ELSEVIER

Journal of Chromatography A, 764 (1997) 35–41

JOURNAL OF CHROMATOGRAPHY A

Rapid separation of enantiomers in perfusion chromatography using a protein chiral stationary phase

H. Hofstetter, O. Hofstetter, V. Schurig*

Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany

Received 9 September 1996; revised 25 October 1996; accepted 25 October 1996

Abstract

For the first time, enantiomer separation was performed using a polymeric flow-through-type chromatographic support (POROS). Bovine serum albumin (BSA) covalently bound to POROS was employed as the chiral stationary phase. Using flow-rates of up to 10 ml/min, enantiomer separation of all investigated compounds, a variety of amino acid derivatives and drugs, could be achieved within a few minutes at medium efficiencies typical for protein chiral stationary phases. It could be shown that the loss in resolution R_s with increasing velocity, u, of the mobile phase was lower than predicted by the theory for conventional supports, but not independent of the velocity, u, as expected by perfusion chromatographic theory.

Keywords: Chiral stationary phases, LC ; Enantiomer separation

1. Introduction

The analytical application of proteins immobilized on chromatographic supports for the separation of enantiomers has been demonstrated by a number of investigators [1]. Since its introduction by Allenmark et al. [2], bovine serum albumin (BSA) immobilized on silica is a versatile and widely used selector for chiral separations of a wide range of drugs and bioactive compounds in high-performance liquid chromatography (HPLC) [3].

The maximum speed of analysis, which is dependent on the flow-rate, is usually limited by the maximum pressure allowed by the pumping system and can be influenced by the choice of particle diameter and column length [4,5]. Using conventional silica sorbents in LC, a dominant limitation of increased speed is the peak broadening due to resistance to mass transfer [6]. Recently, perfusion chromatography was developed as a new technique based on enhanced mass transfer properties, leading to shorter analysis times [7–9]. Large so-called through-pores that allow the mobile phase to flow through the sorbent allow convective transport inside the particles, which is much more rapid than that achieved by diffusion. Support material on the basis of poly(styrene–divinylbenzene), with a high chemical and mechanical stability, is commercially available and rapid separations in most chromatographic modes, e.g., reversed-phase, hydrophobic interaction, bioaffinity, ion-exchange and immobilized metal-affinity, have been reported [10–14].

Rapid separation of racemic practolol on immobilized cellulase has been reported by Hjertén et al. [15], who introduced another support material on the basis of non-disperse polyacrylamide gels permeated by channels, so-called continuous gels [15]. Another approach to more rapid separations is the use of

*Corresponding author.

0021-9673/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0021-9673(96)00936-3

small non-porous particles of 1–2 μm diameter. Enantiomer separations have been reported using Pirkle-type and tetracycline selectors bonded to silica, reducing analysis times to 3 min and less [16].

In this work, we describe the use of perfusion chromatography for the rapid separation of enantiomers using BSA as an immobilized chiral stationary phase. Analyses can be performed with higher flow-rates compared to those with conventional silica supports, due to the significantly reduced flow resistance of the packing. Separation of the enantiomers of a variety of amino acid derivatives and drugs was possible within 10 min. For most of the investigated substances, the increase in the velocity of the mobile phase allows the analysis time to be decreased to 3 min or less using a conventional 120×4.6 mm I.D. analytical column.

2. Experimental

2.1. Chemicals

BSA (Cohn fraction V), Tris, MTH–proline, kynurenine, dansyl–amino acids and dinitrophenyl(DNP)–L-amino acids were purchased from Sigma (Deisenhofen, Germany). Ketoprofen, ibuprofen, carprofen and flurbiprofen were kindly provided by Dr. Zimmermann (Institut für Pharmazie der Universität, Tübingen, Germany). The DNP–D-amino acids and the racemates were synthesized according to Rao and Sober [17]. Phosphate buffered saline (PBS) was prepared according to Ref. [18] and adjusted to the desired pH. All inorganic salts (analytical grade) and organic solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany). Water was purified using a Millipore Q system.

