ELSEVIER

Journal of Chromatography A, 730 (1996) 289–295

JOURNAL OF CHROMATOGRAPHY A

Separation of basic proteins by capillary zone electrophoresis with coatings of a copolymer of vinylpyrrolidone and vinylimidazole

Run Juan Xu[a], Claire Vidal-Madjar[a,*], Bernard Sébille[a], José Carlos Diez-Masa[b]

[a]Laboratoire de Physico-Chimie des Biopolymères, CNRS, Université Paris Val-de-Marne, 2 rue Henri Dunant, 94320 Thiais, France

[b]Institute of Organic Chemistry (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

Abstract

Capillary zone electrophoretic (CZE) separation of basic proteins has been achieved with capillary columns modified with copolymers of vinylpyrrolidone (VP) and vinylimidazole (VI). The copolymerization reaction is performed inside the capillary column and involves chemical bonding of the polymer to silica. The electroosmotic flow (EOF) is greatly decreased by this surface modification. The presence of positive charges on the coating surface, due to the cationic property of vinylimidazole at pH below 7, reduces the adsorption of basic proteins onto the silanol groups of the capillary surface. Acidic proteins are irreversibly adsorbed, but rapid separation and good performance reproducibility are obtained with basic proteins. In the case of capillaries modified with VP, the acidic and basic proteins are eluted within 10 min. In this work, we studied the effects of pH and buffer concentration on the magnitude of the EOF, as well as the effect of copolymer composition on the separation efficiency.

Keywords: Capillary electrophoresis; Capillary columns; Electroosmotic flow; Proteins

1. Introduction

As a separation technique, capillary zone electrophoresis (CZE) affords the possibility of achieving rapid and high-resolution separation of macromolecules such as proteins. However, the application of such a technique in the separation of positively charged samples is complicated by adsorption of protein onto the capillary wall, resulting in band broadening and tailing with reduced separation efficiencies. Various attempts have been made to eliminate this adsorption and optimize the separation of basic solutes. The reported approaches include the use of extreme pH buffers [1,2], high ionic strength buffers [3], zwitterionic buffers [4,5], additives in the buffer [2,6,7], as well as coated columns [1,8–12]. Coating of the capillary surface with a suitable polymer seems to have various advantages, such as stability and reproducibility; it can be much improved by studying surface bonding chemistry and selecting suitable polymers.

Several ionic coatings [12–15] have shown an improved efficiency in controlling protein adsorption and electroosmotic flow (EOF). The capillaries with cationic coatings possess positive charges on the surface which enable reduction of the contribution of silanols to the EOF. With these capillaries high separation efficiencies have been obtained [13] but the analysis time is increased because of a small EOF.

In this paper we present a new polymeric coating of fused silica capillaries in which the basic proteins

*Corresponding author.

0021-9673/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)00960-4

can be separated with high resolution. Polyvinylimidazole, a cationic polymer, has been used as an efficient coating agent for the high-performance liquid chromatographic (HPLC) supports [16]. In the capillary electrophoretic separation of basic proteins the use of copolymers of vinylpyrrolidone and vinylimidazole as capillary coatings was investigated. The role of vinylimidazole is to provide positive charges to hinder the adsorption of cationic proteins on the capillary wall by electrostatic repulsion. This surface modification minimizes protein–capillary wall interactions and maintains a moderate EOF for short analysis times.

2. Experimental

2.1. Apparatus

Capillary electrophoresis was performed on a Spectra Phoresis 1000 instrument (Spectra Physics, CA, USA) controlled by an IBM PS/2 386 computer. The apparatus is equipped with a reversible power supply and a scanning, multiple-wavelength detector. The applied voltage was 20 kV and the detector was positioned near the cathode. Protein elution was monitored at 230 nm. Acetone was also detected at 230 nm for EOF measurements.

