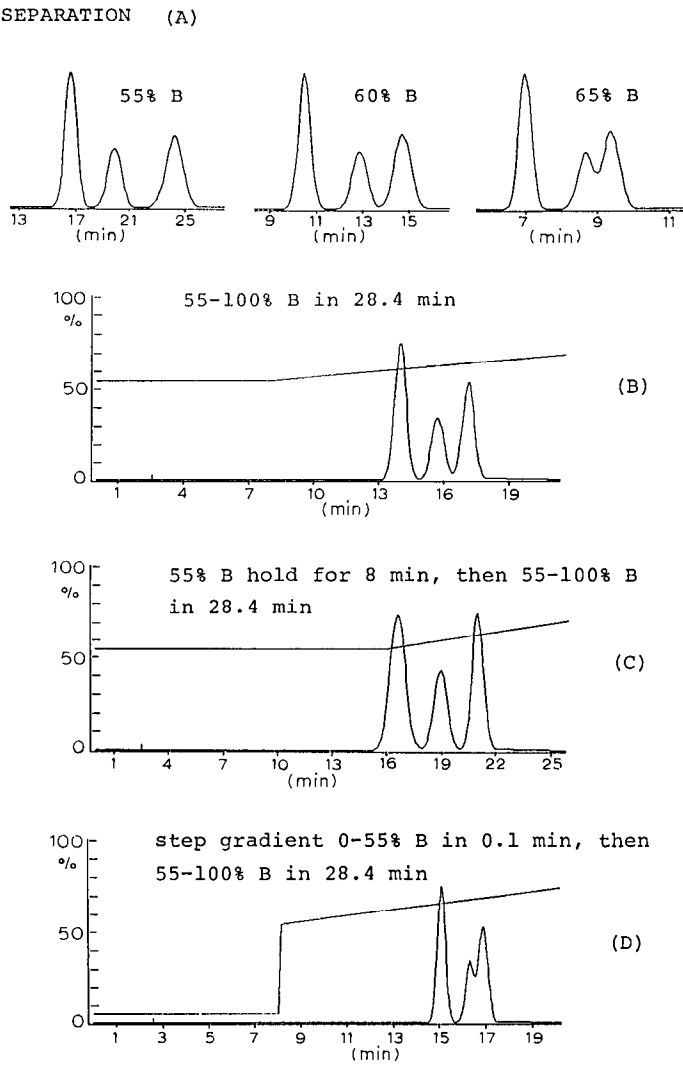

Fig. 9. Effect of isocratic elution in a preceding segment on separation in a following gradient segment. Conditions and sample as in Fig. 6, unless indicated otherwise. Values of S for these three compounds are 5, 5 and 4.5.

spacing and resolution of the three bands. As φ is increased, the middle band (smaller S value) moves toward the late-eluted band.

Now consider the effect of the dwell volume on the corresponding gradient separation (Fig. 9). The initial gradient separation (Fig. 9b, φ_0 = 55% B and V_D = 5.5 ml) shows the middle band slightly closer to the later eluted band, whereas the isocratic separation (Fig. 9a) with φ = 55% B places the middle band closer to the early eluted band. Increasing the value of V_D or incorporating a gradient hold at the

