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PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY UNDER GRADIENT CONDITIONS

III. CRAIG SIMULATIONS FOR HEAVILY OVERLOADED SEPARATIONS

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SUMMARY

Computer simulations for heavily overloaded gradient elution are reported for a limited range of conditions. These simulations show a dependence of separation on sample size and experimental conditions that is similar to that for heavily overloaded isocratic separation. When $\alpha > 1.5$, it appears that relatively large samples ($w/w_s \approx 0.5$ or greater) can often be injected with high recovery (>95%) of purified product. These predictions are corroborated by independent experimental data from peptide and protein samples separated by reversed-phase gradient elution.

Separation in gradient elution as a function of sample size can vary considerably with the nature of the sample. Specifically, the relative dependence of retention [$S = \mathrm{d}(\log k')/\mathrm{d}\varphi$] on mobile phase composition (%B) can have a major effect on overlapping-band separations. For this reason, it is important to measure values of S for the sample of interest prior to completing method development for overlapping-band separations by gradient elution.

INTRODUCTION

Previous papers in this volume[1–5] have presented a comprehensive picture that describes key aspects of preparative high-performance liquid chromatography (HPLC) carried out in either an isocratic or a gradient mode, under conditions of either lightly overloaded (touching-band) or heavily overloaded (overlapping-band) separation. On the basis of this and other work from our group, it appears that there is a general parallelism among all of these separation modes and the corresponding small-sample separations, as summarized in Fig. 1 and Table I. Thus, information from small-sample isocratic separations can be used directly to design corresponding gradient separations (and *vice versa*). Similarly, data from small-sample runs can be used to help infer optimum conditions for touching-band separations. One or two additional runs under lightly overloaded conditions are required (in either an isocrat-

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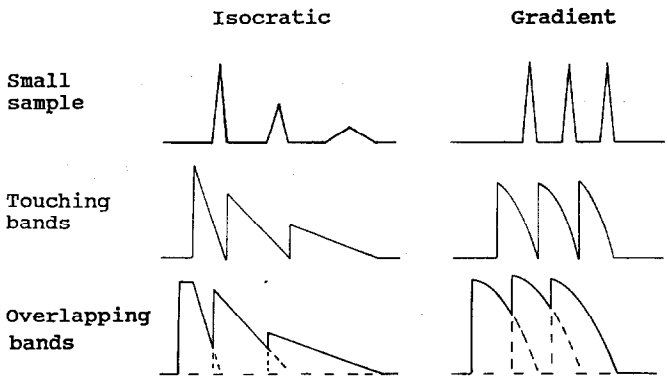

Fig. 1. Different separation modes of interest in preparative HPLC.

ic or a gradient mode), in order to measure the column capacity, w_s, and optimize conditions for touching-band separation. Finally, overlapping-band separations can be developed, using information from preceding small-sample and lightly overloaded runs to guide this process.

TABLE I

SUMMARY OF BASIC PARAMETERS FOR THE DESIGN OF A PREPARATIVE HPLC SEPARATION

Parameters	*Comment*
Small sample, isocratic:	
k_w, S, and N_o for X and Y	Two runs needed with different mobile phases (different %B); isocratic retention and bandwidth can then be predicted as a function of φ; gradient retention and bandwidth can be predicted as a function of gradient steepness (b)
Small sample, gradient:	
k_{og}, S and N_o for X and Y	Two runs needed with different gradient steepness[a]; isocratic retention and bandwidth can then be predicted as a function of φ, and gradient retention and bandwidth can be predicted as a function of gradient steepness (b)
Touching bands, isocratic:	
w_s for X and Y	One additional run needed with a larger sample, *e.g.*, w/w_s = 0.01–0.10. Bandwidth and position can then be calculated as a function of sample weight; the sample weight for touching-band separation can also be predicted (for either isocratic or, gradient elution)
Touching bands, gradient:	
w_s for X and Y	Same comment as for the preceding case
Overlapping bands, isocratic:	
–	No additional information (parameters) required for computer simulation; exact predictions (without computer) not currently feasible, but general rules exist[1] to estimate preferred sample size and plate number N_o when the w_o values of X and Y are similar. General trends can be anticipated for case of unequal w_s values[3]
Overlapping bands, gradient:	
–	Similar to the previous case; also, S values of X and Y are very important

[a] Different value of b, usually involving different values of t_G.

The only case that we have not examined in detail is that of overlapping-band gradient elution. In this paper we report preliminary results from the use of computer simulation (CRAIG4) for the study of overlapping-band separation by gradient elution. We also present findings that are relevant to separations by touching-band gradient elution, and which provide additional insight into the use of a previously described[5] computer program (BIOPREP) for the preparative separation of peptide and protein samples by gradient elution.

EXPERIMENTAL

Equipment, materials and procedures were described in Part II[5]. The CRAIG4 software is described in ref. 1.

