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COLUMN LOADING AND RELATIVE RETENTION IN OVERLOADED ELUTION CHROMATOGRAPHY

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SUMMARY

The influence of the relative retention, α, on the column loading capacity was investigated for compounds having slowly diverging Langmuir isotherms. The same trends as observed previously for a binary mixture with $\alpha = 1.09$ were found. Larger sample sizes were required in order to observe band overlap when α increased. Accordingly, the displacement and tag-along effects observed on the band profiles were stronger. In all instances, the recovery decreased with increasing sample size. The production rates, on the other hand, increased at first, passed through a maximum and then decreased. It is also shown that the optimal production increases with increasing α.

INTRODUCTION

Preparative-scale liquid chromatography is rapidly becoming a major purification technique as the need for large amounts of highly pure compounds has increased dramatically in the last few years. This renewed interest has followed the tremendous growth of the biotechnological and pharmaceutical industries.

When dealing with chromatographic separations, it is important to optimize the resolution, separation speed and sample loading capacity[1]. As these parameters are interrelated, a compromise must be sought. In analytical chromatography, for example, data collection and analysis are emphasized. Accordingly, the separation speed and/or resolution are maximized at the expense of sample loading. In preparative chromatography, on the other hand, the goal is a higher sample capacity and the separation speed and/or resolution are therefore of secondary concern.

The most important factor influencing these characteristics in analytical chromatography is the relative retention, α, which is the ratio between the column capacity factors of the two compounds to be separated. The resolution, R_s, for a very small sample size is given by the classical equation[2]

$$R_s = \frac{1}{4}\sqrt{N}(\alpha - 1)\left(\frac{k'}{1 + k'}\right) \quad (1)$$

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where N is the column efficiency and k' is the column capacity of the second component of the pair. For a given binary mixture, the larger the α value, the better is the analytical resolution and the faster the analysis. Therefore, chromatographic systems are selected in order to maximize the relative retention of the pairs of compounds that are most difficult to separate.

Intuitively, we may conclude that the column loading capacity will also be maximized by the selection of the chromatographic system that affords the greatest α value. This is true if the binary equilibrium isotherms of each of the two compounds are slowly divergent, as seems to be the general case for similar compounds and especially for closely related isomers. This may not be so for compounds that would undergo strong sorbate–sorbate interactions in the stationary phase or for compounds for which the column loading capacities would be very different. If the column capacity is much larger for the lesser retained compound than it is for the more strongly retained compound then the binary isotherms may cross each other, resulting in a practical limit to column overloading which may be abnormally low and lead to paradoxical results at larger loads. This paper deals with what we feel is the general case. A forthcoming paper will discuss an example of the latter case.

THEORETICAL

The semi-ideal model

We calculated elution profiles for large samples of binary mixtures using the previously described and discussed semi-ideal model[3,4]. In this model, a mass balance equation is written for each component of the mixture. It is assumed that the mobile phase is not adsorbed, which is acceptable for a one-component mobile phase and depends on the reference state chosen for adsorption[5]. For binary or more complex mobile phases, the assumption still holds, but only for the weak solvent. Depending on the experimental conditions, dropping the strong solvent mass balance equation may still lead in many instances to correct predictions of the elution band(s) of the solute(s), but complications will arise as it will no longer be possible to account for ghost (or system) peaks.

The system of mass balance equations should be completed by a relationship between the concentrations of each solute in the mobile and stationary phases. This should be given by a kinetic equation written for each component of the binary mixture. In the ideal model of chromatography, it is assumed that the kinetics of mass transfer are so fast that equilibrium between phases is reached instantaneously, so the column has an infinite efficiency. The relationship between the solute concentrations in the mobile and stationary phases is then given by the competitive equilibrium adsorption isotherms. This corresponds to the well studied ideal model of chromatography[6–9]. Finally, the calculations of numerical solutions of the ideal model require a set of boundary conditions describing the injection.

The inherent limitations of the ideal model lie in the appearance of concentration shocks on the elution profiles. The appearance of these shocks and their stability result from the absence of a diffusion term in the partial differential equations. This is a consequence of assuming an infinite column efficiency and does not lead to very realistic results. Further, during the millions of calculation loops, the computer must first locate these shocks. It then calculates the concentration values on both sides of

the discontinuities and finally it extrapolates between these two values. This procedure leads to an unacceptable loss of peak area (*i.e.*, matter) during the calculation[9].

In this work, we used a finite difference method based on the Godunov algorithm[10]. The continuous (z,t) plane is replaced by a $(n\delta z, i\delta t)$ grid defined by a space and a time increment. The space increment, δz, is set equal to the height equivalent to a theoretical plate of the column, H, and the time increment, δt, to twice the time required for a non-retained compound to move a distance equal to $2H$ down the column. It can be shown that with these increment values, the errors resulting from the replacement of the partial differential equations by finite difference equations mimic exactly the effect of a finite column efficiency on the elution profiles[11]. The numerical errors introduced are equivalent to a diffusion term where the diffusion coefficient is equal to the apparent diffusion coefficient of the chromatographic column used. The only assumptions made are that the mass transfer coefficients (such as the molecular diffusion coefficients) are independent of the concentration of solute, which is true in the range used in preparative liquid chromatography, and that H does not depend on the retention time (*i.e.*, k'). Accordingly, the concentration shocks do not appear, the profiles are realistic and there is no loss of matter during the computer calculations.

The simulation

A FORTRAN program allows the calculation of numerical solutions of the system of partial differential equations. It permits the elution profiles of both components of the mixture to be obtained for a well defined set of experimental conditions, provided that the competitive equilibrium adsorption isotherms are known. Eventually, by integrating the elution profiles, the recovery yields and production per unit time for each component of the mixture can be determined[3].

For a two-component mixture, the difficulty of the separation depends on the relative retention, α, of the two compounds and on the ratio of the isotherm curvatures. In this paper, we have assumed that the ratios of the slopes and of the curvatures of the two binary isotherms are equal. This corresponds to two binary isotherms which are slowly diverging. While the precise combination of numbers is arbitrary, the situation seems to be fairly general for pairs of closely related compounds. For others, a change in the chromatographic system will most often transform a separation that is accidentally difficult into an easy one. We have investigated the effect of the relative retention on preparative separations for different relative concentration ratios (1:9, 1:3 and 9:1).

In liquid chromatography, many binary mixtures have adsorption isotherms that can be described reasonably well, to a first approximation, by competitive Langmuir adsorption isotherms of the form

$$q_i = \frac{a_i c_i}{1 + b_1 c_1 + b_2 c_2} \tag{2}$$

where q_i and c_i are the concentrations of component i in the stationary and mobile phases, respectively; a_i and b_i are the Langmuir parameters for component i, a_i being given by the equation

$$a_i = k'_i \cdot \frac{V_m}{V_e} \tag{3}$$

where k_i' is the column capacity factor of component i for a very small sample size and V_m and V_s are the mobile and stationary phase volumes, respectively.

The column is defined by its total porosity, which is set equal to 0.8, a value frequently encountered in liquid chromatographic columns. Hence,

$$\frac{V_m}{V_s} = \frac{\varepsilon}{1 - \varepsilon} = 4 \tag{4}$$

In all instances, the column length is 25 cm while its efficiency, as defined by the number of theoretical plates N, is equal to 5580 for a very small sample size. As shown previously[3,4,6-11], the broadening of the peak with increasing sample size observed experimentally is based on the thermodynamics of the process (non-linearity of the equilibrium adsorption isotherm). It does not reflect a change in the kinetics of mass transfer between phases, *i.e.*, it cannot be explained by a decrease in the actual column efficiency. The characteristics k', a and b of the more strongly adsorbed component of the mixture (2) are kept constant while those of the lesser retained compound (1) are changed. Computer simulation is used to study the influence of the relative retention on the preparative separations of binary mixtures whose competitive Langmuir isotherms are known. The numerical values introduced into eqn. 2 for each solute are reported in Tables I and II. The k_i' values listed correspond to the injection of a very small sample size. The retention time of an unretained solute is 40 s.

