Journal of Chromatography, 502 (1990) 11–19
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 975

Displacement chromatography on packed capillary columns[a]

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(First received April 10th, 1988; revised manuscript received September 4th, 1989)

SUMMARY

A displacement chromatographic microsystem was constructed using packed fused-silica capillary columns and commercially available micro-liquid chromatographic elements. The feasibility of displacement chromatographic separations on the microscale was demonstrated with a reversed-phase system using alkylphenols as test solutes. Successful separations were achieved even with samples as large as 0.1 mg on 200 μm I.D. capillary columns packed with 5-μm particles. UV detection of the separated bands was facile, as sample concentrations in the detector cell reached millimolar levels. Separation conditions were optimized to allow the direct injection of up to 10-μl sample volumes, equivalent to half of the total column volume, without deterioration of the quality of the separation.

INTRODUCTION

Notwithstanding the technical difficulties involved, packed and open-tubular capillary columns are becoming an increasingly accepted means of high-performance liquid chromatographic (HPLC) analysis (for a recent review see, for example, ref. 1). Because of the high mass sensitivity that can be realized, the use of microcolumn liquid chromatograpy systems is especially justified when the amount of sample available is limited. As only nanoliter sample volumes can be injected into packed capillary columns, detectors more sensitive than those currently applied in conventional analytical-scale HPLC systems are needed. The detection problems cannot be eliminated by simply concentrating the sample into a very small volume and injecting all of it into the column because this approach would overload the small sample capacity of the column; resulting in distorted peaks and a decrease in separation efficiency. Thus, in most instances, the bulk of the available sample cannot be utilized for analysis.

Owing to the low flow-rates (μl/min) typically encountered in microcolumn liquid chromatography, the technique is eminently suitable for directly coupled LC–

[a] Presented at the *First International Symposium on High-Performance Capillary Electrophoresis, Boston, MA, April 10–12, 1989*. The majority of the papers presented at this symposium have been published in *J. Chromatogr.*, Vol. 480 (1989).

0021-9673/90/$03.50

mass spectrometric systems. However, as the concentration of the eluted components is low and their amounts are limited, detection may become a problem. Often, comparatively large amounts would be required for post-separation structure elucidation or other sample characterization purposes, or simply for further research on the pure material. Microcolumns as used today are not very effective for micropreparative separations.

Displacement chromatography is a non-linear, preparative-scale separation technique that has attracted considerable attention lately. Although displacement chromatography has been known for many years[2,3], it was revived only recently, when efficient separations were achieved using HPLC equipment[4–6]. Since then, there has been increasing interest in the utilization of displacement chromatography for preparative separations and several groups are pursuing its theoretical [7–13] and practical aspects[14–19].

In displacement chromatography, the column is first equilibrated with the carrier solution which has the least affinity for the stationary phase. Then the sample, whose components are adsorbed more strongly, is introduced, followed by the displacer which has the strongest affinity for the stationary phase. As the front of the displacer moves down the column, it moves the sample components which, in turn, move each other according to their adsorption strength. As the leading front of the displacer is extremely sharp owing to the non-linear nature of its isotherm and as the sample components can move down the column only as they are removed from the adsorption sites by the front of the displacer, the sample bands also assume the very sharp shape of the displacer front (template effect). Eventually, if the components have sufficiently different adsorption strengths and the column has the necessary efficiency, the components will occupy adjacent zones and move with the same velocity in the displacement train. The concentration of each component in the fully developed train depends only on the respective adsorption isotherms and the concentration of the displacer.

Solute concentrations and column loadings orders of magnitude higher than in elution chromatography can be realized in the displacement mode. The sample capacity of the column is better utilized, and higher fraction purities and yields can be achieved than in the alternative elution-mode separations.

Although much has been learned about the role of the operating parameters (column efficiency, capacity, dispersion, mass transfer rate, relative sample loading)[9,10,13], still relatively little is known about the rules of displacer selection and the control of separation selectivity. The lack of well characterized displacers and the lack of knowledge of the adsorption isotherms of both solutes and displacers are the main factors which hamper the wider acceptance of displacement chromatography. Displacer selection is still done by trial-and-error.

Most modern displacement chromatographic separations used either an alkyl-silica-type reversed-phase system to separate small polar molecules, antibotics, oligopeptides and small proteins[12–18], or cyclodextrin silicas to separate chiral components[19].