THEORY[a]

A theory of touching-band gradient separations can be inferred from the discussion in refs. 4 and 6; this background applies equally for separation in an overlapping-band mode. However, an important aspect of preparative gradient elution was ignored in this treatment, *viz.*, changes in band spacing (α) with change in gradient steepness (b), that is, isocratic values of α for various band pairs in a sample often vary when the mobile-phase strength (%B) is changed in reversed-phase HPLC[7]. These changes in α in isocratic elution lead to corresponding changes in α with b in gradient elution, which is also well documented[6,8–10]. The result is that gradients of intermediate steepness are often optimum for a given sample.

Retention (k') in reversed-phase isocratic elution can be represented by

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

where k_w is the value of k' (small sample) for water as mobile phase (0%B), φ is the volume fraction of organic component (B) in the mobile phase and S is a constant that is characteristic of the solute and other experimental conditions. If eqn. 1 is written for solutes X and Y, having k_w values of k_{wx} and k_{wy} and S values of S_x and S_y, we can then derive[11]

$$\log \alpha = \log (k_{wy}/k_{wx}) + (S_x - S_y)\varphi \tag{2}$$

That is, α (and the band spacing) will remain constant for changes in φ when the S values of two adjacent bands are equal. This has been discussed in some detail in ref. 7. This is illustrated in Fig. 2, where $\log k'$ is plotted against %B (or φ) for two compounds having equal S values. The difference in $\log k'$ for these two compounds at a given value of %B is equal to $\log \alpha$, and the latter quantity is seen to remain constant for any value of %B.

Previous papers[8,9,11,12] have shown that gradient retention and separation can be inferred from isocratic plots of $\log k'$ *vs.* %B as in Fig. 2. Thus, in gradient elution

[a] A list of all symbols used in Parts I–III is included in ref. 4.

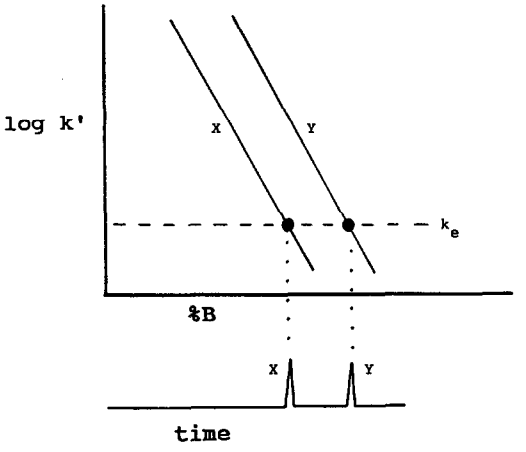

Fig. 2. Dependence of retention on mobile-phase composition in reversed-phase isocratic elution (top). Corresponding gradient elution separation (bottom); see text.

each band leaves the column with a final k' value (k_e) which is constant (for $S_x = S_y$), corresponding to the intersection of horizontal lines (equal k') with the plots for each solute (see dashed line in Fig. 2). Since %B is assumed to change linearly with time in gradient elution (linear gradient), a corresponding chromatogram can be aligned with the plots of log k' *vs.* %B as shown in Figure 2; the vertical dotted lines in Fig. 2 relate the %B at elution for each band with its retention time.

For the corresponding case of gradient elution, eqn. 2 can be written as[11]

$$t_y - t_x = (t_o/b)(\log \alpha) \tag{3}$$

where t_x and t_y are small-sample retention times in gradient elution. We can therefore see that α affects the band spacing in gradient elution in a similar fashion as for isocratic elution. Plots of log k' *vs.* %B (or φ) are shown in Fig. 3 for three different cases: $S_x = S_y$, $S_x < S_y$ and $S_x > S_y$. The small-sample resolution, R_s, is assumed to

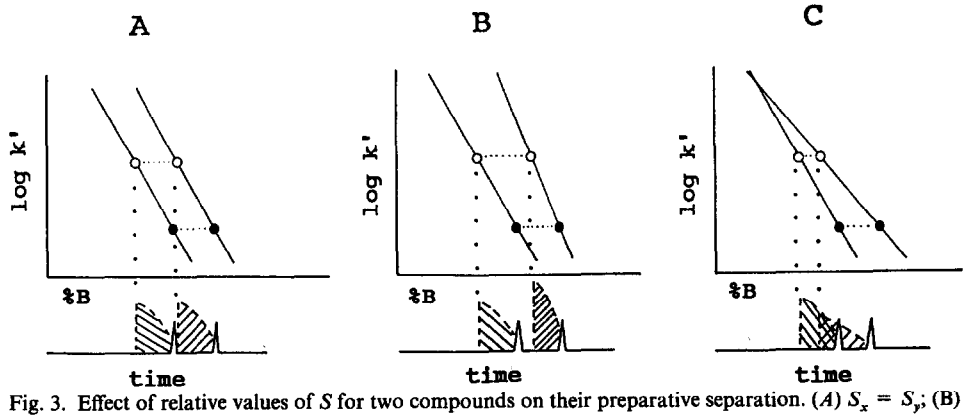

Fig. 3. Effect of relative values of S for two compounds on their preparative separation. (*A*) $S_x = S_y$; (B) $S_x < S_y$; (C) $S_x > S_y$.

be equal for each of these three examples owing to the equal values of α and k_e at elution.

