Journal of Chromatography, 450 (1988) 13–25
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1420

ANALYSIS OF THE INFORMATION IN A PREPARATIVE CHROMATOGRAM FOR FURTHER OPTIMIZATION OF THE OPERATING CONDITIONS

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SUMMARY

It was demonstrated how the most salient theoretical aspects of linear and non-linear elution liquid chromatography can be used, without the aid of sophisticated optimization software, to analyse the information in a preparative chromatogram. It is important, first, to be able to recognize whether a given preparative chromatogram was obtained under volume- or mass-overload conditions or both. This permits further optimization of the injection conditions (size, volume and concentration of the sample), depending on the preparative objectives, and improvement of the stationary phase particle size and of the mobile phase flow-rate. This approach can also provide insight into the performance characteristics of the injection device, the efficiency of the column packing and the stationary phase capacity. Some examples taken from the recent literature are discussed.

INTRODUCTION

The many systematic studies undertaken in the past few years in all areas of preparative liquid chromatography (PLC) have resulted in greater understanding of the behaviour of preparative columns. It can now be said that the well established theory of linear elution satisfactorily describes separations obtained under volume-overload conditions[1–5]. As it is known that the best performance from a preparative point of view (production rate at a given purity) is often obtained under mass-overload conditions, several theoretical approaches describing the shape of non-linear elution peaks have recently been developed. Most often, the mass-balance differential equation for a single solute has been considered together with a non-linear isotherm. The resulting set of equations has been solved either analytically, in the case of slight isotherm curvature[6–9] (so-called Haarhof–Van der Linde treatment), or numerically, without any restriction[10–21]. The plate model scheme, associated with a Langmuirian isotherm, has also been used to calculate the non-linear elution profile of one solute[13,14] and, lately, that of two unresolved compounds[15,16]. It appears that the Haarhof–Van der Linde treatment is limited to describing slight mass-overload conditions, whereas the other models, the promising preparative applications of which

0021-9673/88/$03.50

are still underway, require sophisticated computer programs. This is why we think that the most straightforward and simplest approach to non-linear PLC at this time is to make use of the semi-empirical model we have developed previously[17] in spite of its inherent limitations. This model was based on a mathematical characterization of strongly non-linear experimental elution peaks[18].

The purpose of this report is to show how some of that linear and non-linear theoretical framework can be used in a very simple practical way to analyse the information in a preparative chromatogram. This analysis will lead to a discussion of operating conditions and, subsequently, to their optimization. This will be simply exemplified by some PLC separations taken from the recent literature, but first the salient theoretical features used in the subsequent discussions will be briefly reviewed.

THEORETICAL

Linearity test

When the injection parameters (quantity, volume and concentration of the sample) are varied, a chromatographic column may present two distinct types of behaviour, which are defined in analogy with the theory of systems[17,19,20]. The linear behaviour corresponds to the usual injection conditions of analytical chromatography and to conditions where the sample volume is the only factor accounting for a peak shape alteration. These conditions are referred to as volume overload in preparative chromatography. The non-linear behaviour corresponds to the other cases in which the peak shape alterations are mainly caused by the curvature of the distribution isotherms (mass overload conditions). While optimizing the operating parameters for preparative purposes, it is of prime importance to characterize the column behaviour for any given experimental injection, because the choice of the optimum conditions is very dependent on whether the column behaves linearly or not. In practice, this characterization can be performed very easily by using a test based on the additivity of the independent contributions to the statistical moments of chromatographic peaks[19]. The procedure consists in checking the following conditions

$V_0/2 \ll V_R$ and $V_0^2/12 \ll \sigma^2$ Linear behaviour (pulse injection)
$V_R' - V_0/2 = V_R$ and $\sigma'^2 - V_0^2/12 = \sigma^2$ Linear behaviour (plug injection)
$V_R' - V_0/2 < V_R$ or $\sigma'^2 - V_0^2/12 > \sigma^2$ Non-linear behaviour

where V_R and σ are the retention volume and standard deviation, respectively, for small-size, small-volume injections, V_R' and σ' are the retention volume and standard deviation, respectively, under the injection conditions being tested and V_0 is the sample volume.

