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# Displacement effects in preparative gradient high-performance liquid chromatographic separations

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ABSTRACT

Experiments were carried out which were designed to elucidate the influence of gradient parameters on the strong displacement effects which have been observed in the preparative chromatography of proteins. Unexpected loading phenomena were observed in which a proportion of the model protein load was not taken up by the column, even though the saturation capacity of the column was not exceeded. This proportion was a function of the mobile phase composition at which the protein was loaded. Low solvent strengths were seen to be essential to allow high preparative sample loads. It was shown that the saturation capacity of the column changed little with gradient parameters and also that the saturation capacities calculated on the assumption of approximate Langmuir isotherm behaviour were in error. Mass overloaded separations of binary mixtures of proteins all demonstrated the existence of displacements between the solutes, even when a substantial amount of the second-eluting protein was not adsorbed on initial loading. It was demonstrated that the gradient slope had little effect on the separation because of the very sharp displacement zones observed. Steeper gradients allowed a higher production rate without incurring a decrease in purity or recovery. Guidelines for the design of reversed-phase gradients for the purification of proteins are given with a view to maximising solute–solute displacements. Such gradients will allow the maximum production rates of purified product.

## INTRODUCTION

Displacement effects between solutes in isocratic mass overloaded chromatography are now well documented [1–4]. Much of this work has been carried out by computer simulation [1,2], although some experimental work which supports these results has been published [1,3,4]. The interactions between the solutes which give rise to these displacements in preparative chromatography are controlled by their adsorption isotherms. Although some conclusions can be drawn from a study of the individual isotherms of the solutes, the interactions can only be correctly predicted if the competitive isotherm between them is known [5].

The determination of these competitive isotherms is at the least both tedious and difficult. Further, it is usually impossible to determine the isotherms where the solutes are not available in a pure form and in reasonable amounts. Predictions can be made if a model based on the competitive Langmuir isotherm is assumed and this has been the basis of much of the computer simulation work. Unfortunately, although a good qualitative understanding may be gained from these results, the competitive Langmuir model is not valid for all cases [6]. This means that the isotherms have to be determined experimentally in order to obtain good predictions of the experimental results. In many practical cases, this requirement renders modelling impractical and only the qualitative results from the simulations are useful.

We have reported experimental results for isocratic separations [3] where the simple simulations based on the competitive Langmuir model do not give valid predictions. These are cases in which the

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saturation capacities of the column for the two solutes of interest are markedly dissimilar. Where the saturation capacity for the first-eluting solute is significantly greater than that for the second (*i.e.*, the isotherms cross), the bands merge. Conversely, the second component may displace the first when it has the greater saturation capacity [$w_{s(2)} > w_{s(1)}$]. It is probable that as the saturation capacities for the two components become closer in value their behaviour will move from the extreme case to one intermediate between this and a case at least qualitatively similar to that predicted by the competitive Langmuir model.

We have also reported [7,8] the modeling of gradient elution separations under conditions of mass overload. The parallels between preparative isocratic and gradient elution separations are many [7]; thus, one would expect to see some of the different types of solute interactions which are observed in isocratic separations carried over into the corresponding gradient chromatograms. The peak shape distortions predicted from Craig counter-current simulations of overloaded gradient separations are similar in nature to those found in the simulations of isocratic separations. We have also shown [8] that in the gradient separations of peptides and proteins there are additional factors influencing the separation, related to the different $S$ values (the slope of a plot of log $k'$ against solvent strength modifier concentration) for the solute molecules.

Both modelling and experimental data indicate that even stronger displacement effects may be seen under gradient elution conditions for large molecules than for small solutes. Evidence for this exists both in our own preliminary results and in the work of Mant *et al.* [9]. In the latter instance, it has been demonstrated that extremely strong displacements can occur between peptide molecules. These purifications were carried out under conditions of strong retention and it was seen that separations of these compounds could be effected under isocratic conditions appropriate for the loading of the sample on to the column rather than elution. This sample displacement could explain the sharp boundaries between the components which we observed in our preparative gradient separations of proteins [8], as these gradients began at low concentrations of organic modifier (*i.e.*, conditions of strong retention).

The purposes of the experimental work reported in this paper were threefold: to extend our initial data, which showed very significant displacement effects occurring in the preparative gradient elution chromatography of protein standards, to verify the parallels between gradient and isocratic separations and to observe the effects on the displacements of the slope and the range of compositional change in the gradient.

## EXPERIMENTAL

### *Equipment*

*LC system.* An HP 1090 M liquid chromatograph (Hewlett Packard, Avondale, PA, USA) fitted with a preparative autoinjector was used for part of this work. The remainder was carried out using an HP 1090 L liquid chromatograph fitted with a diode-array detector. A second system (HP 1090 L) was used for the analysis of fractions taken during preparative experiments.

*Data system.* Initial work was done using a Nelson Analytical Data System, based on an HP 200 computer (Nelson Analytical, Cupertino, CA, USA) for data collection and storage in addition to the system built into the HP 1090 M chromatograph. The Nelson software was modified in-house to incorporate the calculation of the capacity factors, efficiencies and skews of detected peaks. In later work an HP 3365 Chem Station was used.

### *Chromatography*

*Columns.* All columns were constructed from stainless steel and were packed by a downward slurry technique. Separations of proteins were carried out using either an experimental Zorbax PSM 1000 $C_8$ packing (particle size 7 $\mu$m, pore size 1000 Å) (DuPont, Wilmington, DE, USA) packed into 15 cm × 4.6 mm I.D. columns or Zorbax PRO-10 Protein Plus (10 $\mu$m, pore size 300 Å) packed into 25 cm × 4.6 mm I.D. columns. Small molecule separations were performed using Zorbax ODS (5 $\mu$m, pore size 70 Å) packed into a 15 cm × 4.6 mm I.D. column.

*Chemicals and mobile phases.* Methanol and acetonitrile were obtained as high-performance liquid chromatographic (HPLC)-grade solvents from J. T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (TFA) was obtained from J. T. Baker, protein

standards from Sigma (St. Louis, MO, USA) and phenol, benzyl alcohol, cresol and 2-phenylethanol from Aldrich (Milwaukee, WI, USA).

Chromatography of proteins was performed using gradients made from mixtures of 0.1% aqueous trifluoroacetic acid and 0.1% trifluoroacetic acid in acetonitrile. Gradients for the other separations were formed from methanol and water.

## RESULTS AND DISCUSSION

### *Small molecules*

Our earlier discussion of the effects of elution gradients on separations [8] did not address the situation where the saturation capacities of the column for the components of interest differ. This is inherent in the simulation model in that the equations based on the Langmuir model assume equal $w_s$ values. This and the effects of deviations from Langmuir behaviour are discussed in more detail in the Appendix. In order to investigate this aspect, gradient separations were carried out for compounds which had been shown earlier to have different saturation capacities under isocratic elution conditions. It was assumed for this part of the study that although the isotherms would clearly change with the eluent composition owing to the changes in the distribution coefficients, the saturation capacities would remain constant. Other studies [10] have shown that the saturation capacity for benzyl alcohol remains constant over a moderate range of solvent composition.

Our previous work [3], which reported the effects of unequal saturation capacities in isocratic mass overloaded separations, used mixtures of either phenol and benzyl alcohol or of *p*-cresol and 2-phenylethanol as solutes. It was found that the saturation capacities of the aromatic alcohols were approximately double those of the phenols. The same solute pairs were chosen for the gradient elution studies. Table I shows the separation conditions for these experiments together with the $S$ values for the components, determined from the gradients reported here and from others with 2.5 times the gradient run time.