The sample size is given in arbitrary units. The column saturation capacity, as defined by Eble *et al.*[12], is equal to 100 so that a sample size of 10 units corresponds to a 10% column capacity loading.

TABLE I

NUMERICAL VALUES USED IN EQN. 2 FOR $\alpha = 1.25$

Component	k'	a	b
1	5	20	2.07
2	6.25	25	2.56

TABLE II

NUMERICAL VALUES USED IN EQN. 2 FOR $\alpha = 1.7$

Component	k'	a	b
1	3.675	14.7	1.52
2	6.25	25	2.56

RESULTS AND DISCUSSION

Influence of the relative retention on the preparative separation of a 1:3 mixture

For $\alpha = 1.25$, a 5-unit sample size of the mixture (5% of the column saturation capacity) is sufficient to allow for some interaction between the two bands (Fig. 1a). With a 20-unit sample size, there is a considerable interaction between the two bands (Fig. 1b). The concentration discontinuities on both chromatograms are extremely sharp, even with the smoothing effect of diffusion; the shock layer (*i.e.*, the region where the concentrations of both components 1 and 2 vary rapidly) has become thin[13]. Compound 1 is concentrated in a narrow band in front of the second band. This phenomenon increases with increasing sample size (see Fig. 1a and b), as indicated by the increase in the peak maximum of component 1 from approximately 0.78 to 2.8. The lower part of the diffuse side of the first peak, however, continues to drag behind, underneath the second peak profile well after the more strongly adsorbed compound has started to elute.

The front of the peak of compound 2 elutes faster than if it were alone and a hump forms on its tail that becomes larger with increasing sample size.

What happens here is that, at the beginning of the column, the concentration of component 2 is very large and its molecules occupy most of the sites on the stationary phase. As a result, component 1 is pushed in the mobile phase and moves faster than if it were alone in the column. This signals the onset of a displacement effect[3,14]. After the peak maximum of component 2 has been eluted, however, its concentration decreases, thus freeing some sites on the stationary phase on which component 1 can be adsorbed. Component 2 then competes less strongly with 1 which, in turn, is less strongly displaced, *i.e.*, it lags behind and an isotachic train can never form. This phenomenon is characterized by the strong tailing of the lower part of the diffuse rear of the peak of component 1.

These phenomena are dramatically illustrated by a comparison of Fig. 2a and b, which correspond to a larger α value of 1.7. Fig. 2a shows that for a 15-unit sample size (15% of the column saturation capacity) completely resolved (albeit overloaded) peaks are eluted. This is the best way to achieve complete recovery of both components of the mixture, as illustrated by Knox and Pyper[15].

A comparison of Figs. 1a, 1b and 2a confirms that the loading capacity of a column increases with increasing α. Fig. 2b shows the superimposition of the chromatograms obtained successively for pure components 1 and 2, in amounts equal to those used for Fig. 2a (3.75 units of 1, then 11.25 units of 2). It can be seen that the profiles of component 2 injected alone and in the mixture are identical. This demonstrates the stability of the band profile. Its shape, which is imposed by solution thermodynamics and by the dynamics of band migration in non-linear chromatography, is restored after perturbation of the frontal shock. Compound 1, however, is displaced from a retention time of 134 s (pure) to a time of 111 s (mixture). Its band width narrows from 57 to 37 s and its maximum concentration increases from 1.6 to 2.1 m*M*. This phenomenon, which has been called the "blockage" effect[12], appears to be the residual consequence of the displacement effect which takes place earlier in the column.

The chromatogram in Fig. 2a is very similar to that corresponding to the reversed-phase separation of two xanthines [β-hydroxyethyl- and 7-β-hydroxypro-

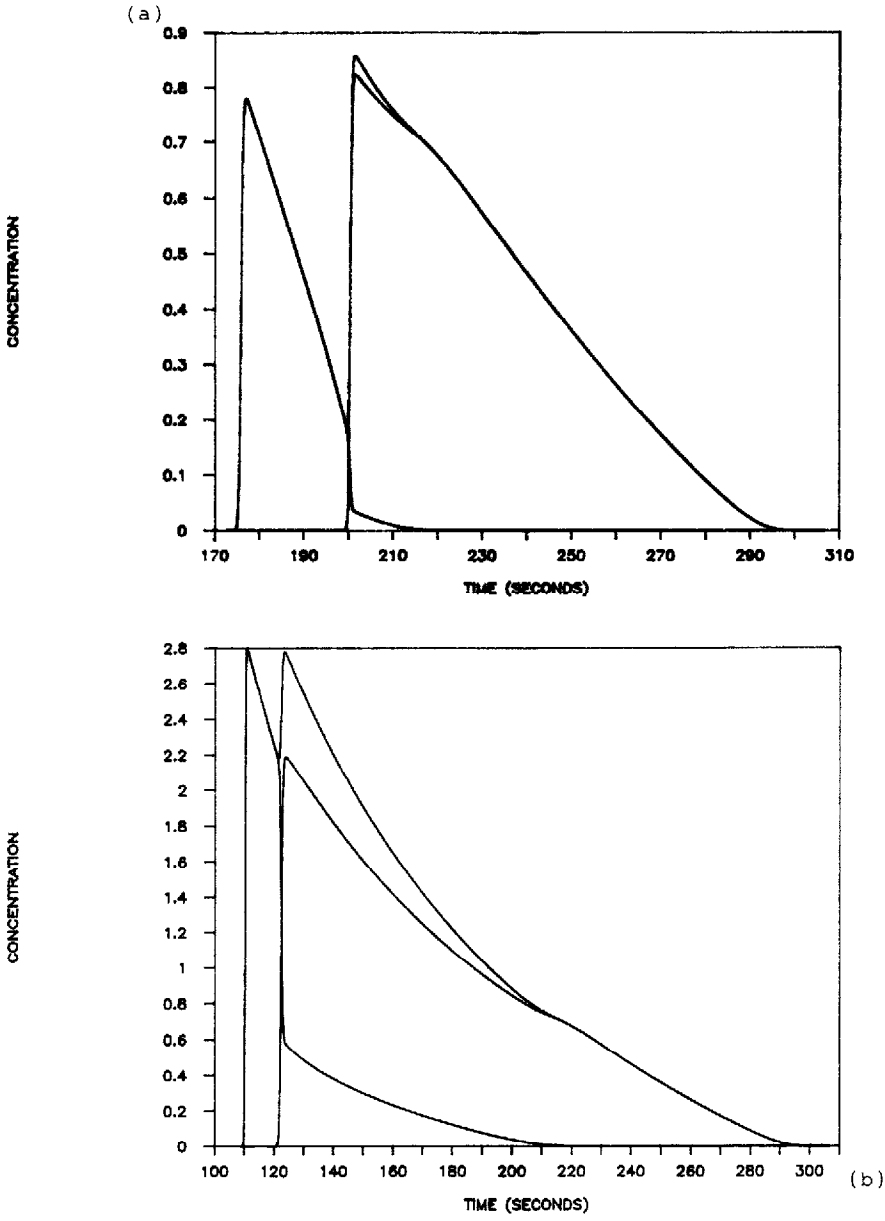

Fig. 1. Overloaded chromatograms of a 1:3 mixture with $\alpha = 1.25$. (a) Sample size 5% of column capacity; (b) sample size 20% of column capacity.