2.2. Materials

The proteins were purchased from Sigma (St. Louis, MO, USA): cytochrome *c* (bovine heart, p*I* 10.6), lysozyme (chicken egg white, p*I* 11.0), ribonuclease A (bovine pancreas, p*I* 9.3), α-chymotrypsinogen A (bovine pancreas, p*I* 9.2), myoglobin (horse skeletal muscle, p*I* 7.1), β-lactoglobulin B (bovine milk, p*I* 5.2), and α-lactalbumin (bovine milk, p*I* 4.8). γ-Methacryloxypropyltrimethoxysilane (MAPT), 1-vinyl-2-pyrrolidone (VP), 1-vinylimidazole (VI) and sodium phosphate were from Aldrich-Chemie (Steinheim, Germany); α,α'-azobisisobutyronitrile (AIBN) and epichlorohydrin (EPh) were, respectively, obtained from Fluka (Buchs, Switzerland) and Prolabo (Paris, France).

Polyimide-coated fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75 μm I.D. and 360 μm O.D. were used. The optical window was made by removing a small section of the polyimide coating from the fused silica capillary. The total length of the coated capillary was about 43 cm with an effective separation length (distance from injection to detection position) of 35 cm. Double deionized water used in the preparation of coated capillary and buffer was prepared using a Milli-Q system (Millipore, Bedford, MA, USA). The samples were prepared by dissolving the appropriate proteins in the running buffer solution with a concentration of 1 g/l. In this work, the phosphate buffers used were prepared by dissolving the weighed amount of sodium dihydrogenphosphate in Milli-Q-purified water and adding 1 *M* sodium hydroxide to adjust pH values.

2.3. Capillary modification

Polymer-coated capillaries were prepared according to a modification of McCormick's procedure [1] as described in Fig. 1. The fused silica capillary was first pretreated with γ-methacryloxypropyltrimethoxysillane and then dynamically coated under nitrogen pressure with the polymerization reagents using AIBN as a free-radical initiator.

A 1.2-m long capillary was washed with CH_2Cl_2, CH_3OH, then etched with 1 *M* NaOH for 3 h and rinsed with deionized water for 30 min. Next the capillary was flushed with 0.1 *M* HCl to remove Na^+ ions from the surface. The capillary was then washed with deionized water for 30 min. After another washing with methanol the capillary was dried at 200°C for 5 h by gentle flushing with nitrogen. After the above capillary treatment, the capillary was first flushed with an acetic acid solution (pH 3.5) for 30 min and then filled with a silane reagent mixture, 0.4% (v/v) MAPT in the same acetic acid solution. The reaction was kept for 3 h at room temperature. The capillary was then flushed with methanol for 30 min and dried by flushing with nitrogen for 1 h. Subsequently the reagent mixture containing 12.3% (v/v) VP, VI (ratios in moles of VP and VI given in Table 1) and 1% (w/w of monomers) AIBN in methanol was pushed through the silane-derivatized capillary by applying an inlet pressure of 0.5 bar. The solution was left to react at 60°C for 4 h. After polymerization the unreacted reagent was removed from the capillary with methanol and the capillary