These considerations, and the need for larger amounts of pure, high-molecular-weight biomolecules in mass spectrometric investigations prompted us to explore the idea of miniaturized displacement chromatographic separation using packed capillary columns. To the best of our knowledge, no such separations have previously been

reported. Our objectives included the construction of an operating microcolumn displacement chromatographic system, the demonstration of the feasibility of the approach by separating model mixtures of phenolic compounds and, if successful, the determination of preliminary performance characteristics and the identification of the main areas for further research.

EXPERIMENTAL

The miniaturized displacement chromatographic system built for this study is shown in Fig. 1. It consists of an SFC 500 micropump, a μLC 10 variable-wavelength UV detector (both from ISCO, Lincoln, NE, U.S.A.), a pneumatically activated, computer-controlled Type 7001 switching valve with a 2-ml loop and a Type 7125 injection valve with 4.3- and 10-μl loops (both from Rheodyne, Cotati, CA, U.S.A.), an 85-cm long fused-silica capillary column packed in our laboratory with 5-μm reversed-phase material, and an NEC Powermate II personal computer (Computerland, College Station, TX, U.S.A.) equipped with a Metrabyte II chromatographic A/D board (Metrabyte, Taunton, MA, U.S.A.).

Columns were made of 200 μm I.D., 280 μm O.D. (Hewlet-Packard, Avondale,

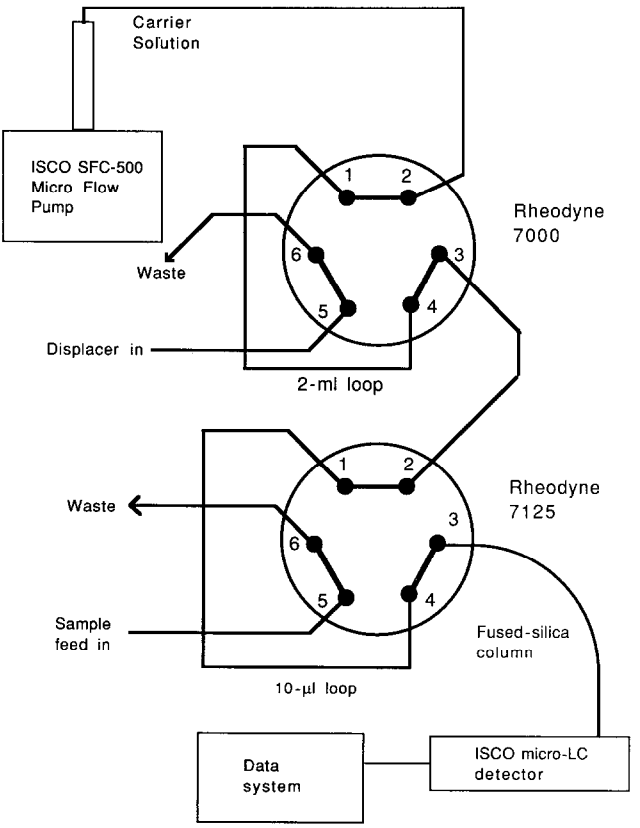

Fig. 1. Schematic diagram of the miniaturized displacement chromatographic system.

PA, U.S.A.) and 250 μm I.D., 340 μm O.D. (Polymicro Technologies, Phoenix, AR U.S.A.) fused-silica tubes. The inner surface of the 200 μm I.D. column was coated with a 0.33 μm thick layer of cross-linked methylsilicone gum[20]. The columns were terminated using a modified version of the method published by Gluckman *et al.*[21] by a Type A/E glass-fiber filter (Gelman, Ann Arbor, MI, U.S.A.) and a piece of 192 μm O.D., 100 μm I.D. fused-silica (Polymicro Technologies) tube glued into the end of the column with a fast-curing epoxy resin (Epo-Tek 353ND; Epoxy Technology, Billerica, MA, U.S.A.).

The columns were slurry packed with 5-μm Nucleosil C_{18} (Macherey, Nagel & Co., Bad Durkheim, F.R.G.) reversed-phase spherical silica. Columns made from uncoated fused-silica tubing were extremly fragile when packed with the reversed-phase silica. Columns coated with the immobilized silicone layer were very stable, and have been used without fracture. A custom-designed 150-μl slurry reservoir and the SFC micropump were used to pack the columns.