It may be noted that the $S$ value for benzyl alcohol is larger than that of phenol, whereas those of *p*-cresol and 2-phenylethanol are equal under the separation conditions chosen. From our earlier discussion of the effect of $S$ values on gradient separations [8], the phenol–benzyl alcohol separation should be characterised by an increase in selectivity as the load increases. The other separation should show a constant selectivity with load. In the isocratic experiments, benzyl alcohol was shown to displace the earlier eluting phenol very strongly, whereas *p*-cresol merged with the earlier eluting 2-phenylethanol. Following the principle that for corresponding separations the data from isocratic and gradient elution experiments are related, then the former pair of solutes should show displacement and the latter pair should merge in the gradient experiments.

The result of loading a mixture of 3 mg of phenol and 1 mg of benzyl alcohol is shown in Fig. 1a. As would be predicted from the isocratic experiment, there is a pronounced displacement of phenol by benzyl alcohol. This is demonstrated by the rapid

TABLE I

OPERATING CONDITIONS FOR SMALL-MOLECULE GRADIENT SEPARATIONS

Flow-rate: 1.0 ml/min. Detector: UV, wavelengths 254 and 280 nm.

| Solute | Column | Gradient | | $S$ |
|---|---|---|---|---|
| | | Range | Time (min) | |
| Phenol | Zorbax ODS | 0–40% | 20 | 2.69 |
| Benzyl alcohol | (15 cm × 4.6 mm I.D.) | $CH_3OH$–$H_2O$ | | 2.99 |
| 2-Phenylethanol | Zorbax ODS | 10–70% | 17 | 2.27 |
| *p*-Cresol | (15 cm × 4.6 mm I.D.) | $CH_3OH$–$H_2O$ | | 2.27 |

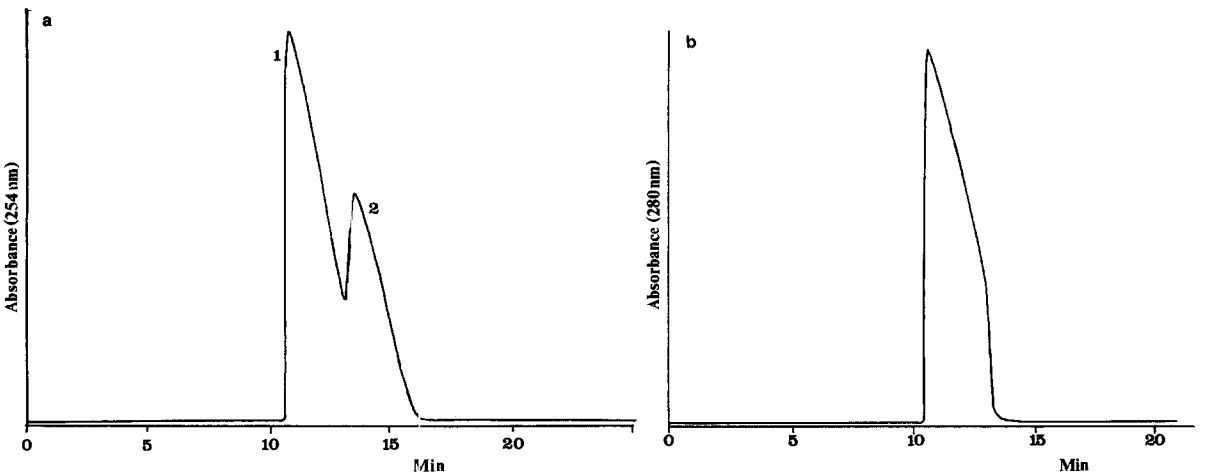


Fig. 1. (a) Gradient elution preparative chromatograms of 3 mg each of phenol and benzyl alcohol. Flow-rate, 1 ml/min; detection, UV, 254 nm. For other conditions, see Table I. Peaks: 1 = phenol; 2 = benzyl alcohol. (b) As for (a), with UV detection at 280 nm.

drop of the trailing edge of the peak envelope of phenol (monitored at 280 nm, where benzyl alcohol has a very small UV absorption) to the baseline (Fig. 1b). It is also of interest that the resolution between the peaks for the given sample load is slightly larger for the gradient separation as compared with the isocratic chromatogram [3]. From the relationship between selectivity and solvent composition derived in the Appendix, this difference is not surprising, as the solutes will certainly be eluting at a mobile phase concentration different from that used isocratically.

With the other solute pair studied, *p*-cresol and 2-phenylethanol have equal $S$ values and hence no such increase in selectivity should be noted. In fact, the two peak maxima move closer as the sample load is increased. The separation, in this instance with the injection of 3 mg of *p*-cresol and 1 mg of benzyl alcohol, is shown in Fig. 2a. Because of the difficulty in deconvolution of the chromatograms taken at the two wavelengths, the separation was repeated, collecting fractions through the peaks at 5-s intervals. The reconstructed chromatogram (Fig. 2b) shows

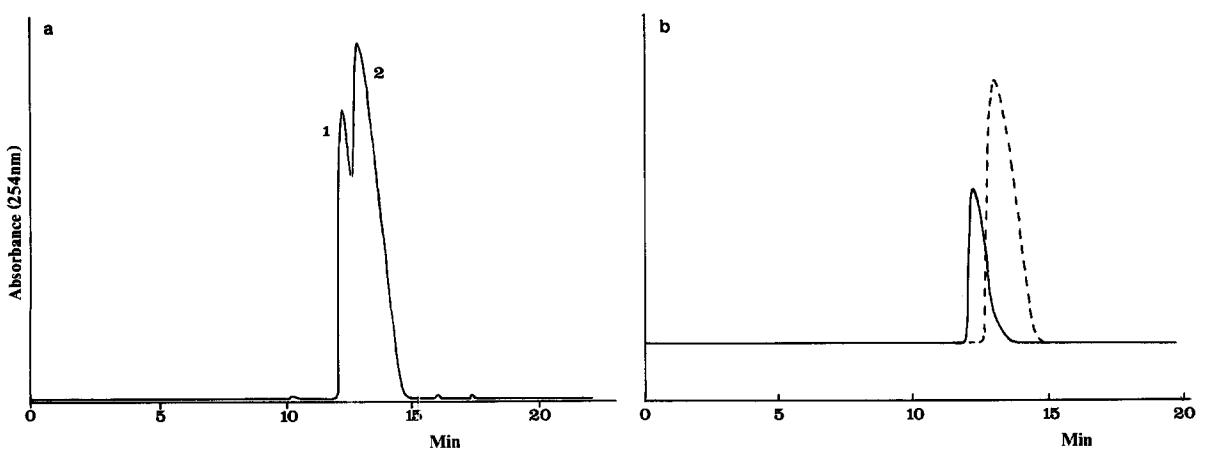


Fig. 2. (a) Gradient elution preparative chromatograms of 3 mg each of *p*-cresol and 2-phenylethanol. Flow-rate, 1 ml/min; detection, UV, 254 nm. For other conditions, see Table I. Peaks: 1 = 2-phenylethanol; 2 = *p*-cresol. (b) As for (a), chromatogram reconstructed from fraction analysis. Solid line, 2-phenylethanol; dashed line, *p*-cresol.

clearly that no displacement effects are present and the peaks merge, just as was seen for the previously reported isocratic separation. Again, the degree of overlap of the peaks was less under the gradient conditions when compared with isocratic chromatograms using the same sample load, where the two components were completely merged. It would be expected from eqn. 11 in the Appendix that the selectivity might decrease for these solutes. This result may reflect the conclusions drawn elsewhere [11] that the required resolution in gradients for optimum separations in preparative chromatography were seen to be less than for the corresponding isocratic case.

