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PREPARATIVE-SCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON ANALYTICAL COLUMNS

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SUMMARY

The capacity of analytical high-performance liquid chromatographs for preparative-scale separations in the adsorption as well as in the reversed phase mode is shown to be about ten times greater than generally believed. A preparative column of 50 × 0.68 cm which can be handled by an analytical instrument can separate 0.1–1 g of relatively complex mixtures. The displacement and elution chromatographic modes are compared.

INTRODUCTION

In the present paper we show that preparative-scale separations can be achieved on analytical columns much more easily and efficiently than is generally believed. In a recent review[1] on preparative-scale liquid chromatography (PLC) an attempt was made to distinguish different classes of preparative applications. Columns up to 25 × 0.46 cm in size were classed in the PLC-1 category; those only slightly larger, *e.g.*, 50 × 0.6–1 cm, which can be handled by analytical instruments for needed flow-rate and detector characteristics, belong to the PLC-2 class. The capacity of such columns was stated to be a few mg for PLC-1 up to maybe 10–20 mg for PLC-2. Such an estimation of capacity is prevalent in this field.

It is obvious that the capacity of any chromatographic system is determined by the separation factor (α) of the pair of compounds to be separated and by the efficiency of the column under high sample loads[2]. In principle, for preparative-scale separations, the column efficiency at various sample loads should be known and therefore determined. The maximum allowable sample size, MASS, of a real mixture that the column can handle is then given by

$$\mathrm{MASS} = 100 \cdot \mathrm{SS}(n')/x$$

where x is the percentage of the component present in the highest concentration of the two components with lowest α value. $\mathrm{SS}(n')$ is the single-component sample size giving the experimental efficiency required for the separation. This efficiency is calculated with the well known equation:

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$$n' = 16\left(\frac{\alpha}{\alpha - 1}\right)^2 \cdot \left(\frac{k + 1}{k}\right)^2$$

For high k and for $\alpha = 1.7$ as in, for example, the isobutoxycyclohexenones of Fig. 1, the n' value is therefore only ≈ 110. From a previously established graph of sample size against efficiency, the amount giving these 110 plates can then be deduced.

This theoretical approach assumes that the column efficiency is unaffected by compound identity. When the polarity of the test compound used to establish the relation between sample size and experimental plate number is very different from the polarity of the mixture to be separated this assumption may not be valid. Although correct, this approach to sample size evaluation before carrying out the separation is therefore not really practical.

The following more empirical approach to the problem of column capacity in PLC is favoured by the different groups of workers in our laboratory. A rule of thumb is to use 30 g silica gel per g of mixture to be separated if the separation is not too difficult, *e.g.*, well separated spots on a thin-layer chromatographic (TLC) plate. For difficult cases, *e.g.*, adjacent spots on TLC, the amount of silica gel is increased to 100 g per g of mixture*. This approach generally works remarkably well. It was applied to a PLC-2 column of 50 × 0.68 cm having a total volume of 18 ml. Packed under pressure, this column takes about 12 g of 10-μm RSiL (a 10-μm irregularly shaped particle silica gel from Alltech-RSL), and should be capable of separating 0.4 g of a simple mixture. The test was carried out with a synthesis product mixture containing the two isobutoxycyclohexenones as shown in Fig. 1. The α value was 1.7. The effect of increasing the sample size is also shown in Fig. 1 which presents some chromatograms selected from a whole series. The RI detector does not produce a useful tracing at the 250-mg sample size, while with a 128-mg sample an entirely acceptable chromatogram was obtained. The detector is overloaded, which does not mean that the separation has not been achieved. The effluent from the column was collected in fractions of 0.3 ml (ten drops). After dilution each fraction was analysed to establish its composition. In this way the true elution pattern was revealed. This is given in Fig. 1f, showing that separation is still complete even at the 250-mg loading.

Overloading occurs sooner with a UV detector than with an RI detector. In order to avoid such overloading it has been suggested[1] that measurements be made on the absorption tail of UV active peaks. However, in our experience this is most difficult and rarely successful. With still larger samples the RI traces are even worse, but analysis of collected fractions reveals the situation. This is shown for 750- and 1500-mg samples in Fig. 2. There is some indication of displacement of peak 1 by peak 2, but this is not as efficient as might have been expected. The mixtures are not satisfactorily separated. Peak 2 is contaminated over nearly its whole width by peak 1.