The packed capillary columns were connected to a regular Type 7125 Rheodyne injection valve through a 'T' splitter (in the elution mode), or through a common zero-dead volume union (in the displacement mode). Short pieces of a tightly fitting PTFE tube, compressed by segments of a stainless-steel capillary and beads of epoxy glue, were used as ferrules. A 1-mm long section of the polyimine layer was removed from the 100 μm I.D. fused-silica tubing below the column terminator using a laboratory-made electric device. This section was used, together with a 0.25-mm diameter aperture, as the detector cell (replacing the original detector cell of the μLC 10 UV detector). The system was controlled and chromatographic data (in both the elution and displacement modes) were collected by the NEC Powermate II computer, using an interactive system control/data acquisition and evaluation software package developed in our laboratory.

HPLC-grade ChromAr solvents were obtained from Malinckrodt (Paris, KY, U.S.A.). All solutes (1-naphthol, 2-naphthol, 4-isopropylphenol and 3-*tert.*-butylphenol) were of analytical-reagent grade and used as received (Aldrich, Milwaukee, WI, U.S.A.). Eluents were prepared by the weighing method[16] and freshly degassed before use.

The adsorption isotherms of all solutes were determined by the breakthrough method, as described [16]. Elution-mode capacity factors and the adsorption isotherms were used to select the type of displacer and the concentration of its solutions.

RESULTS AND DISCUSSION

Selection of the operating conditions: retention and isotherm data

In order to achieve a good displacement chromatographic separation, the injected sample must accumulate at the top of the column. Thus, a carrier solution composition in which the k' values of the solutes are larger than 10 (even for the least retained components) must first be determined. Therefore, the k' values of the alkylphenol model compounds were determined as a function of the methanol concentration of the eluent, as shown in Fig. 2. The k' values are larger than 10 in the eluent containing 45% (v/v) of methanol. Therefore, methanol–water (45:55, v/v) was selected both as the carrier and as the base solvent of the displacer.

The elution-mode analytical separation of the test solutes is shown in Fig. 3.

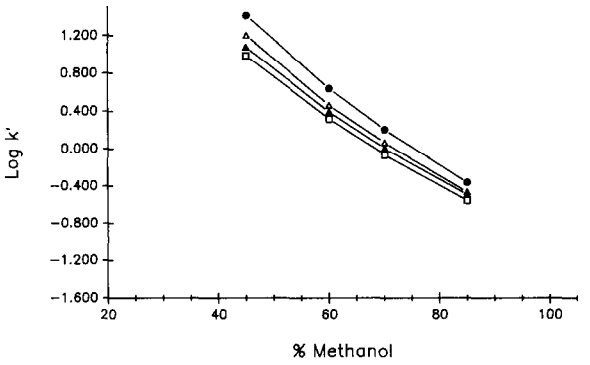

Fig. 2. Logarithm of the capacity factors of the test solutes as a function of the methanol concentration of the eluent on the Nucleosil C_{18} reversed-phase column. ● = 3-*tert.*-Butylphenol; △ = 4-isopropylphenol; ▲ = 1-naphthol; □ = 2-naphthol.

There is adequate, but not excessive, resolution between the first three solutes. The selectivity factors between the neighboring peaks are sufficiently large for analytical-scale separations (1.23 for the 1-naphthol–2-naphthol pair, 1.44 for the 4-isopropylphenol–1-naphthol pair and 1.51 for the 3-*tert.*-buthylphenol–4-isopropylphenol pair), but not large enough to permit easy preparative separations in the elution mode.

For displacement chromatographic separation to occur, the displacer must be sufficiently soluble in the carrier, more retained than the sample and have a convex isotherm that does not intersect the isotherms of the sample components. To select the displacer, the *k' vs.* methanol concentration diagram (Fig. 2) is consulted again. With 45% methanol 3-*tert.*-butylphenol is more retained than any of the other components, and consequently it may prove to be a suitable displacer. (This step can eliminate those components which cannot be used as displacers, but a successful passing of the test does not guarantee that the prospective displacer is actually a suitable one.)

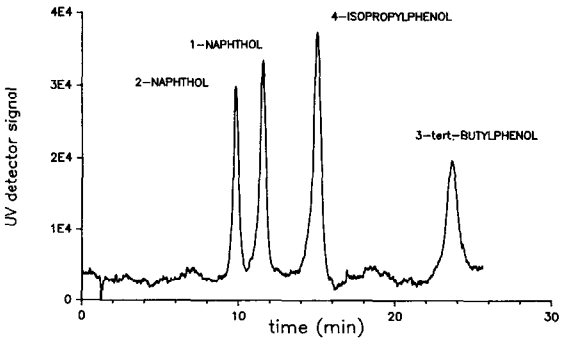

Fig. 3. Elution-mode analytical separation of the test solutes using methanol–water (45:55, v/v) as eluent on the Nucleosil C_{18} reversed-phase column.

