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Journal of Chromatography A, 894 (2000) 337–343

JOURNAL OF CHROMATOGRAPHY A

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Pressurized gradient capillary electrochromatographic separation of eighteen amino acid derivatives

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Abstract

A pressurized gradient capillary electrochromatography (pCEC) instrument was developed to separate 18 amino acid derivatives. A reversed-phase C_{18} column (3 μm, 130 mm×75 μm I.D.) and an acetate buffer (50 mmol/l NaAc, pH 6.4) with an ion-pair reagent (1% *N*,*N*-dimethylformamide) were used to separate derivatized amino acids from a standard solution (2 μg/ml), and the wavelength of the UV–Vis detector was 360 nm. The pressure on the capillary column was kept at approx. 70 Pa and 3 kV positive voltage was added on the outlet end of column. The effect of voltage on the eluting order of amino acids and the resolution of separation were studied, and it was found that when the voltage was higher than 3 kV, the adsorption of amino acids in the porous C_{18} column occurred. The effect of salt concentration, injection volume, and column length on the separation of amino acids was determined. The amino acid sample was separated by pCEC, and RSDs of the migration times of each amino acid were all less than 2.5%.

Keywords: Gradient elution; Pressurized gradient electrochromatography; Electrochromatography; Amino acids

1. Introduction

Capillary electrochromatography (CEC) combines the two well-known analytical techniques high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). It is performed by applying high voltage to a column (typically 25–50 cm long and 50–150 μm I.D.). Besides being a combination of HPLC and CE principles, this technique brings new qualities into separation science by enabling both above mentioned separation principles to operate side-by-side. CEC will thus have the ability to provide high selectivity (due to the variety of stationary and mobile phases), the ability to handle highly polar and neutral compounds, and the ability to possess the well characterized retention mechanisms of HPLC [1–4]. The technique has the potential of providing 5–10-times higher efficiencies [5,6] than HPLC alone primarily due to the plug-like flow profile created by the electroosmotic flow (EOF) compared to the parabolic flow of HPLC [7–9]. The combination of HPLC and CE gives the analyst a larger number of parameters to adjust in order to obtain and optimize a separation than either of the two techniques provides on their own.

So far, CEC experiments have been performed on commercial CE or laboratory-made CE instruments. One disadvantage of this method is that when the concentration of buffer is too high, bubbles can be generated in the capillary column, which will stop the current and destroy the separation. Another

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00712-3

serious problem is that a linear solvent-gradient elution cannot be performed on this instrument though step gradient CEC has been performed by using a commercial CE instrument [10]. We have developed a pCEC instrument comprised of two pumps, a high-voltage power supply and a UV–Vis detector, a micro-fluid manipulation system and a data system, which can perform linear gradient elution during CEC separation.

The unique characteristic of this instrument is that the EOF can be combined with pressure, therefore, the separation mechanism is based on both electrophoretic mobility and chromatographic partitioning. For example, separation of a mixture of both neutral and charged compounds can be achieved by simply changing the ratio of the voltage to the pressure without changing the mobile phase composition.

It was well known that CEC was good for the separation of neutral samples, but there are a few reports on the CEC applications in the biochemical field [11]. Huber et al. realized the separation of 12 amino acid derivatives by pressurized gradient CEC [12]. Here, the separation of 18 L-amino acid derivatives was performed on our home-made pCEC instrument. Amino acids could be separated by many methods such as HPLC–mass spectrometry (MS) [13], micellar electrokinetic chromatography (MEKC) [14], and CE [15], typical separation times of these methods range from 30 to 50 min. Here, the separation of 18 L-amino acids with linear gradient elution pCEC was achieved in 20 min. The results proved that both the pCEC instrument and the separation method were sensitive, stable and accurate.