According to eqn. 2, α will be constant as %B changes when $S_x = S_y$, α will decrease with %B when $S_x < S_y$ and α will increase with %B when $S_x > S_y$. Now consider the case of a touching-band separation, as illustrated in Fig. 3A for the cross-hatched bands at the bottom. The band maxima elute at %B values indicated by the vertical dotted lines in Fig. 3A. The small-sample separation (closed circles and triangular bands) in Fig. 3B is identical with that in Fig. 3A, but when a sample of similar size is injected the band maxima (dotted lines) leave the column with a larger value of α owing to their elution in a weaker solvent and the fact that $S_x > S_y$ (*cf.*, eqn. 3). The result is a better separation of the sample in Fig. 3B than in Fig. 3A. Finally, in Fig. 3C the opposite result is seen, *i.e.*, a poorer separation owing to the convergence of the log k' *vs.* %B plots.

The conclusions from Fig. 3 can also be stated in another way. When $S_x < S_y$, a larger sample can be separated with touching bands than in the case of equal S values and an equivalent small-sample separation. Similarly, a smaller sample will give touching bands when $S_x > S_y$. These conclusions appear to be correct in a qualitative sense, but a more detailed analysis (to be presented later) will show that unequal values of S for two compounds may imply unequal values of w_s (see the discussion in the Appendix I in ref. 3). This requires a more complex expression for the two-component retention isotherm than is currently used in CRAIG4.

In a previous paper[1] it was noted that large values of α are very important in overlapping-band separations. This suggests that unequal S values for two adjacent sample bands will lead to large differences in column loadability compared with the case of equal S values. We shall test this conclusion in the following section.

RESULTS AND DISCUSSION

Computer simulations ($S_x = S_y$)

Sample size effects. Fig. 4 illustrates the effect of increasing sample size in gradient elution, from small-sample to touching-band to overlapping-band separation. Here the gradient steepness $b = 1.0$, the initial k_0 values are $k_x = 10$ and $k_y = 15$ ($\alpha = 1.5$) and $N_0 = 1800^a$. The numbers above each band pair correspond to the recovery of 99% pure product; thus 94/78 (for $w/w_s = 0.20$) indicates that 94% of compound X can be recovered in 99% purity and 78% of compound Y can be recovered in 99% purity. Several observations can be made concerning the simulations in Fig. 4. First, with increasing band overlap ($w/w_s > 0.16$), a fairly sharp boundary appears between the two bands, followed by a tail of X into Y. This can be compared with the simulated and experimental chromatograms shown in Fig. 2 in ref. 1; it confirms the essential similarity of overlapping-band separations in isocratic or gradient elution, which was observed also for small-sample[1,2] and touching-band[4] separations.

Second, consider the total *weight* of compounds X and Y that can be recovered

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[a] Values of N_0 are reported for the first band; $N_0 = [(k' + 1)/k']n_c$, where n_c is the number of Craig stages (see ref. 1); the value of k' assumed is equal to $1/1.15b$. Sample sizes (w/w_s) have not been adjusted (see discussion of Fig. 3 in Part I[4]).

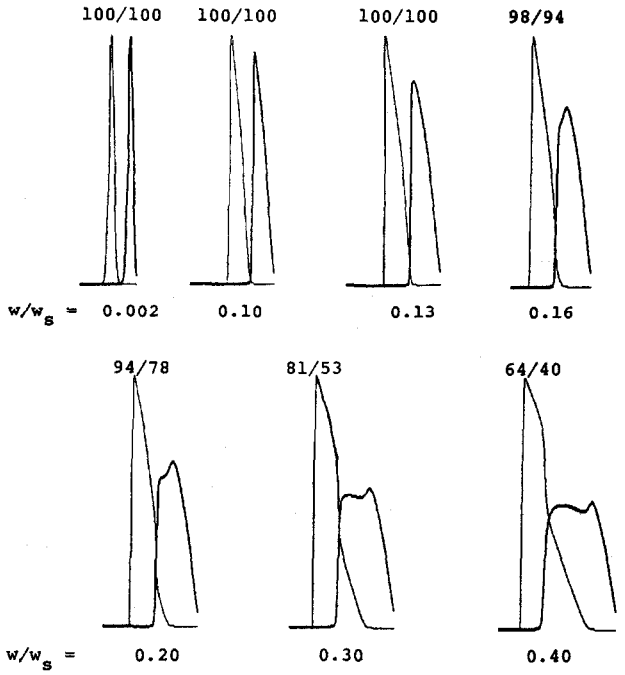

Fig. 4. CRAIG4 simulations of the separation of two compounds (X and Y) by gradient elution with sample size (w/w_s) varying. Conditions: $N_0 = 1800$, $b = 1$, $k_x = 10$ and $k_y = 15$ at beginning of gradient (values of k_{os}). Equal amounts of X and Y in sample.

as a function of the injected sample size. This relationship is shown in Fig. 5 for each band, together with the recoveries. For both X and Y, the weight of pure product recovered increases with increasing sample size, well beyond the sample weight for