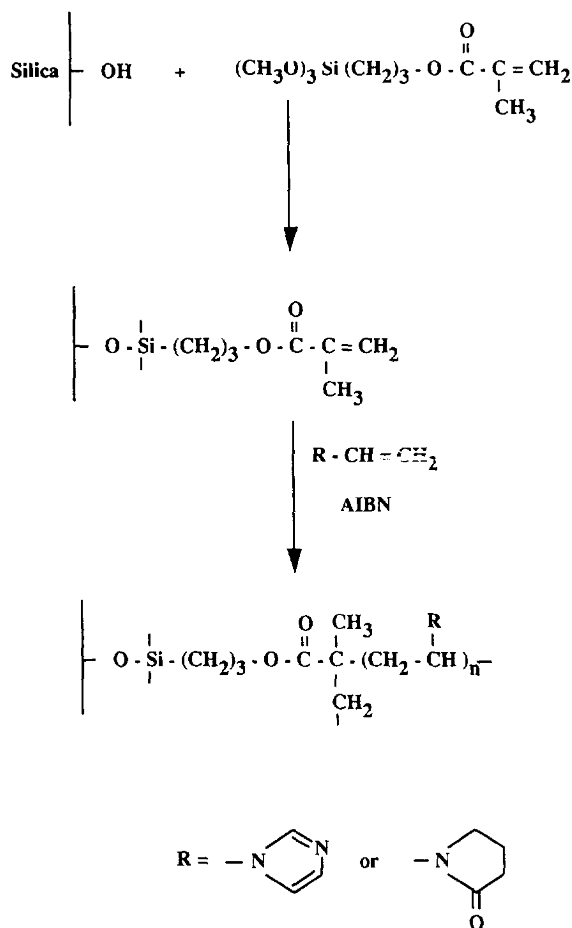

Fig. 1. Schematic illustration of the modification of fused silica capillaries with vinylpyrrolidone and vinylimidazole.

was kept at 60°C for 2 h. Finally, the capillary was rinsed for 30 min with methanol and then with deionized water. For the cross-linked layer column preparation, the cross-linking reaction was performed by passing through the column a solution of 10% (v/v) epichlorohydrin in methanol at 60°C for 20 h before a final rinsing with methanol and then water.

2.4. Electrophoresis

Before analysis the coated capillaries were preconditioned by flushing through the running buffer. They were rinsed with water and buffer between runs. The assay was carried out at 20 kV and the sample was injected by means of the electrokinetic technique at 10 kV for 5 s at the positive side. Since the performance was tested in terms of efficiencies, both methods for sample introduction could be used, electromigration or hydrodynamic introduction. The electroosmotic flow was evaluated with acetone as neutral marker. Such test of the EOF was performed for following the effect of various buffers on the capillary coatings. All experiments were conducted at 20°C.

3. Results and discussion

3.1. Electroosmotic flow

The EOF of the treated capillaries is significantly different from that of uncoated fused silica tubings. First, the capillary is deactivated with silane reagent to yield bonded phases on the capillary wall via siloxane bonds. Then the polymerization reaction produces a layer of polymeric coating on the bonded

Table 1
Effect of polymer coating on EOF. Conditions: capillary, 43 cm (35 cm effective length) × 75 μm I.D.; buffer, 30 m*M* phosphate pH 5; applied voltage, 20 kV; acetone as neutral marker

Coating	VP (%)[a]	VI (%)[a]	EOF × 10^4 (cm^2/V.s.)	Cross-linked
Uncoated	–	–	2.25	No
PVP	100	–	0.44	No
P(VP/VI)	90	10	1.82	No
P(VP/VI)	75	25	1.75	No
PVI	–	100	1.69	No
P(VP/VI)	75	25	0.53	Yes
PVI	–	100	0.17	Yes

[a]Ratios in moles.

phases. The positively charged groups and the unreacted silanols maintain the electroosmotic flow across the capillary. Fig. 2 illustrates the results obtained at 20 kV; it shows a plot of EOF as a function of phosphate concentration in the buffer, at pH 5 and pH 6. Acetone was used as an inert tracer. At pH 5 the EOF drastically changes with the ionic strength for an uncoated capillary column. As can be observed, the EOF with PVI coatings is much lower and it only slightly decreases with increasing buffer ionic strength. At 30 m*M* phosphate buffer, the EOF is ten times lower with cross-linked PVI coatings. For comparison, Fig. 2 also gives the results obtained with a capillary modified with PVP polymer that does not carry positive charges. The EOF is then close to that observed with the cross-linked PVI coating and is almost constant with varying buffer ionic strengths.

