Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1018 (2003) 97–103
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Two-dimensional capillary electrophoresis involving capillary isoelectric focusing and capillary zone electrophoresis

Chun Yang, Lingyi Zhang, Hechun Liu, Weibing Zhang, Yukui Zhang*

National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 161 Zhong Shan Road, Dalian 116011, PR China

Received 8 April 2003; received in revised form 5 August 2003; accepted 8 August 2003

Abstract

Capillary isoelectric focusing (cIEF) and capillary zone electrophoresis (CZE) was on-line hyphenated by a dialysis interface to achieve a 2D capillary electrophoresis (CE) system. The system was used with just one high-voltage power supply and three electrodes (one cathode shared by the two dimensions). The focused zone in the first dimension (i.e. the cIEF) was driven to the dialysis interface by electroosmotic flow (EOF), besides chemical mobilization from the first anode to the shared cathode. And then in the second dimension (i.e. the CZE), the separated zone was further separated and driven by an inverted EOF, which originated from the charged layer of a cationic surfactant adsorbed onto the inner wall of the capillary. Finally, a solution of ribonuclease was rapidly separated to assess the feasibility of the two-dimensional CE implement.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Two-dimensional capillary electrophoresis; Capillary isoelectric focusing; Capillary electrophoresis; Dialysis interface; Electroosmotic flow

1. Introduction

Complex biological systems challenge the technique of analysts all over the world, especially after the introduction of the concept of proteomics, which studies the whole protein contemplate expressed by a genome [1]. Arduous proteomics tasks require techniques with high throughput and high efficiency in order to screen a certain proteome expression and to monitor the effects of environmental conditions and time on the expression. There seldom is, at present, a single separation mode sufficient enough to deal with such complex samples. Multidimensional chromatographic and capillary electrophoresis (CE) protocols provide powerful methods to accomplish ideal separations [2–4]. Among them the most important ones are the integrated systems containing complementary dimensions, where different dimensions separate components on the basis of independent or orthogonal principles [5–8]. In such a multidimensional system, the peak capacity is the product of the peak capacities of each dimension [9,10].

CE is a significant tool for the separation of proteins and peptides [11–13]. To finish complicated separation jobs, great efforts have been concentrated on the development of 2D CE [14–17]. These two-dimensional

* Corresponding author. Tel.: +86-411-3693427; fax: +86-411-3693427.
E-mail address: ykzhang@dicp.ac.cn (Y. Zhang).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.08.031

systems can be carried out in capillary formats or on chips to obtain higher efficiency and capacity than 1D methods. And they are also potentially automated for high-throughput purposes.

We previously reported a two-dimensional capillary isoelectric focusing–capillary gel electrophoresis (cIEF–CGE) system just with one high-voltage power supply and three electrodes. Chemical mobilization was utilized to drive the sample zones of the first dimension [18]. To actualize 2D cIEF–CGE performance, coated and gel-filled capillaries were needed to eliminate the undesired EOF. In a gel-filled capillary the emergence of bubbles is tedious. So it is valuable to exploit a convenient and robust 2D CE system. In this paper we present a two-dimensional capillary isoelectric focusing–capillary zone electrophoresis (cIEF–CZE) system in which EOF was utilized to transport the analyte. This two-dimensional construction makes it easy to achieve a rapid two-dimensional separation for ampholytic biological macromolecules.

2. Experimental

2.1. Materials and instrumentation

The electrophoretic experiments were performed on a TriSep^{TM} 2000 GV system (Unimicro Technology, Pleasanton, CA, USA) equipped with a Data Module UV-Vis detector (wavelength continuously adjustable) and a CEC Control Module high-voltage power supply. A Workstation Echrom 98 of Elite Co. (Dalian, China) was used for data acquisition. The hollow fibers were taken out of a polyethersulfone hollow-fiber dialyzer (Diapes-R12, 30 μm i.d., Caswasumi Laboratories, Inc., Tokyo, Japan) with a molecular weight cutoff (MWCO) of 8000. The silica capillaries (50 μm i.d., 375 μm o.d.) were purchased from Ruiyang Chromatographic Device Co. Ltd. (Yongnian, Hebei, China).

