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EXPERIMENTAL UTILIZATION OF A DISPLACEMENT EFFECT FOR THE OPTIMIZATION OF THE SEPARATION OF A TWO-COMPONENT MIXTURE

JOAN NEWBURGER^a

Wyeth-Ayerst Research, Inc., Princeton, NJ 08540 (U.S.A.)

and

GEORGES GUIOCHON*

**Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600, and Division of Analytical Chemistry, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120 (U.S.A.)*

SUMMARY

Using a mixture of two substituted cyclohexanone epimers, sample loading, binary mixture composition and column efficiency were investigated for their influence on the displacement effect observed between the first and the second eluted components ("sample self-displacement effect"). This effect occurs and can enhance production for loads as high as 1 g on a 250 × 21.4 mm I.D. column. Separations over a broad range of epimer mixture compositions, from 10:90 to 75:25, show a displacement effect on the first-eluting component by the second. In loading and composition studies, interference between the tailing portion of the first component and the front of the second component is the predominant factor influencing recovery loss for both components. A high column efficiency is essential for sharpening band zone boundaries and maximizing yields. There is a very good correlation between experimental results and reported computer simulations of the band profiles of binary mixture components.

INTRODUCTION

It has long been recognized that preparative chromatography must be carried out under conditions such that the column is at least somewhat overloaded¹. Until recently, however, the origin, nature and consequences of the band broadening and component interference phenomena that take place under such conditions were elusive. It was difficult to control or use to advantage effects that were poorly understood and few detailed investigations of these band interactions were reported.

In 1987, three papers were published independently on this topic²⁻⁵ and interest in this area has been growing steadily since. In a previous contribution^{2,3}, we demon-

^a Present address: Squibb Institute for Medical Research, P.O. Box 4000, Princeton, NJ 08543-4000, U.S.A.

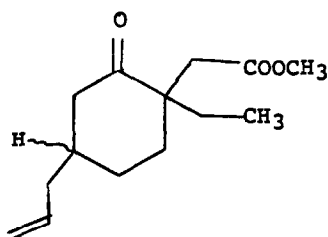


Fig. 1. Structure of the two 1,1,4-trisubstituted cyclohexanone epimers investigated.

strated that column overloading is accompanied not only by the elution of broader, unsymmetrical bands, but also by a high degree of interaction between the bands of closely eluting components. This interaction is not necessarily detrimental. While exploring the relationship between particle size, loading capacity and production rate of a pair of epimers (Fig. 1), the recovery of pure material was found to increase well past the load at which fronting of the second-eluting epimer was expected to reduce production because of overlap with the first component. Fraction collection during the elution of the bands followed by analysis of these fractions revealed that the second epimer was displacing the first, literally pushing this first epimer ahead of itself through the column². Very similar experimental results were described by Parker *et al.*⁴ in gradient elution and by Eble *et al.*⁵ in isocratic analysis. However, in a companion paper⁶, the latter group proposed a new model, termed the "blockage effect", to account for what can be readily explained as a displacement effect^{7,8}.

Displacement of mixture components by a solution of a more strongly adsorbed compound, called the displacer, is a well known mode of preparative liquid chromatography⁷, recently revived by Horváth *et al.*⁸. The fundamental origin of this phenomenon is in the competition between the more strongly adsorbed displacer and the sample components or, more precisely, the last-eluted, most strongly adsorbed component of the mixture. In turn, this component competes with the second most strongly adsorbed component and displaces it. Eventually, if proper experimental conditions are selected (sufficiently long column, displacer concentration sufficiently high and mobile phase velocity sufficiently great), an isotachic train of bands is formed, where each band displaces the previous one and is displaced itself by the next one^{8,9}.

Although the displacement effect cannot lead to the formation of an isotachic train in the case of the elution of a binary mixture under column overloading conditions, the competition between the two components of the mixture for access to the stationary phase is governed by the same mechanism and follows the same law. Therefore, when the degree of column overload is high enough and the two bands interfere, usually because the front of the second tends to be eluted before the tail of the first, a displacement effect on the first band by the second takes place. This phenomenon has been termed the "sample self-displacement effect"². The major advantage of a generalized use of this effect over the standard elution practice of using slightly overloaded columns is the much larger sample size afforded, especially for the purification of the first component of a closely eluting pair. Compared with the classical "touching band" approach¹⁰, the gain is approximately one order of magnitude^{11,12}. The major advantages over conventional displacement with a displacer are

the ability to perform repetitive injections without the need for lengthy column re-equilibration steps in between, and a much faster method development procedure as a search for a suitable displacer is not needed.