2. Experimental

2.1. Instrumentation

Fig. 1 is the photograph of the pCEC instrument. It contains two pumps (1 μl/min–10 ml/min flow-rate, 0–500 Pa), one high-voltage power supply (0~ ±30 kV), one UV–Vis detector (190–800 nm wavelength), one micro-fluid manipulation module, one injector (20-nl volume), one data system and one electrokinetically packed column. The valve combined the pump mixture and the sample injector could keep the pressure on the column at approx. 70 Pa. Polyether ether ketone (PEEK) tubing was used to connect the fluid route. With two pumps and a capillary, micro-column HPLC could be performed. With one pump, a capillary, and a high-voltage electric power supply, CE could be performed. With two pumps, a capillary column, and a high-voltage electric power supply, pCEC could be realized. Therefore, this is an instrument that could carry out three kinds of separation, micro-HPLC, CE and pCEC.

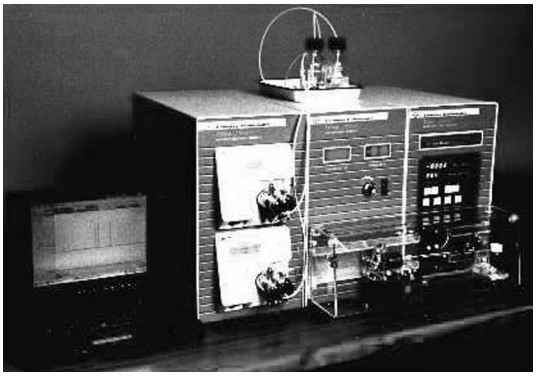

Fig. 1. Photograph of the pressurized gradient capillary electrochromatography (pCEC) instrument.

2.2. Column and mobile phases

C_{18} columns (3 μm, 130 mm×75 μm I.D. or 200 mm×75 μm I.D.) were provided by Unimicro Technologies (Pleasanton, San Francisco, CA, USA). Mobile phase A was 50 mmol/l sodium acetate (NaAc)–1% *N*,*N*-dimethylformamide (pH 6.4), and mobile phase B was 50% aqueous acetonitrile.

2.3. pCEC separation conditions

A linear gradient (60–5% A in 6 min, and then kept at 5%) was used to separate amino acids. The positive voltage 3000 V across the column and approx. 70 Pa pressure were added on the capillary column during the separation. The flow-rate was 50 μl/min. The wavelength of the UV–Vis detector was

360 nm. The injection volume was 20 nl. All experiments were carried out at room temperature.

2.4. Reagents and samples

Eighteen L-amino acid standards were purchased from Sigma (St. Louis, MO, USA), acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Other reagents were obtained from Beijing Reagent Factory (Beijing, China). Samples were provided by Amino Acids Co. (Tianjin, China).

2.5. The derivatization of eighteen L-amino acids

An 18 L-amino acid standard was first dissolved in a 25-ml volumetric flask to prepare the standard solution of 1 mg/ml. A 0.5-ml volume of sodium hydrogencarbonate ($NaHCO_3$, 0.5 mol/l) and 0.1 ml 1% 2,4-dinitrofluobenzene (acetonitrile solution) were added to 0.5 ml of the standard solution, and mixed thoroughly. The mixture was then water-bathed at 60°C for 1 h. After cooling, the mixture was transferred to a 10-ml volumetric flask and dissolved with phosphate-buffered system (PBS, pH 7.0) to prepare the storage solution. Before the separation, it was diluted with PBS to 2 μg/ml.

3. Results and discussion

3.1. Effect of voltage on pCEC separation of amino acids

Fig. 2 shows chromatograms of the 18 amino acid standards separated by pCEC with different positive voltages across the column. With the increase of voltage, the sensitivity and the resolution of separation increased, and the number of detected amino acid peaks increased. Another phenomenon is that with the increase of voltage, the order of amino acid peaks changed too. When the voltage was higher than 3500 V, the peaks of the amino acids all disappeared. We believe that this is due to the adsorption of amino acids on the porous C_{18} column. Therefore, a 3000 V positive voltage across the column was used for pCEC separations.