These results show that the column can indeed separate around 400 mg of a mixture with minor overlap of the two peaks.

A second example is that of a reversed-phase analytical size column (25 × 0.46 cm) filled with 5-μm spherical octadecylated silica gel (5-μm ROSiL-C_{18}-D). About 3.0 g of the packing is forced into the column under pressure. According to the

* In ref. 1 (p. 563) this figure is given as 10 g silica gel per g mixture. This is a printing error for which we apologise.

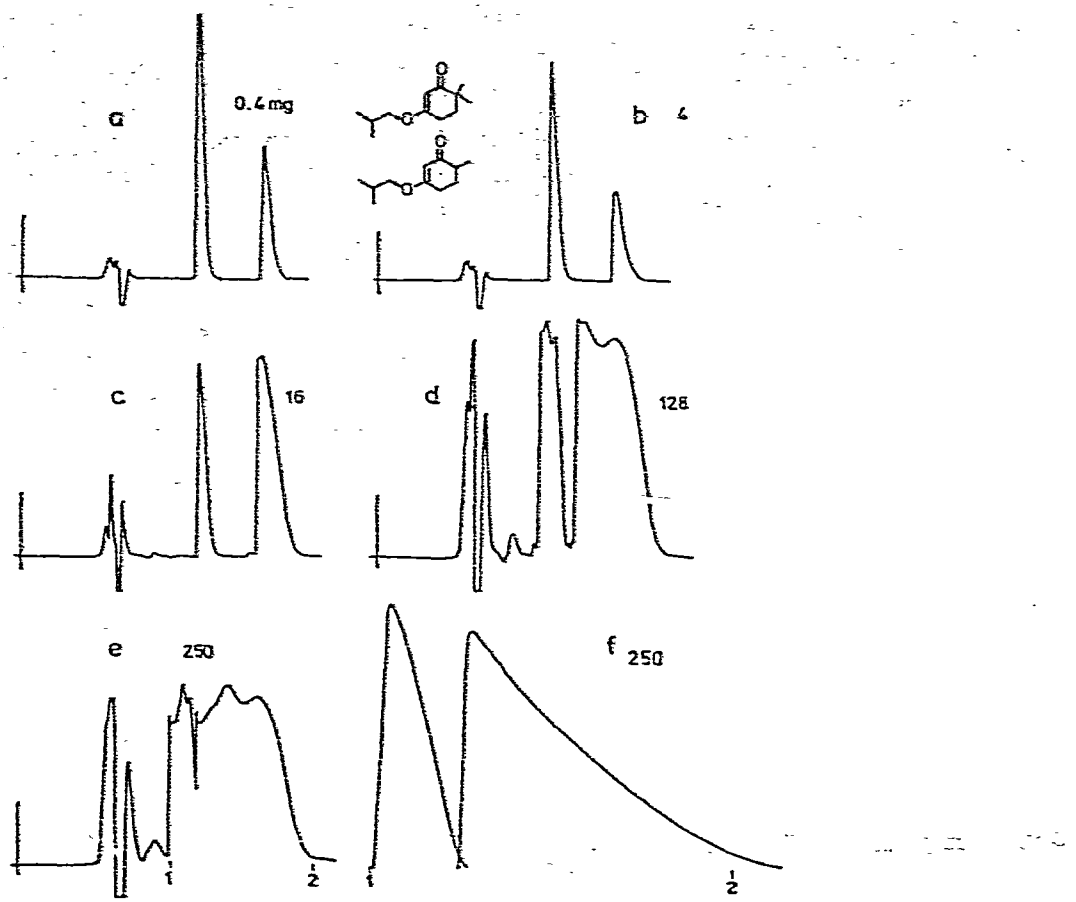

Fig. 1. Effect of sample size on separation. Sample: mixture of cyclohexenones as shown in b. Column: 50 × 0.68 cm, packed with 12 g of 10-μm RSiL. Isocratic elution with hexane–ethyl acetate (77:23) at 4 ml/min. RI detection (Waters Model RI 401). Backpressure: 30 bar. a, b: 0.4-mg and 4-mg samples revealing an α value of 1.7 (component ratio 70:30). c, d, e: 16-, 128-, 250-mg samples (component ratio 30:70), f: separation as in e but graph constructed with results of analysis of collected fractions. f: the real situation. Fig. 1e shows that the RI detector cannot cope with such large concentrations.

above rule of thumb, such a column should be capable of handling 30 mg of a complex mixture. However, this rule applies to adsorption chromatography.