The original data² were limited to injections of 100–500 mg, on a 21.4 mm I.D. column, for a 25:75 mixture of two epimers, where the more retained epimer was in excess. The range of experimental conditions under which sample self-displacement takes place, especially in terms of sample composition and column loading, remained unexplored. The dependence of the effect on column efficiency also needed further investigation.

In the meantime, computer modelling methods were developed that were able to simulate the observed data^{13,14}. Theoretical calculations further predicted the occurrence of sample self-displacement over a broad range of mixture compositions and loadings^{14,15} and a dependence on efficiency^{16,17}. These theoretical results are based on the assumption of competitive Langmuir isotherms for the components of a binary mixture, a fair approximation in most instances¹⁸. A detailed theoretical explanation of the mechanism of the displacement effect in the elution of a binary mixture in chromatography has been published^{19,20}.

In order both to confirm empirically the simulated data and to explore the practical utility of this displacement effect, further work was indicated. The experiments reported here were designed to examine the circumstances under which sample self-displacement occurs, with emphasis on factors integral to production optimization.

EXPERIMENTAL

Apparatus

Preparative experiments were carried out on a Varex (Rockville, MD, U.S.A.) PSLC 100 preparative liquid chromatograph, equipped with a UV detector and a Houston Instruments (Austin, TX, U.S.A.) Model 5000 strip-chart recorder. The mobile phase fluid path of the chromatograph had to be modified to meet specifications for minimum dead volume.

Isomer ratios were determined on an analytical high-performance liquid chromatographic (HPLC) system consisting of a Waters Assoc. (Milford, MA, U.S.A.) Model M6000 pump, a Waters Assoc. Model 710B sample processor, a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 773 variable-wavelength detector and a Spectra-Physics (San Jose, CA, U.S.A.) SP4200 integrator.

The preparative columns were 250 × 21.4 mm I.D. Dynamax HPLC columns packed with 8- μ m, 60-Å silica, 12- μ m, 150-Å silica (Rainin Instruments, Woburn, MA, U.S.A.) or 40- μ m, 150-Å silica (PQ, Valley Forge, PA, U.S.A.). With the exception of the efficiency study, all injections were made on 8- μ m particle columns.

Reagents

Solvents were of HPLC grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). The substituted cyclohexanone mixture was synthesized at Wyeth-Ayerst (Princeton, NJ, U.S.A.).

Procedures

A mixture of two epimeric compounds, methyl *cis*- and *trans*-1-methyl-2-oxo-4-(2-propenyl)cyclohexaneacetate (Fig. 1), was chromatographed using ethyl acetate-*n*-hexane (2.5:97.5) as the mobile phase. For the loading and efficiency studies, an isomer ratio of 1:3 was used, with the minor isomer eluting first. The isomer mixture was dissolved in the mobile phase and injections of 60, 100, 200, 300, 500, 700 or 1000 mg were made. The flow-rate was 40 ml/min and elution was monitored at 300 nm. For the composition study, pure amounts of each isomer were recovered from the loading study, and the mixture ratios were adjusted as needed.

For each injection, the total sample was recycled twice. Fractionation began with the third elution as soon as a slope change was noted. Fraction volumes were monitored indirectly by counting chart-paper units. The first one or two and the last two or three fractions collected were 26 ml in volume. Intermediate fractions were 13 ml in volume. The width of the intermediate band being of the order of 5 min (see below), ten to twelve fractions were collected. This number proved sufficient to sketch the individual band profiles in the mixed zone. Quantitative comparison between the experimental results and the prediction of the model would require the determination of a much larger number of experimental data points. However, such a treatment was not the aim of this work.

It is very important in these experiments to keep negligibly small the contributions of the recycling system and the fraction collection system to minimize band broadening. This is not very difficult with a 20 mm I.D. column.