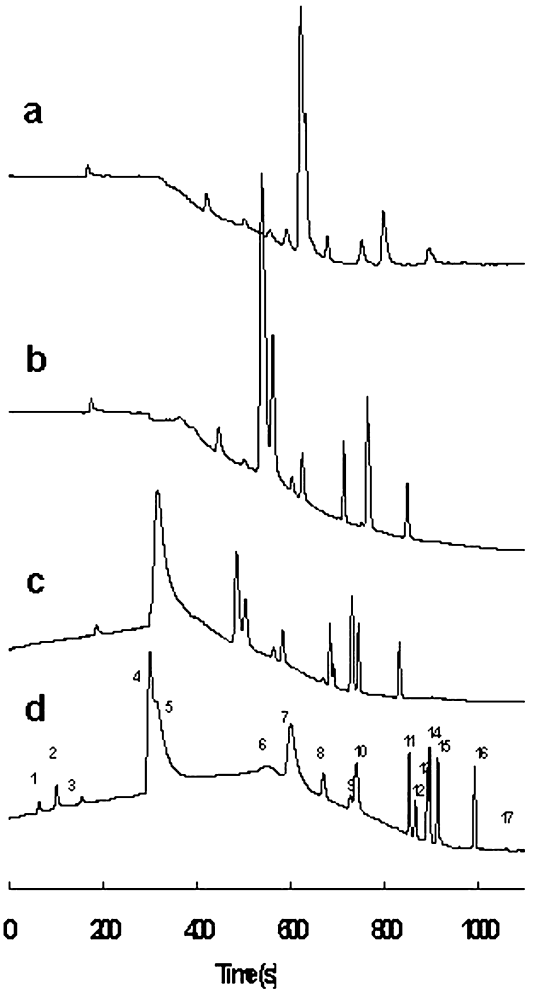

Fig. 2. Effect of voltage on pCEC separation of amino acids. Column: PS-C_{18}, 3 μm, 130 mm×75 μm I.D.; mobile phase: (A) 50 mmol/l NaAc–1% *N*,*N*-dimethylformamide (pH 6.4), (B) 50% acetonitrile in distilled water; linear gradient: 70% A in 4 min, 70–30% A in 1 min, 30–25% A in 4 min, 25–5% A in 3 min, 5–2% A in 1 min, 2% A in 10 min; flow-rate: 50 μl/min; pressure added on-column: approx. 70 Pa; wavelength of UV–Vis detector, 360 nm; injection volume: 20 nl. A 2-μg/ml derived 18 amino acids standard solution was separated at room temperature. Peaks: 1=aspartate (Asp, D); 2=glutamate (Glu, E); 3=tyrosin (Tyr, Y); 4=derivate; 5=serine (Ser, S); 6=tryptophan (Trp, W); 7=glycine (Gly, G); 8=threonine (Thr, T); 9=proline (Pro, P); 10=alanine (Ala, A); 11=valine (Val, V); 12=methionine (Met, M); 13=isoleucine (Ile, I); 14=leucine (Leu, L); 15=phenylalanine (Phe, F); 16=lysine (Lys, K); 17=arginine (Arg, R). (a) Without electric voltage; (b) 1000 V; (c) 2000 V; (d) 3000 V positive voltage across the column.

Table 1
Percentage of acetonitrile needed for elution of amino acids depending on NaAc concentration[a]

NaAc (mmol/l)	Acetonitrile (%)							
	Asp (p*I* 2.77)	Glu (p*I* 3.22)	Tyr (p*I* 5.66)	Ser (p*I* 5.68)	Gly (p*I* 5.97)	Pro (p*I* 6.48)	Lys (p*I* 9.74)	Arg (p*I* 10.76)
10	35.9	39.8	45.3	47.5	47.5	47.5	47.5	47.5
20	29.4	32.8	36.5	42.4	47.5	47.5	47.5	47.5
30	26.1	27.6	30.2	35.0	45.1	47.5	47.5	47.5
40	24.8	26.7	28.6	33.6	43.2	47.5	47.5	47.5
50	24.6	26.5	27.5	32.2	41.9	47.5	47.5	47.5
60	22.1	23.8	24.5	29.1	37.3	42.5	47.5	47.5

[a] Separation conditions were the same as shown in Fig. 6. p*I*=Isoelectric point.