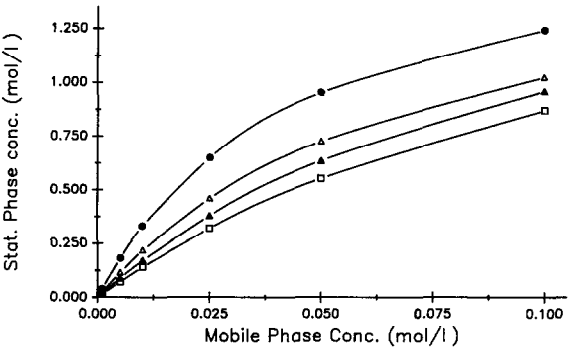

Fig. 4. Adsorption isotherms of the test solutes in methanol–water (45:55, v/v) carrier solution on the Nucleosil C_{18} reversed-phase column. Symbols as in Fig. 2.

Next, the adsorption isotherms of the test solutes and the selected displacer were determined using methanol–water (45:55, v/v) solution (the selected carrier solution composition), as shown in Fig. 4. The individual isotherms can be described well by the Langmuir isotherm equation. As 3-*tert.*-butylphenol fulfils all the requirements listed above, it can be used as a displacer for the other phenolic compounds.

Displacement chromatographic separations

For a displacement chromatographic separation to occur for all components, the operational line must intersect the isotherms of all components. Therefore, the smallest possible displacer concentration is determined by the intersection of the displacer isotherm and the tangent of the isotherm of the least retained solute, 2-naphthol, at infinite dilution. This intersection occurs at *ca.* 80 m*M*, indicating that displacer concentrations above this value may lead to fully developed displacement trains. Therefore, to be on the safe side, a 95.2 m*M* 3-*tert.*-butylphenol concentration was selected as the displacer concentrations to be used throughout the remainder of the experiments.

The displacement chromatogram obtained with direct injection of a large-volume (10 μl), large-mass (0.65 μmol) sample at high flow-rate (1.5 μl/min) is shown in Fig. 5a. (In terms of linear velocity, this flow-rate is equivalent to 0.8 ml/min in a 4.65 mm I.D. column packed with the same stationary phase.) As the UV absorbances of the individual components are different at 280 nm, the wavelength selected for detection, the successive bands can have both higher and lower absorbance signals than the preceding bands, even though the concentrations increase monotonously toward the end of the chromatogram. It can be seen that even though a sample as large as 650 nmol (*ca.* 0.1 mg) was introduced into the capillary column in an injection volume as large as 10 μl (over half of the column volume), the bands of the three solutes are separated from each other. The derivative of the displacement chromatogram is also shown. This helps to locate the inflection points and determine the widths of the fronts. It can be concluded that although the shape of the displacement chromatogram is not yet ideal, separation is possible even with such large-volume, large-mass injections.