Hydroxypropylmethyl-cellulose (HPMC), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, 99%) (both from Acros Organics), Ribonuclease (bovine pancreas, Merck), and Pharmalyte (pH 3.0–10.0, Bio-Chemika) were used directly without further purification. Cetyltrimethylammonium bromide (CTAB) was purchased from Beijing Chemical Reagent Co. (Beijing, China). Other reagents used were of analytical grade.

2.2. Fabrication of the interface

A section of a hollow fiber (10 mm long) was fixed in a reservoir (20 mm × 10 mm × 10 mm, thickness 6 mm) made of polymethyl methacrylate. A thin thread of platinum was plugged into the reservoir to serve as the electrode. All the slots (between fiber and reservoir, and electrode and reservoir) were sealed with cyanoacrylate to protect from leakage. Both ends of the fiber were made expedite and could be later screwed to connect a capillary respectively to establish a separation channel (Fig. 1).

2.3. cIEF and CZE of ribonuclease

An uncoated capillary was equilibrated by the sample buffer of cIEF (40 mmol/l Tris–HCl) containing Pharmalyte (2% (v/v)), HPMC (0.2% (w/v)), and TEMED (0.05% (v/v)) for 10 min before each run. This treatment saturated the inner wall of the capillary with HPMC. The silanol groups were partially covered by the hydrophilic polymer chain. A segment of ribonuclease solution was injected into the capillary at a certain voltage for a fixed period of time. A solution of 15 mmol/l H_3PO_4 was used as the anolyte and 20 mmol/l NaOH as the catholyte. After a voltage was applied, the focusing began. Transported by a residual EOF towards the cathode, the focused sample zones would give signals as they pass the detection window.

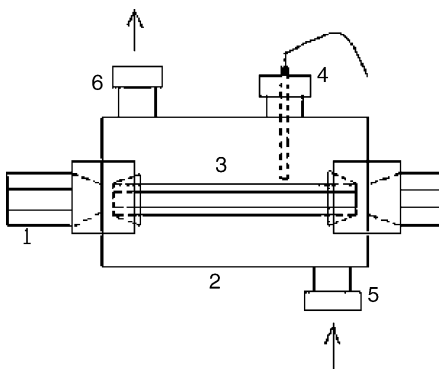

Fig. 1. Dialysis interface: (1) capillaries; (2) buffer reservoir; (3) hollow fiber; (4) electrode; (5) buffer inlet; (6) buffer outlet.

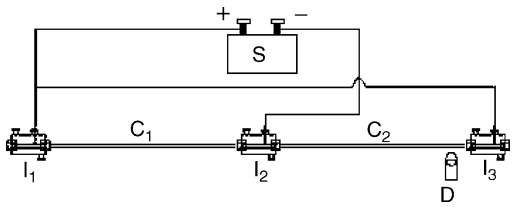

Fig. 2. Construction of 2D cIEF–CZE. S: high-voltage power supply; C_1, C_2: capillaries; I_1, I_2, I_3: interfaces; D: detector.

In the case of CZE, a solution of ribonuclease was injected into a capillary prefilled with CZE buffer. After sample injection, the analysis voltage was applied on the capillary to perform electrophoresis.

2.4. Hyphenation of cIEF and CZE

The two-dimensional system was set up as Fig. 2. Interfaces I_1, I_2 and I_3 contained 15 mmol/l H_3PO_4, 20 mmol/l NaOH, and CZE buffer, respectively. Capillary C_1 was firstly filled with a ribonuclease solution, and C_2 with CZE buffer. The capillaries and interfaces were joined together carefully to avoid bubbles in the separation pathway. Three electrodes were introduced from the high-voltage power supply S. Two anodes were linked to I_1 and I_3. And I_2 was linked to the shared cathode. One minute after the power was turned on, the solution of NaOH in the interface I_2 was displaced by the CZE buffer. Subsequently, till the end of the total two-dimensional operation, CZE buffer (at 0.1 ml/min) was compelled to flow through the shared interface I_2 and rinse the hollow fiber in it.