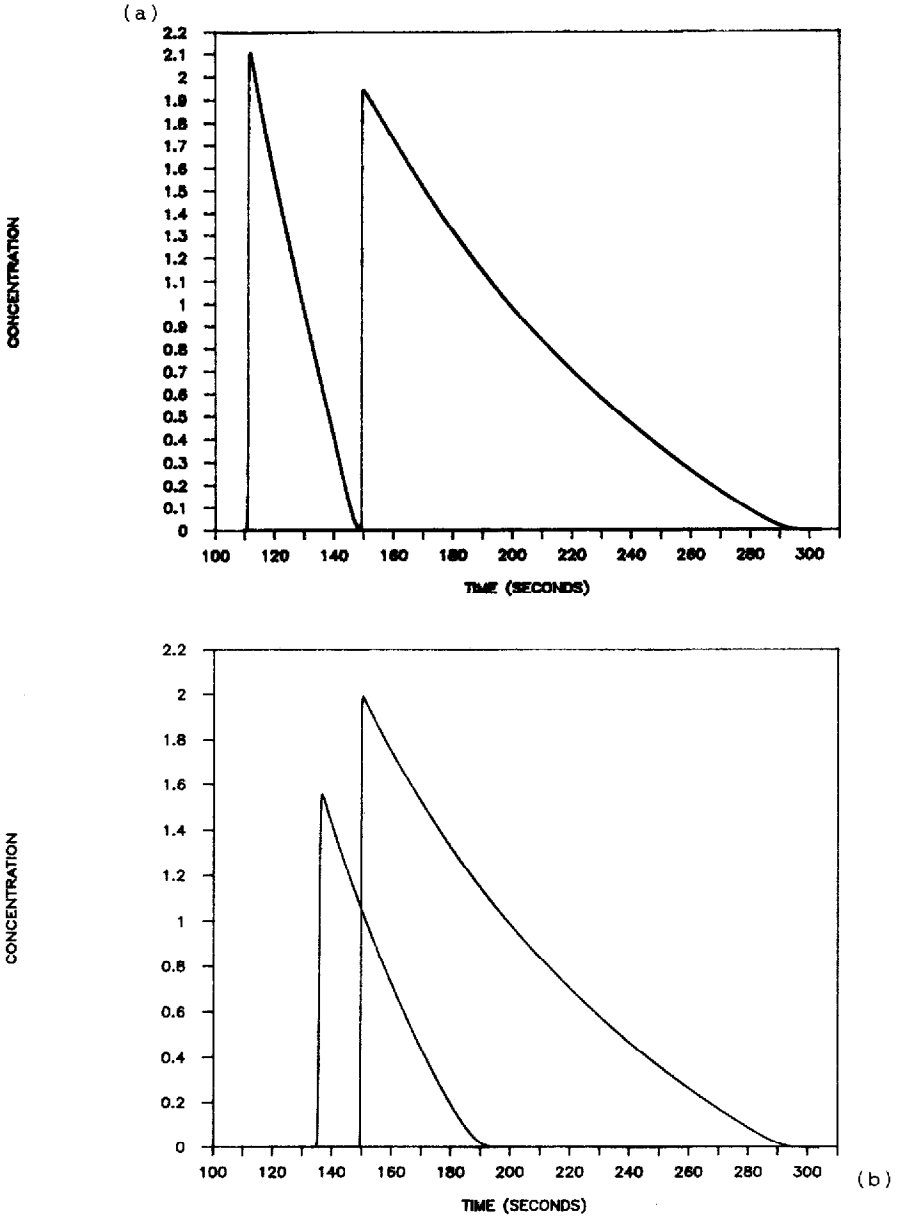

Fig. 2. Overloaded chromatograms of a 1:3 mixture with $\alpha = 1.7$ and a sample size equivalent to 15% of the column capacity (column length 25 cm). (a) 1:3 mixture of compounds 1 and 2; (b) same amounts of compounds 1 (3.75% of the column capacity) and 2 (11.25%), injected successively.

pyltheophylline (HET and HPT)] by Eble *et al.*[16] (see Fig. 4 in ref. 16). The main difference resides in the retention times; in our simulation, compound 1 is less retained and is therefore narrower, and hence more concentrated than in the experimental case. It should be emphasized that although the two bands are completely resolved at the column outlet, they interact very strongly in the column, especially at the beginning of elution. The displacement effect decreases slowly in importance as the bands migrate along the column and slowly become disengaged from one another (see Fig. 3a and b). This effect is more pronounced for smaller values of α as they correspond to compounds whose affinities for the stationary phase are more comparable. Also, the tag-along effect of the second band decreases with increasing migration distance. As the two bands separate, the front of the second band recedes relatively, the profile of this second band becoming progressively more identical with that of a pure sample of compound 2. This is because the velocity of the self-sharpening front of a pure compound band depends only on the concentration of the band maximum and decreases with decreasing maximum concentration. The tag-along effect pulls forward the front of peak 2, decreasing its maximum concentration (see Fig. 2). When the bands separate, the tag-along effect decreases, the front of band 2 recedes and its maximum height increases. Thus, the band returns to the stable profile of pure compound 2 (Fig. 2b).

Fig. 4a and b shows plots of the recoveries for component 1 *versus* amount injected, for $\alpha = 1.25$ and 1.7, respectively. As expected, the yields decrease much faster with increasing sample size for $\alpha = 1.25$ (*e.g.*, 60% compared with 98% for 20% column saturation capacity). At 50% of the column saturation capacity, the recovery of 1 is still greater than 70% for $\alpha = 1.7$. The same general trends are observed for the second component of the mixture. Clearly, a large α value allows for a greater loading capacity. Hence it is certainly good practice to optimize first the analytical resolution before undertaking preparative high-performance liquid chromatography (HPLC). A larger throughput of the mixture of interest will be achieved, provided that the ratio of the curvatures of the isotherms at the origin is larger than 1.

Fig. 5a ($\alpha = 1.25$) and b ($\alpha = 1.7$) shows the production per unit time of component 2 as a function of the total amount of the mixture whose introduction into the column is simulated. In all instances the optimal production shifts towards a higher loading capacity when the required sample purity decreases. This can be understood in terms of the strong tailing of component 1 underneath 2. It is a source of contamination and reduces its yield markedly. In practical terms, the less stringent are the purity requirements, the more overlap, *i.e.*, interactions, can be allowed between the two components of the mixture. Finally, the higher the α value, the greater is the possible throughput and the higher is the optimal production at a given required purity.

Influence of the relative concentration of the components of a binary mixture on their preparative separation for $\alpha = 1.7$

We illustrate the influence of the relative concentration with the chromatograms obtained in two extreme cases, corresponding to relative concentrations of 1:9 and 9:1.