3.2. Effect of gradient and salt concentration on pCEC separation of amino acids

Four gradient elution programs were used to separate amino acid standards. Gradient 1 was 70% of A in 4 min, 70–30% of A in 1 min, 30–25% of A in 4 min, 25–5% of A in 3 min, and 5–2% of A in 1 min. Gradient 2 was 70–30% of A in 5 min, 30–25% of A in 5 min, 25–5% of A in 4 min, 5–2% of A in 1 min, and 2–1% of A in 5 min. Gradient 3 was

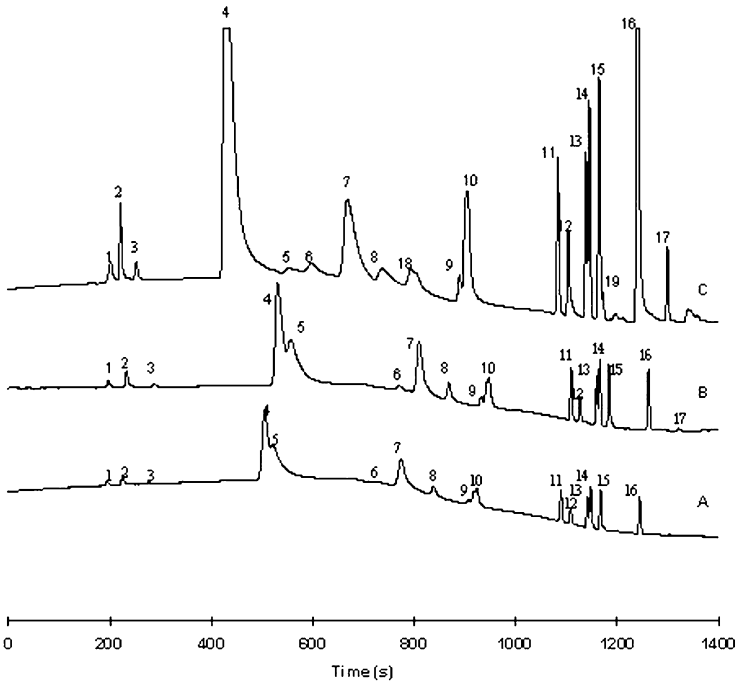

Fig. 3. Effect of injection volume on pCEC separation of amino acids. Column: PS-C_{18}, 3 μm, 150 mm×75 μm I.D.; linear gradient: 60–35% A in 6 min, 35–20% A in 4 min, 20–2% A in 2 min; 3000 V positive voltage across the column; other conditions were the same as shown in Fig. 2. (A) Injection time: 1 s; (B) injection time: 3 s; (C) injection volume, 20 nl. Peak Nos. were the same as shown in Fig. 2; in (C) 18=cysteine (Cys, C); 19=histidine (His, H).

60–35% of A in 6 min, 35–20% of A in 4 min, and 20–2% of A in 2 min. Gradient 4 was 60–5% of A in 6 min. It was found that the separation time of the step gradient mode was longer than that of the linear gradient mode. Besides, there is no big difference in resolution between the step gradient and the linear gradient. Moreover, the baseline of the linear gradient was smoother than that of the step gradient. Therefore, the linear gradient mode (gradient 4) was used for pCEC separations.