The fractions were analyzed to determine isomer ratios using a 5- μ m ODS-2 (Phase Separations, Norwalk, CT, U.S.A.) silica column (250 mm \times 4.5 mm I.D.) and ethyl acetate-*n*-hexane (5:95) as the mobile phase at a flow-rate of 1.7 ml/min. Elution was monitored at 276 nm. Fractions having a purity of 92% or greater were included in the recovery determinations to yield a combined purity of at least 95% A or B epimer. The area under each preparative chromatographic peak was determined by counting the number of chart-paper units. The procedure was repeated and found to be reproducible to within several per cent. The amount of isomer present in each fraction was then determined using the isomer ratio.

Calculations

Theoretical plate numbers were calculated using the conventional equation, $N = 5.54 (t_R/w_{1/2})^2$. This equation was chosen specifically to elucidate particular aspects of peak shape resulting from the self-displacement effect.

RESULTS AND DISCUSSION

Sample self-displacement, or the displacement of the first component band of a mixture by the second, is a phenomenon that occurs for certain relative compositions of a mixture when a sufficiently large sample is injected. The sharp front of the second component desorbs the molecules of the first (less adsorbed) component from the stationary phase and forces their earlier elution. The consequence of sample self-displacement is a concentration and a compression of the first component band. This effect occurs when the relative concentration of the second to the first component is high enough and when the equilibrium isotherms are convex upwards. It takes place

because the amount of each component adsorbed in the stationary phase at equilibrium is a function of the concentration of *both* components in the mobile phase. Initial recognition of this phenomenon was based on the following observations.

The elution behavior of the two epimers injected as a mixture is compared with the chromatograms of these compounds injected alone in Fig. 2a and b. Each of the three chromatograms shows the detector response after the sample has migrated one, two and three times through the column (recycling). The chromatograms obtained for the successive injections of 72 mg of the lesser retained epimer and 228 mg of the more retained are overlaid in Fig. 2a. The column is seriously overloaded in both instances, but obviously it is more so with the second epimer than with the first. The

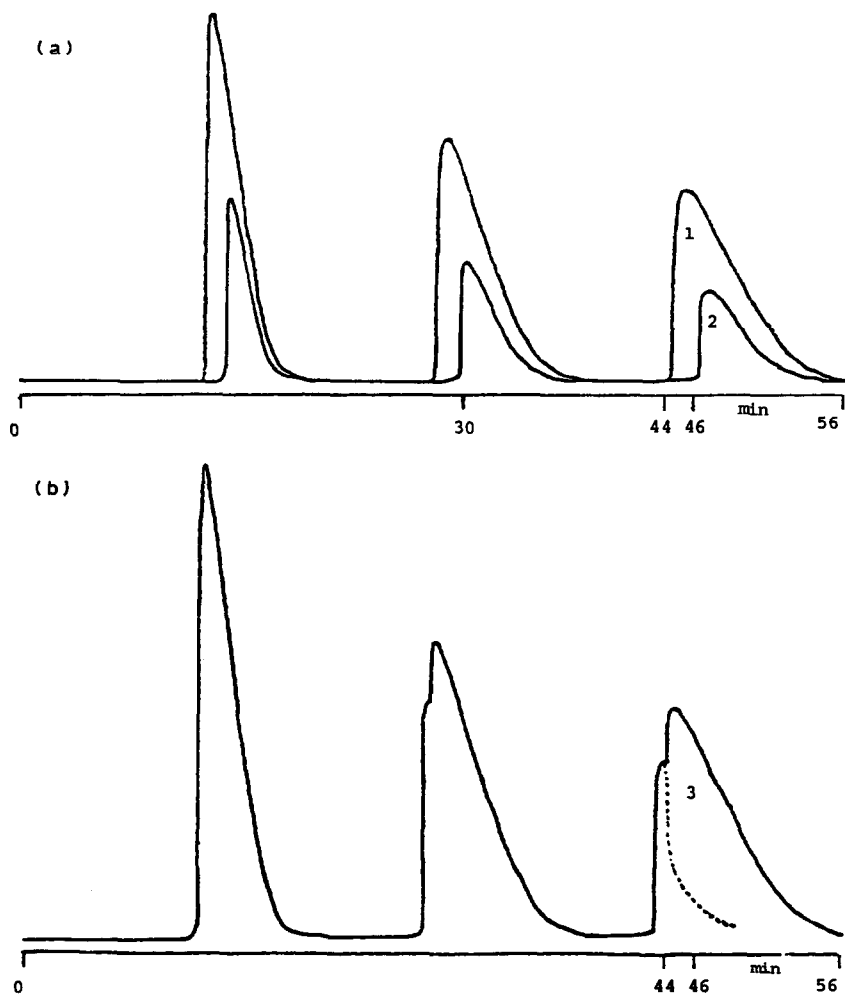


Fig. 2. Elution behavior of the two epimers when injected singly or as a mixture. Chromatograms recorded after each of three cycles (one initial elution, two recycles). For experimental conditions, see text. Column packed with 12- μ m particles. Samples sizes: line 1 (a), 228 mg of epimer B; line 2 (a), 72 mg of epimer A; line 3 (b), 300 mg of a 1:3 mixture.