Fig. 6a and b shows the chromatograms for a 1:9 mixture, with sample sizes

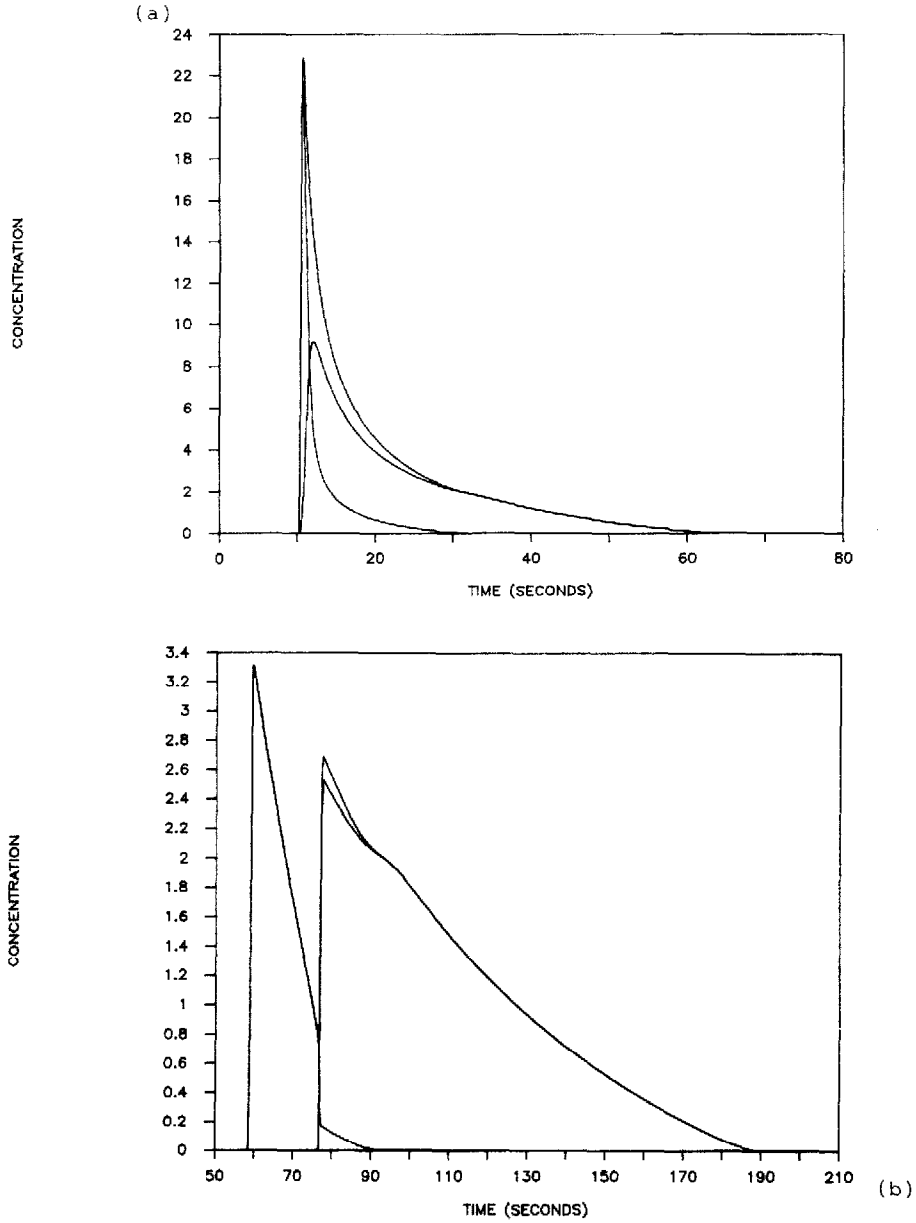

Fig. 3. As Fig. 2a but chromatograms recorded at different column lengths: (a) $z = 5$ cm; (b) $z = 15$ cm.

equal to 20 and 50% of the column capacity, respectively. In Fig. 6a the two bands interact only slightly, whereas they interact very strongly in Fig. 6b. In both instances component 2 is in large excess and it therefore displaces 1 in front of it and concentrates it into a thin zone. This phenomenon is illustrated by the narrowing of the band of 1 with increasing sample size and the increase in the concentration corre-

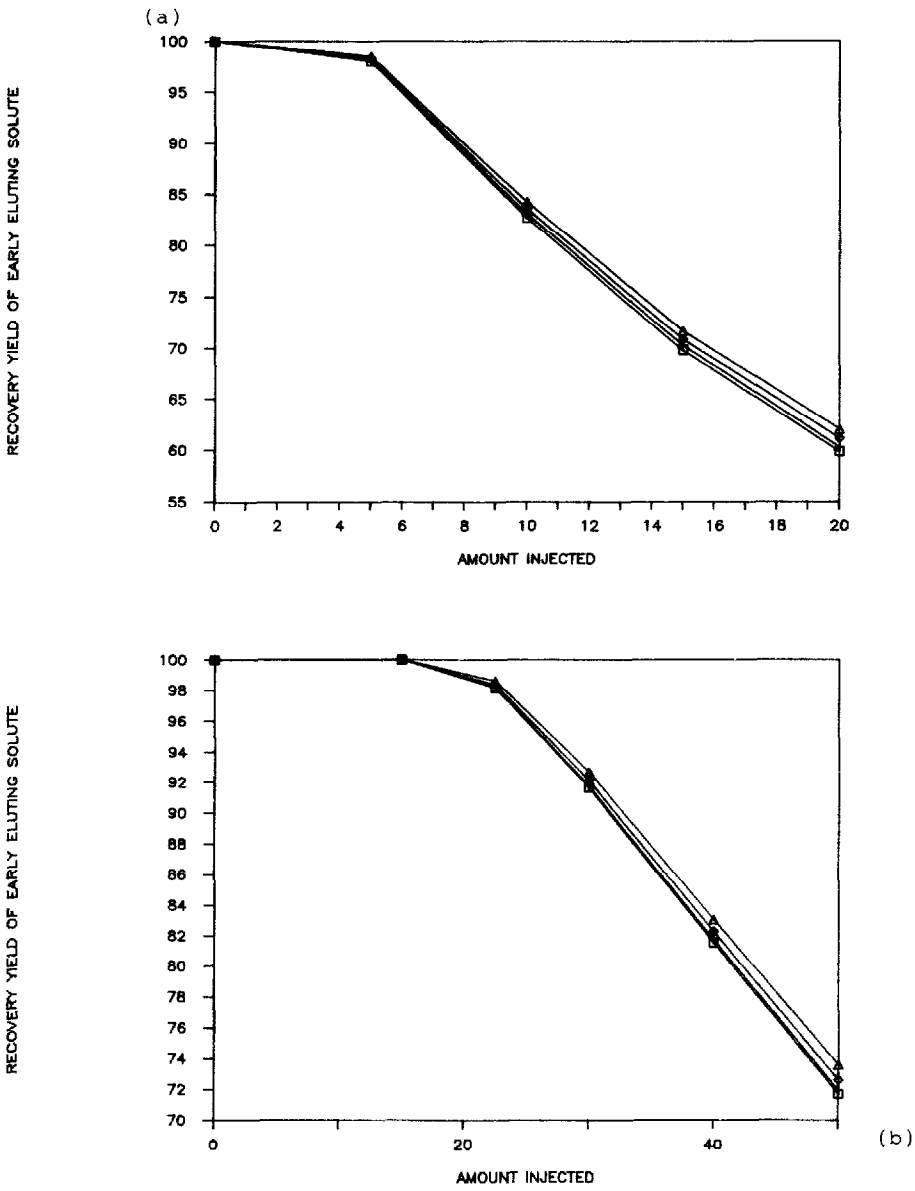

Fig. 4. Plot of recovery of compound 1 *versus* total amount injected (1:3 mixture): (a) α = 1.25; (b) α = 1.7. Purity of the recovered fractions = 99% (□); 95% (◇); 98% (+); 90% (△).

sponding to its peak maximum from approximately 1.9 to 8.3. Moreover, the displacement effect is emphasized by the fact that although the yield of 1 decreased from 98% to 70% (Fig. 7a) when the sample size increased from 20 to 50% of the column capacity, its production per unit time (Fig. 7b) increased by more than 33% from