At pH 5 with the coated columns, the EOF is considerably decreased and stabilized. As shown in Fig. 2, for the PVP coating an increase of the magnitude of EOF is observed at pH 6 and the influence of the ionic strength is important. This indicates that the masking of the silanol groups of the surface is not complete, with a large influence of the unreacted silanol groups at higher pH.

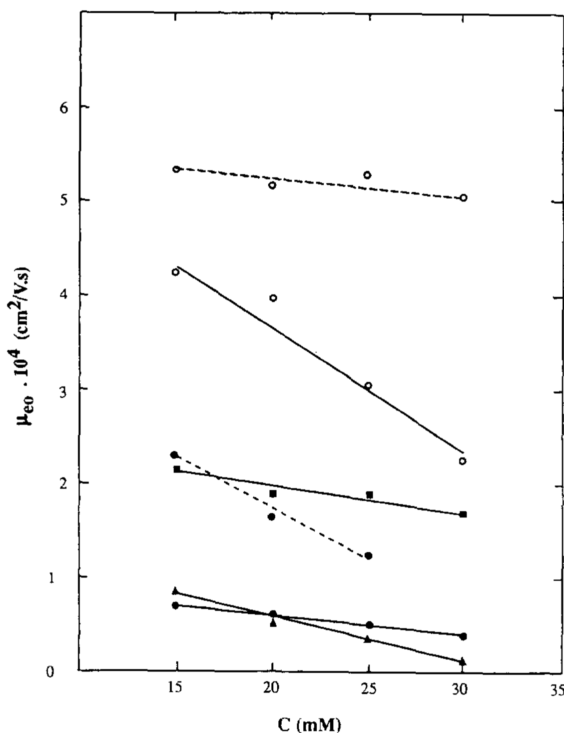

Fig. 2. Plots of electroosmotic mobility, μ_{eo}, versus phosphate buffer concentration. Conditions: capillary, 43 cm (35 cm effective length) × 75 μm I.D.; applied voltage, 20 kV; acetone as neutral marker. Buffer: (———) pH 5; (- - -) pH 6. Columns: (○) uncoated; (●) PVP; (■) PVI; (▲) cross-linked PVI.

The performance of coated columns in CZE not only depends upon the surface immobilization chemistry, but also upon the chemical properties of the polymeric coating. Although the VI groups are ionized at pH below 7, the EOF observed on the PVP coated capillary is much lower than that observed with the non-reticulated PVI coating. This indicates a poor polymeric coating. The relatively high EOF observed with other VP–VI copolymer coatings (Table 1) also indicates an incomplete masking of the surface. With the column having polymeric coatings cross-linked by epichlorohydrin, the magnitude of EOF is considerably decreased. At pH 5 with 30 m*M* phosphate buffer, the EOF with the 75:25 VP/VI cross-linked copolymer column is close to that observed with the PVP column. With similar experimental conditions, it is almost negligible on a cross-linked PVI column at pH 5. However, the EOF is not reversed with the PVI columns, because the coating is not uniform enough. The negatively charged groups of the unreacted silanols still maintain an EOF towards the cathode.

3.2. Evaluation of column performances for protein separations

Because of the electrostatic repulsion, the presence of positive charges on the inner wall of the capillary should decrease the adsorption of positively charged proteins. The direction of the EOF is not changed because the silanol groups of the capillary surface are not totally masked. However, the important decrease of the EOF observed indicates a reduced amount of negative charges. Thus, with capillaries coated with copolymers of VP and VI, the band broadening of positively charged proteins is reduced and the separations are greatly improved. Fig. 3 shows the electropherograms of a mixture of basic proteins on the P(VP/VI) coated columns in which the ratios of VP and VI are, respectively, 75:25 and 90:10. The separation was performed at pH 5 with