2.2. Chiral stationary phase

After washing with water and phosphate buffer (pH 8, 200 m*M*), 0.8 g of POROS-EP (20 μm epoxide-activated particles, PerSeptive Biosystems, Framingham, MA, USA) and 320 mg of BSA were reacted overnight at room temperature in 16 ml of phosphate buffer (pH 8.0, 200 m*M*) using salting out conditions according to the guidelines of the manufacturer. The material was washed with the same buffer and remaining epoxide groups were quenched by treatment with 0.2 *M* Tris buffer for 1 h. Afterwards, the material was washed extensively with PBS, pH 7.4. The BSA content of the packing material was determined by elemental analysis of nitrogen and sulphur. The column contains about 3.9 μmol of protein per gram of POROS.

2.3. Chromatography and instrumentation

A 120×4.6 mm I.D. column was packed with the support at a pressure of 160 bar. The slurry and packing medium was PBS, pH 7.4. No leakage of BSA could be detected during equilibration with PBS.

The chromatographic system consisted of a Chrompack GRAS (CPISOS/LC) pump (Chrompack, Frankfurt, Germany) and a Chrompack UV var variable wavelength detector equipped with a 9-μl detection cell. DNP–amino acids were detected at 340 nm, dansyl-compounds at 320 nm. All other compounds were detected at 254 nm. Injections were performed using a Rheodyne model 7125 injection valve with a 20-μl loop. Elution was performed under isocratic conditions at several flow-rates using PBS of various pH values. The void volume for the determination of the chromatographic data was determined using water [19]. For the short retention times obtained in this work, the inaccuracy in the determination of the dead volume affects the calculation of the retention factor, k, and therefore of the separation factor, α. Slight variations could be explained by this fact. To ensure the reproducibility of the results, all measurements have been carried out at least in triplicate.

3. Results and discussion

Using BSA immobilized on POROS-EP, rapid enantiomer separation of a series of chiral compounds was possible. Table 1 shows the conditions leading to the highest resolutions, R_s, found. Changes in the mobile phase have strong effects on the separation of enantiomers. These can be due to possible changes in the binding site of the protein, by charge as well as by conformational effects. Due to the relatively low binding capacities of protein

Table 1
Chromatographic data for the separation of chiral compounds

Compound	Flow-rate (ml/min)	α	R_s	k_2
Dansyl–α-aminobutyric acid	1[b]	1.71	2.1	5.0
Dansyl–aspartic acid	1[c]	7.21	2.6	5.9
Dansyl–leucine	1[a]	1.62	1.3	4.4
Dansyl–norvaline	1[c]	2.89	2.4	14.9
Dansyl–phenylalanine	1[a]	1.37	0.8	14.1
Dansyl–serine	2[d]	1.63	0.9	15.0
Dansyl–tryptophan	5[b]	1.81	0.8	7.3
Dansyl–valine	1[a]	1.61	1.2	3.3
DNP–alanine	1[d]	1.67	1.5	3.5
DNP–aspartic acid	1[a]	5.43	4.4	7.6
DNP–glutamic acid	1[c]	3.50	3.1	3.5
DNP–proline	1[d]	2.81	1.8	1.8
Carprofen	1[b]	2.88	1.5	0.9
Flurbiprofen	1[b]	2.41	1.9	1.1
Ibuprofen	1[b]	6.29	2.0	1.0
Ketoprofen	1[b]	2.43	2.5	5.1
Kynurenine	1[b]	1.43	1.1	2.0
MTH-proline	5[b]	3.37	1.9	3.0

Conditions: [a]PBS, pH 8.0; [b]PBS, pH 7.4; [c]PBS, pH 6.0; [d]50 m*M* phosphate buffer, pH 6.0; [e]50 m*M* phosphate buffer, pH 8.0, 1.5% 1-propanol.

columns, indicating restricted availability of the binding sites [3], a decrease in sample load resulted in decreasing peak tailing. Protein chiral stationary phases, in particular, show poor efficiency and a large degree of peak-tailing, even at analytical concentrations. A recent study on measurements of equilibrium isotherms reported on the separate determination of the characteristics of the non-selective and the enantioselective binding sites [20]. The kinetics of adsorption and desorption seem to be much slower on the chiral selective sites than on the non-selective ones. The poor efficiency and the peak-tailing can therefore be due to heterogeneous mass transfer kinetics.

