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Joan Newburger[a]; Leonard Liebes[b]; Henri Colin[c]; Georges Guiochon[d]
[a] Ayerst Laboratories Research, Inc., Princeton, New Jersey [b] Sota Chromatography Inc., Crompond, New York [c] The Varex Corporation, Rockville, Maryland [d] Department of Chemistry, University of Tennessee, Knoxville, Tennessee

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Investigation of the Influence of Particle Size on the Productivity of Preparative HPLC Columns

JOAN NEWBURGER

AYERST LABORATORIES RESEARCH, INC.
PRINCETON, NEW JERSEY 08540

LEONARD LIEBES

SOTA CHROMATOGRAPHY INC.
CROMPOND, NEW YORK 10517

HENRI COLIN

THE VAREX CORPORATION
ROCKVILLE, MARYLAND 20852

GEORGES GUIOCHON

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF TENNESSEE
KNOXVILLE, TENNESSEE 37996-1600

Abstract

Changes in band profile, resolution, production, and yield with increasing sample size were determined for 1″ i.d. × 12″ preparative columns packed with 10 or 40 μm silica particles of the same origin. The results for a pure compound and for a mixture are compared. When a pure compound is injected, the efficiency of both columns decreases with increasing sample size. Although the 10-μm particle column is notably more efficient than the 40-μm particle column, for very large samples the plate numbers of both columns eventually become similar. When a binary mixture of isomers is injected, however, the 10-μm particle column provides a much larger yield and production of pure isomers than expected. When the sample size of this mixture is increased, the first eluted peak broadens to a lesser extent than if it were the peak of a pure component. It is better resolved from the more retained isomer than could be predicted on the basis of relative

retentions and the extent of band broadening of single compound zones. The result is a better yield and increased production. This phenomenon is observed on the more efficient column because interaction in the overlapping region between the two incompletely separated bands is dependent on column efficiency. In preparative chromatography, if the efficiency of the column is sufficient, self-displacement of the sample can be adjusted in certain cases and used to improve the performance of the column.

INTRODUCTION

Originally chromatography was developed by Tswett as a preparative technique for the extraction of pure compounds from complex mixtures of natural origin (*1–3*). However, recent advances made in the field of liquid chromatography have essentially dealt with analytical applications. A search of CAS literature for the years 1980–83 shows fewer than 100 references dealing with preparative HPLC out of more than 7000 papers dealing with HPLC (*4*). The usefulness of preparative HPLC in such industries as pharmaceuticals and the life sciences is now being recognized, however, and preparative applications are undergoing a new cycle of strong interest.

The widespread use of preparative HPLC is still hampered by the complexity of the problems of scaling-up, by the lack of present understanding of the behavior of large concentration bands in chromatographic columns, and by problems of applied chemical engineering which are still unsolved, and which may not as yet have been recognized (*5*).

So far, most preparative applications are carried out using low efficiency long columns packed with large size particles (40 μm and larger) and operated at low flow rates with small back pressures. This technique affords separations which, although often satisfactory, require extremely long run times and cannot be easily extrapolated to the large-scale production of many interesting products due to unreasonable costs. More and more problems arise which cannot be solved with the technology presently available. Modern biotechnologies, the pharmaceutical industry, and food and other industries require a major improvement in the production capacity and speed of chromatographic units, together with an increase in the separation power of the method.

At present the dependency on large particle sizes limits the performance of successful separations. Procedures are optimized empirically and the results, even when poor, must be accepted because there is no reasonable alternative. In the chromatography laboratory at Ayerst, 30% of the time is spent carrying out separations which require multiple

tedious injections. Development of preparative methods for isomer separation or product purification can be quite time consuming and, in some cases, an acceptable separation turns out to be impossible with the currently available technology.

A new generation of preparative chromatographs is now being introduced. These instruments permit the use of much larger back pressures (2000 psi or greater) and mobile phase flow rates. Preparative HPLC combining high efficiency large diameter columns and short elution times widely extends the range of economical applications of the method.

Lack of practical and theoretical concepts hinders the development of useful procedures for the extraction of compounds from complex mixtures. The theory of preparative HPLC is still most often approached as an extension of the theory of analytical HPLC. So far, questions about optimum loading capacity, the influence of the average particle size, size distribution and pore volume, etc. have been addressed through extrapolation of data obtained with analytical columns. The definition of the optimum or maximum loading capacity of the column is usually derived from the concept of column efficiency, which is very useful in analytical HPLC but of dubious value in preparative chromatography (*2*, *5*).

The aim of the present study was to achieve an empirical assessment of the advantages of using small particles in preparative HPLC by comparing the loading capacity observed for columns packed with similar materials but having different average particle sizes. A survey of the literature shows extensive contradictions. Some studies conclude that the use of more efficient columns permits an increase in the amount of sample per injection, while other works derive the opposite conclusion, showing that the efficiency of small particle columns falls off rapidly with increasing sample size (*6–9*). Most of the investigations previously published have been conducted using analytical size columns. The question of preparative loading is an important problem which must be solved in order to define the optimum conditions leading to economical production.