Optimization of injection conditions

For a pair of compounds to be separated on a preparative scale the optimum sample volume can be predicted theoretically. If the resolution of the analytical separation is less than 1.3, neither volume nor mass overload is advocated. The sample volume should not exceed the standard deviation of the narrowest peak of the pair[4]. If the analytical resolution is greater than 1.3, the maximum sample volume, $V_{0,\mathrm{lin}}$, allowing a total recovery in the case of a linear behaviour of the column is given by[1-5]

$$V_{0,\mathrm{lin}} = V_{R_2} - V_{R_1} - 2(\sigma_1 + \sigma_2) \qquad (1)$$

in which V_{R_1} and V_{R_2} are the analytical retention volumes of the two compounds and σ_1 and σ_2 the corresponding standard deviations. The maximum sample concentration, $C_{0,\text{lin}}$, consistent with a linear column behaviour, is dependent on the solute retention, molecular size and stationary-phase capacity. Systematic investigations[21] have shown that, in most cases, $C_{0,\text{lin}}$ lies between $5 \cdot 10^{-3}$ and $2 \cdot 10^{-2}$ M. This linear optimization procedure leads to operation under volume overload conditions; it is advocated when the column cannot be operated under mass overload, as discussed below.

If the analytical resolution is rather large and the sample solubility high, a non-linear optimization procedure should be followed. The optimum quantity, $Q_{0,2}$, of the more strongly retained of the two compounds of interest can be assessed by using a model based on the experimental characterization of the shape of strongly non-linear elution peaks[21]

$$Q_{0,2} = C_{\text{m},2}\tau_2 \exp\left(\frac{V_{R_1} + 2\sigma_1 - V_{\text{m}}}{\tau_2}\right) \tag{2}$$

where V_{m} is the column dead-volume and $C_{\text{m},2}$ and τ_2 the model parameters, which can be evaluated as follows: τ_2 is roughly equal to 0.2 V_{R_2} and $C_{\text{m},2}$ in most cases lies in the range of 0.2–0.8 M in the inverse order of solute retention[18]. In addition, it has been observed that this quantity is best injected in a small volume of concentrated solution[21].

DISCUSSION

Mass- and volume overload excluded

We will first exemplify the practical use of the linearity test described above for a separation of two isomeric azo compounds (MW = *ca.* 350) obtained by synthesis[22]. This separation was performed in the reversed-phase mode on Merck Lobar (31 cm × 2.5 cm I.D.) preparative column. The capacity factors of the compounds of interest were determined to be 10.1 and 14.4, and the corresponding analytical resolution was *ca.* 1.2. The effect of sample volume was studied up to 100 ml for a constant sample size (5 mg). It was experimentally determined that the sample volume has no influence on the peak width up to about 70 ml. Table I gives the results obtained for the two extreme values of sample volumes studied, *i.e.*, 10 and 100 ml. Using the methodology mentioned above for linearity testing, it is clear that the chromatographic behaviour of the column under these conditions is linear for these compounds over this range of sample volumes. The chromatogram resulting from a 10-ml injection can be considered as an analytical type impulse response of the column ($V_0/\sigma \approx 0.18$), while the one resulting from a 100-ml injection is a linear chromatographic response to a plug-shaped injection ($V_0/\sigma \approx 1.8$). These results were quite predictable for such a sample size (5 mg, or *ca.* 0.07 mg sample per g stationary phase) and for sample concentrations in the range of from $1.4 \cdot 10^{-4}$ to $1.4 \cdot 10^{-3}$ M, *i.e.*, below the usual values for $C_{0,\text{lin}}$. Owing to the resolution of 1.2, the sample volume, for practical preparative purposes, should not be much in excess of the analytical standard deviation of the narrowest peak, *i.e.*, 60–70 ml. This is in agreement with the experimental results reported and means that the injection device used for large

TABLE I

EFFECT OF THE SAMPLE VOLUME, V_0, ON THE SEPARATION CHARACTERISTICS OF TWO ISOMERIC AZO COMPOUNDS

V'_R, σ' = measured retention volumes and standard deviations. Operating conditions: column, 31 cm × 2.5 cm I.D. (Lobar, Merck); stationary phase, LiChroprep RP 8, 50 μm; mobile phase, methanol–water (3:2); flow-rate, 7.4 ml/min; sample size, 5 mg mixture.

	Solute	*Sample volume (ml)*	
		10	*100*
$V'_R - V_0/2$ (ml)	1	804	804
	2	1103	1096
$(\sigma'^2 - V_0^2/12)^{\frac{1}{2}}$ (ml)	1	56	56
	2	68	70

volumes in this study was working quite well. Despite the rather high capacity factors, the sample concentration could probably be raised to around $2–3 \cdot 10^{-3}$ M, barring solubility limitation, without serious mass-overload effects, and this would allow injections of the order of 50 mg of sample.