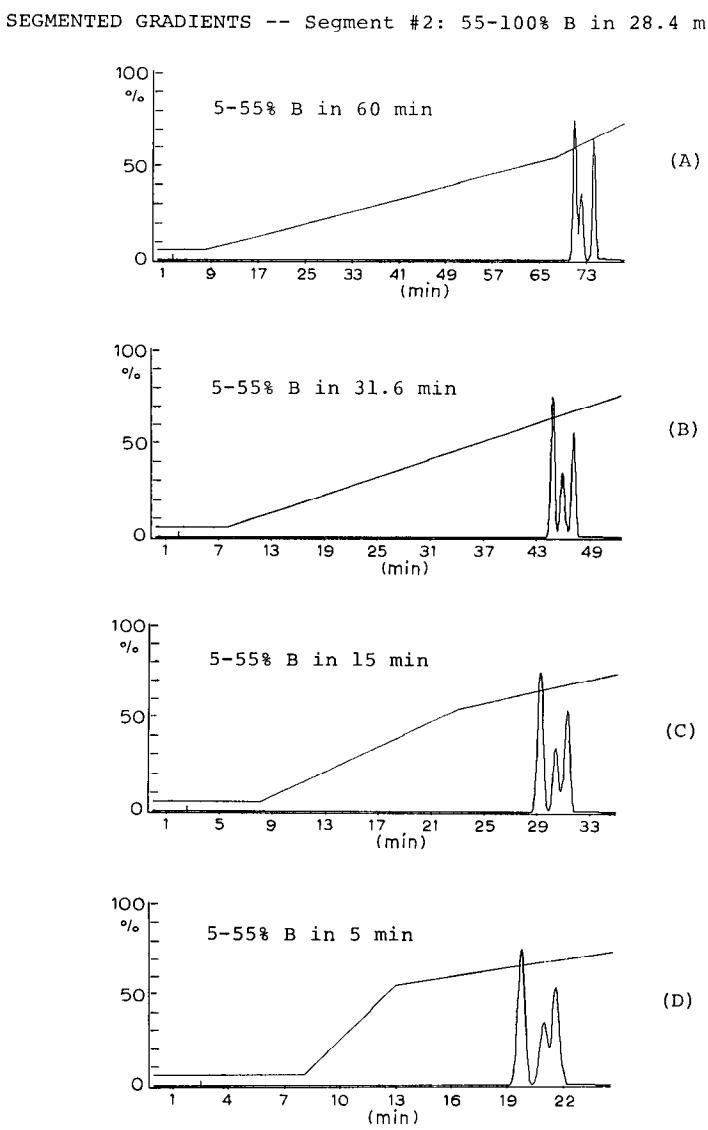

Fig. 10. Effect of prior gradient segment on separation of a critical band pair eluted in a subsequent segment. Conditions and sample as in Fig. 6, unless indicated otherwise.