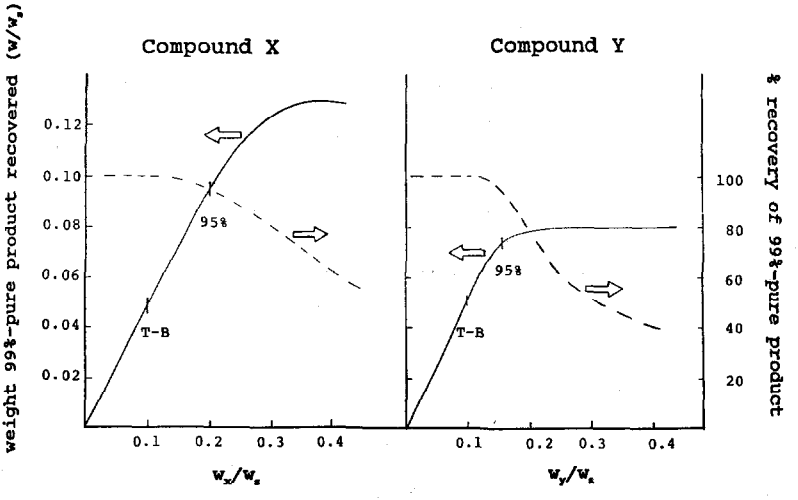

Fig. 5. Dependence of yield and recovery of pure product as a function of sample size. Data from Fig. 4.

touching bands (T-B in Fig. 5). For compound *X* it is possible to recover about twice as much pure *X*, with a recovery of 95% (noted in Fig. 5), as for the touching-band case. A similar situation is observed for compound *Y*, although the advantage with respect to touching bands is not as great. These observations parallel those found for the case of isocratic overlapping-band separation[1] (but note that the separations in Fig. 5 are not optimized in terms of N_o for maximum production rate).

Finally, it is seen that fairly large samples can be charged when α is as large as 1.5 (the value assumed in Fig. 5). Thus, a total sample weight $w/w_s = 0.15$ is possible for 95% recovery of 99% pure *Y*.

Effect of initial %B in gradient. This is illustrated for one set of conditions in Fig. 6. When there is substantial overlap for a gradient that begins with a higher %B (and smaller values of k' at the beginning of the gradient), a decrease in %B (with increase in the initial k' values) appears to give increased recovery (%) and yield (mg) of pure product. This effect levels off for initial k' values >100, and there is no advantage in beginning the gradient with a weaker mobile phase ($k_x > 100$), since the run time increases much faster than the recovery of pure product.

Effect of N_o. This is illustrated in Fig. 7 for conditions similar to those in Figs. 4 and 6: the initial k' values are 10 (*X*) and 15 (*Y*), $w/w_s = 0.40$ and $b = 1$. Separation as measured by the recovery of pure *X* and *Y* initially increases rapidly as N_o increases from 100 to about 800, but then levels off. This pattern is essentially similar to what we have seen for the other preparative HPLC modes in Fig. 1. As a result, there will

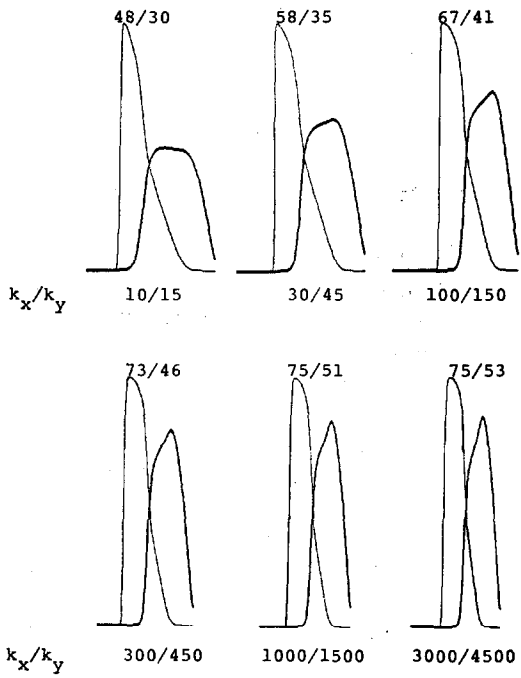

Fig. 6. CRAIG4 simulations of the separation of two compounds (*X* and *Y*) by gradient elution as a function of initial mobile phase composition (which determines initial values of k_{og}: k_x and k_y). Conditions: $N_o = 400$, $b = 1$, $w/w_s = 0.40$ and $\alpha = 1.5$. Equal amounts of *X* and *Y* in sample.