3. Results and discussion

3.1. Characteristics of 2D cIEF–CZE

This two-dimensional system enables a separation based on two conterminous orthogonal criteria. Firstly ampholytic macromolecules such as peptides and proteins are distinguished in accordance with their different isoelectric points (p*I*s) in the first dimension. The EOF in the first dimension acts as an electrophoretic pump to transfer the analyte. And the semi-permeable property of the interface ensures that macromolecules of ampholytic analytes remain in the separation channel. Zero net charged analyte molecules focused in the first dimension are recharged in the interface (I_2 in Fig. 2) according to the pH of the altered buffer. Then the recharged macromolecules are forced to migrate into the second dimension. Further separation according to the ratios of charge and mass (electrophoretic mobility) will be carried out in the second dimension (CZE). The dialysis interfaces play important roles in the electrophoretic experiments. In the 2D CE system, the two capillaries were tightly screwed to connect to the hollow fiber in the interface. This protocol endues the interface with durability and makes for convenient performance. To reduce the dead volume, it is necessary to match the inner diameter of the hollow fiber to that of the capillaries. The tangent surfaces of these units should be made even and smooth.

Isoelectric focusing in a dynamically modified capillary with EOF suppressed to a certain level is termed as dynamical focusing [19,20]. A section of sample solution is injected into the capillary and kept at a certain distance from the detection point. Molecules of carrier ampholytes (CAs) establish a pH gradient under a high voltage. Mixed analytes are focused into various zones due to the differences in the p*I*s and then mobilized by the EOF. It is simple to carry out a fast two-dimensional separation with this cIEF–CZE arrangement. For both cIEF (Fig. 3) and CZE (Fig. 4), it took less than 3 min to deal with a sample of ribonuclease.

When performing isoelectric focusing, one can fill the total volume of a capillary with sample solution. It can be expected that the detection sensitivity of the hyphenated system benefits from the concentration effect of the first dimension of cIEF. This feature holds advantage over other CE modes such as CZE, CGE, micellar electrokinetic capillary chromatography (MECC), and capillary electrochromatography (CEC). Practically, cIEF has a power to concentrate analytes up to several hundred folds in a capillary [21]. Such a condensed and shortened analyte plug in a capillary is appropriate for sample injection to other CE modes. Therefore cIEF is a proper candidate for the first dimension in a multi-dimensional CE system. Apparently, this will improve the sensitivity for mass detection. It is advantageous over those systems in which cIEF was utilized as the second dimension. Nevertheless, the sensitivity of UV absorbance suffers from the necessity of the CAs involved in cIEF. Of course, isotachophoresis (ITP) as a pretreatment

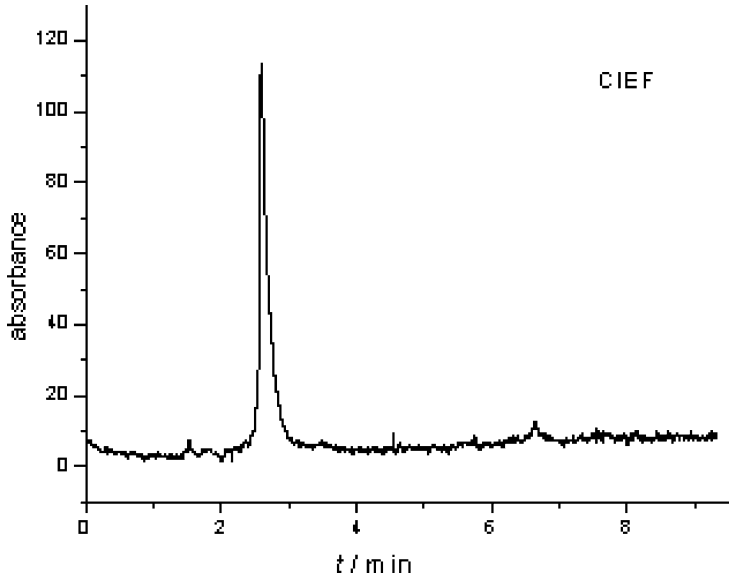

Fig. 3. cIEF of ribonuclease. The capillary is 30 cm long with an effective length of 22 cm from the anode to the detection window. Ribonuclease concentration is 0.2% (w/v) in 40 mmol/l Tris–HCl containing Pharmalyte (2% (v/v)), HPMC (0.2% (w/v)), and TEMED (0.05% (v/v)). Injection: 10 kV for 90 s. The voltage is 10 kV and the detection wavelength is 280 nm.