The experiments reported here were designed to examine the effect of loading on the efficiency of large diameter columns and on the purity of collected fractions, and to examine the column loading limits for real separations.

EXPERIMENTAL

Apparatus

Preparative experiments were carried out on a Varex (Rockville, Maryland) PSLC 100 Preparative Liquid Chromatograph, equipped with

a UV detector and a Houston Instrument (Austin, Texas) 5000 strip chart recorder.

Isomer ratios were determined on an analytical HPLC system consisting of a Knauer (Bad Homburg, FRG) Model 64 HPLC pump, a Waters (Milford, Massachusetts) Model 710B Sample Processor, a Kratos (Ramsey, New Jersey) Spectroflow 773 Variable Wavelength Detector, and a Spectra Physics (San Jose, California) SP4200 Integrator.

Columns were 1 in. by 30 cm stainless steel columns (HP Chemicals, St. Louis, Missouri), packed by Varex Corporation with Davisil 10 μm (5–15 μm) silica or with 40 μm (35–70 μm) Davisil silica (W. R. Grace, Columbia, Maryland).

Reagents

Solvents were HPLC grade purchased from Fisher Scientific (Santa Clara, California). Benzophenone (99%) was purchased from Aldrich Chemical (Milwaukee, Wisconsin). The substituted cyclohexanones mixture was synthetized at Ayerst.

Single Component Study

Benzophenone solutions (20% toluene/80% hexane) were made up volumetrically so that using a 2-mL loop the following quantities would be injected: 25, 50, 100, 200, 300, 500, and 700 mg. The mobile phase was 0.5% ethyl acetate/99.5% hexane and the flow rate was maintained between 43 and 47 mL/min. Attenuation was set at the highest sensitivity which would remain on scale for each injection. The wavelength was set at 340 nm. On each column, injections from 25 to 700 mg were made sequentially and repeated sequentially two more times to insure that poor efficiency due to column failure would be apparent. Percent standard deviations ranged from 2 to 6.5% except for the 500-mg injections on the 40-μm particle column and for the 300-mg injections on the 10-μm particle column where, for no apparent reason, the standard deviations are 11 and 13%, respectively. Retention volumes were larger on the 10-μm column than on the 40-μm column. When the mobile phase composition was adjusted to provide retention volumes similar to the 40-μm particle column, values for the theoretical plate numbers of the 10-μm particle column did not change.

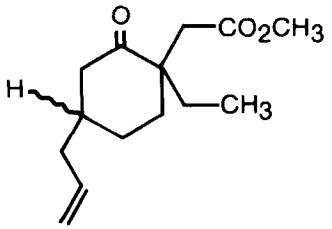

FIG. 1. Structure of the 2,2,5-trisubstituted cyclohexanone epimers.

Dual Component Study

A mixture of two epimeric compounds, the *cis*- and *trans*-1-ethyl-2-oxo-4-(2-propenyl)cyclohexaneacetic acid methyl ester (Fig. 1) (*10*), was chromatographed using a mobile phase of 2% ethyl acetate/98% hexane on the 40-μm particle column and 2.8% ethyl acetate/97.2% hexane on the 10-μm particle column (composition adjusted to give similar retention volumes). The isomer mixture was dissolved in the mobile phase and injections of 50, 100, 200, 300, and 500 mg were made. The flow rate was 66–70 mL/min and elution was monitored at 300 nm. The two isomers are very difficult to separate, having a relative retention of 1.05 in the best conditions we have found. They are present in the mixture in the ratio 1:3, with the minor isomer eluting first.

For each injection the total sample was recycled two times. Fractionation began with the third elution as soon as a slope change was noted. For the 100-mg injections, 40 mL fractions were collected. For the 200 to 500-mg injections, Fractions 1, 11, and 12 were 40 mL and Fractions 2–10 were 20 mL. Fraction volumes were monitored indirectly by following chart paper units.

The fractions were analyzed using a 5-μm Sotaphase (Sota Chromatography, Crompond, New York) silica column (4.5 mm × 25 cm) and a 5% ethyl acetate/95% hexane mobile phase at 2 mL/min. Elution was monitored at 276 nm. The area under each peak was determined by counting the number of chart units. The procedure was repeated and found to be reproducible within several percent. The amount of isomer present in each fraction was then calculated using the isomer ratio.

Calculations

Theoretical plate numbers were calculated using the conventional equation:

$$N = 5.54\left(\frac{tR}{w_{1/2}}\right)^2 \quad (1)$$

Asymmetry values were calculated at 10% of the peak height.