Analytical to preparative column scale-up. Mass overload

A second example taken from the literature[23] is the normal-phase separation of two positional isomers, the 1- and 4-hydroxy-1,2,3,4-tetrahydrophenanthrenes (MW = 197). The mobile phase was first optimized for preparative application by considering the criteria of separation selectivity, sample solubility, and volatility, viscosity and consumption of solvents. A dichloromethane–ethyl acetate (95:5) mixture was selected. The resulting analytical chromatogram is shown in Fig. 1A. The analytical column (30 cm × 4.2 mm I.D.) was next used to determine the optimum sample load experimentally, according to the preparative objectives of high purity and yield of both compounds. The result is shown in Fig. 1B. Then, the separation was scaled up to a 10-g sample load on a Waters Prep LC 500 preparative chromatograph, equipped with a 30 cm × 5.7 cm I.D. column, working with the same phase system.

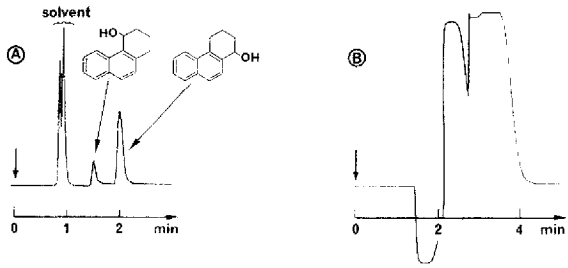

Fig. 1. Analytical (A) and small-scale preparative (B) separations of 1- and 4-hydroxy-1,2,3,4-tetrahydrophenanthrenes (4:1 synthetic mixture). Column: 30 cm × 0.42 cm I.D. Stationary phase, μPorasil, 10 μm. Mobile phase: dichloromethane–ethyl acetate (95:5). Detection: refractive index (RI). (A) Sample size, 100 μg in 10 μl. Flow-rate: 4 ml/min. Sensitivity: × 16. (B) Sample size: 54 mg in 1.75 ml. Flow-rate: 2.5 ml/min. Sensitivity: × 128 (from ref. 23 with permission).

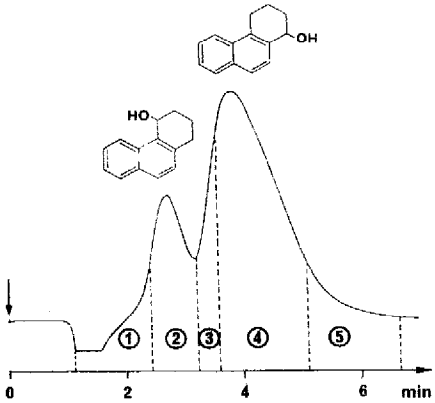

Fig. 2. Scale-up of the preparative separation of the tetrahydrophenanthrene mixture. Column: 30 cm × 5.7 cm I.D. Solvent system as in Fig. 1. Flow-rate: 300 ml/min. Particle size: 75 μm. Sample size: 10 g in 45 ml. Detection: RI (from ref. 23).

A preparative chromatogram, featuring fraction collection, is shown in Fig. 2. The 4-hydroxy isomer was recovered from fraction 2 in a yield of 85%, whereas the 1-hydroxy isomer was recovered from fractions 4 and 5 in a yield of 79%. The purities achieved were greater than 99%.

The analytical and preparative columns used in this study differed only in diameter and particle size. It is clear that the ratio of their volumes ($57^2/4.2^2 = 184.2$) was equal to the ratio of the sample sizes (10:0.054 = 185.2). Thus, the specific loads (defined as the sample to stationary phase ratio) were quite similar for the two columns.

To assess the pertinence of the operating conditions, let us first look into the working linearity of the analytical and preparative columns for the sample size considered as optimum. For the phase system used, the analytical capacity factors of both compounds were reported to be 0.63 and 1.38, which corresponds to a selectivity of 2.19. Assuming a total porosity of 0.80 for the column packing, the analytical retention volumes were calculated. The retention volumes corresponding to the optimal sample size, V'_{R_1} and V'_{R_2}, can be calculated from the flow-rate and the elution

TABLE II

RETENTION VOLUMES OF THE 1- AND 4-HYDROXYTETRAHYDROPHENANTHRENE ISOMERS FOR SMALL SIZE (V_R) AND OPTIMUM SIZE (V'_R) INJECTIONS ON THE ANALYTICAL AND PREPARATIVE COLUMNS