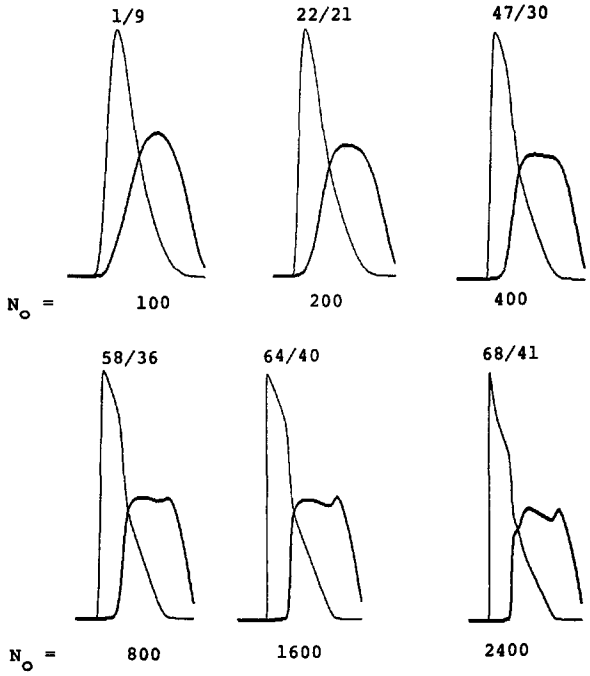

Fig. 7. CRAIG4 simulations of the separation of two compounds (X and Y) by gradient elution as a function of column plate number N_o. Conditions: $b = 1$, $k_x = 10$ and $k_y = 15$ at beginning of gradient (values of k_{og}), $w/w_s = 0.40$. Equal amounts of X and Y in sample.

be an optimum plate number for a given separation, one that yields a maximum production rate.

Effect of gradient steepness b. Separation generally improves as the gradient steepness is reduced and $\bar{k}$ increases, as illustrated in the simulations in Fig. 8. This is again similar to isocratic elution under overload conditions[1], where there is generally an optimum combination of N_o and k_o for maximum production rate. We have carried out a number of simulations where gradient steepness and plate number were changed together, with the general conclusion that separation (as measured by the recovery of pure X and Y) is similar when the product $N_o[k/(1+k)]^2$ is constant.

Computer simulations ($S_x \neq S_y$)

The preceding conclusions apply to the case where the S values of the two bands are equal, which should be approximately the case for most "real" samples, *i.e.*, samples having similar structures and molecular weights. However, the contrary ($S_x \neq S_y$) is often observed in practice[6–10,13,14]. Differences in solute S values can have a profound effect on preparative separations by gradient elution, as illustrated by the example in Fig. 9. Here, experimental conditions are assumed to be the same for two different samples (A and B), and the resolution for small-sample injections is seen to be identical in each instance. On the basis of our previous discussions, it would be expected that the separation of these two samples would also be similar for the injection of large samples of the same size. As seen, however, this is not so. Sample A

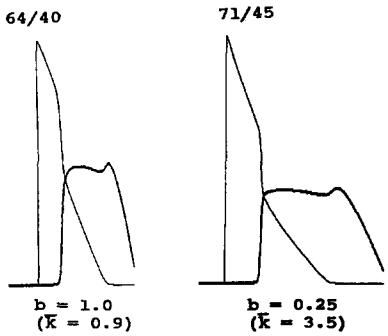

Fig. 8. CRAIG4 simulations of the separation of two compounds (X and Y) by gradient elution as a function of gradient steepness. Conditions as in Fig. 7, except $N_0 = 1600$ and b varies.

shows almost total overlap of the two bands for a large sample ($w/w_s = 0.8$), whereas sample B is totally resolved. The only difference in the two samples is their values of S; $S_x < S_y$ for sample A and $S_x > S_y$ for sample B.

Effect of sample size. Preceding computer simulations (Figs. 4–8) have assumed $S_x = S_y = 10$. Fig. 10 shows simulations for similar conditions to those in the preceding examples, but for samples where X and Y have different values of S. In series A in Fig. 10, $S_x = 10$ and $S_y = 8$ (the unfavorable case). It is seen that extensive band overlap occurs for samples larger than $w/w_s > 0.15$. In series B, $S_x = 10$ and $S_y = 12$ (the favorable case). Here, it is possible to saturate the column almost

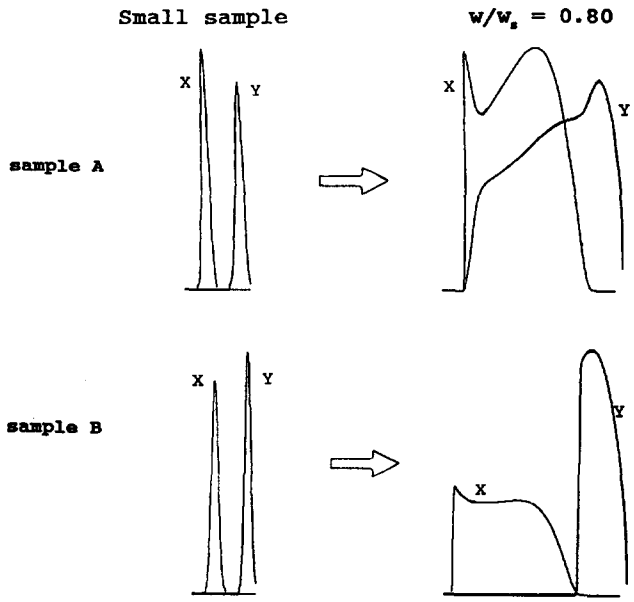

Fig. 9. CRAIG4 simulations of the separation of two compounds (X and Y) by gradient elution as a function of the sample and sample size. Examples taken from Fig. 10.