Hence the preparative isocratic and gradient separations follow similar courses as predicted by the original modelling, even in cases where the displacements and "tag-alongs" are much greater than predicted from the models [6].

It should be pointed out that there is no advantage in the use of gradient elution for the purification of these small molecules by preparative liquid chromatography. The isocratic separation will always give the higher production rate, if only because of the time necessary for re-equilibration of the column. This is not necessarily the case with larger molecules, such as proteins, where elution gradients have an important rôle to play in their purification.

*Protein samples*

We have earlier reported that the gradient elution mass overloaded separations of proteins on a 1000 Å pore size $C_8$ packing result in dramatically large displacement effects [8]. These data were obtained for the solute pairs of lysozyme and cytochrome *c* and of ribonuclease A and cytochrome *c*. In both experiments the first component was strongly displaced from the packing by the second-eluted peak. The latter peak was very little distorted by the presence of the first, moving only slightly to shorter retention time. The boundary between the peaks was very sharp, allowing high recoveries of the pure components.

The displacements observed were significantly greater than are predicted by the Craig modelling and are probably related to differences between the approximate Langmuir behaviour assumed in that model and the real isotherms. Large molecules such as proteins cannot follow a true Langmuir isotherm, as their size dictates that more than one solvent molecule is displaced per molecule adsorbed on the packing material surface [12]. Consideration of some of the effects of this phenomenon have shown [10] that although the saturation capacities determined from the experimental data are lower than those expected from consideration of the surface area of the packing material, the course of the separation should be as predicted by the calculations based on the Langmuir isotherm. It is known, however, that proteins undergo a number of configuration changes during chromatography, and it is not surprising that their behaviour deviates from that expected.

This initial work was carried out using only one gradient profile, of 20 min duration and encompassing a wide range of solvent compositions. In optimising gradients for preparative separations we have previously suggested [13] that a small solvent compositional range should be employed together with a short gradient run time ($t_G$). The purpose of this is to reduce the overall run time and thus to increase the production rate of the purification. In order to calculate the effects of changes in the gradient profile on the separation, it is necessary to determine the values of $S$, the slope of the plot of log $k'$ *versus* solvent composition, and of $k'_w$, the capacity factor at zero percentage of the organic modifier. These can be determined [14] from two gradient experiments carried out at analytical loads. Experiments to determine the gradient parameters for cytochrome *c* and lysozyme were carried out using a 300 Å pore size packing. Under these conditions, the $S$ values for the two proteins were approximately equal, with values close to 20.

Gradient experiments at elevated loadings were carried out using the 300 Å pore diameter packing material. The conditions are shown in Table II. A 20-mg amount of each solute was loaded. This is higher than was used in our earlier study, because the 300 Å packing had a higher surface area (by a factor of *ca.* 10) than the 1000 Å pore packing used then. The chromatograms are shown in Fig. 3. Fig. 3a shows the chromatogram, monitored at 290 and 480 nm (dashed line), for the 20-min run. The sample concentrations in this run were too high to maintain a linear detector response at the peak maxima where typical detector overload response is seen. Fortunately, the area of overlap between the peaks occurs

TABLE II

GRADIENT CONDITIONS FOR PROTEIN SEPARATIONS

| | |
|---|---|
| Column: Zorbax Pro-10 Protein Plus (25 cm × 4.6 mm I.D.) | |
| Gradient: | |
| Solvent A: | 0.1% aqueous trifluoroacetic acid |
| Solvent B: | 0.1% trifluoroacetic acid in acetonitrile |
| Range: | 5 to 70% B |
| Gradient time: | (a) 20 min |
| | (b) 50 min |
| Flow-rate: 1 ml/min | |
| Detection: UV, wavelengths 290 and 480 nm | |

at absorbances where the detector response is reliable. Fig. 3b shows the chromatograms (290 and 480 nm) from the 50-min gradient.

These chromatograms show the same very strong displacement effects as were seen in our earlier experiments. The maxima of the two peaks in the 50-min gradient are better separated than those in the 20-min run, as would be expected from the use of the shallower gradient. Interestingly, because of the displacement, there is little difference in the purity or recovery of the two components between the two experiments. Hence, there is no reason not to use the steeper gradient, as this has a direct influence on the production rate possible from the system. The major difference between the two gradients lay in the cytochrome *c* peak. In both gradients a small tail may be observed which runs into the lysozyme peak. With a gradient time (for a range of 5–70% acetonitrile) of 20 min, the tail consisted of 0.3% of the cytochrome *c* peak area. In the experiment with a

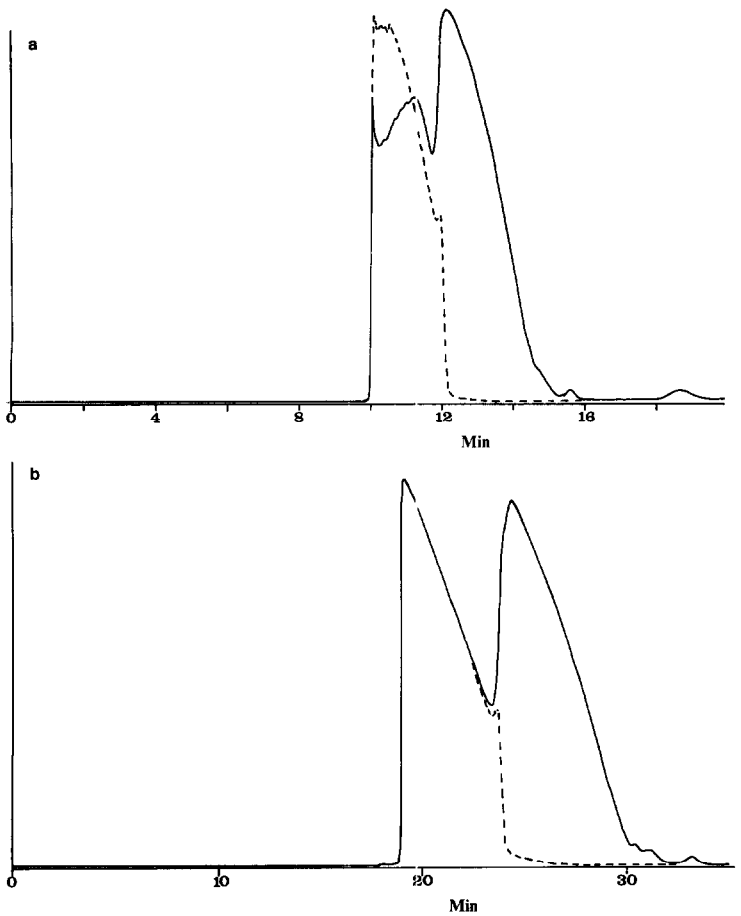


Fig. 3. Preparative gradient separation of 20 mg each of cytochrome *c* and lysozyme. Conditions as in Table II. (a) 20-min run time; (b) 50-min run time. Solid line, 290 nm; dashed line, 480 nm.

shallower gradient, this tail increased to 1.2% of the peak area.

In order to study this further, a number of gradients of different slopes were run under mass overload. Table III summarises the conditions used in the experiments. Columns 15 cm rather than 25 cm in length were used. This reduction in length was not expected to affect the results significantly.