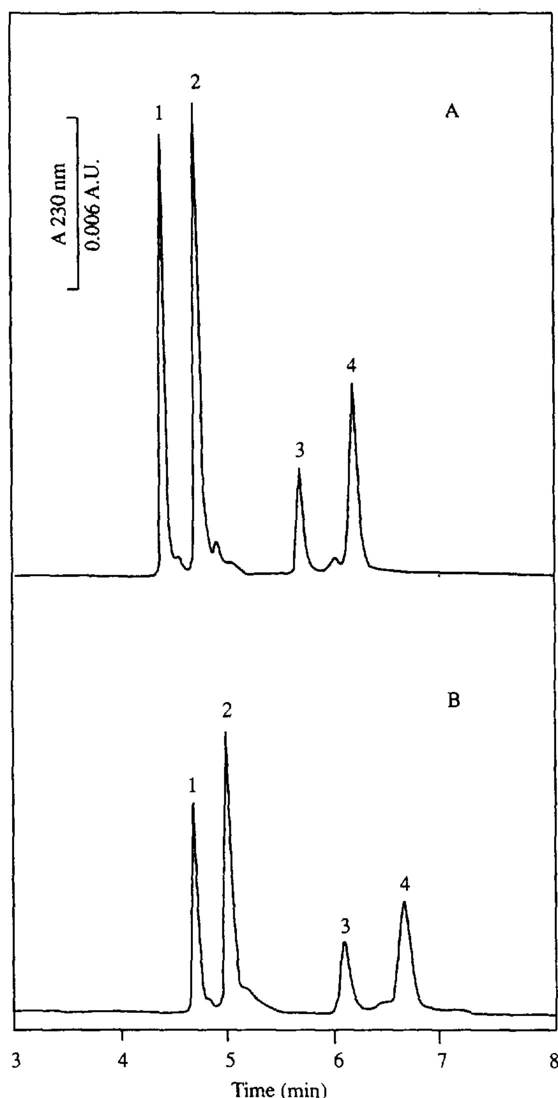

Fig. 3. CZE separations of basic proteins on P(VP/VI) coated columns with (A) VP/VI, 75:25; (B) VP/VI, 90:10. Conditions: capillary, 43 cm (35 cm effective length) × 75 μm I.D.; buffer, 30 m*M* phosphate pH 5; applied voltage, 20 kV. Peak identifications: (1) cytochrome *c*; (2) lysozyme; (3) ribonuclease A; (4) α-chymotrypsinogen A.

moderate ionic strength (30 m*M*). At this pH value, these four proteins are positively charged and have electrophoretic mobilities towards the cathode. The best results for the separation of basic proteins were obtained with the non-reticulated 75:25 P(VP/VI) coating. The separation was completed within 7 min with sharp and symmetrical peaks (Fig. 3). Relatively high efficiencies and good separation time reproducibility were obtained with this column (Table 2).

Separations of basic proteins were also examined on PVI coated columns. Elution was achieved in less than 9 min but the separation efficiency was lower (Table 2). With the cross-linked PVI polymer, the efficiency was better and the separation performances were improved. As previously shown with EOF data, the cross-linking step leads to a more efficient masking of the capillary surface.

Although the lowest EOF is observed with the cross-linked PVI capillary (Table 1), the separation times of basic proteins are close to that observed with the other modified capillaries: the migration velocity of the positively charged proteins towards the cathode is higher than the EOF [17]. The migration time reproducibility and peak symmetry on PVI coated columns are not as good as these observed on the P(VP/VI) coated column. This may be due to an increase of hydrophobic interactions because of a larger number of imidazole groups on the surface. In spite of a lower surface density of positive charges, the use of copolymers of VP and VI results in more uniform and more stable capillary coatings.