RESULTS AND DISCUSSION

The peak profiles for increasing amounts of benzophenone (25 to 700 mg) injected on the two columns, packed with 10 and 40 μm particles, respectively, are shown in Figs. 2 and 3. They demonstrate increasing

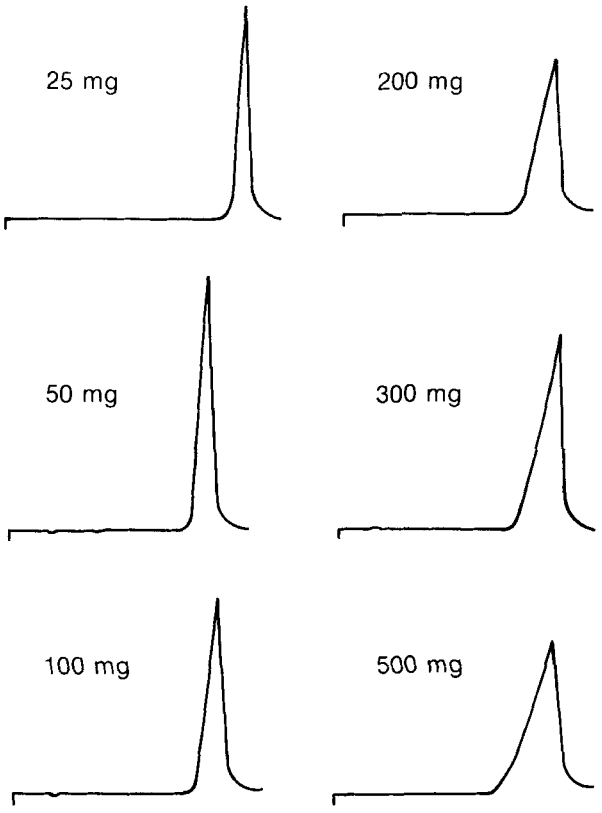

FIG. 2. Band profiles obtained with a 1" × 12" column packed with 10 μm particles of Davisil silica. Sample: Benzophenone. Mobile phase: *n*-Hexane/ethyl acetate (99.5/0.5); flow rate, 45 mL/min. Sample sizes: 25, 50, 100, 200, 300, and 500 mg.

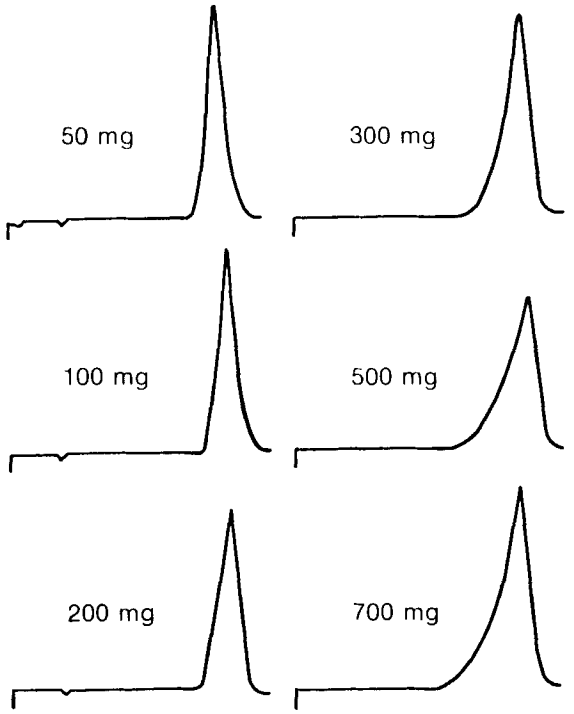

FIG. 3. Band profiles obtained with a 1″ × 12″ column packed with 40 μm particles of Davisil silica. Sample: Benzophenone. Mobile phase: *n*-Hexane/ethyl acetate (99.5/0.5); flow rate, 45 mL/min. Sample sizes: 50, 100, 200, 300, 500, and 700 mg.

column overload. The profiles become increasingly unsymmetrical, with a smooth front and a very sharp tail, indicating that the migration rate of benzophenone decreases with increasing concentration in the mobile phase. In other words, the larger the concentration in the mobile phase, the larger the proportion of the benzophenone sorbed. The equilibrium isotherm shape is thus anti-Langmuir.

The variation in plate number is reported in Fig. 4. The drop in efficiency is more serious for the more efficient column, but it is important to observe that the curves do not cross. The 10-μm particle column remains the more efficient at all loadings although the advantage almost vanishes at the largest sample size. The 10-μm particle column exhibits 50% better efficiency than the 40-μm particle column for a 100 mg sample size and 25% better efficiency for a 300-mg sample, but the difference is no longer significant for a 500-mg sample (cf. Table 1).