The chromatograms were monitored at wavelengths of 295 and 480 nm in order to prevent overload of the detector electronics. The chromatograms of two of the overloaded runs are shown in Fig. 4. Fig. 4a shows the chromatograms (290 and 480 nm) from the 15-min gradient and Fig. 4b those for the 60-min gradient. Subtraction of the normalised 480-nm chromatogram from the 290-nm chromatogram in Fig. 4b results in a difference chromatogram with the response from the cytochrome *c* eliminated. This difference chromatogram (Fig. 4c) indicates that the lysozyme peak is not distorted by the displacement, but appears totally unaffected. The difference chromatogram also shows the presence of a minor component displaced by the cytochrome *c* peak. This material is probably a component of the lysozyme sample used.

In order to quantify the degree of overlap between the two main peaks, a goal purity of 99.5% cytochrome *c* was chosen. Ignoring the minor impurity for the moment, the recovery of cytochrome *c* at cut points appropriate for the collection of 99.5% pure material was calculated from the chromatograms. The results are shown in Table IV. There is very little difference between the different gradients in terms of the purity and recovery and any variation in these parameters with gradient slope presumably results from experimental error. In contrast to the earlier experimental data, no differences in the tailing of the cytochrome *c* peak arising from changes in the gradient time could be seen in these experiments.

TABLE III

GRADIENT CONDITIONS FOR STUDY OF THE EFFECT OF GRADIENT PARAMETERS ON THE MASS-OVERLOADED SEPARATION OF PROTEINS

| | |
|---|---|
| Column: Zorbax Pro-10 Protein Plus (15 cm × 4.6 mm I.D.) | |
| Gradient: | |
| Solvent A: | 0.1% aqueous trifluoroacetic acid |
| Solvent B: | 0.1% trifluoroacetic acid in acetonitrile |
| Range: | 5–70% B |
| Gradient time: | (a) 15 min |
| | (b) 30 min |
| | (c) 45 min |
| | (d) 60 min |
| Flow-rate: 1 ml/min | |
| Detection: UV, wavelengths 295 and 480 nm | |
| Sample: cytochrome *c*, 3 mg; lysozyme, 10 mg. | |

TABLE IV

PURITY AND RECOVERY OF CYTOCHROME *c*

| Gradient time (min) | Purity (%) | Recovery (%) |
|---|---|---|
| 15 | 99.46 | 93.1 |
| 30 | 99.47 | 93.7 |
| 45 | 99.50 | 91.3 |
| 60 | 99.50 | 95.9 |

In addition to the gradient slope, the range of the gradient can also be an important factor in the purification of proteins. As the protein molecules are effectively completely retained for much of the gradient (when the solvent strength is low), the production rate can in theory be increased by elimination of this early part of the gradient [14]. This is a standard technique in analytical protein separations where the analysis time can be appreciably shortened by using only that part of the gradient in which the proteins elute. A study was carried out to investigate how the protein separations and solute–solute interactions were affected by such a reduction in the gradient range.

*Single solutes.* A set of experiments in which the components were introduced singly was first carried out. Details of the gradients used are given in Table V. Analytical (0.01 mg) and preparative (3 and 10 mg) loads of each component were used.

From the $S$ and $k_w$ values for the proteins studied, it was possible to calculate the values of $k'$ ($k'_0$) for the solutes in the starting mobile phase composition of the gradients. These are shown in Table VI and range from 11 for cytochrome *c* at the highest initial composition to over $2 \cdot 10^6$ for lysozyme at the lowest.

Gradients starting at 24 or 31% acetonitrile resulted in incomplete uptake of the solutes. Fig. 5a shows a composite chromatogram assembled from an analytical-scale (0.05 mg) run with a mixture of the two components (dotted line), a chromatogram

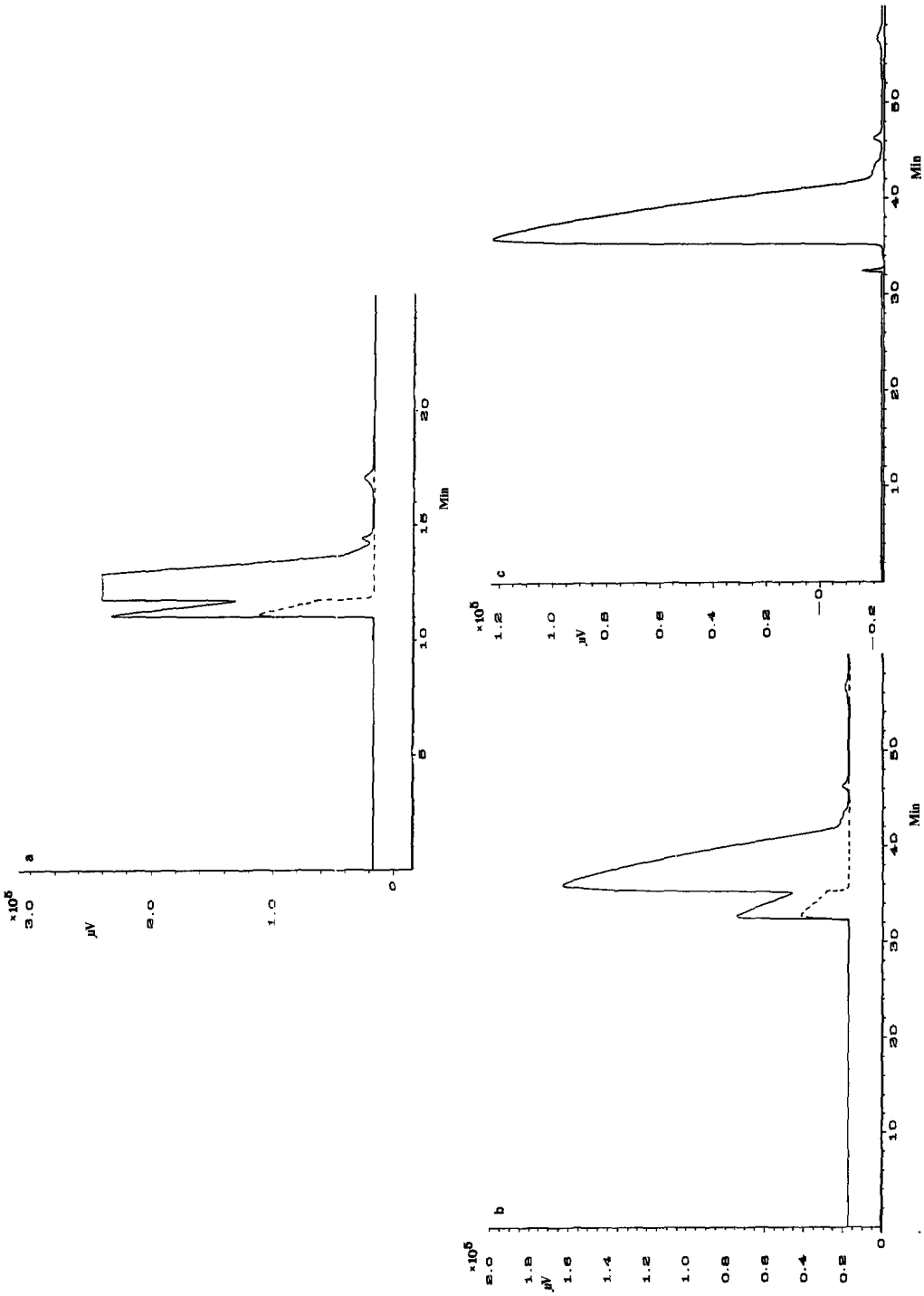


Fig. 4. Chromatograms obtained using two different gradient slopes for cytochrome *c* and lysozyme. (a) Conditions in Table III(a); (b) conditions as in Table III(d); (c) difference chromatogram (normalized 280 nm–475 nm) showing lysozyme peak and impurity.