The effects of the pH of the mobile phase, addition of organic modifier and salt concentration, respectively ionic strength, on the retention were investigated. Depending on the charge of the analyte, a change in the pH value can either increase or decrease the k_2 values [3]. The long-term stability of the column was tested. During a period of several months and after several hundred injections, no significant change in the properties of the column was observed.

Due to the properties of the support material, separations were investigated at flow-rates from 1 up to 10 ml/min, without the limitation of high back pressure. The practical limit was given by the maximum delivery of the pump. Increased flow-rates lead to more rapid separations due to shorter retention times, while the resolution decreases (Table 2). The drug, ketoprofen, showed a strong interaction with the stationary phase. At a flow-rate of 1 ml/min it could be separated within 11 min, with a resolution $R_s = 2.5$, while at a flow-rate of 5 ml/min, baseline separation was possible in 2.5 min, with a decreased resolution $R_s = 1.5$ (Fig. 1). As shown in Table 2, the resolution, R_s, for the separations of ketoprofen and

Table 2
Influence of the flow-rate on the separation

Compound	Flow-rate (ml/min)	α	R_s	k_2
Dansyl–aspartic acid	1[c]	1.79	1.6	6.5
	2[c]	1.86	1.2	6.7
	5[c]	1.75	1.0	6.5
Dansyl–norvaline	3[a]	3.09	1.9	7.9
	5[a]	2.79	1.8	7.9
	8[a]	3.16	1.7	8.3
	10[a]	2.09	1.5	7.9
DNP–aspartic acid	1[a]	5.43	4.4	7.6
	3[a]	5.0	3.0	7.0
	5[a]	5.34	2.4	7.5
	8[a]	4.98	2.1	7.1
	10[a]	4.17	1.8	7.3
DNP–glutamic acid	3[a]	3.2	1.9	2.6
	5[a]	3.45	1.8	2.7
	8[a]	2.69	1.5	2.3
	10[a]	2.64	1.3	2.6
DNP–proline	1[c]	2.81	1.8	1.8
	2[c]	3.11	1.7	1.6
	3[c]	2.89	1.6	1.8
	5[c]	2.94	1.3	1.7
	8[c]	3.01	1.1	1.5
Ketoprofen	1[a]	2.43	2.5	5.1
	3[a]	2.46	1.9	5.5
	5[a]	2.29	1.5	5.1
	8[a]	2.29	1.3	5.2
MTH–proline	3[b]	3.19	1.9	3.1
	5[b]	3.37	1.8	3.0
	8[b]	3.01	1.4	3.0

Conditions: PBS, [a]pH 8.0; [b]pH 7.4; [c]50 m*M* phosphate buffer, pH 6.0.

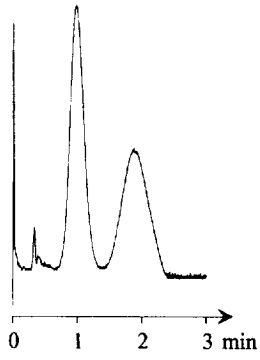

Fig. 1. Enantiomeric separation of ketoprofen. Conditions: PBS, pH 8.0; flow-rate, 5 ml/min.

dansyl–norvaline decrease with higher flow-rates, due to lower efficiencies. Separation of dansyl–norvaline could be achieved in 3.5 min at a flow-rate of 8 ml/min. Increasing the flow-rate to 10 ml/min reduced the separation time to 2.9 min (Fig. 2). Dansyl–aspartic acid was separated within 6 min at a flow-rate of 1 ml/min. Using PBS, pH 8.0, DNP–aspartic acid could be separated showing a resolution R_s=4.4 at 1 ml/min. At a flow-rate of 10 ml/min, the separation time was reduced by a factor of approximately ten, while the resolution, R_s, dropped to 1.8. Baseline separation could be achieved within 1.7 min (Fig. 3). Compared to DNP–aspartic acid, the glutamic acid homologue showed lower k_2 values, resulting in lower separation factors, α, and decreased resolution, R_s. At a flow-rate of 8 ml/min, baseline separation was possible in only 0.8 min (Fig. 4). The separation of DNP–proline could be