	Analytical column (30 cm × 4.2 mm I.D.)	*Preparative column (30 cm × 5.7 cm I.D.)*
V_{R_4} (ml)	5.4	1000
V_{R_1} (ml)	7.9	1455
V'_{R_4} (ml)	4.9	795
V'_{R_1} (ml)	7.0	1145
$[V_{R_4} - (V'_{R_4} - V_0/2)]V_{R_4}$ (%)	8	23
$[V_{R_1} - (V'_{R_1} - V_0/2)]/V_{R_1}$ (%)	11	23

time at the peak apex in Figs. 1B and 2. The results are given in Table II. By use of the linearity test, restricted to the comparison of the first statistical moments (retention volumes), a decrease in retention of *ca.* 10% for the analytical column is seen when it is used under optimum sample load conditions, indicating a slightly non-linear behaviour for this injection. The same treatment shows a more pronounced non-linear behaviour for the preparative column (23% decrease in retention) for a similar specific load. Assuming a packing density of 0.5 g/ml for silica, this sample load corresponds to about 26 mg of sample per gram of packing, which is very high. Thus, the observed non-linear behaviour is not surprising in this case. It might have been even more pronounced with larger sample molecules and higher capacity factors, In addition, at that high specific load there is no reason for using fine particles, for the major source of band spreading lies in the isotherm curvature[18].

Now, let us evaluate the optimum sample size suggested by the semi-empirical non-linear model allowing for mass overload. If we assume $C_m = 0.7\ M$ to allow for the small capacity factor of the more strongly retained isomer ($k'_2 = 1.38$) and $\tau_2 = 0.2\ V_{R_2} = 1.58$ ml, eqn. 2 gives the quantity to be injected as 43 mg for the more strongly retained isomer, *i.e.*, a sample of 54 mg for a 4:1 mixture. This is the quantity that was determined as optimal by the experimental approach.

Some other interesting conclusions may be drawn if we now consider the sample volumes and concentrations used for this separation. Systematic studies have shown that if the sample size which can be injected gives rise to pronounced non-linear behaviour, better resolution will be obtained with a small sample volume and high concentration[21]. To calculate the sample volume, the values of the analytical standard deviations, σ_1 and σ_2, of both peaks, in volume units, are needed. For the small-scale column, they can be evaluated by measuring the baseline intercept of the peak inflection tangents from Fig. 1A, by assuming gaussian peak shapes. Thus, $\sigma_1 = 0.16$ ml and $\sigma_2 = 0.22$ ml. Using these values and those of Table II, eqn. 1 enables one to calculate the maximum sample volume that can be injected in the case of a complete separation of both species under volume overload but without mass overload. A value of 1.73 ml is obtained. Thus, the volume actually injected into the analytical column (Fig. 1B) was obviously chosen so as to fit in with eqn. 1. However, the sample concentration, 0.16 *M* (31 mg/ml), greatly exceeds the highest known values for the upper limit of the linear range of the distribution isotherm. This choice of volume and concentration does not seem to be appropriate here, since the experimental results showed that mass overload can be contemplated. However, it is worth noting that, contrary to what is usually done when scaling up to a preparative column, the sample volume was increased by a factor much lower than the ratio of the column volumes. Taking into account the change in particle size from 10 to 75 μm between the two columns, it can be stated that the standard deviations of both analytical peaks on the preparative column are increased by a factor greater than the ratio of the column volumes. Thus, these standard deviations must be greater than 30 and 40 ml, respectively, and a sample volume of 45 ml like that actually injected into the preparative column did not contribute to bandspreading by volume-overload effects. Conversely, the sample concentration was increased to *ca.* 1.13 *M* (220 mg/ml) so as to keep the sample size proportional to the column volume. The increase in sample concentration explains why the non-linear behaviour of the preparative column is more pronounced than that of the small-scale column (Table II), in spite of quite

similar specific loads. Lastly, the linear velocity, which was very high for the analytical separation, was reduced to a medium value for the preparative column.

Finally, it can be said that the choice of the sample volume and concentration, which seemed questionable for the analytical column, appears to be perfectly adapted to a non-linear optimization strategy for the preparative column. In a case where this separation would have to be performed repeatedly, the flow-rate could be slightly increased to improve the production rate, since the flow-rate does not contribute much to the overall bandspreading under severe mass-overload conditions.