In order to improve the shape of the displacement train, the amount of sample

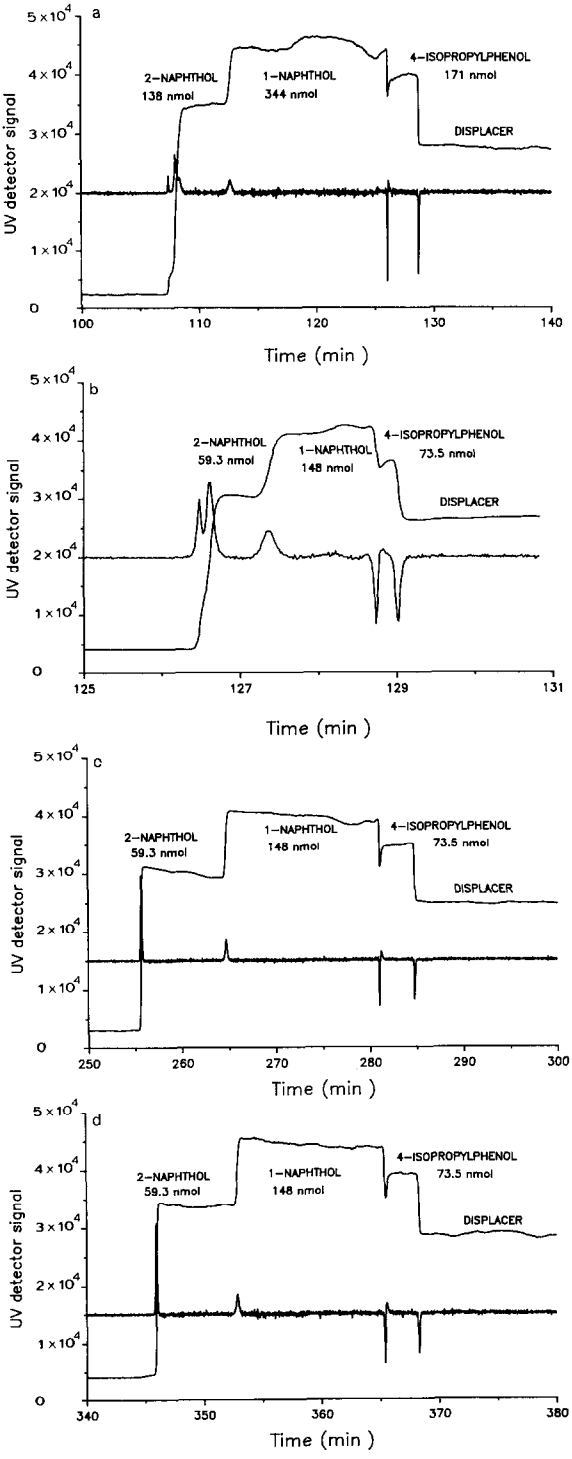

Fig. 5. Displacement chromatograms of the test solutes using a 95.2 m*M* solution of 3-*tert.*-butylphenol in methanol–water (45:55, v/v) as displacer and an 85 cm × 200 μm I.D. packed capillary as separation column. Conditions: (a) injection volume 10 μl, sample mass 65 nmol (0.1 mg), flow-rate 1.5 μl/min; (b) injection volume 4.3 μl; sample mass 280 nmol (43 μg), flow-rate 1.5 μl/min; (c) as (b), except flow-rate 0.5 μl/min; (d) as (c), except carrier solution: methanol–water (20:80, v/v) and displacer solvent methanol–water (45:55, v/v).

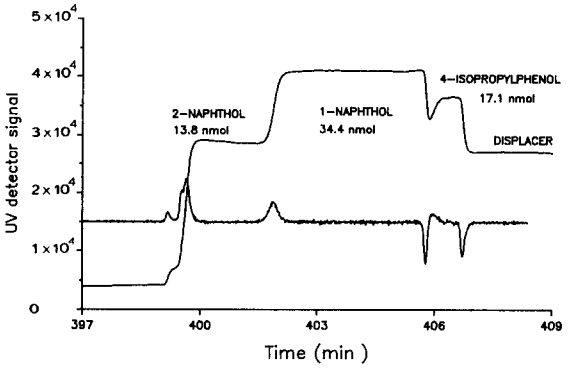

Fig. 6. Displacement chromatogram of the test solutes using a 95.2 m*M* solution of 3-*tert.*-butylphenol in methanol–water (45:55, v/v) as displacer and an 85 cm × 200 μm I.D. packed capillary as separation column. Conditions: injection volume, 10 μl; sample mass, 65 nmol (10 μg); flow-rate, 0.5 μl/min; carrier solution, methanol–water (20:80, v/v).

injected was approximately halved by replacing the manufactured 10-μl sample loop of the Type 7125 Rheodyne valve with a laboratory-made 4.3-μl loop. The displacement chromatogram obtained with the smaller volume (4.3 μl), smaller mass (280 nmol) injection of the same sample solution at the same high flow-rate (1.5 μl/min) is shown in Fig. 5b. It can be concluded that a developed displacement train is present even with the smaller sample amounts, but the sharpness of the front has not improved sufficiently.

Next, we attempted to sharpen the fronts by decreasing the flow-rate of the displacer to one third of the original, *i.e.*, to 0.-5 μl/min. The displacement chromatogram of the same sample as in Fig. 5b is shown in Fig. 5c. The chromatogram clearly shows that at reduced flow-rate the definition of the zones improved significantly.