It might be expected that the reversed-phase silica gel column would have a lower capacity, if only because of the specific surface reduction by derivatization. As sample mixture we chose two steroid acetates, one of which was cholesteryl acetate, the other unknown. The α value for the pair in tetrahydrofuran–methanol (10:90) was 1.22. Variations in solvent composition had only slight influence on this α value. For analytical high-performance liquid chromatography (HPLC) this is not a difficult separation problem. In TLC, however, this is equivalent to two spots with R_F values of 0.35 and 0.40, *i.e.*, lying quite close to each other. The RI trace of a 30-mg sample of this steroid mixture on the 25 × 0.46 cm analytical reversed-phase column is shown in Fig. 3. The peak (?) was collected in ten fractions each of 0.5 ml. Separate

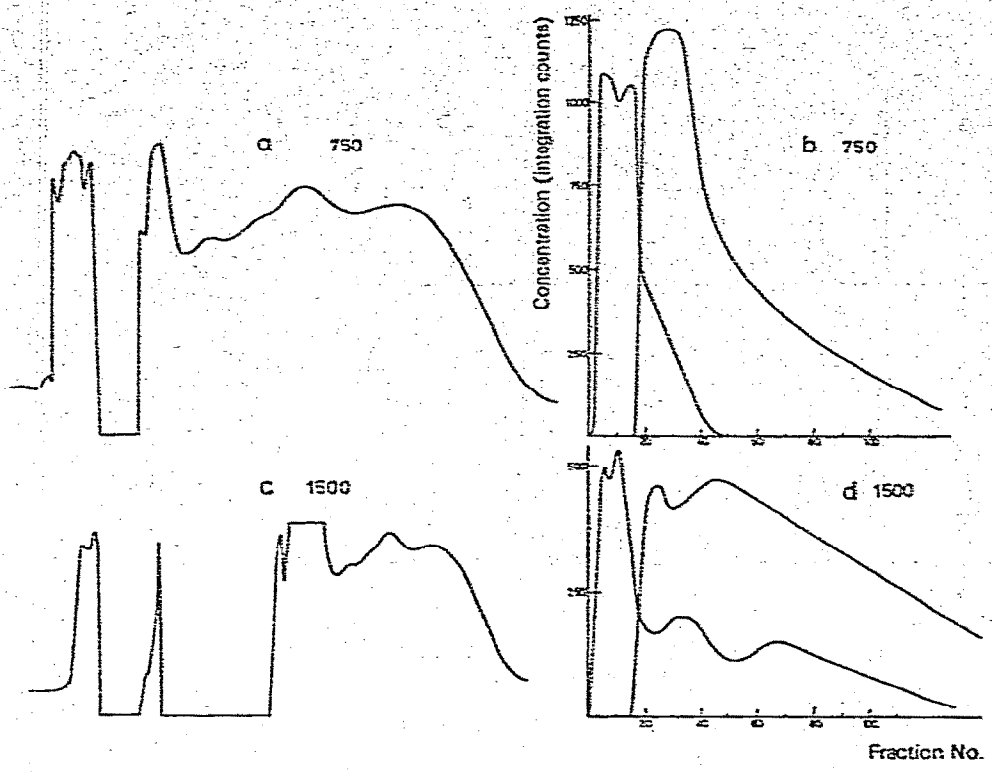

Fig. 2. Separations carried out under the same experimental conditions as in Fig. 1 except at greater sample loading. a, b: RI trace and analysis of individual collected fractions for 750-mg sample. c and 3: as above but for 1500-mg sample.