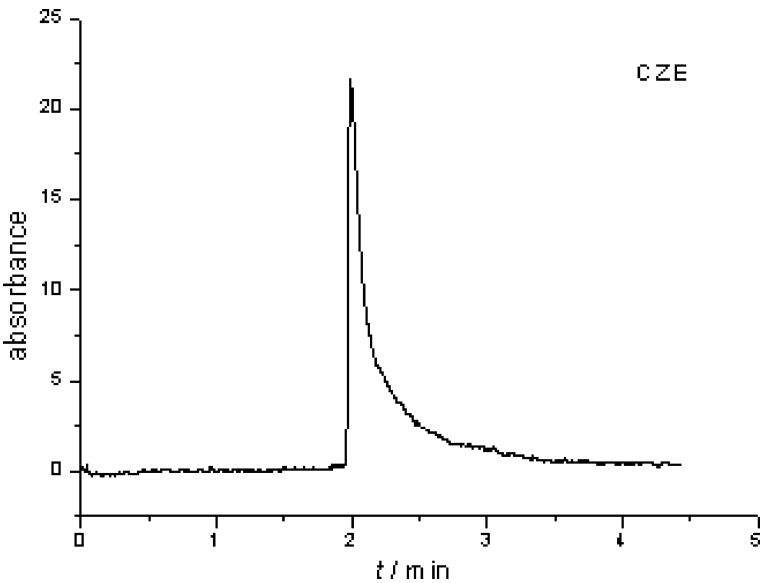

Fig. 4. CZE of ribonuclease. Buffer: 40 mmol/l Tris–HCl containing 0.1% (w/v) CTAB. Injection time is 5 s. Other conditions are the same in Fig. 3.

tool for CZE separation also has a concentration effect [22]. ITP is carried out based on the mobility differences of ions and, IEF, based on different pIs of ampholytic molecules.

As mentioned above, a two-dimensional separation system integrated by a dialysis interface could be used with just one high-voltage power supply. During the two-dimensional operation, the two dimensions shared a cathode. In practice, the two dimensions are flexible to share an anode. In such a way, chemical mobilization towards anode (anodic mobilization) should be run in the first dimension. And the EOF is normally from the anode to the cathode in the second dimension. Additionally, this 2D CE has the expandability to multi-dimensional system in theory [18]. But optimization should be carried through before the utilization in a practical proteome program. Aspects such as compatibility with mass spectrometry (MS) and the ability of identification and quantitation need to be improved.

3.2. Mobilization of the electrolytes from the first to the second dimension

After focusing, the ampholytic molecules possess zero net charge in the capillary. Thus there should be at least one additional force to drive the focused zones other than electric mobility. The semipermeable feature of a dialysis interface supplies convenience to introduce small molecules or to remove them from the separation channel. This provides a method to adjust the properties such as pH, ionic strength, and viscosity in the capillaries [23–26]. In the mean time, electrolytes can be displaced on-line by applying dialysis interfaces. Thus, there is a choice to run chemical mobilization in the dimension of cIEF after focusing.

Under the control of a high voltage, the CAs will move and then establish a stable pH gradient between the acidic anolyte and the basic catholyte. Once an electrolyte is displaced by a salt solution, the pH gradient will be destroyed and the focused zones will begin to migrate. This is known as chemical mobilization [27]. A 2D CE system involving cIEF as the first dimension could apply chemical mobilization as an inherent force for driving the focused zones [18]. In this paper the transfer of analytes is achieved by both EOF and chemical mobilization. In this case, chemical mobilization could be used [18]. But we know that chemical mobilization is inefficient when the pH is extremely basic or acidic. Valves and sample loops were usually used in two- or multi-dimensional separation systems [6,7]. The advantage of a valve or sample loop is their robustness. Naturally separated zones were driven mechanically in those systems. Switching by a valve or sampling by a loop is simple to operate and is easy to be automated. But it is not so easy to precisely collect the whole components within a peak in the first dimension and transfer them to the second dimension. The discontinuity of sample transfer and injection into the second dimension will further result in component loss during the performance. Analytes with molecular mass bigger than the MWCO of the hollow fiber cannot be lost in this cIEF–CZE implement. On the contrary, they will all be injected into the second dimension for further separation. Focused zones could also be hydrodynamically injected into the second dimension by simply elevating the inlet reservoir above the outlet for a certain time [8]. However, on turning off of the high-voltage power applied to the cIEF capillary intermittently, broadening of the zones can be expected. Because there are no solid materials to stack in either of the capillaries, mechanical methods such as nitrogen pressure and siphon could be utilized to drive analyte zones.