Table 1 shows the effect of salt (NaAc) concentration on the migration time of amino acids; the migration time was calculated as the percentage of acetonitrile during the linear gradient. With the increase of salt concentration, the separation became faster, and both the resolution and the efficiency was improved. In order to avoid the occurrence of bubbles during the separation, 50 m*M* NaAc was used.

3.3. Effect of injection volume on pCEC separation of amino acids

The influence of injection volume on the sensitivity was studied. A 2-μg/ml standard solution was injected separately within 1 s (Fig. 3A), 3 s (Fig. 3B), and full volume (20 nl, Fig. 3C). Obviously, the peak height increased linearly with the injection volume. Even peaks of cysteine and histidine could

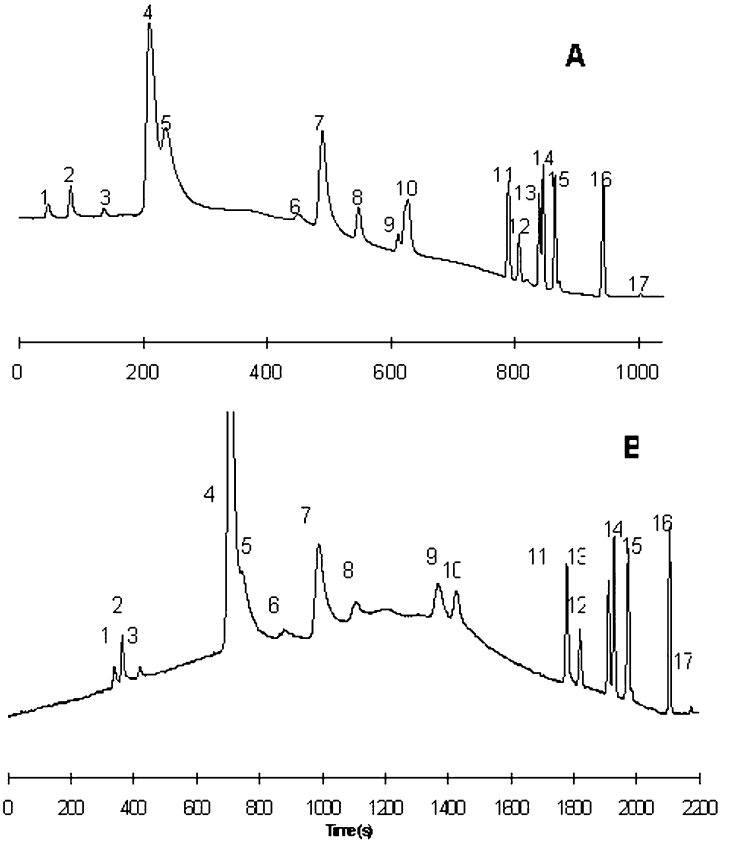

Fig. 4. Effect of column length on pCEC separation of amino acids. Linear gradient: 70–30% A in 5 min, 30–25% A in 5 min, 25–5% A in 4 min, 5–2% A in 1 min, 2–1% A in 5 min, then kept at 1%; 20 nl injection; 3000 V positive voltage across the column; other conditions and peak Nos. were the same as shown in Fig. 2. (A) PS-C_{18} column, 3 μm, 130 mm×75 μm I.D.; (B) PS-C_{18} column, 3 μm, 200 mm×75 μm I.D.

be detected with a 20-nl injection, while the peak width of the amino acids did not increase. Therefore, the full injection volume (20 nl) was used.