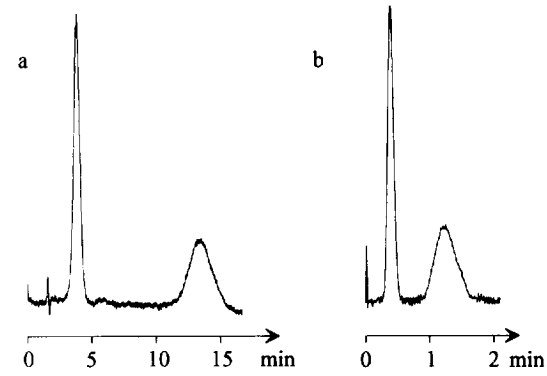

Fig. 3. Influence of the flow-rate on the enantiomeric separation of DNP–aspartic acid. Conditions: PBS, pH 8.0; flow-rate, (a) 1 ml/min and (b) 10 ml/min.

obtained within 1.9 min at a flow-rate of 3 ml/min with a resolution R_s=1.6.

An increase of the flow from 3 to 5 ml/min had almost no effect on the separation of MTH–proline; the resolution was not altered essentially.

As reported in previous publications by others [7,21], POROS-packed columns can be operated at higher mobile phase velocities with lower loss of resolution compared to conventional HPLC packings of smaller pore diameter. According to theory [5,22], the resolution, R_s, is proportional to the square root of the plate number, N, which itself is proportional to $1/H$ (Eq. (1)).

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k}{1+k} \quad \text{with } N = \frac{L}{H} \tag{1}$$

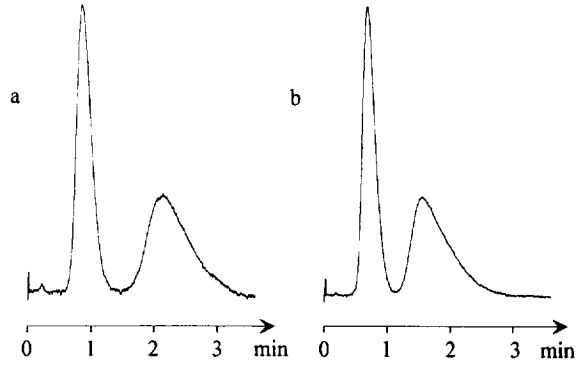

Fig. 2. Enantiomeric separation of dansyl–norvaline. Conditions: PBS, pH 8.0; flow-rate, (a) 8 ml/min and (b) 10 ml/min.

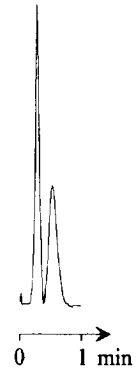

Fig. 4. Enantiomeric separation of DNP–glutamic acid, Conditions: PBS, pH 8.0; flow-rate, 8 ml/min.

The resolution, R_s, is therefore proportional to $1/\sqrt{H}$.

The theoretical relationship between the plate height, H, and the fluid velocity, u, is expressed by the plate height equation, which can be considerably simplified by the use of reduced parameters (h, u). Then, the equation can be expressed in a form that does not explicitly contain particle size or diffusion coefficients. Geometrically similar columns should produce identical curves of h versus u. The condensed form of the conventional plate height equation can be written as Eq. (2).

$$h = A + \frac{B}{u} + C \cdot u \quad \text{with } A = \omega \cdot d_p \text{ and } B = 2 \cdot \gamma \cdot D_m \tag{2}$$

The plate height, h, is proportional to the velocity, u, in the range of medium-to-high flow-rates ($u > u_{opt}$), usually used for analyses [23]. At higher velocities, the C-term of the plate height equation dominates and is responsible for the increase of h as the velocity rises [24] (Eq. (3)).

$$h = A + \text{const.} \cdot \frac{d_p^2}{D_p} \cdot u \tag{3}$$

This term refers to band spreading caused by resistance to mass transfer. Since the velocity, u, is proportional to the column's flow-rate, F [25], the resolution, R_s, is directly proportional to $1/\sqrt{F}$. Comparing separations at two different flow-rates, this leads to Eq. (4).

$$R_{s,2} \cdot \sqrt{F_2} = R_{s,1} \cdot \sqrt{F_1} = \text{const.} \tag{4}$$

On the basis of these considerations, the product of $\sqrt{F'}$, representing the changes in flow-rates, and the changes in the actual resolution, R_s', at two different flow-rates should have a value of one (Eq. (5)).

$$\frac{R_{s,2}}{R_{s,1}} \cdot \frac{\sqrt{F_2}}{\sqrt{F_1}} = 1 \tag{5}$$

The data obtained for the perfusive packing used here are shown in Table 3. According to Table 3, an increase in the flow-rate leads to less loss in resolution, R_s, for the perfusive packing than predicted by conventional theory. This suggests an advantage of the perfusive packing over conventional supports.