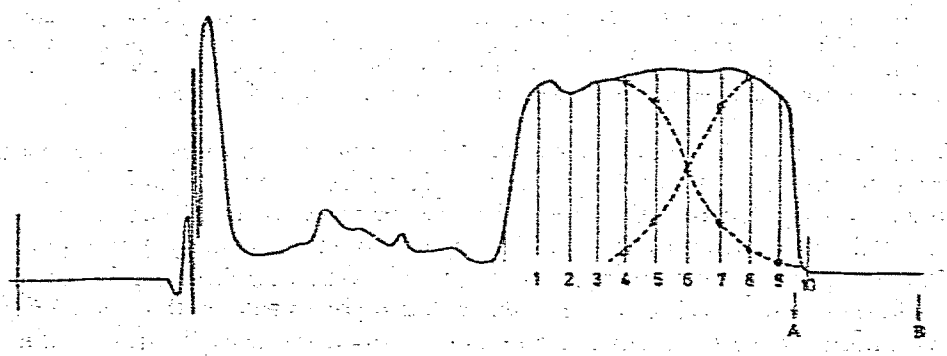

Fig. 3. Separation of a 30-mg sample of cholesteryl acetate and an unknown steroid; $\alpha = 1.22$. Column: 25 × 0.46 cm, packed with octadecylated silica gel (ROSiL-C_{18}-D). Solvent: tetrahydrofuran–methanol (10:90) at 1 ml/min. Varian 5020 LC with Waters RI. Full line, RI trace; dotted lines, detailed analysis of ten collected fractions. Fractions: 1–4 = 10 mg cholesteryl acetate; 5–7 = 10 mg of mixture; 8–10 = 10 mg unknown. These amounts are sufficient for full spectral examination. A and B indicate positions of peaks in analytical sample.

analysis of each fraction allows one to draw the partial curves for each compound of the binary mixture (dotted line in Fig. 3). Fractions 1–4 together gave practically pure compound 1 (10 mg), fractions 8–10 gave practically pure compound 2 (also 10 mg). Fractions 5–7 of course contained 10 mg of the mixture which could be recycled if necessary.

Recently, displacement by adding a strongly adsorbed "displacer" was advocated by Horváth *et al.*[3] for preparative-scale liquid chromatography. It was shown that the potential of analytical HPLC systems for preparative-scale separations was also much greater than could be expected, 160 mg of a mixture of resorcinol and pyrocatechol being separated in one run on a 50 × 0.46 cm column.

Considering the general interest in PLC we thought it worthwhile to compare displacement and partition modes with regard to sample capacity. For this we adopted Horváth's sample mixture of resorcinol and pyrocatechol. Our column was only 10 × 0.46 cm or 1/5 the size of that of Horváth and therefore the sample was also only 1/5 —more precisely, 12 mg resorcinol and 20 mg pyrocatechol in 100 µl water. The α value for this pair in the analytical mode on 5-µm ROSiL-C_{18}-D with water–methanol (30:70) plus 1% H_3PO_4 as eluent was 1.5. Fractions of the preparative separations were collected and analysed separately, giving the concentration profiles.

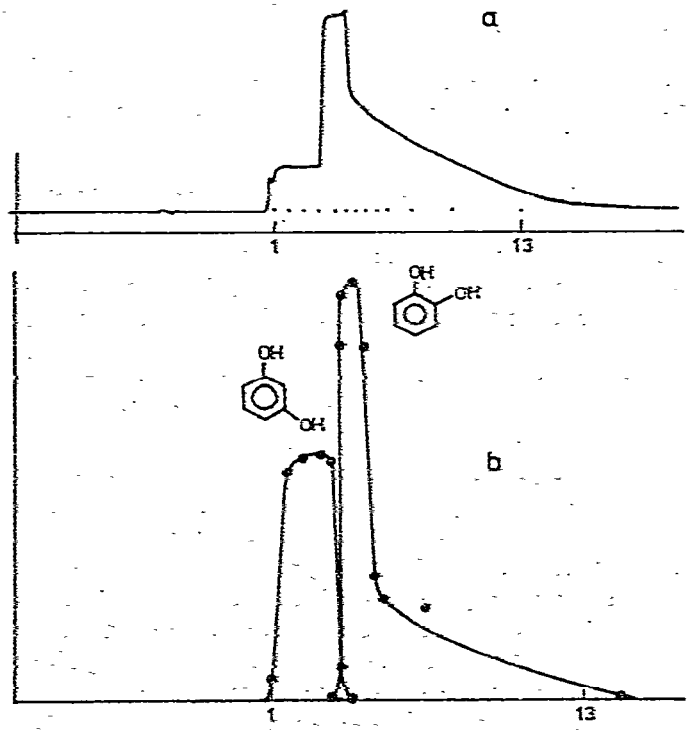

Fig. 4. Displacement chromatography of resorcinol (12 µg) and pyrocatechol (20 mg) in water (100 µl). Column: 10 × 0.46 cm, packed with octadecylated silica gel (5-µm ROSiL-C_{18}-D). The column was saturated with water; the sample was dissolved in water, injected with a 100-µl loop and eluted with 5% *n*-propanol in water at 0.2 ml/min. a, UV trace recorded at 300 nm. b, graph constructed from detailed analysis of thirteen collected fractions as indicated in a.