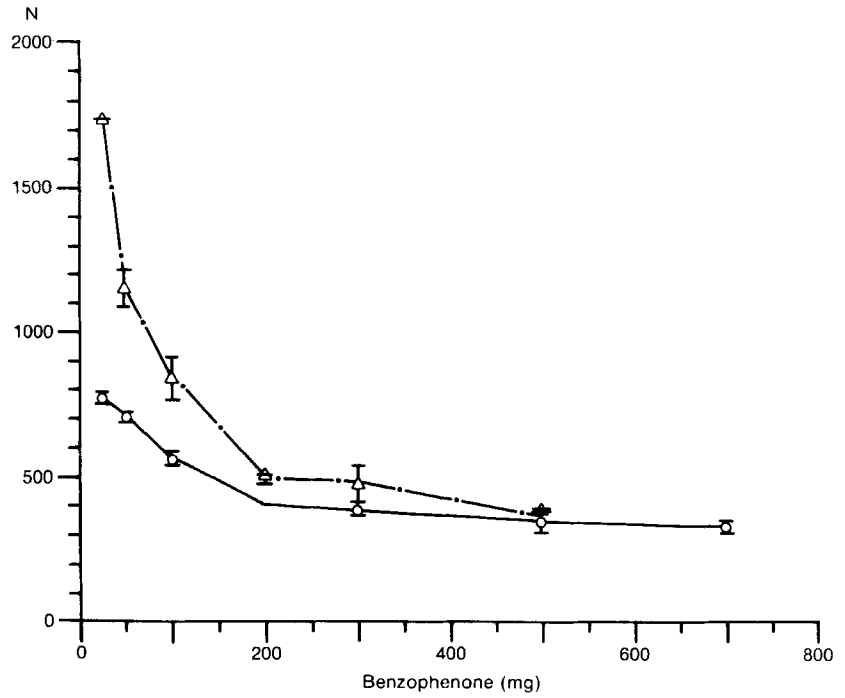

FIG. 4. Plot of the column efficiency versus the sample size (cf. Table 1). Dotted line: 10 μm particle column. Solid line: 40 μm particle column.

TABLE 1
Comparison of Column Performance[a]

	Plate number		$\sqrt{N}$		Peak asymmetry	
dp (μm):	40	10	40	10	40	10
Sample size (mg):						
25	771	1736	27.8	41.7	1.13	—
50	707	1153	26.6	34.0	0.85	0.65
100	565	840	23.8	29.0	0.67	0.61
200	404	494	20.1	22.2	0.47	0.35
300	388	477	19.7	21.8	0.40	0.30
500	348	375	18.7	19.4	0.34	0.24
700	331	—	18.2	—	0.31	—

[a]Benzophenone on Davisil Silica. Solvent: *n*-hexane/ethyl acetate (99.5/0.5).

There is still, however, a great difference between the two columns: the 10-μm particle column was operated below its optimum flow rate, so its efficiency could be increased by using a larger mobile phase flow velocity.

The asymmetry of the bands increases with increasing sample size (cf. Table 1). The variation is more important for the more efficient column, which again is expected, since the thermodynamic effects which are responsible for the change in band profile are less relaxed by band broadening than on the lesser efficient 40 μm particle column.

The chromatograms obtained for a 50-mg sample of the mixture of epimeric cyclohexanones on both columns are reproduced in Fig. 5. There is a marked difference between these chromatograms and the previous ones observed on the same columns for benzophenone (compare Figs. 1, 2, and 5). With benzophenone the peaks have a smooth front and a sharp tail, indicating an anti-Langmuir isotherm, at least at

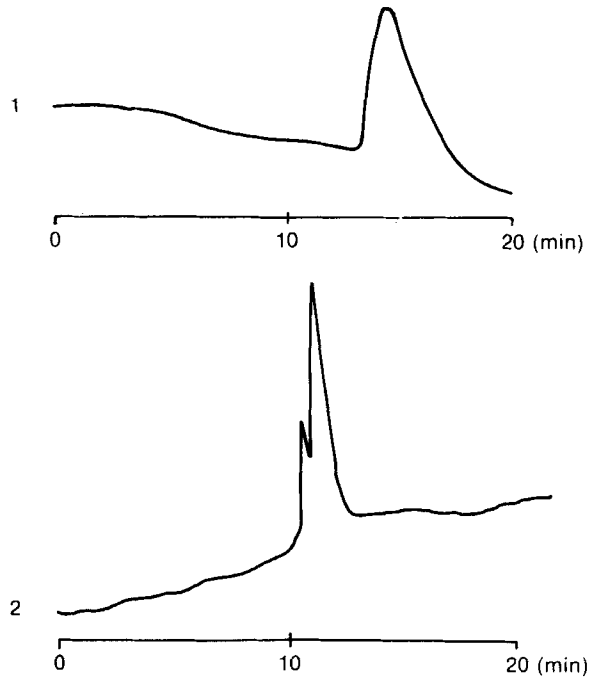

FIG. 5. Chromatograms obtained with the two columns for the 25:75 mixture of the two epimers (Fig. 1). Small sample size (50 mg); one pass. Time in minutes. (1) 40 μm particle column. (2) 10 μm particle column.

low concentrations. With the cyclohexanones the bands have a very sharp front and a smooth tail, indicative of either a Langmuir isotherm or an isotherm having a similar curvature toward the axis of concentration in the mobile phase. The 10-μm particle column yields some separation, with a valley observed between the peaks of the two epimers, while the 40-μm particle column does not give any qualitative resolution between the peaks of the two components. On the latter column the second band tails markedly.

When samples of more than 100 mg were injected, it was necessary to recycle the band twice (equivalent column length: 90 cm) in order to achieve sufficient separation for fraction collection. Ordinarily, product yield would be increased by using the shave/recycle technique, i.e., by collecting the two extreme wings of the band after each pass. However, in order to standardize fractionation, 20 mL fractions were collected only at the end of the third and final pass through the column. Isomer ratios were determined by HPLC analysis.