The directions of the electric fields in the two dimensions of this three-electrode setup are in reverse to each other. It is necessary to modify the second-dimensional capillary before utilizing the EOF to transfer separated zones. Quaternary ammonium cationic surfactants could be adsorbed onto silica and will drastically alter the polarity of the hydrophilic surface. Hence the compounds such as CTAB were used to coat silica capillaries and reverse the direction of EOF [28–30]. Thus, a solution of 0.05% (w/v) CTAB was added to the buffer to initiate the EOF from the shared cathode (in the interface I_2) to the anode (in I_3). This adapts to the direction of the EOF from the anode (in I_1) to the shared cathode (in I_2) in the first dimension.

The EOF in the first dimension varies with the position in the gradient while running cIEF. Low EOF appears at acidic pH in a capillary due to a deficiency in the ionization of the silanol groups and vice versa. Verification experiment was carried out to evaluate whether all the CAs could be swept through the two-dimensional separation pathway. In such an

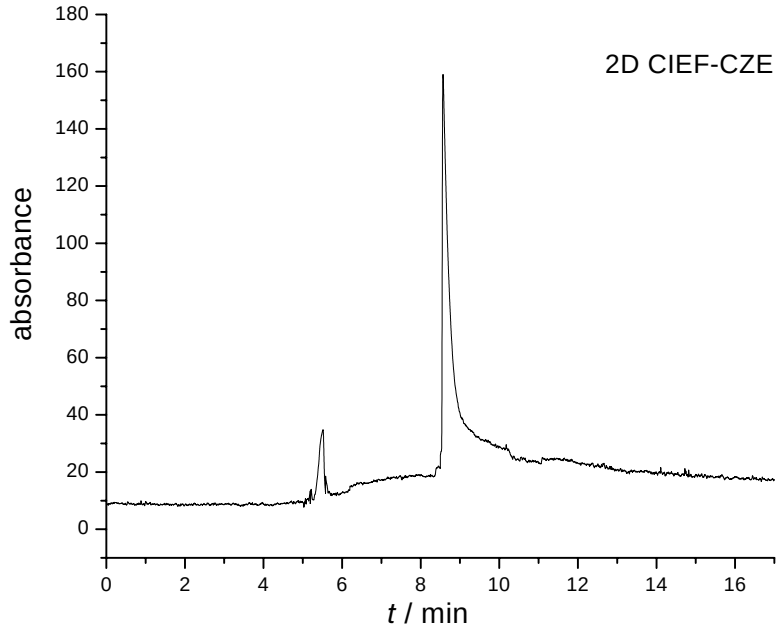

Fig. 5. Electrophoregram of 2D cIEF–CZE. The detection wavelength is 280 nm. Other conditions are the same as in Fig. 3 or 4.

experiment the detection wavelength was 254 nm, which was strongly absorbed by the CAs. Other conditions were the same in Fig. 5 but without ribonuclease. The result showed it would take more than 100 min to transfer all the gradient carriers through the two dimensions (data not showed). It proves that the remainder EOF is sufficient to migrate all focused zones in a wide pH gradient (3–10), even though there are different pH values in the capillary.

Only a reproducible method is valuable in practical analytical experiments. The reproducibility of this cIEF–CZE system depends on the stability of high-voltage power supply, the properties of the capillary inner walls, and the components of the buffers. Repeat experiments exhibited acceptable reproducibility of the elution times. The result of six continuous runs of 2D cIEF–CZE gave an R.S.D. value of 2.7% for the first peak and 3.1% for the second in Fig. 5.