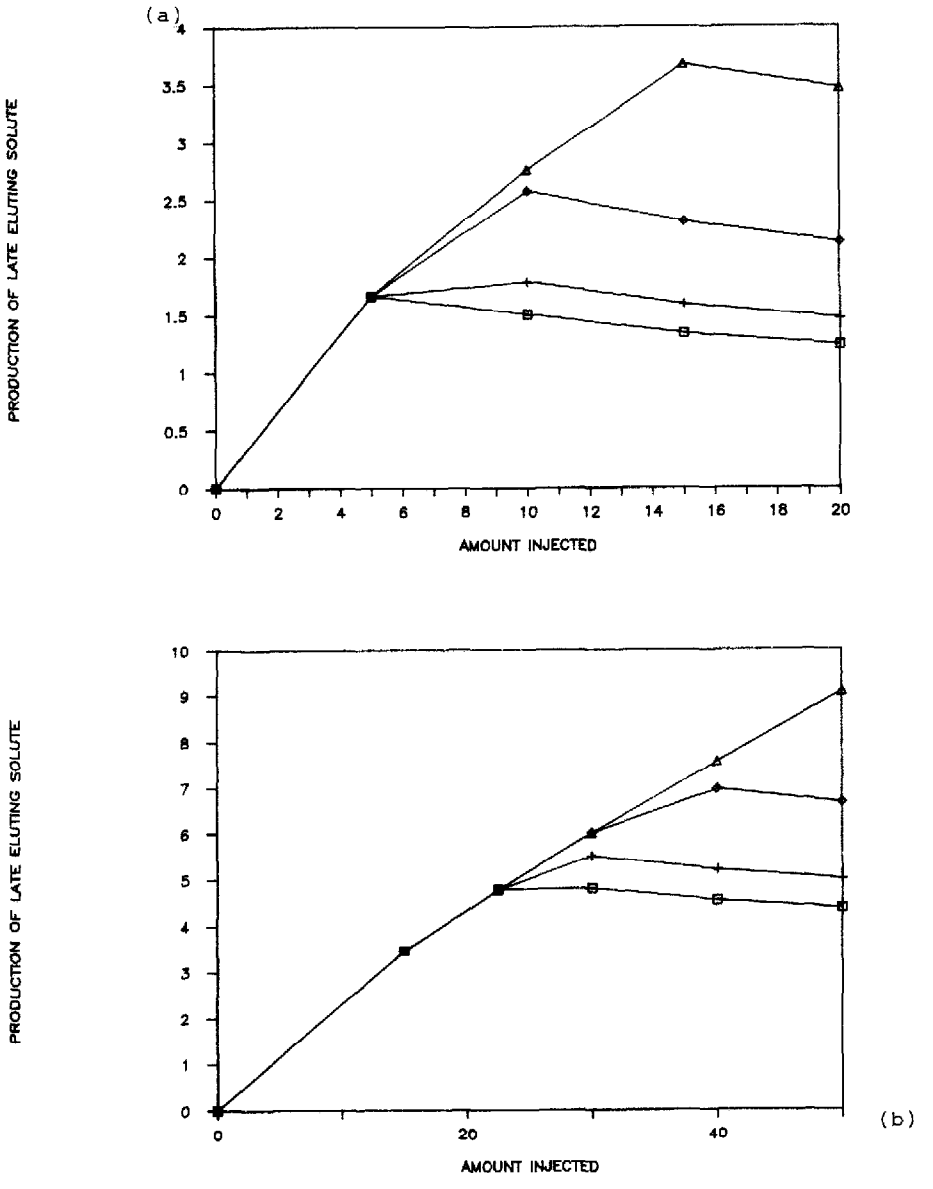

Fig. 5. Plot of production rate of compound 2 *versus* total amount injected (1:3 mixture): (a) $\alpha = 1.25$; (b) $\alpha = 1.7$. Key as in Fig. 4.

less than 0.6 to 0.8. Fig. 7b clearly indicates that a column loading equivalent to 50% of the column capacity leads to an optimal production per unit time for the lesser retained compound.

Finally, we note that the production and recovery of compound 1 depend very little on the required purity (see Fig. 7a and b). In contrast, the production and

recovery of component 2 depend very much on the required purity of the product. Fig. 8a shows a recovery of almost 100% for a required purity of 90% at 80% of the column saturation capacity compared with 30% for 99% purity. This can be traced back to the tail of 1 behind the steep part of the tail, at the border separating the bands of 1 and 2 (see Fig. 7a and b). The production of component 2 increases with increasing throughput until a very weak maximum is observed (Fig. 8b). The optimal throughput shifts to higher loading capacity as the required purity decreases. Again, it should be noted that the optimal production always occurs far after the two bands have merged together.

The chromatograms obtained for a 9:1 mixture are very different. For a sample corresponding to 20% of the column capacity, the bands of compounds 1 and 2 are completely resolved. There is hardly any difference between the profiles obtained for component 1 with this mixture or with a pure sample, and a size corresponding to 18% of the column capacity (same amount of 1). No displacement effect is observed, as illustrated by the lack of a sharp front between the profiles of the two compounds (Fig. 9a). The band of 2, in contrast, has a profile that is very different from the profile of a pure compound band. It exhibits a strong "tag-along" effect[12], *i.e.*, the lesser retained compound 1 drags component 2 forward. Peak 2 is very shallow and presents a much larger band width than when injected alone on the column. As the column loading is increased compound 2 interacts increasingly with 1, as these effects are non-linear, and its band is therefore dragged forward more and more. As a result, its band becomes shallower to the point of becoming unnoticeable, *i.e.*, undetected as seen in Fig. 9b. For a 50% column capacity sample of a 9:1 mixture, the ratio of the maximum height of band 1 to the nearly constant height of band 2 is about 50. To the chemist unaware of non-linear effects, it might seem that compound 2 has disappeared. Nevertheless, it is striking that the recovery (Fig. 10a) is still more than 50%. For an injection corresponding to 80% of the column saturation capacity, more than 30% of 99% pure 2 can still be recovered (Fig. 10a). This is because, with the large difference between the adsorption isotherms of compounds 1 and 2, compound 2 never interacts with 1 (Fig. 9b). Fig. 10b further indicates a levelling off of the production rate of 2 between 30 and 80% of the column capacity. In this specific instance, a 30% column loading would be advisable. It corresponds to the optimal production and a still high recovery (80% for $P = 90\%$, 72% for $P = 99\%$).

It might seem surprising to observe (Fig. 9a) the two compounds being completely resolved, the band profile of compound 1 being nearly identical with that obtained with a pure compound sample and the band profile of 2 so considerably broadened. This is, however, merely the converse of the displacement effect observed in Fig. 1a. The bands of the two components interact considerably at the beginning of the separation process, in the first part of the column, as they are not resolved until they elute (see Fig. 3a and b). In Fig. 1a, the major effect is displacement and band 1 is moved forward and compressed. In Fig. 9a, the tag-along effect is predominant and band 2 is moved forward and spread. In this instance, there is a marked dilution of compound 2 in the process. It decreases the extent of non-linear phenomena and prevents the band profile from recovering and returning to the classical single component profile. The opposite is true when the displacement effect is predominant (see Fig. 1a).