column overloading for these pure compound samples results in the classical band shape associated with the elution of compounds experiencing single-compound Langmuir equilibrium isotherms between the mobile and stationary phases²¹. The bands have a very steep front and a diffuse, smooth rear profile which extends to the retention time of an extremely small pulse, *i.e.*, to the analytical retention time. Owing to the greater degree of overloading for the second epimer, its band has fronted to such an extent that its retention time is now shorter than the retention time of the band of the first, less concentrated epimer.

Based on the individual band profiles of the epimers in Fig. 2a, successful purification of large samples of a mixture of the two epimers (with $\alpha = 1.04$) would have been hard to predict, but rather coelution would be expected. However, the chromatogram of a 300-mg sample of the mixture, shown in Fig. 2b, reveals that a substantial recovery of pure material is possible. The band profile of the smaller component can be interpolated from the analyses of the fractions collected during the elution of the third band in Fig. 2b. The dotted line shows that the first band is strongly compressed, that most of the first epimer elutes prior to the second component and that a sharp boundary separates the top of the two elution zones. Total band separation is not achieved as the first component trails into the second, but the mixed zone in between the zones of pure components is not very wide. The discrepancy between the chromatograms in Fig. 2a and b occurs because the second epimer significantly influences the behavior of the first as they migrate down the column, displacing it as predicted¹³⁻²⁰.

A series of experiments were performed to investigate the influence of sample size and composition and also of column efficiency on this effect.

Influence of column loading

Loading experiments were conducted and analyzed to give both gross data on production and yield and a more specific evaluation of displacement effects. Table I presents the overall variation in production and yield for injections from 60 to 1000 mg. In the following discussion, the first eluting epimer is designated the A epimer and the second the B epimer. The increase in sample load is accompanied by a substantial decrease in yield, a 58% relative loss for the A epimer and 72% for the B epimer. Neither the production of A nor that of B, however, actually declines. It appears to peak between the 500- and 700-mg injections, but then rises again with the

TABLE I
RECOVERY AS A FUNCTION OF LOAD

Load (mg)	Yield (%)		Amount recovered (mg)		Total production (mg)
	A	B	A	B	
60	89	92	13	42	55
100	82	92	20	70	90
300	67	64	48	135	183
500	49	44	59	167	226
700	36	29	60	154	214
1000	37	26	89	198	287

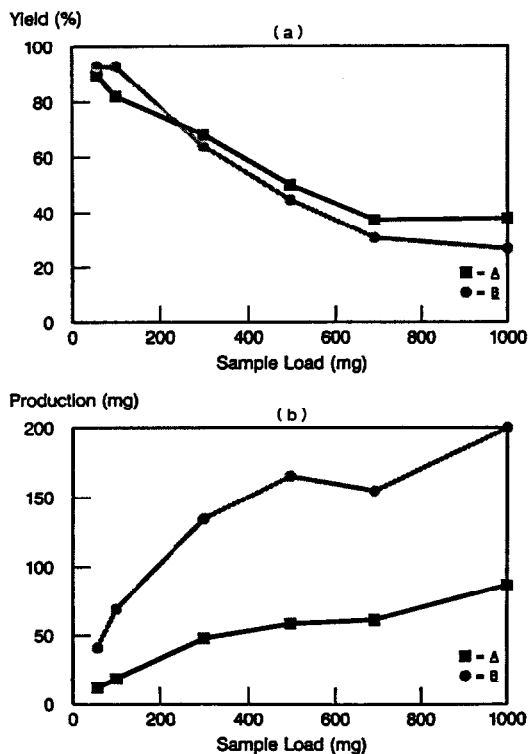


Fig. 3. Plot of (a) yield and (b) production for the two trisubstituted cyclohexanone epimers *versus* sample size. ■, epimer A; ●, epimer B. Conditions as in Fig. 2.