Working with materials with large pores, intraparticle diffusivity, depending on the Peclet number, λ, has to be considered. An extended plate height equation for large-pore packings was developed by Rodrigues [26] (Eq. (6)).

$$h = A + \frac{B}{u} + C \cdot f(\lambda) \cdot u \tag{6}$$

The C-term becomes a function ($f(\lambda)$) of the interparticle convective velocity inside large pores and the mass transport through the pores, which is related to superficial fluid velocity. At low values of u, $f(\lambda) \approx 1$, the equations for conventional supports and for the perfusive material give similar results. At high values of u, the term of the Rodrigues equation becomes a constant. This means that at high velocities, the plate height, h, is independent of the velocity, u [7,21,27,28]. The resolution, R_s, is therefore expected to be independent of the flow-rate. However, as shown in Table 2, the R_s values obtained here are not independent of the flow-rate, as was expected. With increasing flow-rates, R_s decreases. This could be due to limitations resulting from finite kinetics of adsorption and desorption

Table 3
Relation between the change in flow-rate and the change in actual resolution, R_s'

Compound	$F' = (F_2/F_1)$ (change in flow-rate)	$\sqrt{F'}$	$R_s' = (R_{s,2}/R_{s,1})$ (change in actual R_s)	$R_s' \cdot \sqrt{F'}$
Dansyl–aspartic acid	5/1 = 5.0	2.2	0.6	1.3
Dansyl–norvaline	10/3 = 3.3	1.8	0.8	1.4
DNP–aspartic acid	10/1 = 10.0	3.2	0.4	1.3
DNP–glutamic acid	10/3 = 3.3	1.8	0.7	1.3
DNP–proline	8/1 = 8.0	2.8	0.6	1.7
Ketoprofen	8/1 = 8.0	2.8	0.6	1.7
MTH-proline	8/3 = 2.7	1.6	0.8	1.3

from the stationary phase. Because of the specific interactions between the chiral selector, BSA, and the investigated compounds, the system has to be considered as an affinity chromatographic system. There, slow processes of non-covalent molecular interaction are of special importance [21,26,28,29]. Slow kinetics are directly evident by the peak-broadening for the second-eluted enantiomer [20]. According to theory, slow kinetics will contribute to an extra term in the plate height equation, which is proportional to the velocity, u. Therefore, the resolution is no longer independent of the mobile phase velocity [6,28].

4. Conclusions

In this work, we were able to demonstrate for the first time that enantiomer separations are possible using a perfusion chromatographic support (POROS). Immobilized BSA was used as the chiral stationary phase for the separation of a variety of amino acid derivatives and drugs. Due to the properties of the support material, separations could be investigated at flow-rates from 1 up to 10 ml/min, without the limitation of a high back pressure. Speeding up the separations by increasing the flow-rate did not lead to a significant decrease in column efficiency and rapid separations within a few minutes could be achieved. Working with an affinity chromatographic system, the decrease in resolution was not independent of the velocity, u, of the mobile phase, as expected by perfusion chromatographic theory, but the loss in resolution with increasing velocity was lower than predicted by theory for conventional supports.

5. Abbreviations

D_m	Solute diffusion coefficient in the mobile phase
d_p	Particle diameter
D_p	Pore diffusion
F	Flow-rate
H	Plate height
h	Reduced plate height
k	Retention factor
k_2	Retention factor of the second peak
L	Column length
N	Plate number
R_s	Resolution
$R_{s,1}, R_{s,2}$	Resolution at two different flow-rates
u	Mobile phase velocity
Greek symbols	
α	Separation factor
γ	Obstructive factor, B-term
λ	Intra-particle Peclet number
ω	Structural parameter, A-term

Acknowledgments

The authors would like to thank Dr. D. Schmalzing from PerSeptive Biosystems for kindly providing the POROS support material and Dr. M. Fluck for helpful discussion. The support of this work by the "German–Israeli Foundation for Scientific Research and Development", "Deutsche Forschungsgemeinschaft" and "Fonds der Chemischen Industrie" is gratefully acknowledged.