Finally, we have simulated chromatograms corresponding to the separation of

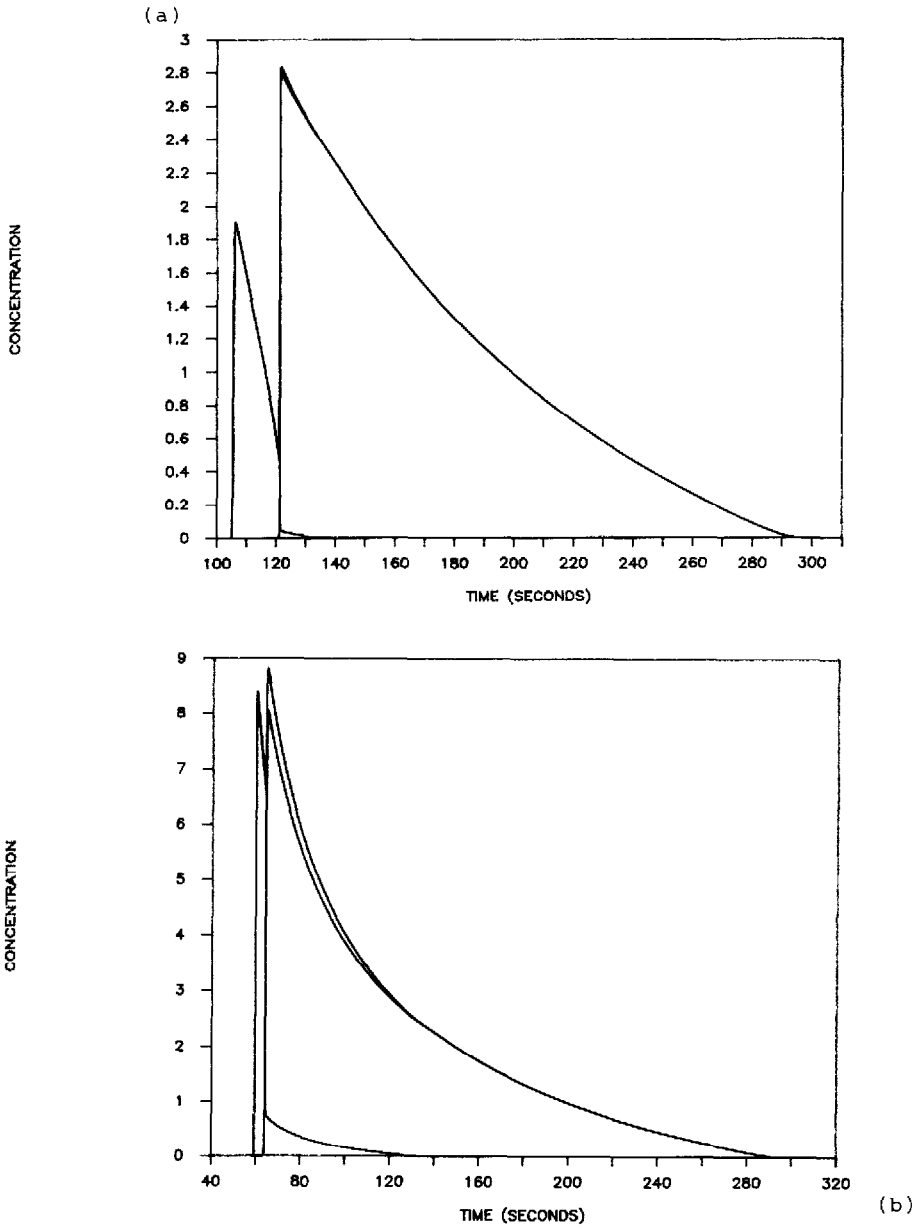

Fig. 6. Overloaded chromatogram of a 1:9 mixture and a sample size corresponding to (a) 20% of the column capacity and (b) 50% of the column capacity.

similar mixtures with a relative retention of 3.0. The results obtained exhibit the same phenomena, with stronger non-linear effects of displacement and tag-along effects due to the large sample size needed to achieve band interaction.

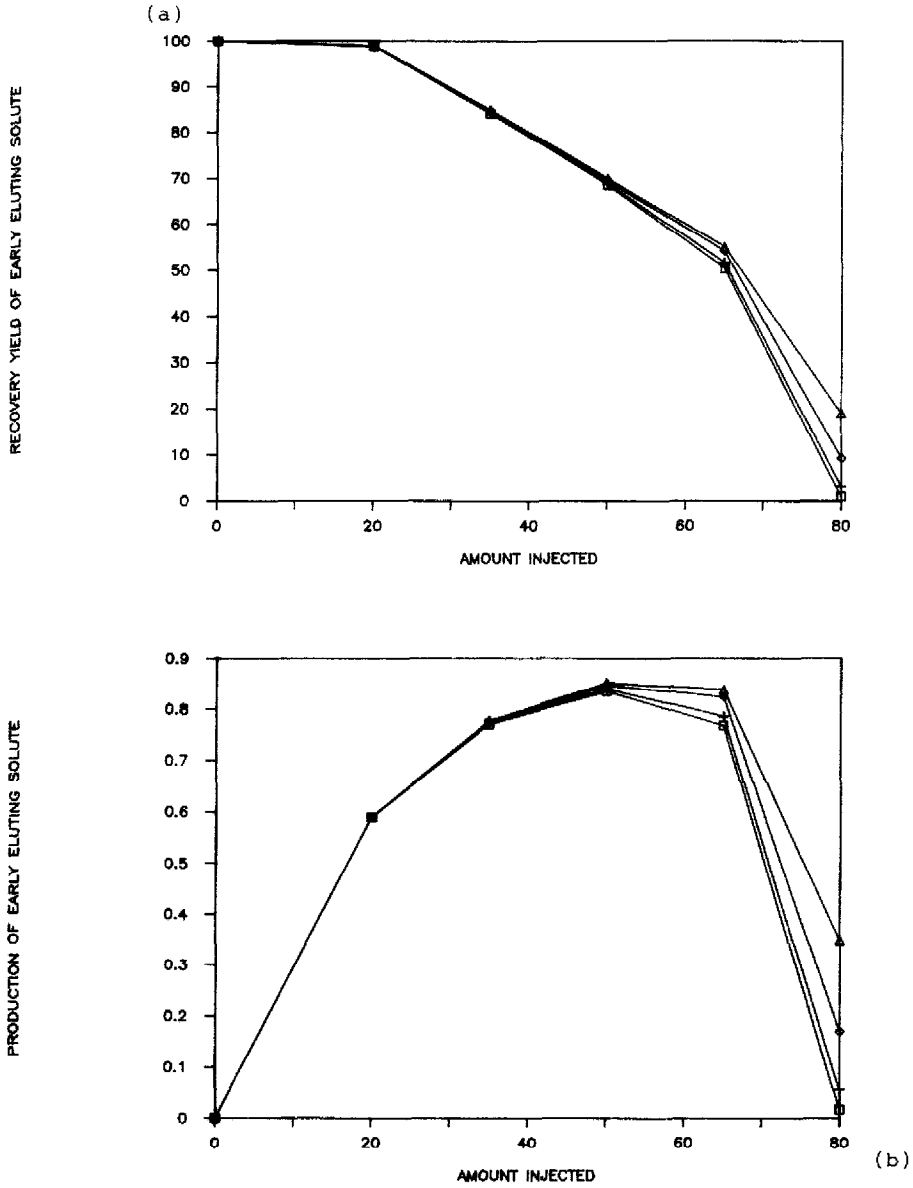

Fig. 7. Component 1 of a 1:9 mixture (α = 1.70): (a) plot of recovery *versus* sample size; (b) plot of production rate *versus* sample size. Key as in Fig. 4.

CONCLUSION

Comparison of Figs. 1 and 2, 4a and 4b and 5a and 5b and with the results of our previous work[3,5,14] shows that the same non-linear efffects take place during the separation of a binary mixture whether the relative retention is 1.09, 1.25, 1.7 or 3.0.