Stationary phase capacity limitation. Volume overload

Another significant example is the gram-scale separation of monensin A and B sodium salts described by Beran *et al.*[24]. These compounds are polyether antibiotics differing only by a methylene group (MW = 648 and 660) and were obtained from a fermentation broth. Their separation was carried out in the reversed-phase mode. An analytical chromatogram is presented in Fig. 3A. In order to determine the sample loadability of the chromatographic system, the sample size was progressively increased on the analytical column by varying the sample concentration and keeping the injected volume constant. For this study, the ratio, P, of the depth of the valley between the two peaks, f, to the mean peak height, g, was taken as the resolution criterion (Fig. 3B). Accordingly, the optimum sample size, determined as that corresponding to a P value of 0.9, was found to be 3 mg with a 500-μl sample volume. From these results, the separation was directly scaled up on a Jobin Yvon Chromatospac Prep 100 preparative chromatograph, using a 33.6 cm × 8 cm I.D. axially compressed column. The P values were measured as a function of sample size in the range from 0.85 to 2 g for a 25-ml sample volume. It was found that a 1-g sample size resulted in a P value of 0.8. Subsequently, this sample size was considered as optimum for this column (Fig. 3B). It

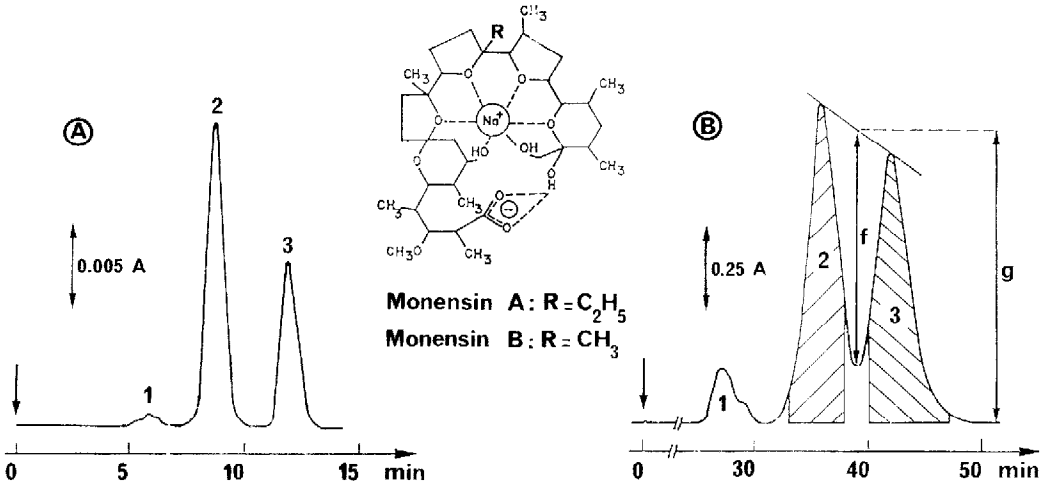

Fig. 3. Analytical (A) and preparative (B) separations of monensin A and B by reversed-phase chromatography on Separon C_{18} octadecyl silica with methanol–water (88:12). Detection: absorbance at 215 nm. Peak identification: 1 = unknown compounds; 2 = monensin B; 3 = monensin A. (A) Column: 34 cm × 0.4 cm I.D. Stationary phase particle size: 10 μm. Flow-rate: 1 ml/min. Sample size: 0.1 mg (500 μl). (B) Column: 33.6 cm × 8 cm I.D. Particle size: 15 μm. Flow-rate: 80 ml/min. Sample size: 1 g (25 ml) (from ref. 24 with permission).

TABLE III

CHROMATOGRAPHIC DATA FOR THE SEPARATION OF MONENSINS A AND B

	Analytical column (34 cm × 0.4 cm I.D.), 0.1 mg sample (500 µl) (measured)	*Preparative column (33.6 cm × 8 cm I.D.), 1 g sample (25 ml)*	
		Measured	*Calculated*
Retention volumes (ml)			
B	9.1	2855	3510
A	12.3	3375	4775
Standard deviations (ml)			
B	0.4		
A	0.4		
Hold-up volume* (ml)	2.4	944	
Total porosity	0.56	0.56	

* Given in the original paper[24].

allowed the recovery of 0.4 g of monensin B and 0.5 g of monensin A of 99.7 and 98.6% purity, respectively.

Using the data for the analytical column reported in Table III, a separation selectivity of 1.48 and an analytical resolution of about 2 can be calculated for the two compounds of interest. The chromatogram shown in Fig. 3A can be considered as the impulse response of the analytical column, in spite of the slight volume overload expected by comparing the injected volume with the measured standard deviations. The sample load regarded as optimal for that column (3 mg) was injected in a 500-µl volume of a *ca.* $9.3 \cdot 10^{-3}$ *M* mixture in the mobile phase. This concentration corresponds to the usual values of maximum concentrations, consistent with a linear distribution mechanism. However, eqn. 1 suggests that a volume of 1.6 ml can be injected without serious band overlapping due to volume overload. It would have been interesting to study the injection of *ca.* 4 mg mixture in 1.3–1.5 ml of a slightly more diluted solution, say about $5 \cdot 10^{-3}$ *M*.