1000-mg injection. The data show a certain spread, due to the potential inaccuracy of these determinations inherent in the manual fraction collection procedure. A plot of the data (Fig. 3) shows that further increases in production with a possible leveling off in yield are predicted. Injection problems related to increasing sample viscosity with increasing feed concentration prevented continuation of the experiment.

The features of sample self-displacement that control the production of pure components are the sharpness of the boundary between the two component bands and the tailing of the first component into the second. Increasing the load from 200 to 1000 mg does not substantially alter the profile of the elution front of the second component as measured by the analysis of the collected fractions. This front remains nearly vertical and merely moves towards shorter retention times with increasing sample size. On the other hand, increasing the sample load does cause greater tailing of the first component, as seen in Fig. 4. Note that, in contrast to the tail of the second component which always ends at the k'_0 value, the tail of the first component is not anchored; it ends at a time that depends on the sample size, the relative concentration of the two components and the isotherm coefficients^{19,20}. These experimental results are in excellent agreement with the theoretical predictions of the ideal¹⁸⁻²⁰ and the semi-ideal models¹³⁻¹⁷ of non-linear chromatography.

The anatomy of loading effects on the displacement mechanism can be studied

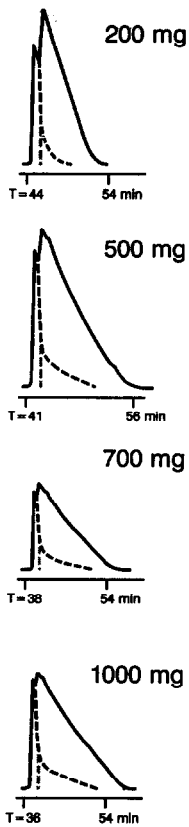


Fig. 4. Effect of increasing sample size on the elution profile of the A epimer. The third pass only of three cycles is shown for injections of (a) 200, (b) 500, (c) 700 and (d) 1000 mg. Conditions as in Fig. 2.

by determining the individual elution profiles of the A epimer under various experimental conditions. Values of the apparent plate number, N (ref. 2), the apparent column capacity factor, k' , and the apparent relative retention, α , were derived, using classical relationships, from the retention times of the two band maxima and the band width of the first peak at half-height. The results are reported in Table II. As the

TABLE II
LOAD DEPENDENCE OF THE SEPARATION PARAMETERS

Load (mg)	k'_A	k'_B	α	N	A_v/A_t
1	6.50	6.80	1.045		N/A ^a
200	6.25	6.47	1.035	4889	4.07
500	5.75	5.93	1.03	6410	1.64
700	5.29	5.42	1.03	6843	1.30
1000	4.90	5.03	1.03	7300	0.95

^a N/A = Not available.

column is loaded, k' decreases by 22%, whereas α remains virtually constant. Because of the difficulty in acquiring highly precise experimental data, the observed variation of $1 - \alpha$, which is of more fundamental importance, is probably not significant. Fronting is due to the thermodynamic properties of the equilibrium isotherms and the existence of a finite value of the column saturation capacity. The larger second component band, however, does not overlap into the less overloaded first component. In fact, as we have shown above, the upper part of the band of the first component is narrow and even seems to narrow down as the sample size is increased, again in agreement with theoretical results¹⁰. This is confirmed by the positive trend observed for the number of theoretical plates measured for the A band.

The values of the parameters N , k' and α are not diagnostic of the actual loss in recovery yield. The tailing of the A epimer into the B one accounts for all the decrease in the production of both epimers. An arbitrary parameter was defined, in an effort to determine the relative importance of the tailing of the first component under the second component and to study the influence of various parameters. The definition of this parameter is based on the L-shaped profile of the A band, reported previously¹³⁻¹⁹. A vertical line is dropped to the baseline from the point at which the band slope of the A band begins to change. This is shown as the dashed lines in Fig. 4. These lines divide the A band into a vertical front part containing pure material and a triangular portion which represents the amount of A in the mixed zone of the chromatogram¹⁸. The areas of each portion, denoted A_v and A_t respectively, are measured. The value of the ratio A_v/A_t is indicative of the extent of tailing and consequently of the decrease in recovery.