TABLE V

GRADIENT CONDITIONS: STUDY OF GRADIENT RANGE

Other conditions as Table III.

| Slope (%/min) | Start %B | End %B | Duration (min) |
|---|---|---|---|
| 2.8 | 31 | 45 | 5 |
| 1.4 | 31 | 45 | 10 |
| 0.93 | 31 | 45 | 15 |
| 0.7 | 31 | 45 | 20 |
| 2.8 | 24 | 52 | 10 |
| 2.8 | 17 | 59 | 15 |
| 2.8 | 10 | 66 | 20 |

TABLE VI

INITIAL CAPACITY FACTORS IN GRADIENTS

| Initial %B | $k_0$ | |
|---|---|---|
| | Cytochrome *c* | Lysozyme |
| 10 | 216 000 | 2 660 000 |
| 17 | 7800 | 77 300 |
| 24 | 287 | 2249 |
| 31 | 11 | 65 |

from injection of 10 mg of cytochrome *c* (dashed line) and a chromatogram from injection of 10 mg of lysozyme (solid line). These were all run using a

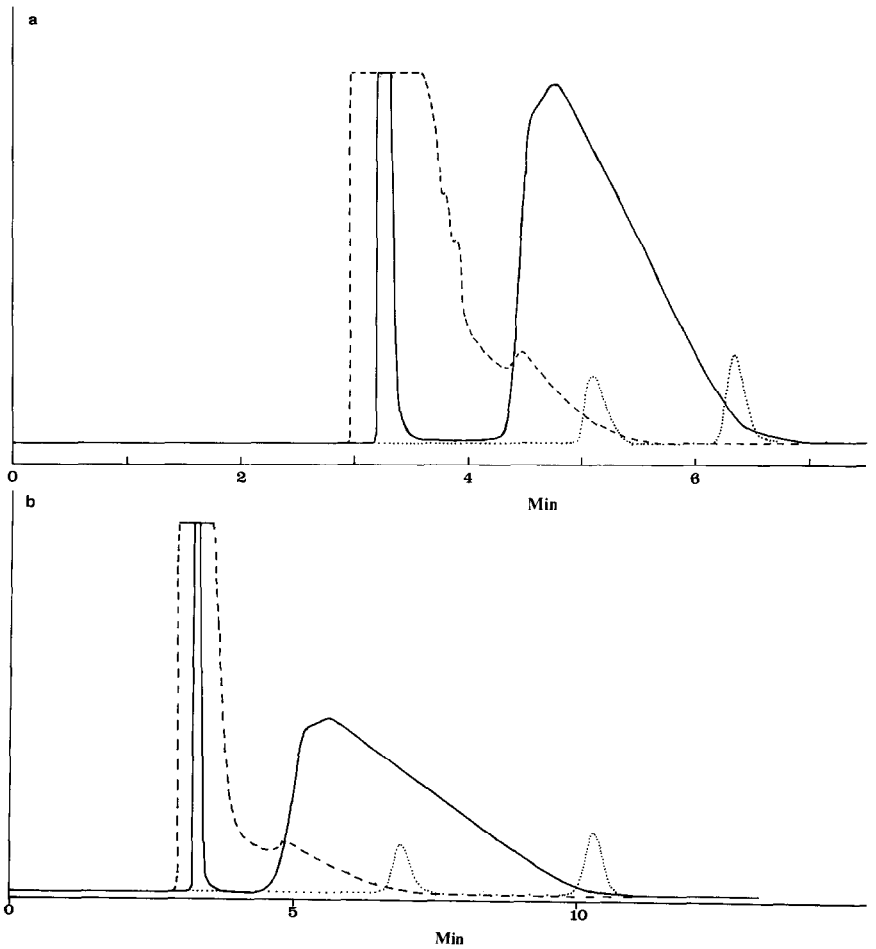


Fig. 5. Composite chromatograms from separate injections of cytochrome *c* and lysozyme. 10 mg of each compound, gradients from 31 to 45% acetonitrile in 0.1% TFA in (a) 5 min and (b) 15 min. Dashed lines, cytochrome *c*; solid lines, lysozyme; dotted lines, analytical (0.05 mg) injection of a mixture under the same conditions. Other conditions as Table III.

starting concentration of 31% acetonitrile and a gradient slope of 2.8%/min. It should be noted that the analytical chromatograms shown in all of the figures here are not on the same scale as the preparative chromatograms.

The cytochrome *c* peak begins elution at $t_0$ of the column and ends, as expected, at the retention time of the non-overloaded peak. The lysozyme peak is split. Only 8.6 mg of lysozyme, as estimated from peak-area data, were retained by the column regardless of the subsequent slope, the excess being eluted as a non-retained peak. A capacity even lower than this, estimated as slightly over 1.5 mg, was seen for cytochrome *c*. Fig. 5b shows similar chromatograms with a gradient slope of 0.93%/min. Other than the longer time scale, the two sets of chromatograms are essentially identical in all important respects.

With an initial acetonitrile concentration of 24% and the same gradient slope as for Fig. 5a, higher capacities for cytochrome *c* and lysozyme (7 and 9.4 mg, respectively) were seen. For gradients, still with the same slope as in Fig. 5a but commencing at 17% and 10% initial concentration of acetonitrile, all the material injected was retained.

From the known loads and the band widths of the peaks in the analytical and preparative runs, it was possible to calculate the saturation capacities of the columns by the procedure described elsewhere [15]. In addition, the apparent saturation capacity for each solute was determined by calculation of the mass retained by the column. This was done by taking the ratio of areas of the non-retained and the retained peaks and distributing the known mass injected between them. The calculated (band-width data) and apparent (peak-area ratio) saturation capacities for the two solutes under the different gradient conditions are shown in Table VII.

The values of the saturation capacity calculated from the band width for lysozyme range between 12.5 and 19 mg. If these values were accurate, then a significant amount of unretained protein should have been eluted in those earlier experiments where 20-mg loads of each protein were used. This is also true for the data for cytochrome *c*, where the saturation capacity is calculated to be less than 10 mg under all conditions. As suggested in an earlier paper [15], these saturation capacity values represent a minimum value rather than a true figure owing, perhaps, to the non-Langmuir adsorption of the proteins. The calculated saturation capacities should be approximately doubled to reach a more appropriate value. More importantly, the band-width data suggest that the actual column saturation capacity does not significantly change with the initial solvent composition. This is in contrast to the "apparent" values of saturation capacity (determined from the ratio of retained and non-retained solutes) which show a strong dependence on the initial solvent composition in the opposite direction from the much smaller trend in the values calculated from band width. It follows that the change in protein uptake with initial solvent strength is not a

TABLE VII

COMPARISON OF SATURATION CAPACITIES: CALCULATED *VS.* APPARENT

| Gradient | | $w_s$ (mg) | | | |
|---|---|---|---|---|---|
| Start | Slope | Calculated | | Apparent | |
| | | Cytochrome *c* | Lysozyme | Cytochrome *c* | Lysozyme |
| 31 | 2.8 | – | 18.5 | 2[a] | 8.6 |
| 31 | 1.4 | – | 18.9 | 2[a] | 8.6 |
| 31 | 0.9 | – | 18.6 | 2[a] | 8.6 |
| 24 | 2.8 | 8.2 | 15.7 | 7 | 9.4 |
| 17 | 2.8 | 9.1 | 12.9 | 10+ | 10+ |
| 10 | 2.8 | 8.4 | 12.6 | 10+ | 10+ |

[a] This value was obtained from the chromatography by assuming a likely shape for the retained cytochrome *c* peak and subtracting it from the overall peak envelope. The associated error is less than 0.5 mg.

phenomenon due to changes in the saturation capacity of the solutes in the column.