A comparison of the separations achieved and the purity of the fractions collected when using the two columns with 100 mg samples is shown in Fig. 6. Neither column is able to resolve the two epimers after one pass with this sample size. After two passes the 10-μm particle column gives a partial resolution of the two compounds, with a valley between the two bands. The resolution increases and the valley deepens

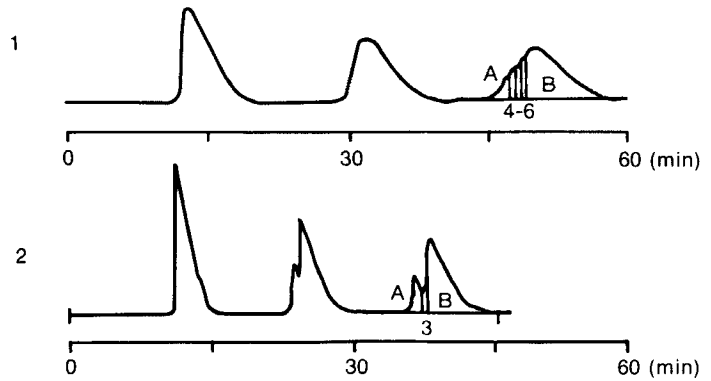

FIG. 6. Chromatograms obtained with the two columns for the 25:75 mixture of the two epimers (Fig. 1). Moderate sample size (100 mg); first, second, and third passes. Time in minutes. Solvent composition: *n*-hexane/ethyl acetate (97.5/2.5) for the 40-μm particle column, (97.2/2.8) for the 10-μm particle column. Recovery data in Tables 2 and 3. The numbers on the chromatograms indicate the collected fractions of unacceptable purity (see Table 2). (1) 40 μm particle column. (2) 10 μm particle column.

after the third pass. With the 40-μm particle column only a marked broadening of the common band is observed after two passes. A broad shoulder is observed with this column after three passes. To achieve a purity of 95% for each isomer (a product requirement), three fractions of the 40-μm particle column have to be rejected, while only one fraction is unacceptable with the 10-μm particle column (cf. Table 2). This column also gives more concentrated fractions, since a total of 8 fractions is recovered, instead of 12 fractions, with the less efficient column.

Similar experiments were repeated with different sample sizes on the 10-μm particle column (cf. Fig. 7). The yield of the separation was derived from the volume of each fraction and its isomer composition as measured by analytical HPLC. The results are summarized in Table 3. The reproducibility of these calculations is within several percent. No attempt was made to improve the yield of the 10-μm particle column by reducing the width of the intermediate fraction and optimizing its position with respect to the maximum of the first peak. A significant increase of that yield could probably have been obtained.

The increase in column loading, although resulting in a severe loss of column efficiency, as with benzophenone, does not cause a dramatic decrease in the resolution between the two isomers: Even with a sample size of 500 mg (corresponding to a load of 20 mg on an analytical column), a valley is still observed between the bands of the two isomers. Quantitatively, the loss in yield of the minor isomer is small, only 15%. The decrease in the yield of the major isomer, eluted second, is more

TABLE 2
Composition of the Fractions Collected (% epimer)

Column dp:	40	10	10	10	10
Sample (mg):	100	100	200	300	500
Fraction:					
1	96	100	99	98	97
2	95	100	99	98	100
3	99	67	99	96	99
4	64	7	96	75	92
5	31	4	65	28	49
6	15	4	21	16	21
7	8	4	10	12	17
8	5	4	8	11	15
9	3	—	7	8	13
10	3	—	5	7	11
11	2	—	4	5	9
12	2	—	4	4	7

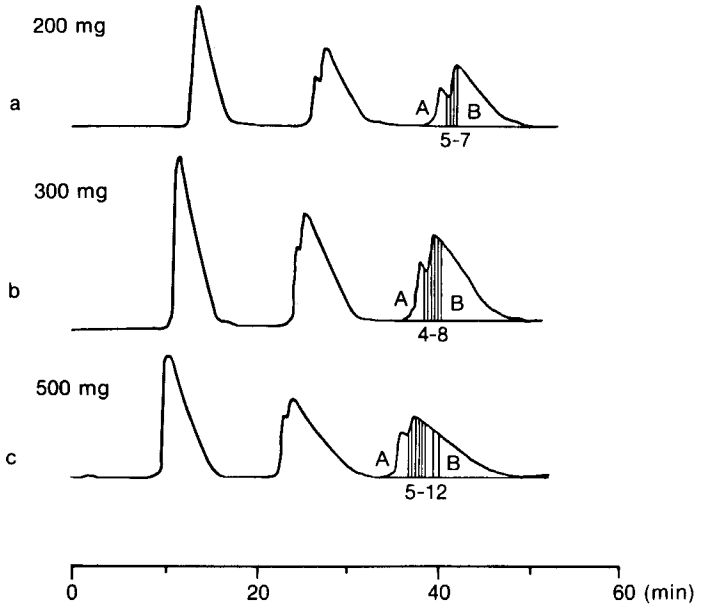

FIG. 7. Chromatograms obtained with the 10-μm particle column for the 25:75 mixture of the two epimers (Fig. 1). Large sample sizes (a) 200 mg, (b) 300 mg, (c) 500 mg; one, two, and three passes. Time in minutes. Experimental conditions as for Fig. 6. Recovery data in Table 2. The numbers on the chromatograms indicate the collected fractions of unacceptable purity (see Table 2).