If we now consider the scale-up of these injection conditions on the preparative column, it can be shown that the resolution parameter, *P*, decreases as a function of the specific load more rapidly on the preparative column than on the analytical one. This discrepancy cannot be ascribed to a difference in packing density, since both columns had the same total porosity (Table III), but rather to the fact that the sample load was increased by merely increasing the sample concentration at constant volume, while the ratio of the sample volume on both columns was lower than the ratio of the column volumes. Consequently, the preparative column exhibits a more pronounced non-linear behaviour than the analytical column for identical specific loads. Eventually, the optimum sample size and sample volume were increased by factors of 1000/3 = 333 and 25/0.5 = 50, respectively, whereas the column volume was increased by a factor of 395. Simultaneously, the sample concentration was raised to *ca.* $6.2 \cdot 10^{-2}$ *M* (40 mg/ml). This is probably above the maximum concentration allowed for linear distribution. Using the multiplicative factor approach[25], it also appears that, when scaling up the analytical conditions, the linear velocity was reduced by a factor of

$$\frac{F_{anal}}{F_{prep}}\left(\frac{d_{c\ prep}}{d_{c\ anal}}\right)^2 = 5$$

where d_c and F are the column diameters and flow-rates, respectively. This is expected to result in an improved resolution and a drastic decrease in pressure drop, as shown below, at the expense of separation time

$$R_{s\ prep}/R_{s\ anal} = \frac{1}{b}\sqrt{\frac{l}{u}} = 1.5$$

$$\Delta P_{prep}/\Delta P_{anal} = ul/b^2 = 0.088$$

where u, l and b are the ratios of the preparative- to analytical linear velocities, column lengths and particle sizes, respectively. Finally, it should be stressed that the measured values of retention volumes on the preparative column deviate from the calculated ones for the case of linear chromatography by about 19% for monensin B and 29% for monensin A, eluted later (Table III). This deviation, mainly due to the above-mentioned non-linear effects, appears to be a rather serious one for a sample size corresponding to a moderate specific load of about 1.2 mg sample per g of stationary phase (assuming a packing density of 0.5 g/ml). We think that this should be considered together with the low value of the column total porosity that can be calculated from the measured hold-up volumes (Table III). This might indicate a partial pore clogging and a decrease in specific surface area during the silica bonding process. The net result is, of course, a decrease in the available capacity of the stationary phase. A non-linear chromatographic behaviour more pronounced than expected was also observed by us in the case of the gram-scale enantiomer separation of a tertiary phosphine oxide on an aminopropyl silica, bonded with (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine moieties[26]. For a specific loading of only 2 mg/g, a decrease in retention volume by 32 and 35% was obtained with the two optical isomers, compared with their analytically determined values. This pronounced non-linear behaviour was related to the low number of chiral sites (34% of the aminopropyl sites, *i.e.*, 0.3 mmol/g) available on the stationary phase, resulting in a low capacity.

In the separation of monensins, with this phase system, the preparative column can certainly accommodate a much larger sample volume than that actually injected. This would allow reduction of the sample concentration and thereby, non-linear effects. Another approach might be to use a bonded phase having a more common value of the total porosity, in order to improve column loadability.

Sample volume; column packing; injection device

Another large-scale separation of interest is that of an equimolar mixture of two menthyl methylphenylphosphinate diastereoisomers, performed in our research group[27]. An appropriate phase system was first investigated on a 20 cm × 0.48 cm I.D. analytical column. The best conditions are given in Fig. 4A. The separation was next scaled up on a 70 cm × 1.0 cm I.D. column, packed with the same silica gel and operated with the same mobile phase. A sample size of 30 mg, corresponding to

a specific load of 1 mg/g, was first injected by use of a syringe. The resulting chromatogram is shown in Fig. 4B. Still using a syringe, the sample size was then increased to 90 mg (0.3 ml of a 300 mg/ml solution in the mobile phase). For such a load, the purities of the recovered isomers were 95 and 97%, respectively, in a yield of 88%. Twelve identical injections of this size were performed without waiting for the elution of both isomers between injections (short cycling technique), so that 0.48 and 0.47 g, respectively, of each isomer were obtained in only 5 h.