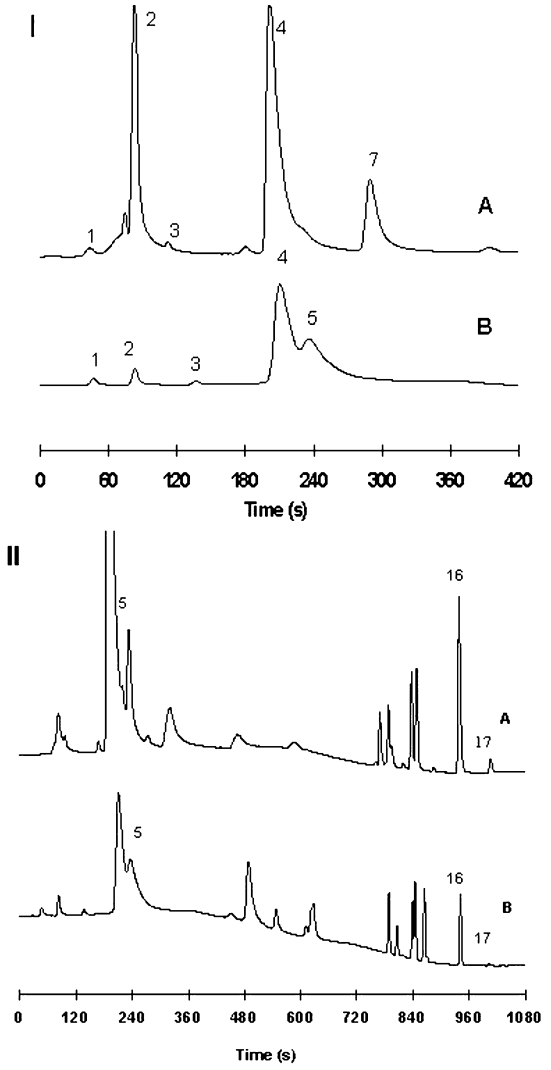

Fig. 5. Identification of peaks of 18 amino acids separated by pCEC. Linear gradient: 60–5% A in 6 min, and kept at 5%; 3 s injection time; 3000 V positive voltage across the column; other conditions were the same as shown in Fig. 2. (I) A, 50 μg/ml Glu (peak 2) was mixed with 2 μg/ml amino acid standards (1:1, v/v). B, 2 μg/ml amino acid standards. (II) A, 20 μg/ml Ser (peak 5), 20 μg/ml Lys (peak 16), and 20 μg/ml Arg (peak 17) were mixed with 2 μg/ml amino acid standards (1:1:1:1, v/v). B, 2 μg/ml amino acid standards.

3.4. Effect of column length on pCEC separation of amino acids

Fig. 4 shows that the separation of amino acids could be completed in 20 min with a 130 mm column, and in 40 min with a 200 mm column. Because the longer column did not improve the resolution of separation dramatically, we therefore chose the 130 mm column for the subsequent experiments.

3.5. Identification of amino acid peaks separated by pCEC

Each single amino acid was derivatized separately, and then mixed with a standard solution of 18 amino acids in a constant ratio. The mixtures were separated by pCEC one by one. Based on the increase of peak heights, the peaks of all 18 were identified. The identification of the acidic amino acid glutamine, the neutral amino acid serine, and the basic amino acids lysine and arginine are shown in Fig. 5.

3.6. Determination of 18 amino acids sample by pCEC

The amino acids standard solution was separated by pCEC five times, and the relative standard deviations (RSDs) of the migration times of all 18 amino acids were less than 2.5%. The content of each amino acid was calculated with the percentage of each peak area to the total peak area, and total amount of 18 amino acids was taken as 100%. RSDs of all peak area percentage were less than 2%. Then, the amino acids sample solution was separated by pCEC (Fig. 6A). Compared with that of the standard solution (Fig. 6B), the content of 15 amino acids (except cysteine, arginine and histidine) basically reached the level of quality control.