TABLE 3
Variation of Yield and Production with Sample Size

Sample size (mg)	dp	Yield		Production[a]	
		A	B	A	B
100	40	50	82	12.5	63
100	10	68	98	17.0	74
200	10	67	83	34.0	125
300	10	58	72	44.0	162
500	10	58	55	73.0	206

[a]At a purity greater than or equal to 95%.

serious, but the production continues to rise and would certainly have continued to increase if loads larger than 500 mg had been applied (see Fig. 8). Obviously the impure intermediate fractions increase in amount too, and recycling these fractions would further augment the yield.

A study of these data shows that it is not possible to predict the yield or the production of pure fractions from the mere consideration of efficiency measurements. The band broadening of a single compound was observed. It indicates how the column reacts to overload, i.e., what changes in band profile are associated with a deviation of the equilibrium isotherm from linear behavior. Depending on the nature of the compound, the band becomes more or less unsymmetrical, with tailing (cf. Fig. 5) or fronting (cf. Figs. 2 and 3) depending on the curvature of the isotherm at the origin (zero concentration) and on the existence of inflection points. These are characteristic of the solute(s) studied, and different results can be obtained with the same column (cf. Figs. 3 and 5). However, other considerations, not intrinsic efficiency, influence yield.

Furthermore, when the separation of a mixture of closely related compounds is considered, the chromatogram obtained for the mixture is not the sum of the chromatograms obtained separately for the injections of each pure compound. The chromatogram in Fig. 5 is different from that obtained by the addition of two chromatograms, one of a 12.5-mg sample of the first isomer (A) and the other of a 37.5-mg sample of the pure second isomer (B). This is further illustrated by several observations made when comparing Tables 2 and 4. Table 4 gives the efficiency for the benzophenone band extrapolated to three passes through the column for different sample sizes on the two columns used. In comparing these efficiencies with the recovery of the purified isomers under similar experimental conditions, the efficiency for the 100-mg sample of benzophenone is 22% greater with the 10-μm particle column than with the 40-μm particle column, while the yield for the first isomer is 36% greater. The efficiency for a 200-mg sample of benzophenone on the 10-μm particle column is comparable to the efficiency for a 100-mg sample on the 40-μm particle column (actually it is 7% smaller). Nevertheless, the yield for the first isomer with a 200-mg sample is 34% greater on the 10-μm column than it is for a 100-mg sample with the 40-μm column. For a 500-mg sample the efficiency for the benzophenone band on the 10-μm column is 23% less than the efficiency for the 100-mg sample on the 40-μm column, yet the yield for the first isomer is still 16% greater with the 10-μm column. The yield does not vary in the same manner as the efficiency. A more complex phenomenon is at work.

Table 5 gives data on the retention volume measured for the two isomers on the 10-μm column at different sample loads. Although there is

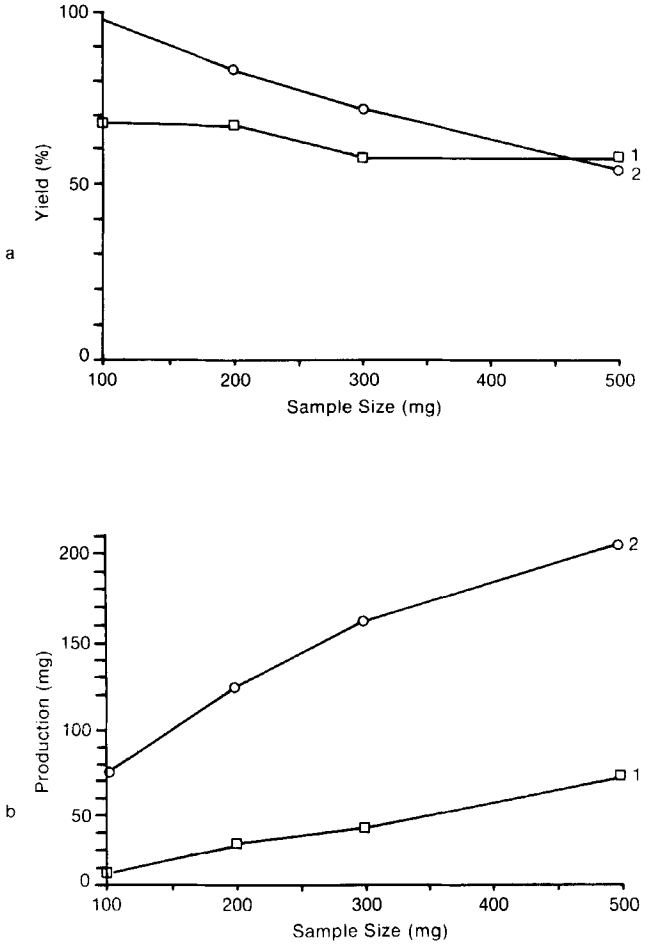

FIG. 8. Plot of the yield (a) and the production (b) for the two trisubstituted cyclohexanone epimers versus sample size. Experimental conditions as for Fig. 6. (1) Yield or production of Compound A. (2) Yield or production of Compound B.