The data obtained from a small-size injection on the analytical column (Fig. 4A) and from a 30-mg injection on the preparative column (Fig. 4B) are given in Table IV. Assuming a total porosity of 0.75 for these columns, we can calculate a selectivity of 1.12 from the analytical results, which indicates a rather difficult separation. The resolution calculated from the values of retention volumes and standard deviations was only 1.03. This resolution was improved by choosing a preparative column longer than the analytical one. The chromatographic characteristics of a small-size injection into the preparative column, which were not given in the original paper, were calculated, assuming that, at constant linear velocity, the retention volumes are proportional to the column volume, and the plate number and pressure drop are proportional to the column length. Thus, a resolution close to 1.9 is expected on the preparative column.

However, it appears that the retention volumes of both compounds on the preparative column for a 30-mg sample injection (0.1 ml) are 23–24% higher than those predicted on this column for a small size sample (Table IV). This increase in retention volume cannot be ascribed to a volume- or mass-overload effect. An unexpected change in solvent composition may be responsible, but this seems very doubtful. A more likely explanation would be that the analytical and preparative columns do not have identical total porosities, the porosity of the preparative column

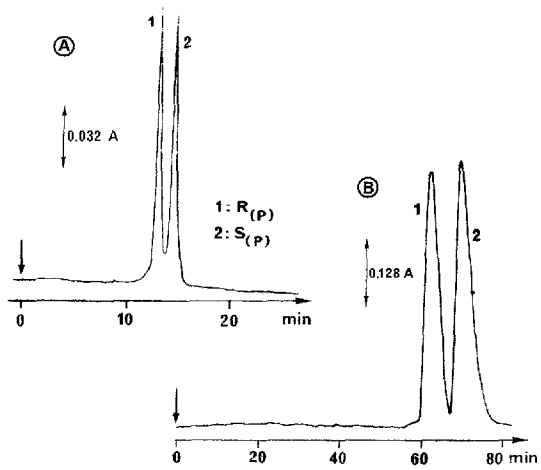

Fig. 4. Analytical and preparative separation of two menthylphenylphosphinate diastereoisomers. Stationary phase: Partisil 5 μm. Mobile phase: hexane–methanol (98.5:1.5), water content 0.01%. (A) Column: 20 cm × 0.48 cm I.D. Flow-rate: 100 ml/h. Pressure drop: 40 bar. Sample: 5 μl of a 10 mg/ml solution. (B) Column: 70 cm × 1.0 cm I.D. Flow-rate: 400 ml/h. Pressure drop: 110 bar. Sample: 100 μl of a 300 mg/ml solution (from ref. 27 with permission).

being higher than that of the analytical one. This explanation is consistent with the value of the ratio of the preparative to analytical column permeability, 1.18, which can be calculated from the pressure drop measurement given in Table IV. These remarks suggest that the preparative column was not packed densely enough. This is not surprising for a column 70 cm in length, packed by conventional methods. Likewise, the standard deviations, measured for a 30-mg injection, are higher than the predicted values for a small-size injection (Table IV) and their ratios are similar for the two compounds. This discrepancy can be attributed to less efficient packing of the preparative column rather than to extra-column or non-linear elution band broadening.

Using eqn. 1, the maximum sample volume that can be injected in the case of linear behaviour of the column was found to be about 17.6 ml. The corresponding maximum solute concentration was estimated to be of the order of $7\text{–}8 \cdot 10^{-3}$ M (strongly retained solutes having capacity factors of 7.1 and 8). Thus, using the linear approach, the optimum sample size of the isomer mixture (MW = 294) would be *ca.* 75 mg. If we take into account non-linear distribution effects, the maximum sample size will amount to about 370 mg. For this last calculation, 0.5 M and 74 ml = 0.2 V_{R_2} were taken for $C_{m,2}$ and τ_2, respectively, and substituted into eqn. 2. As expected from theory, this sample size should be injected as a concentrated solution, *e.g.*, 1.25 ml of a 300 mg/l solution.

If we now consider the actual experimental results, we can see that the sample size considered optimal from a practical point of view is close to that calculated for linear elution, whereas the small sample volume and the high concentration would correspond to a non-linear elution separation. At this point, one wonders why the sample volume actually injected was so small. It is likely that it is impossible to inject a larger volume by means of a manual syringe without detrimental effect on band broadening or simply because of the pressure resistance of the column packing.