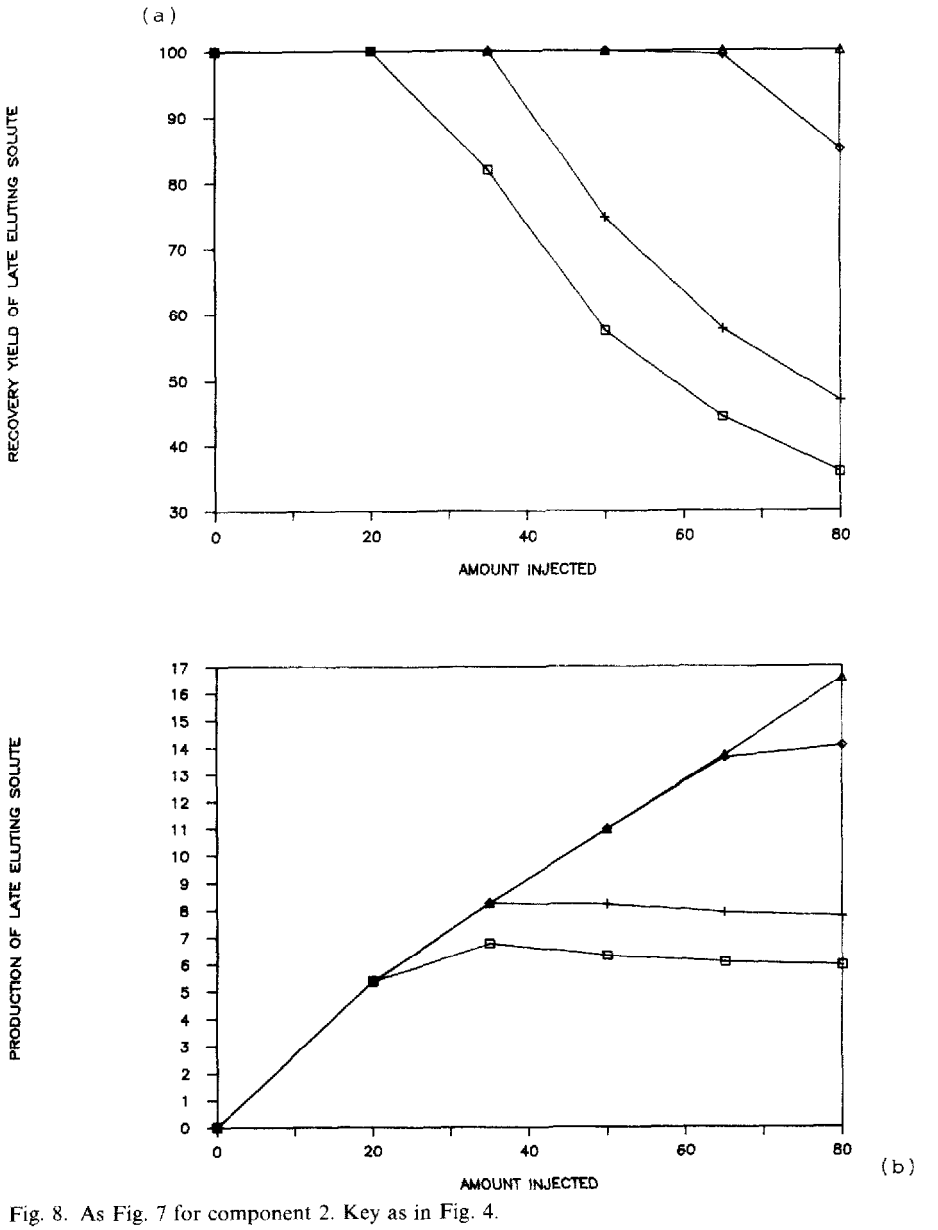

Fig. 8. As Fig. 7 for component 2. Key as in Fig. 4.

The only differences come from the relative intensities of these efffects. In order to achieve significant band overlap during most of the migration of the sample compounds along the column, the sample size must be increased rapidly with increasing α. Then, for a certain degree of band interference, the displacement and the tag-along effects become stronger.

This is observed when the separation layer between bands 1 and 2 for 1:3, 1:1 and 3:1 mixtures especially becomes narrower, and the tail of compound 1 behind

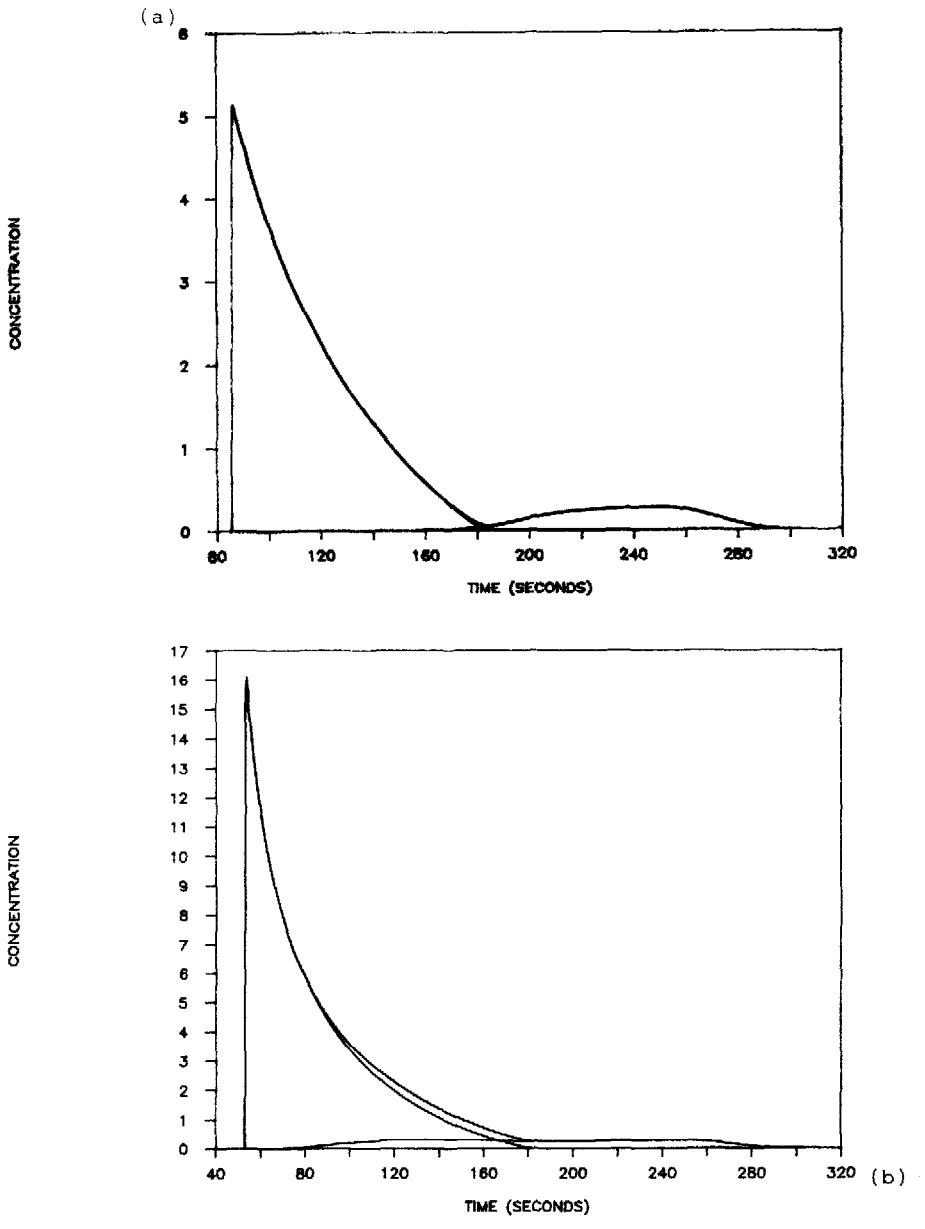

Fig. 9. As Fig. 6 for a 9:1 mixture.

this layer becomes smaller. This is also shown by the considerable tag-along effect resulting in the spread of the band of an impurity over a considerable range of retention volumes (see Fig. 9b, where the band of compound 2 goes almost from $k' = 1$ to 6). This is also illustrated by the important distortion exhibited by bands that are just resolved (resolution ≈ 0.9–1.2) at the column exit. Depending on the