TABLE 4
Variation of Column Efficiency with Sample Size[a]

Sample size (mg)	Plate number		$\sqrt{3N}^b$		Ratio[d]
	40[c]	10[c]	40[c]	10[c]	
100	565	840	41.2	50.2	1.22
200	404	494	34.8	38.5	1.11
300	388	477	34.2	37.8	1.11
500	348	375	32.3	33.5	1.04

[a]Solute: benzophenone. Conditions as for Fig. 2.
[b]Assuming there is no loss of efficiency due to the use of three recycling passes.
[c]This number indicates the column particle size.
[d]Ratio of $\sqrt{(3N(10)/3N(40))}$.

a marked decrease in the retention volume consistent with a Langmuir isotherm, (approximately 10%), it is remarkable how constant the relative retention of the two band maxima remains: the variation of the relative retention is of the same order of magnitude as the error of measurement, and hardly significant. At the same time, the overall change in retention volumes is of the order of two column volumes (cf. Table 5), i.e., the change in apparent column capacity factor is of the order of 2, indicative of strong column overload.

Further information can be derived from the efficiency determined for the band of the first isomer. This has been done by reconstituting the elution profile of this compound from the composition of the different

TABLE 5
Variation of the Retention Volumes with Increasing Sample Size

Sample size (mg)	VR (mL) (A)	VR (mL) (B)	Relative retention	VB − VA (mL)	Peak migration[a]	
					A	B
100	2645	2776	1.050	131	0	0
200	2637	2740	1.039	103	8	36
300	2521	2624	1.041	103	124	152
500	2395	2488	1.039	93	250	288
Total volume change: in mL				38		
	in column volume			0.27	1.76	2.03

[a]Difference between the retention volume observed for that sample and the one measured for a 100 mg sample.

fractions collected. This determination is approximate, since we measured the average composition for 20 mL fractions and thus have only 5 points for the second half of the profile. Nevertheless, the results are quite informative. The reconstructed profiles for the four sample sizes used are given in Fig. 9. The values of the band plate number derived from these profiles are given in Table 6 under the heading "apparent plate number." They are compared with the efficiencies measured for samples of benzophenone having the same weight (cf. Table 1), extrapolated to a recycling mode with three passes, derived from the efficiencies, and given in Table 6 under the heading $\sqrt{N3}$.

The results in Table 6 are striking. The values of the apparent plate number obtained for the first isomer, 25% of the sample mixture, are much larger than those derived for the comparable benzophenone band. The difference *increases* with increasing sample load, as the first peak is compressed because it is pushed out of the column by the second one. What we are really seeing here is the onset of displacement.

The conclusions derived from a consideration of the data in Tables 5 and 6 are consistent. When the column is overloaded and the bands of the two compounds interfere during their entire migration through the column, they do not behave independently but interact. When the stationary phase is overloaded by one compound, i.e., if the equilibrium isotherm of this compound deviates appreciably from a linear behavior in the concentration range covered by its elution band, the stationary phase cannot behave linearly for another compound which migrates with the first one and whose band overlaps with the band of the first compound. If a large fraction of the adsorption sites of the stationary phase are occupied by the molecule of one compound, they are not available to sorb the molecules of the other one. The compound eluted second is more strongly sorbed than the one eluted first. Thus its molecules displace those of the first eluted one which migrates faster than it would if it were pure.

This phenomenon is quite similar to displacement, but it differs in that the second compound is not very strongly sorbed. It is eluted itself by the solvent. Elution of the displacing agent does not take place during displacement chromatography where the displacer is chosen to be strongly sorbed by the stationary phase and must usually be eliminated by thorough column regeneration. This difficult step, which is long and tedious and often results in loss of column efficiency, has been eliminated. However, the second compound of the mixture being eluted gives a strongly unsymmetrical band, usually tailing. As the difference between the adsorption free energies of the two compounds being