The data in Table II show that for a 200-mg injection, 80% of the material is accounted for by A_v and is recoverable. In contrast, for a 1000-mg injection, only 50% of the A epimer is in the front part of the band. Plotting $\ln(A_v/A_t)$ versus the logarithm of the amount of A epimer injected gives a straight line (Fig. 5). Although the validity of this relationship remains questionable at this stage because it has not yet been tested on any other mixture, it might prove useful in predicting optimum loads should it apply for other mixtures.

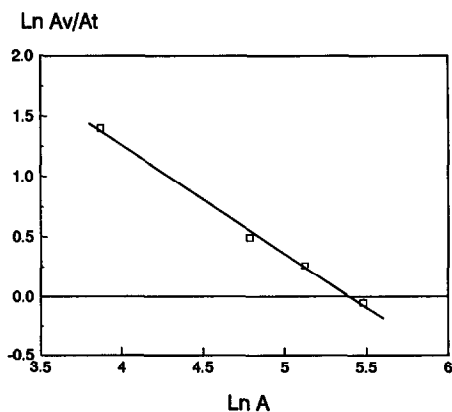


Fig. 5. Plot of $\ln(A_v/A_t)$ versus $\ln m$ (mg), where m is the amount of the A epimer injected. Data shown in Fig. 4. A_v is defined as the area under the A band to the left of the dashed line in Fig. 4 and A_t as the area under the A band to the right of the dashed line. Regression line: $y = 4.88 - 0.904x$, $R^2 = 0.996$.

Influence of sample composition

The loading experiments reported here and also the previously published results show sample self-displacement for a mixture ratio of 25:75. The elution profiles of 200-mg samples of mixtures of various compositions (see Fig. 6a) indicate that the phenomenon can occur over a wide range of sample composition. Tailing of the A epimer becomes more pronounced as its concentration increases from 10% to 75%. Some displacement of the A by the B epimer remains significant for an A concentration of 50% (Fig. 6a). Even with a mixture containing 75% of the A epimer, the profile of the B epimer begins with a very sharp front. The concentration jump is insufficient, however, to cause a noticeable drop in the A concentration and a significant displacement effect.

Material that elutes prior to the break in the slope of the rear profile of the A band can be collected as pure product. As found in the column loading study, loss of production is due to the tailing of the A band, not to the fronting of the B band. The displacement effect vanishes completely when the mixture composition becomes 90:10 and the second-eluting component trails along through the entire elution band, demonstrating a "tag-along" effect^{13,15}. In the latter instance, no pure material is obtained.

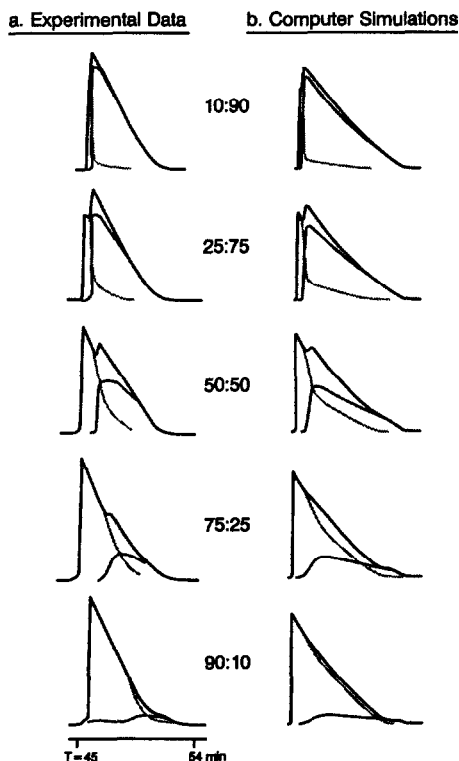


Fig. 6. Self-displacement effect on different mixture compositions of the two epimers. Conditions as in Fig. 2. (a) Experimental results, third pass only of three cycles; 200-mg injections at A:B mixture ratios of 10:90, 25:75, 50:50, 75:25 and 90:10. (b) Computer simulations for a 300-mg injection of two components in the same mixture ratios as in (a) (note: the time scale is different).

The mixture band profiles shown in Fig. 6b are computer simulations generated following the method previously described⁵. Their pattern is strikingly similar to that of the profiles derived from experimental determinations. The data constitute an experimental validation of these theoretical results, on a qualitative basis. An investigation of the degree of quantitative agreement would require the determination of the competitive equilibrium isotherms of the two epimers in the chromatographic system used here, which was beyond the scope of this work.