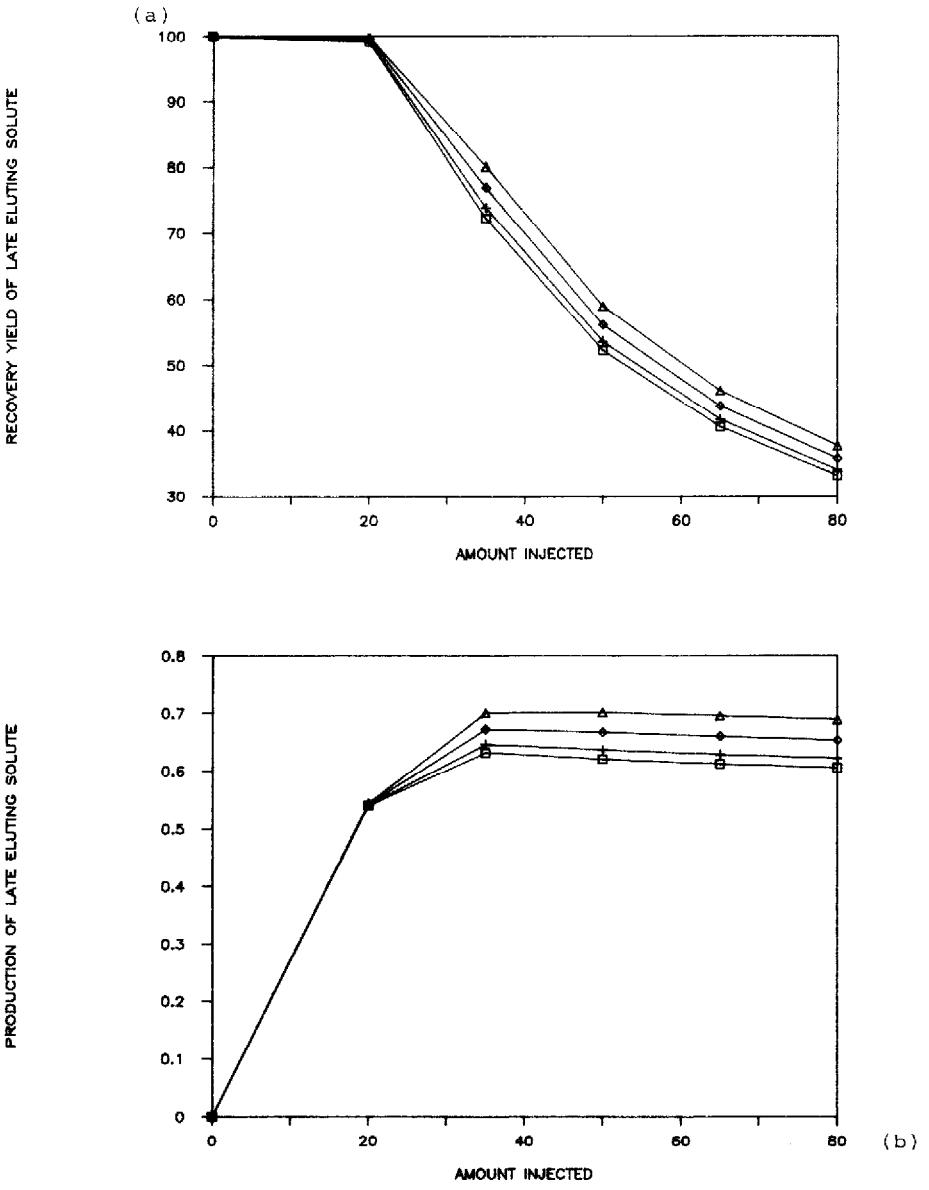

Fig. 10. As Fig. 8 for a 9:1 mixture. Key as in Fig. 4.

relative concentration, the effect is seen mainly either for the first compound (Fig. 1a) or the second compound (Fig. 9a).

The importance of the relative retention with respect to the optimal production rate of a preparative separation is considerable, at least when the two binary isotherms slowly diverge, as discussed here. This is illustrated by Fig. 11. Both the recovery and production rate increase rapidly with increasing α for both compounds

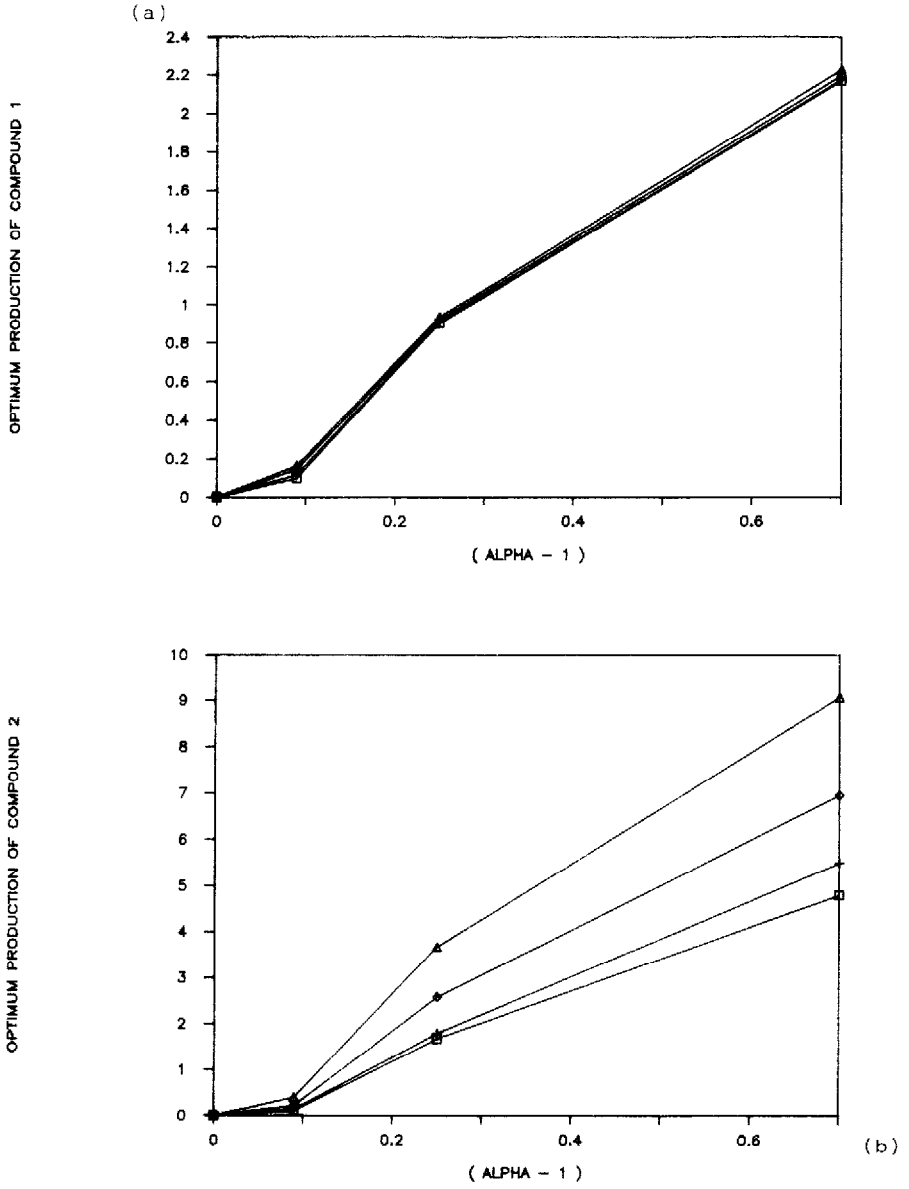

Fig. 11. Optimal production rate *versus* $\alpha - 1$ for a 1:3 mixture for (a) compound 1 and (b) compound 2. Key as in Fig. 4.

1 and 2. This result was obviously expected. It must be emphasized, however, that in all instances the production rate keeps increasing with increasing size whereas the yield decreases markedly. The maximal production is obtained for values of the yield that depend to some extent on the experimental conditions, but mainly on the relative retention (*i.e.*, *ca.* 90% for $\alpha = 1.7$ and *ca.* 70% for $\alpha = 1.25$).

The selection of the experimental conditions should stress the importance of selecting a chromatographic system in which the most important compound is eluted first (unless the equilibrium isotherms are concave), the binary (single component) equilibrium isotherms diverge and the relative retention at zero sample size is as large as possible. Then, the selection of the sample size will depend on whether a maximal production rate is sought or whether a compromise between the recovery and production rate is desired.

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