With the positively charged polymeric coatings, the basic proteins are eluted within a few minutes, but the acidic proteins are not eluted and remain irreversibly adsorbed on the capillary surface. For comparison, an electropherogram of proteins on the PVP columns at pH 5 in 30 m*M* phosphate buffer is shown in Fig. 4. An efficient and reproducible capillary electrophoretic separation is observed for both basic and acidic proteins. For lysozyme and cytochrome *c* the efficiencies are 160 000 plates/m. For other proteins the efficiencies found are lower, about 80 000 plates/m. The separation of proteins with isoelectric point ranging from 4.8 to 11.0 was achieved in less than 10 min at pH 5. Both acidic and basic proteins are eluted, because this polymer coating does not contain positive charges and also because the p*I* of the acidic proteins tested is slightly lower than the pH used for this experiment. McCormick has already reported the CZE separation of proteins in capillaries modified with vinylpyrrolidone [1] and that the separation of acidic and basic

Table 2
Average migration times and efficiencies of basic proteins analyzed on polymer-coated capillaries

Column	Cytochrome c			Lysozyme			Ribonuclease A			α-Chymotropsinogen A		
	t_m (min)	R.S.D. (%)	N (plates/m)	t_m (min)	R.S.D. (%)	N (plates/m)	t_m (min)	R.S.D. (%)	N (plates/m)	t_m (min)	R.S.D. (%)	N (plates/m)
P(VP/VI) (75:25)	4.6	0.2	155 540	4.9	0.4	114 830	5.9	0.1	125 250	6.4	0.1	118 530
PVI	5.6	2.5	92 230	6.0	2.0	47 730	7.7	2.8	85 750	8.5	3.1	88 690
Cross-linked PVI	5.2	1.3	158 680	5.5	0.3	133 540	7.1	1.2	94 250	7.9	1.4	81 520
PVP	5.1	2.1	158 840	5.4	2.4	164 690	5.9	3.0	54 460	6.6	3.0	68 700

Same experimental conditions as in Fig. 3; R.S.D. of elution times calculated from six consecutive runs.

proteins was obtained in about 25 min at low pH values (pH 1.5–3.0) and high ionic strength.

The efficiency of basic proteins is higher on the P(VP/VI) column than on the PVP column (Table 2) because the first polymer coating, with positive charges, not only acts as a masking agent for the silanol groups of the surface, but also hinders the adsorption of the positively charged proteins and improves the efficiency.

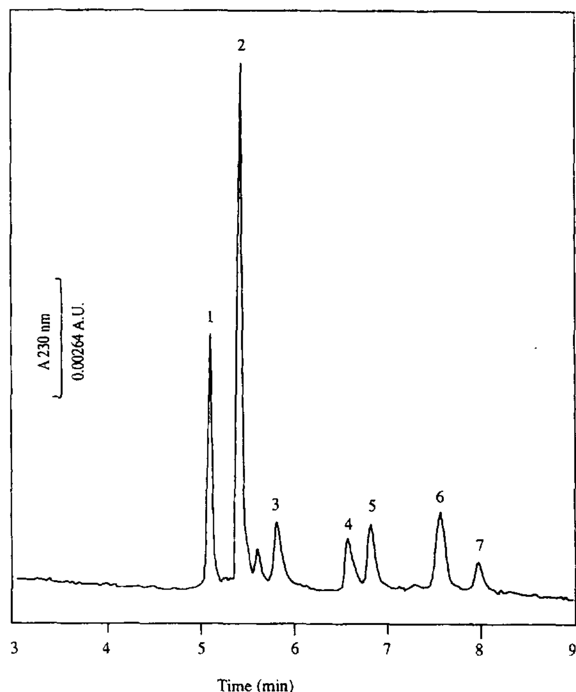

Fig. 4. CZE separation of a mixture of proteins on PVP-coated columns at pH 5. Conditions: capillary, 43 cm (35 cm effective length) × 75 μm I.D.; buffer, 30 m*M* phosphate pH 5; applied voltage, 20 kV. Peak identifications: (1) cytochrome *c*; (2) lysozyme; (3) ribonuclease A; (4) α-chymotrypsinogen A; (5) myoglobin; (6) β-lactoglobulin B; (7) α-lactalbumin.