To explore the origin of the loss of the displacement effect with a 90:10 mixture, and whether it is due to the amount of the B epimer (too low) or to the composition of the mixture (too large a relative concentration of A), injections of both the 90:10 and the 75:25 mixtures were made at lower loads. The band profiles are shown in Fig. 7. Comparing chromatograms corresponding to the same amount of the B epimer, *e.g.*, a 200-mg injection of the 90:10 mixture and a 100-mg injection of the 75:25 mixture containing 20 and 25 mg of B, respectively, a much sharper boundary is observed with the latter mixture. A similar result is seen on comparing the profiles of a 100-mg injection of the 90:10 mixture and a 40-mg injection of the 75:25 mixture, where 10 mg

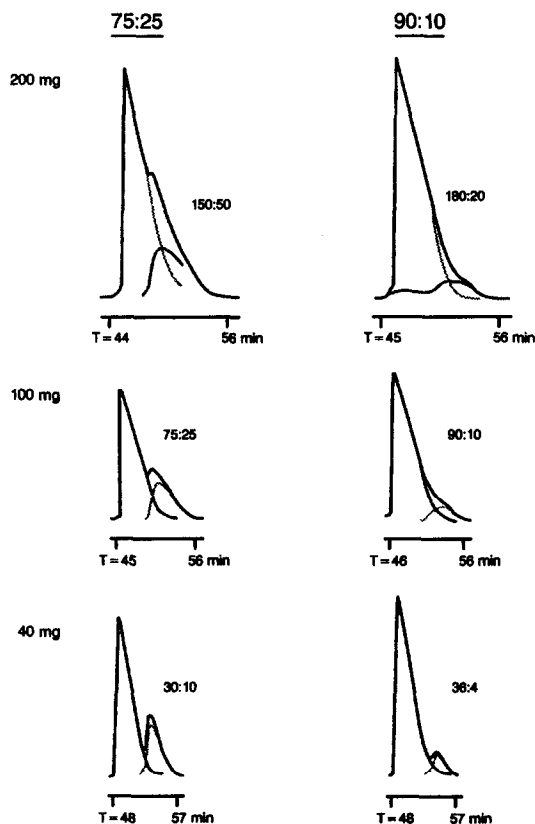


Fig. 7. Relative importance of mixture ratio and amount of the second-eluting component in causing a displacement effect. Third Pass of three cycles. Left: A:B mixture ratio of 75:25 for 200-, 100- and 40-mg samples. Right: A:B mixture ratio of 90:10 for 200-, 100- and 40-mg samples.

of B are present. Therefore, the relative composition of the sample, not the amount of second component injected, affects the relative intensity of the displacement and the tag-along effect.

It is important to emphasize that the two individual band profiles and the interaction between the two bands depend on two parameters, the total loading factor and the concentration ratio of the two components in the feed¹³⁻²⁰. The first parameter determines the intensity of the non-linear effects and the second the relative importance of the displacement and the tag-along effects. Admittedly, the values of the loading factors for each component are important, as the total sample load decreases when the bands separate, but these factors, which are included in the definition of the effective sample loading factor^{19,20}, have to be taken into account only for quantitative predictions of the band profiles.

Influence of column efficiency

The phenomenon of sample self-displacement was first observed during a study of the relationship between production, sample size and the average particle size of the packing material used. In this work, we investigated this relationship again by