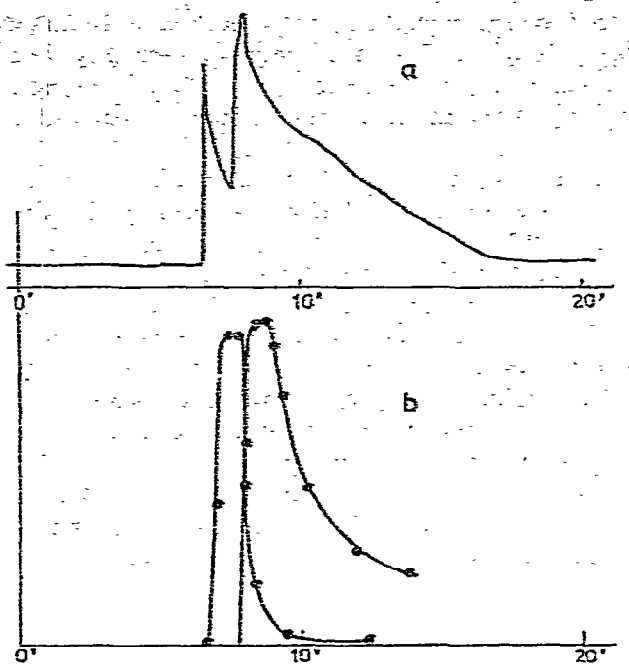

Fig. 5. Elution chromatography of the same sample as in Fig. 4. The same column was saturated with water–methanol (70:30) and the sample eluted with the same solvent. a, UV trace; b, graph constructed from detailed analysis of thirteen collected fractions.

The two separations were run at the same speed of 0.2 ml/min. For displacement chromatography this slow elution is essential. For partition, even a speed ten times higher produces the same result. In the displacement mode (Fig. 4), the sample is injected on the column in the carrier (water) and is immediately followed by the displacer solution. The separation in the normal adsorption-partition mode is shown in Fig. 5.

Our results show that Horváth's separations can easily be reproduced and indeed practically pure compounds can be collected. Reconditioning of the column with water is very fast. The UV trace can even be used for fraction collection. The tailing of the pyrocatechol peak was more pronounced than observed by Horváth *et al.* In the partition mode the separation is not as good; in particular, the UV trace is practically useless. Detailed analysis of the collected fractions shows however that there is little difference in capacity between the two chromatographic modes. Most of the first peak in Fig. 5 can be collected in high purity. The second peak is contaminated by tailing of the first peak. This is not the case in the displacement mode. These facts could lead to the conclusion that the displacement mode is to be preferred for PLC. However, finding the optimum conditions for efficient preparative HPLC displacement chromatography is not always easy. For example, the difference in experimental conditions between Figs. 4 and 5 is very small.

Considering the greater capacity of underivatized silica gel and especially the higher solubility of most mixtures in the solvents used in silica gel adsorption PLC, we also attempted displacement experiments in this chromatographic mode. With ac-

etophenone–benzophenone as sample mixture and with different displacers at various concentrations, no clear displacement effect could be obtained in the 2 days spent on this particular experiment. It is therefore not easy to find the appropriate conditions. For dedicated, replicable separations, displacement chromatography may be the method of choice for preparative HPLC. The effort necessary to find the optimum conditions may then be warranted. For exploratory, once only separations, it seems to us that partition (or adsorption) chromatography is still to be preferred. For a research laboratory, adsorption on silica gel still remains the most practical technique, combining large capacity and high solubility in the non-aqueous solvents used in this chromatographic mode.