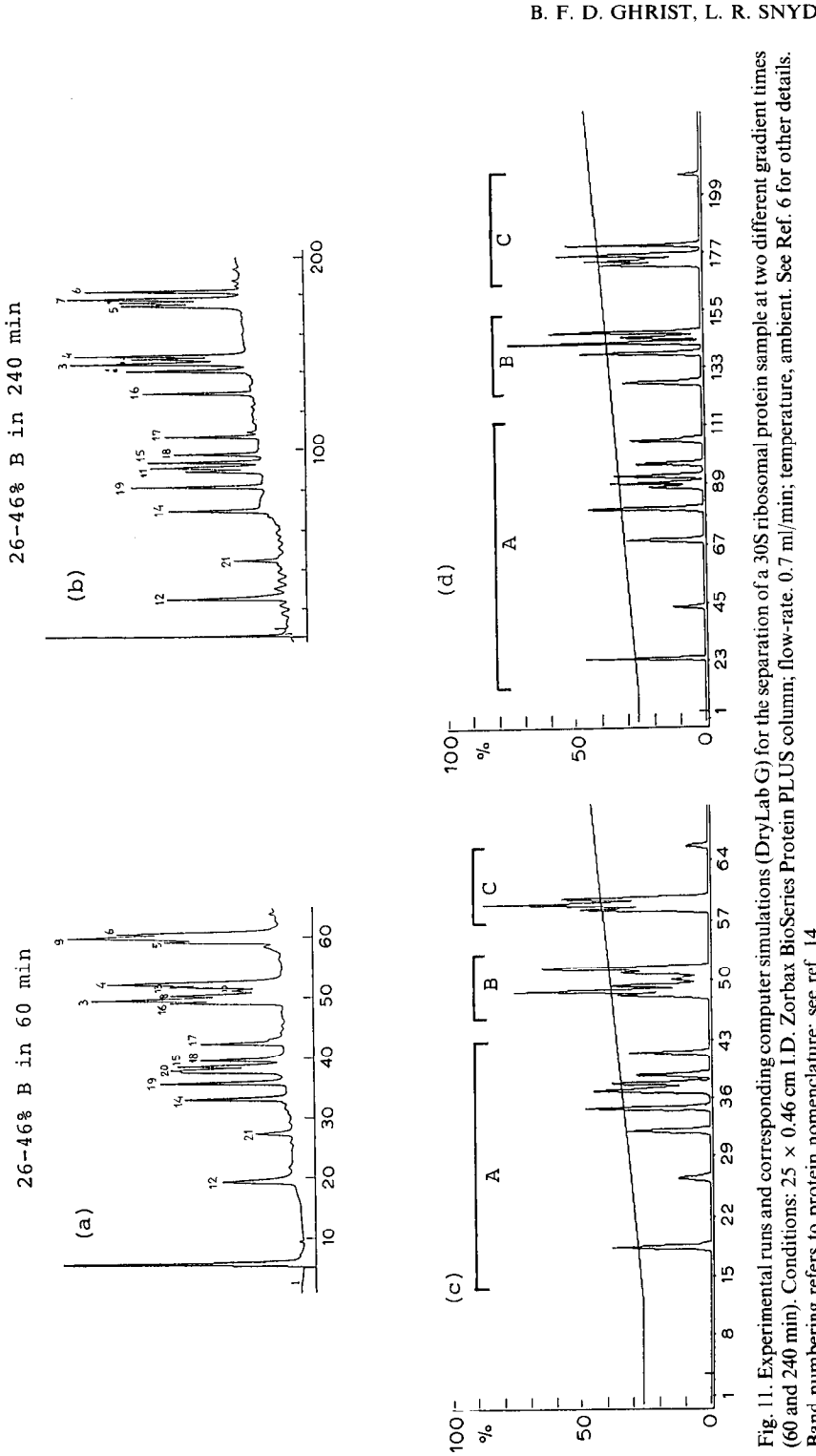

Fig. 11. Experimental runs and corresponding computer simulations (DryLab G) for the separation of a 30S ribosomal protein sample at two different gradient times (60 and 240 min). Conditions: 25 × 0.46 cm I.D. Zorbax BioSeries Protein PLUS column; flow-rate. 0.7 ml/min; temperature, ambient. See Ref. 6 for other details. Band numbering refers to protein nomenclature; see ref. 14.

beginning of the separation should lead to a separation more like that for isocratic elution with 55% B, and this is seen to be the case in Fig. 9c.

Conversely, the use of an initial step gradient freezes the sample at the column inlet until the actual start of the gradient. This should be equivalent to eliminating the dwell volume, giving the separation shown in Fig. 9d (movement of middle band toward later eluted band).

Segmented gradients: effect of prior segment. The effect of a prior gradient segment on separation by multi-segmented gradients is illustrated in Fig. 10. In each of these examples the second gradient segment (during which the sample bands are eluted) is held fixed at 55–100% B in 28.4 min. The preceding gradient segment varies from shallower (Fig. 10a) to equal (Fig. 10b) to steeper (Fig. 10c and d). These results (same sample as for Fig. 9) can be understood in terms of the similar examples in Fig. 9. The use of a shallower preceding segment will give rise to effects similar to those produced by more extensive pre-elution (larger value of V_D). Likewise, the use of a steeper preceding segment yields results like those obtained with a step gradient (Fig. 9d).

The examples in Figs. 6–10 plus the above discussion suggest that the effect of a previous gradient segment on the separation of bands eluted in a following segment can be complicated. From a practical standpoint, however, this is relatively unimportant. Changes in relative peak position (and resolution) as a result of the preceding segment can to a considerable extent be offset by changing the slope of the segment in which the bands of interest are eluted (as in Fig. 7). This is next illustrated by the separation of the 30S ribosomal proteins (Fig. 11).

The separation of the 30S ribosomal proteins by reversed-phase gradient elution yields chromatograms which can be subdivided into three distinct groups of bands: A, B and C in Fig. 11 [experimental (a and b) and simulated (c and d) chromatograms]. Bands 10–15 are eluted as a marginally resolved cluster (B), over a φ_e range of 38–39%. The closest preceding bands which are difficult to resolve (bands 5–7 in group A) are eluted before 35% B. We shall see that it is profitable to use different gradient slopes for the separation of bands 1–7 (A) and 10–15 (B); this means a change in slope will be required at about 35% B. As the value of $\varphi_e - \varphi^*$ for this sample (see Fig. 11) is about 7%, this suggests that the initial gradient segment (to elute bands 1–7) may have a significant effect on the resolution of bands 10–15 in the following segment. The question is whether we can still obtain the same resolution (regardless of the first segment) by varying the slope of the second segment, that is, whether slope adjustments can compensate for changes due to the preceding segment.

We can answer this question for the sample in question by constructing resolution maps for just the six bands of interest (bands 10–15) as a function of gradient range. The results for different values of φ_0 are shown in Fig. 12. It can be seen that there is no significant change in these maps until φ_0 exceeds 34%. Interestingly, the adjustment of gradient time for larger values of φ_0 leads to the same minimum resolution ($R_s = 0.8$) for $0 < \varphi_0 < 37\%$. Only when φ_0 exceeds 37% (Fig. 12g) and the sample is eluted isocratically does the minimum resolution drop below a value of $R_s = 0.8$. This example confirms that adjustments in the slope of a gradient segment can be used to largely overcome the effect of a preceding segment, even for $\varphi_e - \varphi^*$ values much smaller than predicted by Fig. 8. Hence $\varphi_e - \varphi_0 > 1\%$ is acceptable in the present case.