The observed relationship between the calculated saturation capacity of the proteins and the solvent strength at the beginning of the gradient may well be due to changes in the isotherms caused by changes in the interaction of the proteins with the surface of the packing material. At lower solvent strengths this interaction would be expected to be stronger and the protein conformation on the surface would be likely to change, bringing more of the hydrophobic groups within it into contact with the surface of the stationary phase. This probably would have the effect of making a larger "footprint", which in turn would result in the displacement of more solvent molecules per protein. According to our understanding of the changes in isotherm thus expected, this would result in a lower measured saturation capacity.

The foregoing implies that the lack of take-up of the proteins by the column on loading at relatively high solvent strength is due to reasons other than a decreased saturation capacity. One problem which has been seen in the past is that too high a water concentration in the mobile phase can lead to poor wetting of the packing, leading to only partial uptake of the solute. This is clearly not the case here; increasing the water concentration leads to a better uptake. From Table VI, at 24% acetonitrile the $k'$ of lysozyme is over 2000. Hence the equilibrium in the column is expected to favour uptake of the solute rather than its exclusion. Nevertheless, a small amount of material is not retained on introduction of the sample. One possibility [16] is that the adsorption of the large amount of protein on the surface of the packing displaces acetonitrile. The local increase in acetonitrile concentration may well be sufficient to desorb a certain amount of the protein, which is then swept down the column in the acetonitrile-rich band. Adsorption of the protein at low initial solvent strengths would not create a high enough local acetonitrile concentration to cause this desorption. The effect would be expected to increase as the initial acetonitrile concentration in the gradient increases. Experiments to verify this possibility are in progress.

*Mixed solutes.* Similar experiments to those above were carried out with samples consisting of mixtures of the proteins using the same packing material and gradients. Chromatograms from a mixture of 3 mg of cytochrome *c* and 10 mg of lysozyme are shown in Figs. 6–8 as overlays of analytical and preparative chromatograms. Again, the analytical chromatograms are displayed on a greatly increased scale.

The cytochrome *c* peak in the 31–45% gradient (Fig. 6) is displaced by the front of the lysozyme envelope. This is seen by the tail of the peak, which now ends considerably earlier than the elution time

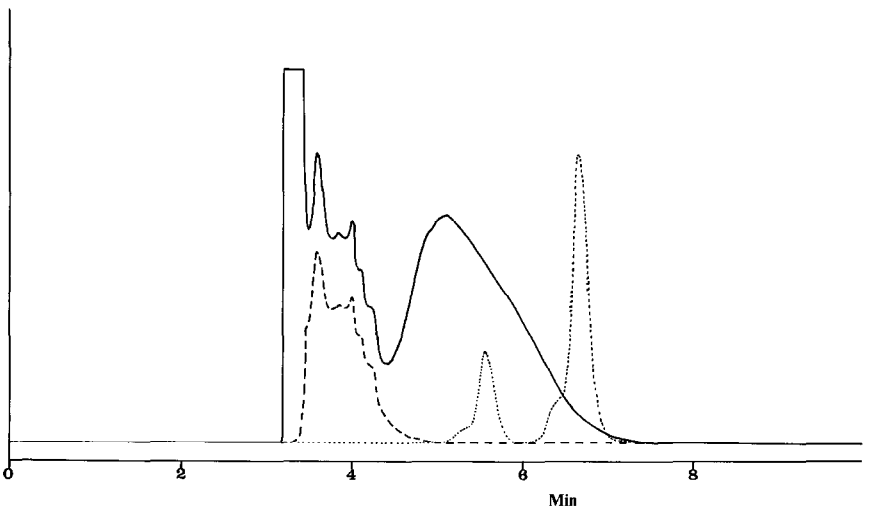


Fig. 6. Chromatograms of a mixture of cytochrome *c* and lysozyme. Sample, 3 mg of cytochrome *c* and 10 mg of lysozyme. Other conditions as in Fig. 5. Solid line, 290 nm; dashed line, 480 nm; dotted line, mixture of cytochrome *c* and lysozyme, 0.05 mg each.

of the same component under analytical load. There is a noticeable tail to the peak, which corresponds to the relatively slow increase in concentration of lysozyme as it begins to elute. As seen in later chromatograms, as the peak front of lysozyme becomes closer to the vertical, the tail of the cytochrome *c* peak diminishes.

Strangely, it appears that the non-retained lysozyme peak may be displaced from the column by the cytochrome *c* at the same time; certainly the non-retained lysozyme elutes earlier than the cytochrome *c* peak envelope, unlike the situation seen in Fig. 5 where the unretained lysozyme elutes at the same retention time as the unretained cytochrome *c* when injected separately. This could be ascribed to the initial uptake of protein being at the surface of the particle, thus blocking the pores and preventing their penetration by the unsorbed lysozyme molecules. These molecules, in being excluded from the pore volume within the particle, would then elute earlier than adsorbed or permeating species. Such a mechanism does not explain why cytochrome *c* is apparently not excluded from the packing. If the process is purely one of building a crust of adsorbed protein at the surface of the particle, then a proportion of cytochrome *c* should also be excluded by the same mechanism. In addition, it would be expected that the formation of such a crust would be favoured by increased energy of interaction of the solutes with the packing (the molecules would "stick" faster and then be less likely to move) and that there would be less protein (rather than more) adsorbed at lower solvent strengths.

Similar displacement effects, coupled with the non-adsorption of a proportion of the injected lysozyme, are seen in gradients with the same range of solvent composition but longer duration (lower slope). Although the separation between the peak maxima increases as expected, the band widths also increase, giving qualitatively very similar separations at the different slopes. The actual separation between the bands is controlled more by the displacement between them than by the gradient profile, just as was seen in the initial experiments (Fig. 3). As the initial acetonitrile concentration is decreased to 24 and 10%, but with constant gradient slope (Figs. 7 and 8), the peaks maintain approximately a constant width but move to longer retention time. At the same time, the amount of non-retained material decreases, as expected from the saturation capacities measured for single solutes.

*Optimization of the gradient*

Although the band-width data suggest that the true column capacity does not change with the gradient, in practice the load which may be applied to the column depends on the initial solvent composition. In order to make maximum use of the column

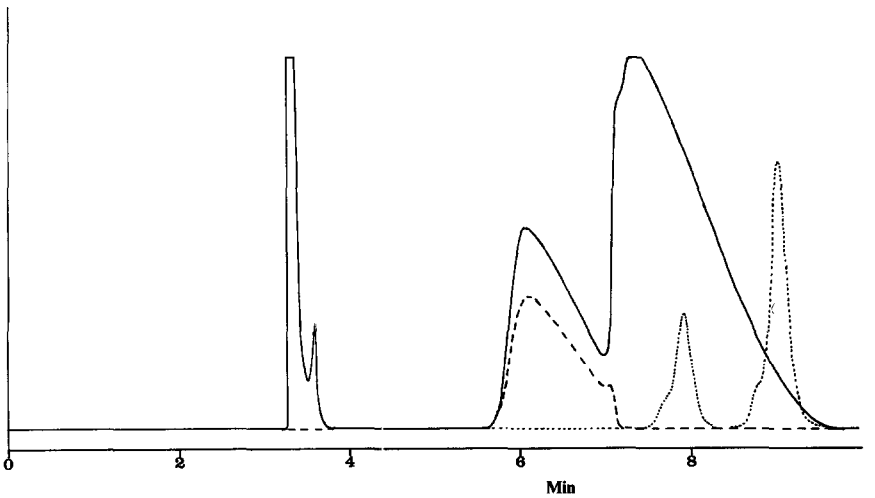


Fig. 7. Chromatograms of a mixture of cytochrome *c* and lysozyme. Gradient conditions, 24 to 52% acetonitrile in 10 min. Other conditions as in Fig. 6.