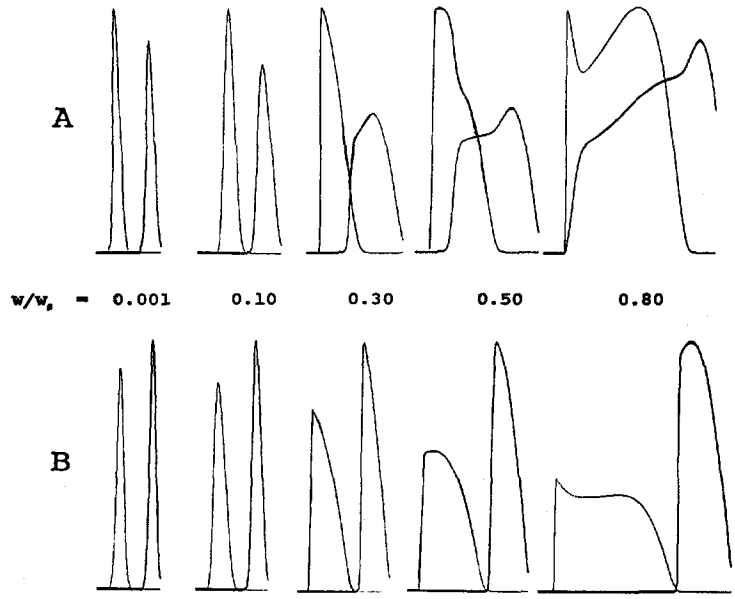

Fig. 10. CRAIG4 simulations of the separation of two compounds (X and Y) by gradient elution as a function of the sample and sample size. Conditions: $b \approx 1.0$, $N_0 = 400$; (A) initial $k' = 100$ (X) and 101 (Y), $S_x = 10$, $S_y = 8$; (B) initial $k' = 100$ (X) and 840 (Y), $S_x = 10$ and $S_y = 12$.

completely with sample ($w/w_s = 0.80$) without seeing significant band overlap. The reason for this differing behavior should be clear from the examples in Fig. 3, but it is nevertheless impressive.

Effect of initial mobile phase composition. When the S values for two compounds are equal, there is not a very large effect of the starting mobile phase composition on the resulting overlapping-band separation, as seen in Fig. 6 for initial k' values >100. The reason is that under conditions of large k' the bands (even under heavy-overload conditions) do not migrate very rapidly through the column, and therefore little separation occurs during this phase of the separation. However, when the S values for the two compounds differ appreciably, the situation can be completely different. This is illustrated in Fig. 11 for the (unfavorable) case where $S_x > S_y$. Simulated separations are shown for different sample weights and different initial mobile phases. As the initial mobile phase is made weaker (the initial k' value, k_x, increases from 10 to 1000), there is little effect on the separation of a small sample ($w/w_s = 0.002$). This is also the case when S values for the two compounds are equal, as discussed above. However, for larger samples ($w/w_s = 0.30, 0.50$), it is seen that an increase in the initial k' values of the sample results in a pronounced degradation of the separation. This reflects the fact (see Fig. 3C) that the initial α value is much smaller for a smaller %B and larger value of k_x, combined with the fact that a large sample migrates more rapidly in a given mobile phase than does a small sample. Consequently, the choice of the best starting mobile phase appears to be important.

When $S_x < S_y$ (favorable case), an opposite trend of separation *vs.* starting mobile phase composition (and k_x) will be observed. In this case, the separation can

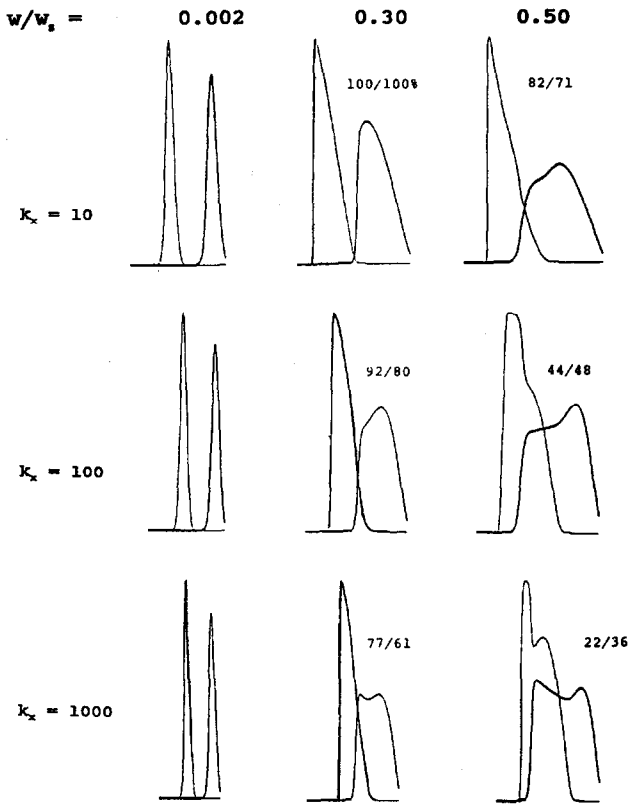

Fig. 11. CRAIG4 simulations of the separation of two compounds (X and Y) by gradient elution as a function of the starting mobile phase composition and sample size. Conditions: $b \approx 1.0$, $N_0 = 400$; initial $k' = 10$ (X) and 12.8 (Y), 100 (X) and 101 (Y) and 1000 (X) and 780 (Y); $S_x = 10$, $S_y = 8$.