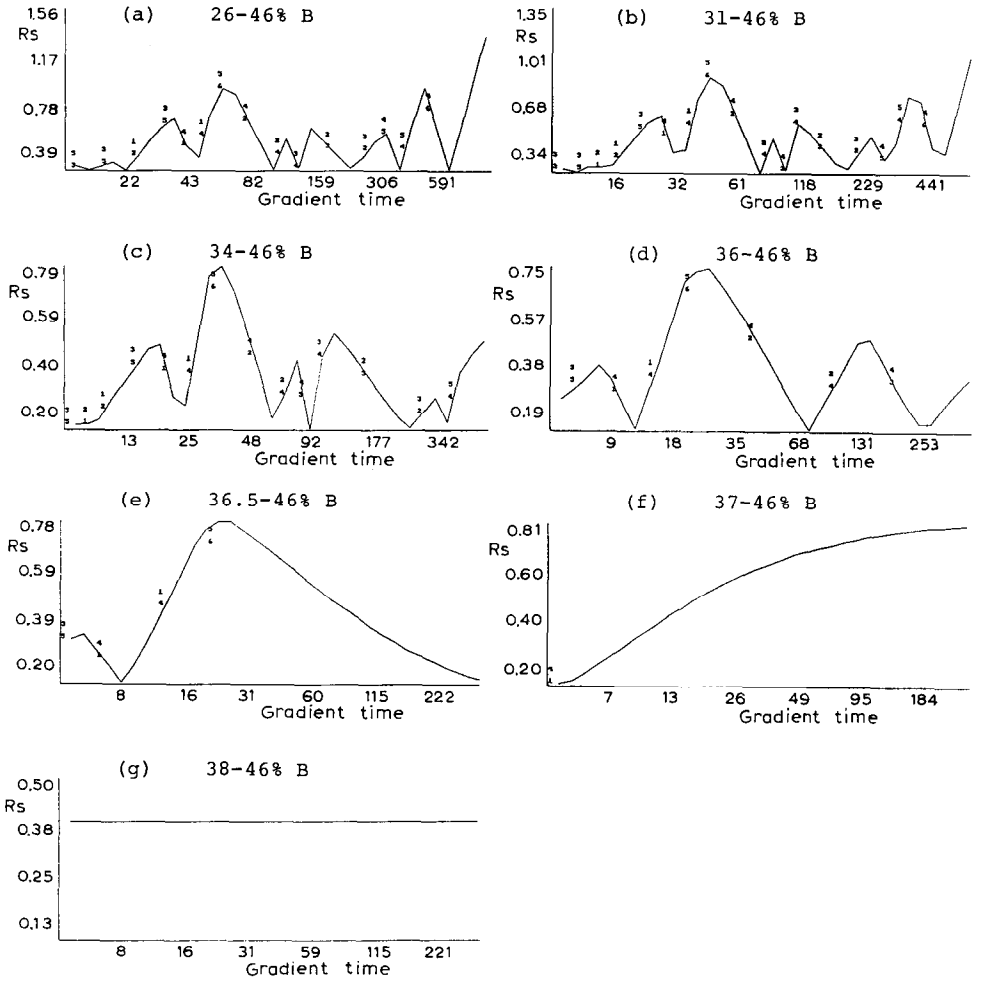

Fig. 12. Separation of bands 10–15 of a ribosomal protein sample as a function of initial %B (φ_0). Relative resolution maps ($N = 2500$) as a function of gradient time. DryLab G simulations. For conditions, see Fig. 11.

CONCLUSIONS

The various factors that contribute to maximizing resolution in a gradient elution separation have been examined in detail. Various samples can be categorized according to the appearance of two initial (linear gradient) chromatograms with different t_G values for the two runs, *e.g.*, see Figs. 3 and 4. Some samples exhibit minimum changes in band position as the gradient time is changed. The selection of optimized gradient conditions for these samples is relatively straightforward (see the following paper[8]).

Other samples (these include many peptide and protein mixtures) exhibit

pronounced changes in band spacing as the gradient steepness (or time) is varied. Typically, the resolution of such samples can be optimized by choosing gradients of different steepness for different parts of the chromatogram (multi-segmented gradients). However, the separation in any one region of the chromatogram will be dependent on some fraction of the (multi-segmented) gradient that precedes the elution of that part of the sample. Criteria for evaluating the magnitude of this effect are given and illustrated with various examples.

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