References

[1] J.G. Dorsey, W.T. Cooper, B.A. Siles, J.P. Foley and H.G. Barth, Anal. Chem., 68 (1996) 515.
[2] S. Allenmark, B. Bomgren and H. Borén, J. Chromatogr., 264 (1983) 63.
[3] S. Allenmark, Chiral Liquid Chromatography in Chromatographic Enantioseparation, Methods and Applications, Ellis Horwood Series in Analytical Chemistry, Chichester, 2nd Ed., 1991.
[4] J.H. Knox and M. Saleem, J. Chromatogr. Sci., 7 (1969) 614.
[5] M. Martin, G. Blu, C. Eon and G. Guiochon, J. Chromatogr. Sci., 12 (1974) 438.
[6] C. Horvath and H.-J. Lin, J. Chromatogr., 126 (1976) 401.
[7] N.B. Afeyan, N.F. Gordon, I. Mazsaroff, L. Varady, S.P. Fulton, Y.B. Yang and F.E Regnier, J. Chromatogr., 519 (1990) 1.
[8] F.E. Regnier, Nature, 350 (1991) 634.
[9] N.B. Afeyan, F.E. Regnier and R.C. Dean, U.S. Patent No. 5019270 (1991).
[10] N.B. Afeyan, S.P. Fulton, N.F. Gordon, I. Mazsaroff, L. Varady and F.E. Regnier, Bio/Technology, 8 (1990) 203.
[11] N.B. Afeyan, S.P. Fulton and F.E. Regnier, J. Chromatogr., 544 (1991) 267.
[12] S.P. Fulton, N.B. Afeyan, N.F. Gordon and F.E. Regnier, J. Chromatogr., 547 (1991) 452.

[13] S.P. Fulton, M. Meys, L. Varady, R. Jansen and N.B. Afeyan, BioTechniques, 11 (1991) 226.
[14] S.P. Fulton, M. Meys, J. Protentis, N.B. Afeyan, J. Carlton and J. Haycock, BioTechniques, 12 (1992) 742.
[15] S. Hjertén, Y.-M. Li, J.-L. Liao. J. Mohammad, K. Nakazato and G. Pettersson, Nature, 356 (1992) 810.
[16] A. Kurganov, T. Issaeva, K. Unger, K. Bischoff and T. Barder, Book of Abstracts of the 7th International Symposium on Chiral Discrimination, Jerusalem, Israel, November 1995, p. 66.
[17] K.R. Rao and H.A. Sober, J. Am. Chem. Soc., 76 (1954) 1328.
[18] M. Holtzhauer, Biochemische Labormethoden, Arbeitsvorschriften und Tabellen, Springer Verlag, Berlin, Heidelberg, 1988.
[19] G.J. Kennedy and J.H. Knox J. Chromatogr. Sci., 10 (1972) 549.
[20] T. Fornstedt, G. Zhong, Z. Bensetiti and G. Guiochon, Anal. Chem., 68 (1996) 2370.
[21] A.I. Liaps and M.A. McCoy, J. Chromatogr., 599 (1992) 87.
[22] L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York, NY, 1974.
[23] L.R. Snyder, J. Chromatogr. Sci., 7 (1969) 352.
[24] J.H. Knox, J. Chromatogr. Sci., 15 (1977) 352.
[25] L.S. Ettre, Pure Appl. Chem., 65 (1993) 819.
[26] A.E. Rodrigues, LC·GC Int., 6 (1993) 20.
[27] A.E. Rodrigues, Z.P. Lu and J.M. Loureiro, Chem. Eng. Sci., 5 (1991) 2765.
[28] A.E. Rodrigues, J.C. Lopez, Z.P. Lu, J.M. Loureiro and M.M. Dias, J. Chromatogr., 590 (1992) 93.
[29] A.E. Rodrigues, Z.P. Lu and J.M. Loureiro, J. Chromatogr., 653 (1993) 189.