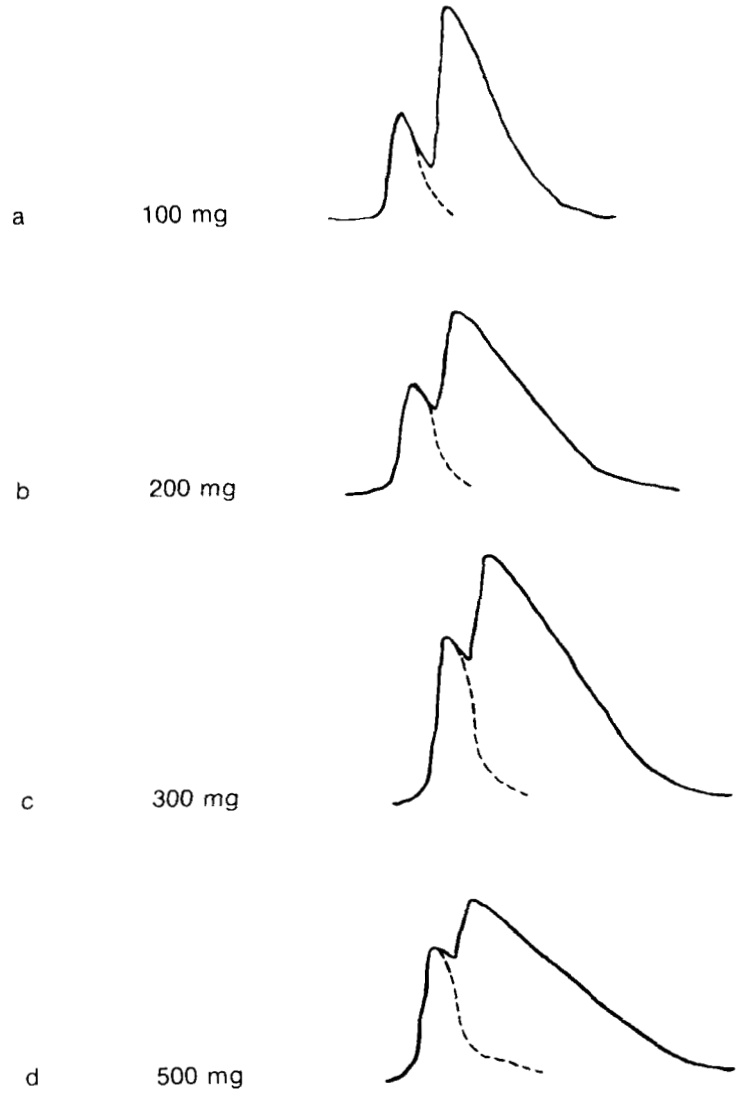

FIG. 9. Reconstructed elution profiles for the two epimers from the chromatograms in Fig. 7 and the results of the analysis of the collected fractions. 10 μm particle column, 25:75 mixture of the two epimers (Fig. 1). Sample sizes: (a) 100 mg, (b) 200 mg, (c) 300 mg, (d) 500 mg; profiles of the third pass. Time in minutes. Experimental conditions as for Fig. 6.

TABLE 6
Comparison between Two Measures of Column Efficiency

Sample load						
Total	1st isomer	Na[a]	$N3$[b]	Na/$N3$	$\sqrt{Na}$	$\sqrt{N3}$
100	25	5800	5210	1.13	80.0	72.2
200	50	4280	3460	1.24	65.4	58.8
300	75	4160	2520[c]	1.65	60.2	50.2[c]
500	125	3560		1.41	59.7	

[a]Apparent plate number, derived from the reconstructed profile of the first isomer (see text).
[b]Derived from the plate number measured for the benzophenone band (Table 1).
[c]One measure was made for 100 mg of benzophenone.

considered is rather small, the concentration effect normally associated with displacement chromatography is not seen for the second isomer.

Nevertheless, it is very important to recognize the nature of the self-displacement phenomenon, as it can be manipulated to achieve much larger yields for a given preparative separation and much larger production for a given chromatograph. The production and the yield achieved are much larger even though the column is seriously overloaded and the recorded chromatogram shows only a very small degree of resolution. As in displacement chromatography, but to a much larger extent, the degree of band overlap between the displaced band and the displacing one, which is responsible for the effect, depends on the column efficiency. *Therefore, there is a strong advantage in using columns which have a high maximal efficiency* (a large number of theoretical plates for a very small sample size).

CONCLUSION

As the loading of a preparative column increases, its efficiency decreases. Eventually, the efficiency becomes very low and approaches a value independent of the performance of the column at very small sample sizes (cf. Fig. 4 and Table 1). At the same time, the bands become strongly unsymmetrical. Depending on the nature of the compound under investigation, it may exhibit either a very sharp, almost vertical front and a quasi-Gaussian tail or the reverse, a normal, quasi-Gaussian front and a very steep tail (cf. Figs. 2, 3, and 5–7).

Regardless of peak symmetry, however, two compounds which are very closely related chemically and are difficult to resolve on any chromatographic system would be expected to have isotherms with the same shape and with very similar values of their numerical parameters (there are exceptions, cf. Ref. *11*). For such a mixture, when the column is overloaded for one component, it would be, *ipso facto,* overloaded for the other one in the part of the column where the two bands overlap. The behavior of the column would no longer be linear and the elution profile of each compound would be influenced by the other and would depend on the relative concentration of the two compounds.

Most often in HPLC, a Langmuir or quasi-Langmuir isotherm is involved. When the column is overloaded, the interaction described above results in self-displacement of the sample, the displacement of the first eluted compound by the second one. As a consequence, the band of the first compound is eluted earlier and is narrower than it would be if the same amount of the first eluted compound were injected pure. This phenomenon is of major importance and can be exploited with the use of highly efficient columns to achieve considerably increased production with reasonable or high yields.

Although the numerical results described in this report are specific to the particular mixture studied, the conclusions are sufficiently in agreement with the results of theoretical studies (*12*) to be of general applicability. Application of these results to other mixtures is under current investigation.

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