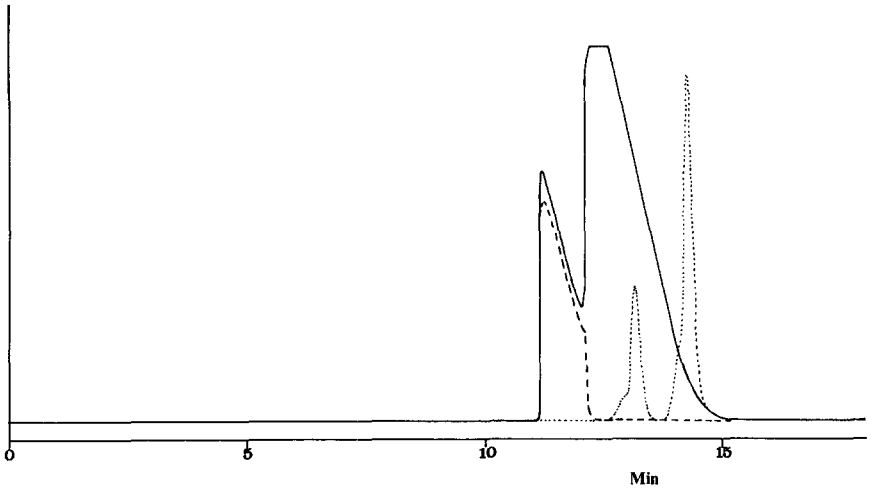


Fig. 8. Chromatograms of a mixture of cytochrome *c* and lysozyme. Gradient conditions, 10 to 66% acetonitrile in 20 min. Other conditions as in Fig. 6.

capacity, it is necessary to begin the gradient under conditions of very strong retention. This means that, even with a steep gradient, the retention time of the solute will be long. This will impact on the production rate of the separation. If the non-retention of the solutes by the column is a loading phenomenon, then it should be possible to load the column under strong retention conditions, quickly change the solvent strength to a high value and elute the components rapidly. If the effect is due in some way to a change in the dynamic capacity of the column, then it would be expected that the "excess" protein would be rapidly eluted from the column as the solvent composition was changed from the loading to the starting composition.

An experiment was performed to investigate this by loading the column at 10% acetonitrile, holding the acetonitrile concentration at 10% for 5 min and then rapidly changing the mobile phase (over 1 min) to 31% acetonitrile and subsequently running an elution gradient from 31 to 45% acetonitrile over 5 min (Fig. 9). No protein eluted during the period of rapid change, and the chromatogram shows no non-retained peaks, but is closely similar to the others which started at low solvent strength and maintained a constant gradient slope. The cytochrome *c* peak is clearly displaced by the lysozyme, as seen from comparison with the analytical chromatogram obtained under identical conditions.

In order to maximise the production rate for the preparative HPLC purification of proteins, it appears that a number of aspects need to be addressed. The sample must be loaded on the column under strongly retaining conditions. It should be noted that often a small concentration of organic component must be present in the mobile phase to ensure wetting of the packing material, such that the solutes will be able to interact with its surface. Where the solutes are hydrophilic and elute at low concentrations of organic component, this starting concentration will have to be determined with care.

Once the sample has been introduced, the mobile phase solvent strength is increased in a rapid gradient. This allows the displacements to occur. It is not yet known if there is an optimum for this gradient slope to maximise the displacements. This short gradient is intended to bring the mobile phase to the correct solvent strength for the beginning of a second, shallower gradient during which the components are eluted. The slope and range of this gradient are chosen to allow an adequate analytical resolution between the components, but should be as steep as possible. As it is highly probable that the actual gradients needed for the solution of any one problem will be separation dependent, the actual values of the parameters for both gradients will need to be determined experimentally on a case by case basis.

The displacements are maximised by the opera-

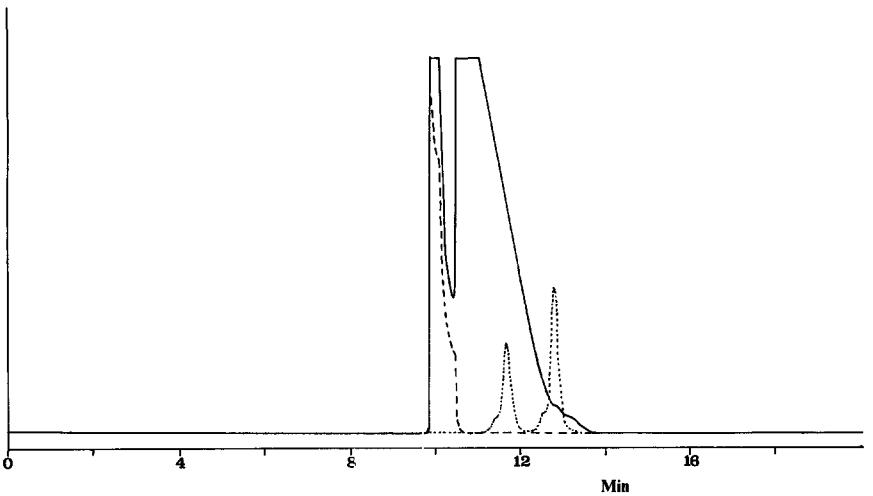


Fig. 9. Chromatogram of a mixture of cytochrome *c* (3 mg) and lysozyme (10 mg). Gradient, 5 min at 10%, from 10 to 31% in 1 min, from 31 to 45% acetonitrile in 5 min. Other conditions as in Fig. 6.

tion of the column such that the elution front of the displacing peak has a sharp increase in solute concentration. The column must therefore be operated under conditions of high efficiency. Not surprisingly, this is the same conclusion as has been reached for isocratic chromatography of small molecules where the column efficiency controls the width of the overlap zones of the displacing solute bands. Work designed to investigate the effects of column efficiency on solute–solute displacements in preparative elution chromatography is ongoing.

## CONCLUSIONS

The mass overloaded gradient elution separation of small molecules is closely similar to the corresponding isocratic separation. This extends to the displacement effects observed between mass-overloaded components. Where the second-eluted component has the higher saturation capacity, displacement effects are observed, just as in the isocratic case. Where the first component has the higher saturation capacity, the peaks merge.

The previous report that equations for the relationship between band width and sample load yield low values of saturation capacity for proteins has been confirmed. This is probably due to the assumption of Langmuir isotherm behaviour. Proteins cannot follow this precisely; even in the most favourable case their size dictates multiple displacement of solvent molecules on adsorption.

Displacement effects within preparative elution gradients occur between protein solutes under a wide range of conditions. The initial solvent strength affects the practical loadability greatly; at (relatively) high initial solvent strength the loadability is severely compromised by the inability of the column to retain samples injected at loads well below the saturation capacity. Samples should therefore be introduced at low solvent strength.

The slope of the latter part of the gradient was demonstrated to have little influence on the overlap of well separated bands. The gradient profile adopted for heavily overloaded preparative gradient separations of proteins must be carefully designed to allow high loading, solute–solute displacement effects and a rapid elution of the peaks of interest to maximise the production rate of the purification.