From the foregoing discussion it may be concluded that, in order to increase the sample througput of this separation, further investigations are needed in two directions: first, to obtain a more efficient preparative column with a denser bed by a more appropriate packing method, *e.g.*, a method involving axial and/or radial

TABLE IV

CHROMATOGRAPHIC CHARACTERISTICS OF THE SEPARATION OF THE MENTHYL PHOSPHINATE ISOMERS ON THE ANALYTICAL AND PREPARATIVE COLUMNS

V_R = Retention volume; σ = standard deviation.

	20 cm × 0.48 cm I.D. column, small-size sample (measured)	*70 cm × 1.0 cm I.D. column*	
		Small-size sample (calculated)	*Sample: 0.1 ml of a 300 mg/ml solution (measured)*
V_{R_1} (ml)	22.0	333	415
V_{R_2} (ml)	24.4	370	465
σ_1 (ml)	0.52	4.2	10.6
σ_2 (ml)	0.64	5.2	12.4
Pressure drop (bar)	40	140	110
Flow-rate (ml/h)	100	433	400

compresion; secondly to make use of a sample loop valve together with a device affording an even sample distribution over the column cross-section at the column inlet.

Mass overload; sample introduction

The last example chosen to illustrate this methodology is a separation of 1- and 4-diamentanol isomers, reported by Kříž *et al.*[28] on a 27 cm × 4 cm I.D. axially compressed column, packed with 10–20 μm silica gel (Fig. 5). Because these compounds were poorly soluble in potential mobile phases, a method of solid sample introduction was used. A silica layer of about 1-cm thickness was removed from the top of the column and mixed with approximately half the amount of solid sample before being packed into the column. This was easy to do with axially compressed columns, although difficult to automate, and it was shown to provide higher purities and recovery yields for sample sizes of the order of 2–3 g. However, for a sample size of 5 g, the elution pattern displayed long tails as evidenced by gas–liquid chromatographic analysis of small fractions. The authors wondered about the contributions of isotherm non-linearity and of solid-sample introduction to this peak tailing, which prevented them from recovering any pure fraction, of the later-eluted isomer. To answer this question, it is worth noting that the tails of both peaks extended far beyond the retentions corresponding to $V_R + 3\sigma$ (V_R and σ being the retention volume and standard deviation, respectively, determined under analytical conditions), as if there

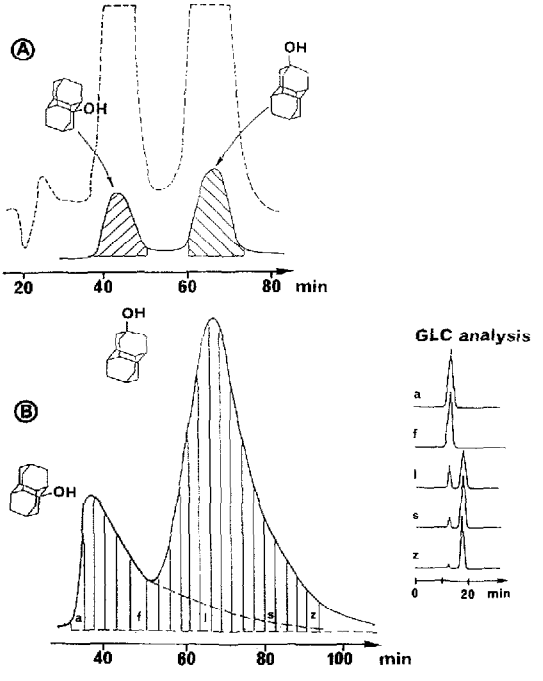

Fig. 5. Preparative separation of 1- and 4-diamantanol isomers. Column: 27 × 4 cm I.D. Stationary phase: 10–20 μm silica gel. Mobile phase: *n*-pentane–2-propanol (98:2). Flow-rate: 22 ml/min. Sample size introduced in solid state: (A) 1 g; (B) 5 g. RI detection (from ref. 28 with permission).

were a large volume overload. Although the specific load was high in that case (25 mg/g), such a phenomenon is not consistent with the known peak shapes obtained with non-linear, convex adsorption isotherms[11,16–18,29]. To our mind, it can be explained only by a slow dissolution of the sample in the mobile phase. It seems likely that, beyond a certain amount of sample introduced in the solid state, peak tailing is controlled by the rate at which the sample dissolves. Furthermore, an amount of silica equal to twice the sample amount seems inadequate to accommodate all of the sample. Based on literature capacity data[16,30], the amount of silica should be *ca.* 4 to 5 times greater than the sample amount.

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