3.3. Stability of the polymer coatings

The separation reproducibility of the capillaries modified with VP and VI has been investigated by measuring the migration times of the proteins with a buffer of 30 m*M* phosphate at pH 5 and at a voltage of 20 kV. The results are given in Table 2 with the relative standard deviation (R.S.D.) in terms of elution time for six consecutive runs. For the copolymer coatings, it was found to be less than 0.4%. Comparing PVI and cross-linked PVI coatings, it is noted that the cross-linked PVI coating gives more reproducible separations (Table 2). The capillaries with polymeric coatings of P(VP/VI) have been tested using buffer pH 5–6 with moderate ionic strength and more than 200 injections were performed. These coated capillaries exhibit constant solute migration time and good reproducibility of the separation performance with high-resolution at pH 5.

The effect of pH on capillary stability has also been investigated. As previously reported, pH change can cause substantial modification in the magnitude of the electroosmotic flow. Silanols on the capillary wall become progressively more ionized as the buffer pH is made more basic. At higher pH values a significant increase in electroosmotic flow was observed on the capillaries with polymeric coatings of VP and VI, which means that the surface silanols are contributing to the net charge of the capillary wall under this condition. At pH 7 the polymeric coatings are not stable.

Similar behaviour was observed with the PVP coating. The column may be used at pH 6 in the

same ionic strength but the separation efficiency is lower than that observed at more acidic pH, due to the presence of ionized silanol groups of the inner wall. As the pH is further raised, the stability of the coatings is poor and protein separation is not possible. In fact, it is known that the stability of coating prepared by silanization is poor at higher pH values because of the low stability of the siloxane bond.

In this study, we have shown that the copolymers of VP and VI are very useful for capillary coating in the separation of basic proteins by CZE. The capillaries modified with these copolymers exhibit constant solute migration time and good reproducible separation performance at pH 5. The preparation of these capillary coatings is simple, reproducible and easy to control.

References

[1] R.M. McCormick, Anal. Chem., 60 (1988) 2322.
[2] H.H. Lauer and D. McManigill, Anal. Chem., 58 (1986) 166.
[3] J.S. Green and J.W. Jorgenson, J. Chromatogr., 478 (1989) 63.
[4] M.M. Bushey and J.W. Jorgenson, J. Chromatogr., 480 (1989) 301.
[5] M. Zhu, R. Rodriguez, D. Hansen and T. Wehr, J. Chromatogr., 516 (1990) 123.
[6] J.K. Towns and F.E. Regnier, Anal. Chem., 63 (1991) 1126.
[7] A. Cifuentes, J.M. Santos, M. de Frutos and J.C. Diez-Masa, J. Chromatogr. A, 652 (1993) 161.
[8] S. Hjertén, J. Chromatogr., 347 (1985) 191.
[9] G.J.M. Bruin, J.P. Chang, R.H. Kuhlman, K. Zegers, J.C. Kraak and H. Poppe, J. Chromatogr., 471 (1989) 429.
[10] G.J.M. Bruin, R. Huisden, J.C. Kraak and H. Poppe, J. Chromatogr., 480 (1989) 339.
[11] J.K. Towns, J. Bao and F.E. Regnier, J. Chromatogr., 599 (1992) 227.
[12] J.K. Towns and F.E. Regnier, J. Chromatogr., 516 (1990) 69.
[13] J.T. Smith and Z. El Rassi, Electrophoresis, 14 (1993) 396.
[14] J.T. Smith and Z. El Rassi, J. High. Resolut. Chromatogr., 15 (1992) 573.
[15] P. Sun, A. Landman and R.A. Hartwick, J. Microcol. Sep., 6 (1994) 403.
[16] R. Lemque, C. Vidal-Madjar, M. Racine, J. Piquion and B. Sébille, J. Chromatogr., 553 (1991) 165.
[17] H. Engelhardt, W. Beck, J. Kohr and T. Schmitt, Angew. Chem. Int. Ed. Engl., 32 (1993) 629.