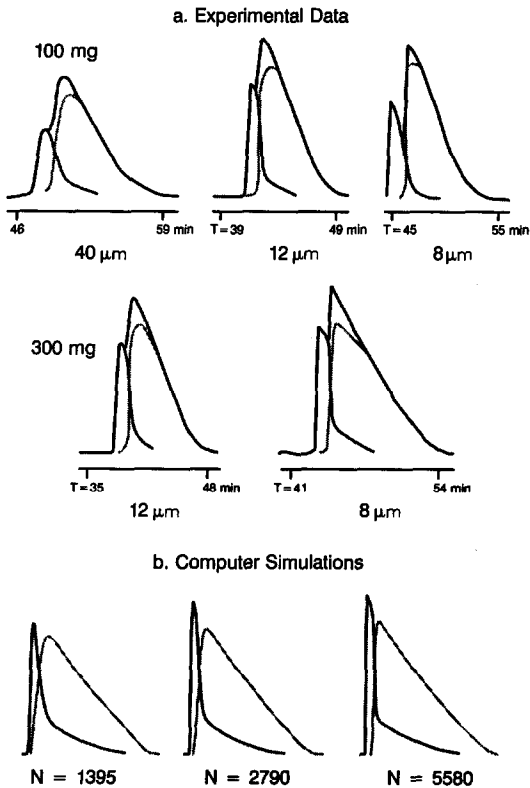


Fig. 8. Effect of column efficiency on elution profiles. (a) Experimental results. Third pass of three cycles. Injections of 100- and 300-mg samples of the epimer mixture at an A:B ratio of 1:3. Conditions as in Fig. 2, except packing material (40, 12 and 8 μm). (b) Computer simulations of 300-mg injections on columns having efficiencies of 1395, 2790 and 5580 theoretical plates.

determining the individual band profiles for each component of the eluting mixtures. The dependence of the displacement effect on column efficiency is illustrated in Fig. 8a.

The comparison between the production rates and the recoveries obtained with these three columns should take into account the difference between the values of the column saturation capacities that are likely to result from the different silica batches used and, with the 8- and 12- μm particles, the difference in average pore sizes and hence in specific surface area. The relative retentions with small sample sizes were nearly the same, however. Similarly to single-compound band fronts²¹, the slope of the two individual bands in the mixed region depend little on the loading factor, but essentially on the column efficiency, provided that the latter exceeds a few thousand plates^{19,20}. As is recognized, the proper prediction of any of the characteristic phenomena arising in non-linear chromatography depends on the possibility of simulating columns having at least several thousand plates¹¹⁻²¹.

Proceeding from a 40- to a 12- and an 8- μm packing material, we found increasingly sharp band interfaces, for both a 100-mg and a 300-mg sample size. The use of more efficient columns results in very similar chromatograms, with a single, but major, difference, *i.e.*, the sharpness of the boundary between the two components. This factor has the important effect of reducing the number of mixed, impure fractions that have to be collected between the two pure fractions, decreasing their total volume. Higher recoveries can be achieved.

The experimental results shown in Fig. 8a are again in excellent agreement with the predictions of the semi-ideal model of non-linear chromatography⁵. This similarity confirms the ability of computer modeling to predict experimental results correctly. The accuracy of these predictions is discussed independently¹⁸.

CONCLUSIONS

This work confirms our previous results regarding the separation of binary mixtures of closely eluting compounds^{2,3,13-20}. The interaction between the two epimers considered here gives rise to an important displacement effect of the first-eluted epimer by the second. Production of important amounts of pure material, especially of the first-eluted component, can be achieved under a high degree of column overloading. The production is greater than could be predicted by a direct extrapolation from the individual band profiles. The loading factor, the mixture composition and the column efficiency together determine the production per cycle.

Efficient columns are essential for maximizing the recovery, because column efficiency has been shown to determine the sharpness of the demarcation of band boundaries. In studies of loading factor and mixture composition, the loss of yield has been ascribed to the tailing of the first band into the second. This feature clearly distinguished the band profiles obtained in overloaded elution chromatography from the more rectangular zones found in actual displacement chromatography. The tailing is due to the decrease in concentration of the second component on the rear part of its zone, contrasting with the constant concentration of a displacer throughout the whole run. An awareness of the first-component band profile is critical for an accurate prediction of the optimum sample load for maximum production of a pure fraction from a feed having a given composition.

There are a number of instances where optimum conditions are achieved by first collecting a significant amount of the first-eluting component to attain a more desirable relative composition of the mixture used as the feed. In practice, this first collection step is easy to accomplish as pure material can be identified as that portion eluting prior to the obvious onset of the second component, eliminating the need for fraction cutting and purity determinations of mixed zone fractions.

The close similarities between the experimental and calculated chromatograms emphasize the usefulness of modeling in prediction and optimization. Moreover, as the equations used to generate the calculated band profiles rely on very general principles and on a fundamental model of chromatography, the ability to predict accurately these particular experimental data implies that the phenomenon of sample self-displacement is not limited to the pair of epimers studied so far. Further experiments are in progress to show that these results are valid for other compound mixtures and that they can be used as a routine model of separation²².

Finally, we wish to emphasize the importance of selecting the chromatographic system that gives the optimum elution order for the mixture investigated.

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