improve dramatically if the starting mobile phase is made weaker. We carried out further modeling studies in which separation was studied during migration on the column. It appears that the choice of mobile phase composition for the initial injection of the sample is not very important to the final separation. Rather, it is the migration on the column *following* injection that most affects the final separation. We shall report further on this in a later paper.

Experimental gradient separations under overlapping-band conditions

The previous examples (Figs. 4–11) suggest that under certain conditions it is possible to apply fairly large samples ($w/w_s > 0.5$) in gradient elution and obtain almost complete recovery of highly pure product. In some instances this is possible even when the α values for a comparable small-sample separation are fairly small ($\alpha < 1.5$). This observation must seem surprising to most workers (it was surprising to us!), but previous reports from the literature tend to support this possibility.

Literature examples. Using displacement chromatography, Horvath *et al.*[15] reported almost complete recoveries of pure product ($\alpha = 1.07$) from the reversed-

phase separation of 110 mg of a mixture of phenylacetic acids on a 25 × 46 cm I.D. column ($w_s \approx 400$ mg). Even more striking is the separation reported by Mant *et al.*[16] for a decapeptide sample ($\alpha \approx 1.7$) by reversed-phase gradient elution. In this example, reproduced in Fig. 12, the column capacity w_s can be estimated to be about 50 mg. Touching-band separation occurs for a sample of 1 mg, but a 20-mg sample yielded 99% of pure *X* and 98% of pure *Y*. Injecting a sample equal to the column capacity (50 mg) gave 87% pure *X* and 61% pure *Y*. It is hoped that these results are now easier to understand on the basis of the preceding discussion.

Protein separations. We carried out some preliminary gradient elution separations under overlapping-band conditions, using various proteins as samples. Fig. 13 shows a series of such separations for the proteins cytochrome *c* (*X*) and lysozyme (*Y*), where $N_o = 1750$, $S_x = 29.6$ and $S_y = 30.5$ (favorable values of *S*) and $\alpha = 2.3$ in these separations. The numbers (*e.g.*, 0.5/10.0) refer to the weight (mg) of *X* and *Y* in

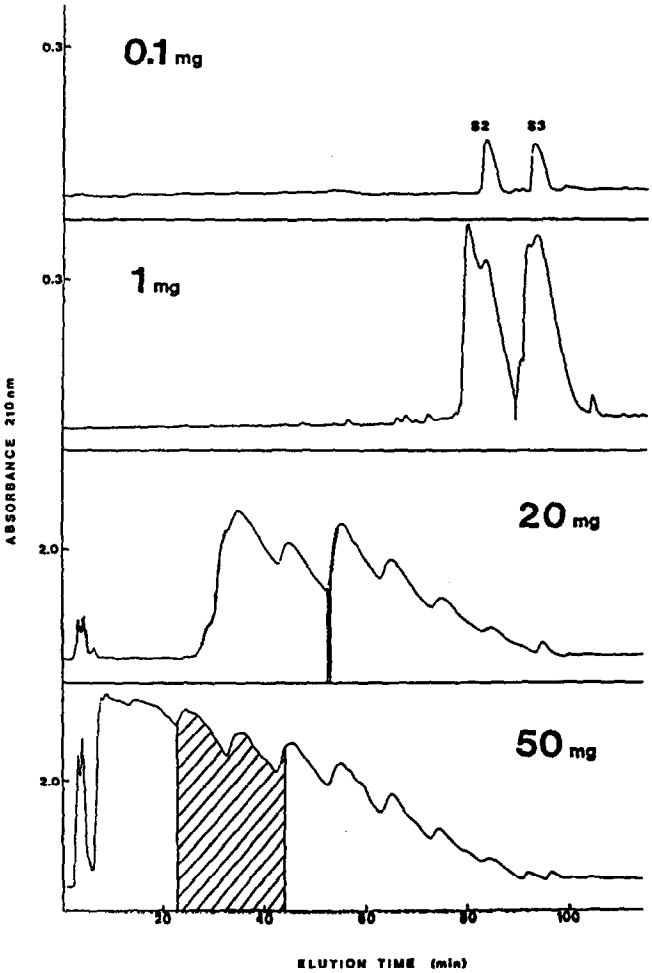

Fig. 12. Separation of decapeptide sample by reversed-phase gradient elution[16].