Two further examples, shown in Figs. 6 and 7, illustrate this. These examples come from the daily routine of the synthesis division of our laboratory[4,5]. The limiting capacity of the system is far from reached, but these conditions seem to be preferred by the practising synthesis chemists. Their interest is not in the optimization of a separation, but is rather in its reliability and ease of achievement. Even in these separations, the capacity of the analytical chromatographic systems is much higher than generally expected. It is important to use good quality demineralized silica gel for high recovery of the samples. It should also be noted that the stability of silica gel columns in the non-aqueous solvents is much higher than for derivatized materials in aqueous solvents. The column employed in the separations of Figs. 6 and 7 has now

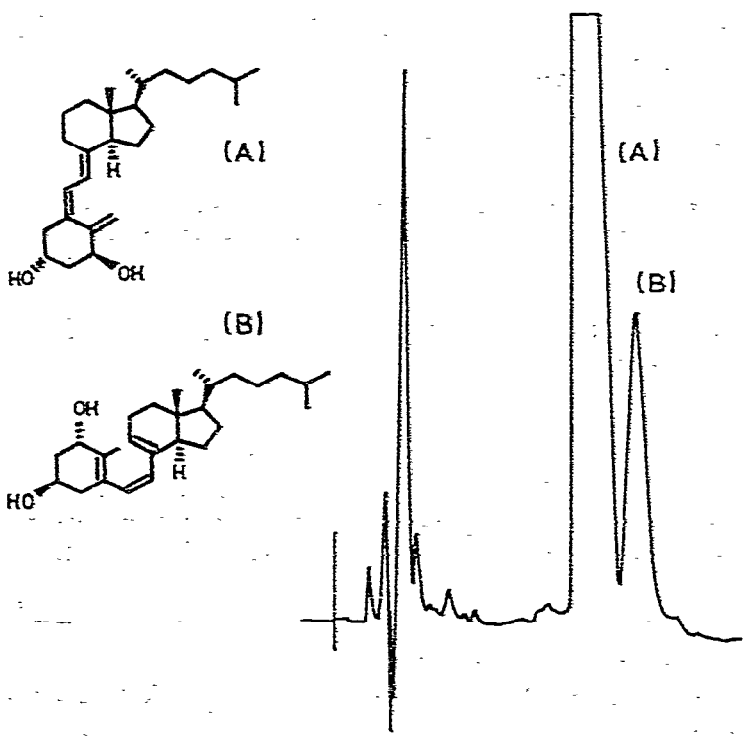

Fig. 6. Silica gel adsorption PLC. Same system as Figs. 1 and 2 (column 50 × 0.68 cm, 10-μm RSiL, silica gel). Sample: 75 mg of the mixture shown. Eluent: *n*-hexane–acetone (80:20) at 4 ml/min. RI detection. Working pressure: ≈60 bar. Time required: about 20 min. TLC of the mixture revealed only one spot.

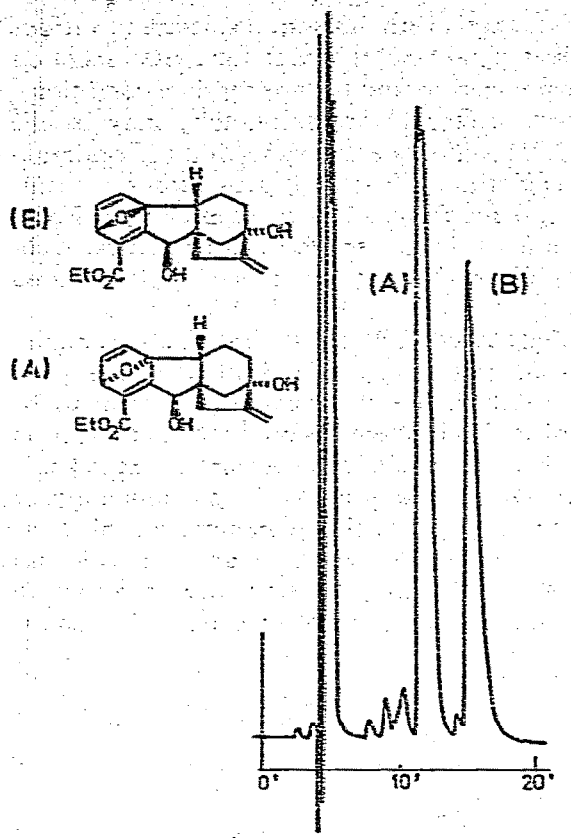

Fig. 7. Silica gel adsorption PLC as in Fig. 6 with 50 mg of the sample mixture shown. RI detection. Eluent: diethylether–*n*-hexane (80:20) at 4 ml/min. Working pressure: ≈20 bar. A much larger sample could be handled by the column. Time required: about 20 min.

been in use for nearly one year and still shows no signs of deterioration. This would not be the case with reversed-phase systems.

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