In trying to improve the band shape even further, the same sample was injected onto the microcolumn after pre-equilibration with methanol–water (20:80, v/v) carrier solution to improve the focusing of the sample on the top of the column. The previous methanol–water (45:55, v/v) solution was still used as the solvent of the displacer. The displacement chromatogram obtained is shown in Fig. 5d. As a result of depositing the sample in a narrow at the top of the column, an ideal displacement train was obtained.

Finally, in order to see if the concentrating effect of the sample solvent is strong enough to cope with the larger injection volume (a case of practical significance), the sample used in Fig. 5d was diluted 10-fold and 10 μl were injected onto the column pre-equilibrated with methanol–water (20:80, v/v) carrier solution. This means that the sample load was now almost five times lower than in Fig. 5d. Nevertheless, the displacement chromatogram in Fig. 6 indicates that a good separation and a good zone shape are obtained even though the dilute sample had an injection volume as large as half of the column volume.

CONCLUSIONS

It has been shown that displacement chromatographic separations are possible on microcolumns. Successful separations were achieved with 200 μm I.D. packed capillary columns and sample loadings as high as 0.1 mg. Injection volumes up to 10 μl, the equivalent of half of the column volume, were successfully used. UV detection of the separated bands was facile, as sample concentrations in the detector cell reached millimolar levels. It is expected that by decreasing the column I.D. to 10 μm and using micropellicular stationary phases, the amounts injected can be decreased significantly without losing band definition or sacrificing millimolar exit concentrations. However, we are still far away from the time when this promising technique will be readily available to life scientists to help them solve their pressing micropreparative separation needs.

ACKNOWLEDGEMENTS

Financial support by the Texas Coordinating Board of Higher Education TATR Program (Grant Number 3376) is acknowledged. The authors are grateful to Dr. J. Teherani of ISCO (Lincoln, NE, U.S.A.) for the loan of the micro-LC system used in this study.

REFERENCES

1 M. Novotny, *Anal. Chem.*, 60 (1988) 500A.
2 A. Tiselius, *Ark. Kemi Mineral. Geol.*, 16A (1943) 1.
3 F. G. Hellferich and G. Klein, *Multicomponent Chromatography — Theory of Interference*, Marcel Dekker, New York, 1970.
4 H. Kalász and Cs. Horváth, *J. Chromatogr.*, 215 (1981) 295.
5 Cs. Horváth, A. Nahum and J. H. Frenz, *J. Chromatogr.*, 218 (1981) 365.
6 Cs. Horváth, J. H. Frenz and Z. El Rassi, *J. Chromatogr.*, 255 (1983) 273.
7 Cs. Horváth and W. R. Melander, in E. Heftmann (Editor), *Chromatography, Part A, Fundamentals and Techniques (Journal of Chromatography Library*, Vol. 22A), Elsevier, Amsterdam, 1983, p. A27.
8 J. Jacobson, J. H. Frenz and Cs. Horváth, *J. Chromatogr.*, 316 (1984) 53.
9 J. H. Frenz and Cs. Horváth, *AIChE J.* 31 (1985) 400.
10 Cs. Horváth, in F. Bruner (Editor), *The Science of Chromatography (Journal of Chromatography Library*, Vol. 32), Elsevier, Amsterdam, 1985, p. 179.
11 G. Guiochon and A. Katti, *Chromatographia*, 24 (1987) 165.
12 J. Jacobson, J. H. Frenz and Cs. Horváth, *Ind. Eng. Chem. Res.*, 26 (1987) 43.
13 S. M. Cramer, Z. El Rassi, D. M. LeMaster and Cs. Horváth, *Chromatographia*, 24 (1987) 881.
14 Gy. Vigh, Z. Varga-Puchony, G. Szepesi and M. Gazdag, *J. Chromatogr.*, 386 (1986) 353.
15 S. M. Cramer, Z. El Rassi and Cs. Horváth, *J. Chromatogr.*, 394 (1987) 305.
16 G. Subramanian, M. W. Phillips and S. M. Cramer, *J. Chromatogr.*, 439 (1988) 341.
17 S. M. Cramer and Cs. Horváth, *Prep. Chromatogr.*, 1 (1988) 29.
18 M. W. Phillips, G. Subramanian and S. M. Cramer, *J. Chromatogr.*, 454 (1988) 1.
19 Gy. Vigh, G. Quintero and Gy. Farkas, *J. Chromatogr.*, in press.
20 O. Ettler and Gy. Vigh, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7 (1984) 432.
21 J. Gluckman, D. Shelly and M. Novotny, *Anal. Chem.*, 57 (1985) 1546.