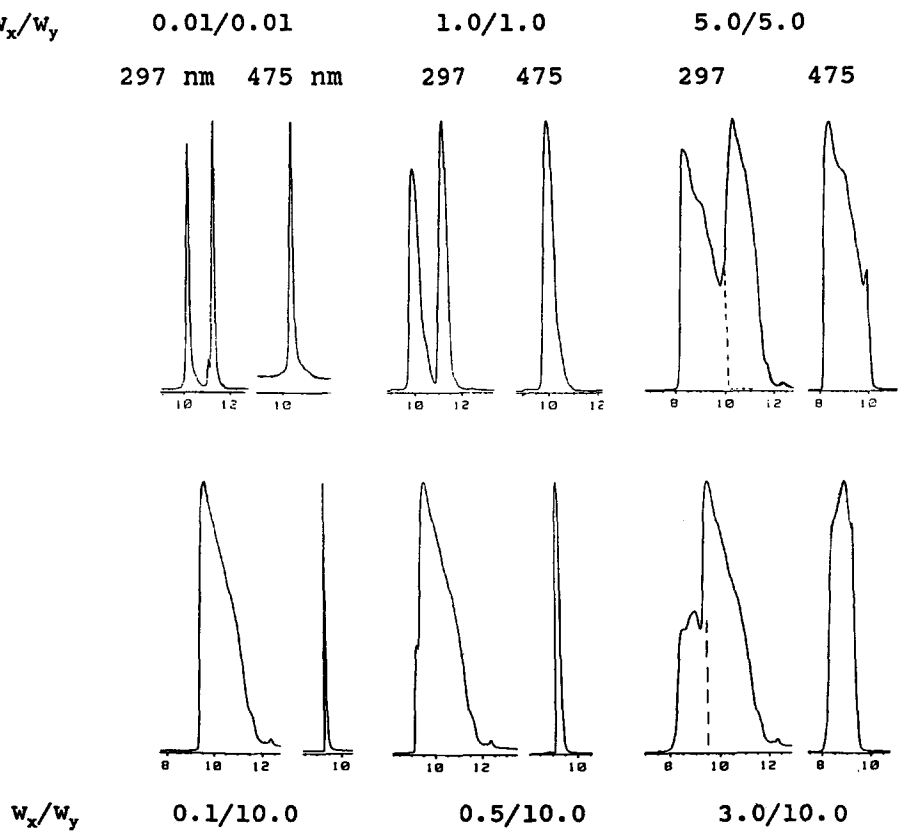

Fig. 13. Experimental separations of cytochrome *c*–lysozyme mixture by reversed-phase gradient elution. Sample size varies as indicated (*e.g.*, 0.1/10.0 signifies 0.1 mg of cytochrome *c* plus 10 mg of lysozyme in the injected sample). Conditions: 15 × 0.46 cm I.D. column of 100-nm pore-size C_8 packing (w_s = 11–20 mg, see Table II in ref. 4); flow-rate, 1 ml/min; gradient, 5–70% acetonitrile–water (plus 0.1% TFA) in 20 min; detection at either 297 or 475 nm.

the injected sample (*e.g.*, 0.5 mg of *X* and 10 mg of *Y*). Detection was carried out at two wavelengths (297 and 475 nm); cytochrome *c* absorbs at both wavelengths, but lysozyme absorbs only at 297 nm. Therefore, the 475-nm chromatograms show the cytochrome *c* band, whereas the 297-nm tracings track both bands. The dashed lines in some of these chromatograms (5.0/5.0 and 3.0/10.0 runs) indicate the superimposed plot of the cytochrome *c* band.

We note first that there is a sharp boundary between the two bands when they overlap appreciably, as a result of sample displacement. This is reminiscent of the computer simulations in Figs. 4 and 6, but is much more pronounced. Presumably this is due to the larger value of α for this sample (α = 2.3) compared with the smaller value of α (α = 1.5) assumed in the various simulations. As a result, we see again (Fig. 13) that fairly large samples can be separated with good recovery of purified product when the sample characteristics and separation conditions are favorable.

We have observed similar results to those in Fig. 13 for the separation of a

mixture of cytochrome *c* and ribonuclease A (results not shown). Later we shall report on the agreement between these experimental results and computer simulations based on CRAIG4. One complication in these comparisons, which requires further study, is the difference in the values of w_s determined by column saturation and chromatographic data under conditions of partial overload (for protein samples see Table II in Part I[4]).

CONCLUSIONS

This preliminary study reports computer simulations for the overlapping-band separation of various hypothetical samples by gradient elution. When the S values [$S = \mathrm{d}(\log k')/\mathrm{d}\varphi$] of two compounds are similar, the resulting separations parallel those obtained by corresponding isocratic separations. When the S values of two adjacent bands differ appreciably, this can have a profound affect on the separation. For the favorable case (S for the first band less than S for the second band), very large samples can be separated, even when α (and resolution) is fairly small for the small-sample separation. The optimum sample size can then be an order of magnitude greater than for touching-band separation. For the unfavourable case (S for the first band larger than S for the second), sample sizes larger than those which give touching bands lead to extensive band overlap and low recoveries of purified product. For this reason, it is important to know the values of S for compounds that are to be separated by gradient elution (both touching-band and overlapping-band sample sizes). Values of S can be readily determined from two experimental small-sample runs[6,17].

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