4. Conclusion

A pressurized gradient CEC instrument and method was developed. The pCEC instrument was applied for the separation of 18 amino acids, and the results proved that both the instrument and the method were reliable, sensitive and accurate. The

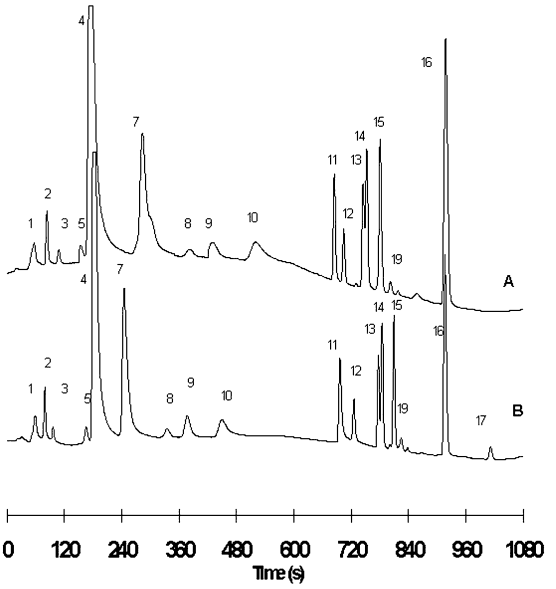

Fig. 6. The separation of 18 amino acids sample by pCEC. Column: PS-C_{18}, 3 μm, 130 mm×75 μm I.D.; linear gradient: 60–5% A in 6 min, and kept at 5%; 20 nl injection; 3000 V positive voltage across the column; other conditions were the same as shown in Fig. 2. (A) a 2-μg/ml derived 18 amino acids sample solution; (B) a 2-μg/ml derived 18 amino acids standard solution.

unique characteristic of the pCEC instrument is that both the voltage and the pressure can be used separately or simultaneously, so that both electrophoretic mobility as well as chromatographic partitioning can be utilized for the separation of complex mixtures. The instrument can be also used in micro-HPLC and CE modes. The further improvement would focus on the decrease of dead volume, the addition of the temperature control, and the development of more application methods in the biochemical research field.

Acknowledgements

This research was supported by the National Natural Science Foundation of China and by the Ministry of Science and Technology.

References

[1] I.S. Lurie, R.E. Meyers, T.S. Conver, Anal. Chem. 70 (1998) 3255.
[2] R. Dadoo, C. Yan, R.N. Zare, D.J. Rakestraw, D. Anex, Anal. Chem. 70 (1998) 4787.
[3] L.A. Colon, Y. Guo, A. Fermier, Anal. Chem. 69 (1997) 461A.
[4] M.R. Euerby, D. Gilligan, C.M. Johnson, S.C. Roulin, P. Myers, K.D. Bartle, J. Microcol. Sep. 5 (1997) 373.
[5] M. Dittmann, G. Rozing, J. Chromatogr. A 744 (1996) 63.
[6] C. Yan, R. Dadoo, R.N. Zare, D.J. Rakestraw, D.S. Anex, Anal. Chem. 68 (1996) 2726.
[7] M. Dittmann, G. Rozing, J. Microcol. Sep. 5 (1997) 399.
[8] N.W. Smith, K.D. Altria, C.H. Tumbull, Chromatographia 46 (1997) 664.
[9] S.E. Bosch, S. Heemstra, J.C. Kraak, H. Poppe, J. Chromatogr. A 755 (1996) 165.
[10] N.C. Gillott, M.R. Euerby, C.M. Johnson, D.A. Barrett, P.N. Shaw, Chromatographia 51 (2000) 167.
[11] T. Helboe, S.H. Hansen, J. Chromatogr. A 836 (1999) 315.
[12] C.G. Huber, G. Choudhary, Cs. Horváth, Anal. Chem. 69 (1997) 4429.
[13] H.M.H. Eijk, D.R. Rooyakkers, P.B. Soeters, N.F.P. Deutz, Anal. Biochem. 271 (1999) 8.
[14] G. Nouadje, N. Simeon, F. Dedieu, M. Nertz, Ph. Puig, F. Couderc, J. Chromatogr. A 765 (1997) 337.
[15] S. Oguri, K. Yokoi, Y. Motohase, J. Chromatogr. A 787 (1997) 253.