## APPENDIX

### *Non-Langmuir adsorption: consequences for solute interactions*

Langmuir retention is represented in Fig. A1. Initially a solute molecule X (cross-hatched cube) is in the mobile phase, and a solvent molecule M (white cube) is in the stationary phase. The retention of X requires a displacement of M. The area required for

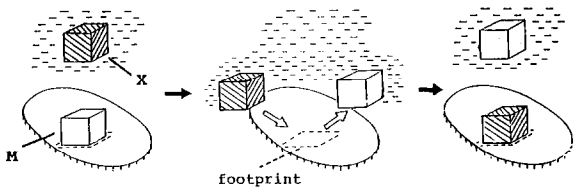


Fig. A1. Representation of sample retention in HPLC by a displacement process. Sample (X) and mobile phase (M) molecules of equal size.

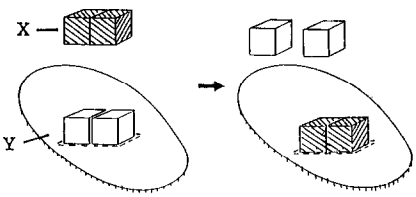


Fig. A3. Representation of sample retention in HPLC by a displacement process. Sample molecules (X and Y) of different sizes, equal saturation capacities but unequal footprints.

the retention of either X or M (known as the "footprint") is the same and the thickness of a monolayer of X or M is also the same. From this it follows that the column saturation capacities for X and M are the same, assuming equal densities.

A similar retention process involving a second solute molecule Y can be visualised, wherein the white cube in Fig. A1 might now represent Y instead of M. Again, there is a one-for-one displacement process, so that the $w_s$ values for X and Y are the same. This is essentially the model which has been assumed in most computer simulations of preparative LC. This model gives rise to the Langmuir isotherm.

If the footprints of the two solutes are the same, it can be seen (Fig. A2) that even if one of the solutes is twice the size of the other, the Langmuir equations will still hold. In this instance, the saturation capacity ($w_s$) values of the two solutes taken on a weight basis will differ, although the saturation capacities determined on a molar basis will be equal.

Fig. A3 shows the case where the molecule of X has twice the footprint of solute Y. For adsorption of X to occur (assuming that the surface of the packing is uniformly filled with the two solutes) two molecules of solute Y must be displaced. Equally, for each molecule of X to be displaced, two molecules of Y may be adsorbed. Thus, on a molar basis, the saturation capacity of Y will be double that of X. If solutes X and Y are of equal molecular weight, then the weight-based saturation capacities will also be unequal. In the situation where X has twice the molecular weight of Y, the weight-based saturation capacities will again be equal. It follows that conclusions as to the likelihood of displacement or peak merging should be made based on molar saturation capacities rather than weight-based saturation capacities.

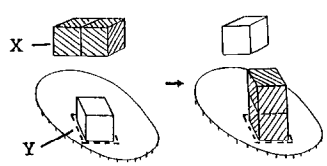


Fig. A2. Representation of sample retention in HPLC by a displacement process. Sample molecules (X and Y) of different sizes (and $w_s$ values), but equal footprints.

The two solutes, X and Y, each displace a certain number of solute molecules. These numbers are represented by $n$ and $p$ respectively. If X and Y have different footprints, then $n$ and $p$ are not equal and $n/p$ molecules of Y will displace one molecule of X. Assuming that the activity coefficients are constant, the equilibria occurring at this point on the surface can be represented by

$$X_m + nM_a \rightleftharpoons X_a + nM_m \quad (1)$$

$$Y_m + pM_a \rightleftharpoons Y_a + pM_m \quad (2)$$

$$X_a + (n/p)Y_m \rightleftharpoons X_m + (n/p)Y_a \quad (3)$$

$$K_X = \Theta_X C_m^n / \Theta_m C_X^n \quad (4)$$

$$K_Y = \Theta_Y C_m^p / \Theta_m C_Y^p \quad (5)$$

$$\Theta_X = w_{Xs}/w_s \quad (6)$$

$$\Theta_Y = w_{Ys}/w_s \quad (7)$$

where, $K_X$ and $K_Y$ are equilibrium constants for eqns. 1 and 2, $\Theta_i$ refers to the fraction of stationary phase sites occupied by molecular species $i$, $C_i$ is the mobile phase concentration of $i$ (g/ml), $w_{is}$ is the weight of $i$ in the stationary phase (g/ml) and $w_s$ is the weight of the adsorbed monolayer.

If we first assume that $n = p = 1$, that $C_X$ and $C_Y \ll C_m$, $V_m$ is the volume of mobile phase in the column and that all activity coefficients remain constant, the selectivity is given by

$$\alpha = \frac{[(\text{weight of Y in stationary phase}) \cdot (\text{weight of X in mobile phase})]}{[(\text{weight of X in stationary phase}) \cdot (\text{weight of Y in mobile phase})]}$$

$$= w_{Ys} C_X V_m / w_{Xs} C_Y V_m = K_Y / K_X \quad (8)$$

In this situation, the selectivity does not change with load.

When $n \neq p$, the mobile phase modifier concentration becomes important. We assume that in a binary mobile phase mixture the concentration of the stronger solvent does not change in the mobile phase with sample load and that the stationary phase contains only molecules of the solutes and the strong mobile phase component, *i.e.*,

$$\Theta_X + \Theta_Y + \Theta_b \approx 1 \quad (9)$$

where $\Theta_b$ is the fraction of stationary phase sites occupied by the strong mobile phase component.

Calculation of $\alpha$ in the same fashion as earlier results in a changed relationship:

$$\alpha = (K_Y/K_X)(\Theta_b/C_b)^{p-n} \quad (10)$$

Combination of eqns. 11 and 12 results in

$$\alpha = [(K_Y/K_X)(C_b^{n-p})](1 - \Theta_X - \Theta_Y)^{p-n} \quad (11)$$

This equation shows that $\alpha$ will change with sample size, in that the term $(1 - \Theta_X - \Theta_Y)$ will decrease with increasing sample size. This term is less than unity. If $p > n$, the selectivity will decrease with increasing sample size, *i.e.*, at some point the peak maxima will merge and change elution order. Because $p > n$, the individual solute isotherms (measured on a molar basis) will cross. Conversely, selectivity will increase with sample size if $n > p$ and the isotherms will diverge. These two cases correspond to the situations in Fig. A3 where the second-eluting component has respectively a smaller and a larger saturation capacity.

If we now assume that the solute concentrations are very high and the entire surface of the packing is covered with solute molecules, then eqn. 3 is particularly relevant to the discussion. The equilibrium constant for this process is

$$K = [X_m][Y_s]^{n/p}/[X_a][Y_m]^{n/p} \quad (12)$$

Comparing the cases for $n = p$ and $n > p$, it is clear that (at our given $k'$ value) the concentration of Y in the stationary phase will have a much greater influence in the latter situation and, in comparison with the former instance, the concentration of X in the stationary phase will be much reduced. If X is the earlier eluted species, the peak will be much more displaced than when $n = p$ (equal molar saturation capacity values) and the recovery of X and Y will be higher. If X is the later eluted species, its displacement from the surface by Y means that it will elute earlier. The extent of the interaction will determine if the solutes will simply show a strong tag-along, if they will merge or if the second-eluted component will be displaced to the front of the first-eluting peak.

In practice, the entire surface of the packing is not filled only with solute molecules. As a significant proportion is taken up by solute molecules in strongly overloaded separations, the phenomena predicted will be observed to an extent less than may be assumed from the above equilibria. This will be determined by the relative surface area occupied by the solvent molecules.

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