3.3. An example: 2D cIEF–CZE electrophoregram of ribonuclease

A solution of ribonuclease was used to verify the effectiveness of the 2D cIEF–CZE system. Firstly the sample was separated in both cIEF and CZE modes. Due to the poor absorbance sensitivity at 280 nm and limited sampling volume of the two modes, cIEF and CZE, electrophoregrams of both cIEF and CZE gave a single peak (Figs. 3 and 4). By the agency of the concentration effect from cIEF the cooperation of cIEF–CZE could reveal different components in the sample solution. Once the sample was subjected to CZE after being focused by cIEF, two peaks were clearly observed (Fig. 5). It can be concluded that the 2D cIEF–CZE system possesses higher resolving power than each of the single modes.

Acknowledgements

This work was supported by the State Key Fundamental Research with a Ministry of Science and Technology (People's Republic of China) grant, number 001CB510202.

References

[1] V.C. Wasinger, S.J. Cordwell, A. Cerpa-Poljak, M.R. Wilkins, M.W. Duncan, R. Harris, K.L. Williams, L. Humphery-Smith, Electrophoresis 16 (1995) 1090.

[2] L.R. Zieske, D. Fu, S.H. Khan, J. Chromatogr. A 720 (1996) 395.
[3] M. Hanna, C. Simpson, D. Perrett, J. Chromatogr. A 894 (2000) 117.
[4] L. Krivankova, P. Bocek, J. Chromatogr. B 689 (1997) 13.
[5] A.W.J. Moore, Anal. Chem. 67 (1995) 3456.
[6] A.V. Lemmo, J.W. Jorgenson, J. Chromatogr. 633 (1993) 213.
[7] L. Sheng, J. PawliszynHille, Analyst 127 (2002) 1159.
[8] D. Mohan, C.S. Lee, Electrophoresis 23 (2002) 3160.
[9] J.C. Giddings, J. High Resolut. Chromatogr. Chromatogr. Commun. 10 (1987) 319.
[10] G. Guiochon, L.A. Beaver, M.F. Gonnord, A.M. Siouffi, M. Zakaria, J. Chromatogr. 255 (1983) 415.
[11] C. Schoneich, S.K. Kwok, G.S. Wilson, S.R. Rabel, J.F. Stobaugh, T.D. Williams, D.G. Vander Velde, Anal. Chem. 65 (1993) 67R.
[12] I. Messana, D.V. Rossetti, L. Cassiano, F. Misiti, B. Giardina, M. Castagnola, J. Chromatogr. B 699 (1997) 149.
[13] V. Dolnik, K.M. Hutterer, Electrophoresis 22 (2001) 4163.
[14] D. Kaniansky, M. Masar, J. Bielcikova, F. Ivanyi, F. Eisenbeiss, B. Stanislawski, B. Grass, A. Neyer, M. Joehnck, Anal. Chem. 72 (2000) 3596.
[15] N. Gottschlich, S.C. Jacobson, C.T. Culbertson, J.M. Ramsey, Anal. Chem. 73 (2001) 2669.
[16] X. Chen, H. Wu, C. Mao, G.M. Whitesides, Anal. Chem. 74 (2002) 1772.
[17] F. Kvasnicka, M. Jaroš, B. Gaš, J. Chromatogr. A 916 (2001) 131.
[18] C. Yang, H.C. Liu, Q. Yang, L.Y. Zhang, W.B. Zhang, Y.K. Zhang, Anal. Chem. 75 (2003) 215.
[19] W. Thormann, J. Caslavska, S. Molteni, J. Chmelik, J. Chromatogr. 589 (1992) 321.
[20] J.R. Mazzeo, I.S. Krull, Anal. Chem. 63 (1991) 2852.
[21] Y. Shen, S.J. Berger, G.A. Anderson, R.D. Smith, Anal. Chem. 72 (2000) 2154.
[22] D. Kaniansky, J. Marák, J. Laštinec, J. Reijenga, F.I. Onuska, J. Microcolumn Sep. 11 (1999) 141.
[23] J. Wu, J. Pawliszyn, Anal. Chem. 67 (1995) 2010.
[24] M.W. Lada, G. Schaller, M.H. Carriger, T.W. Vickroy, R.T. Kennedy, Anal. Chim. Acta 307 (1995) 217.
[25] M.H. Lamoree, U.R. Tjaden, J. van der Greef, J. Chromatogr. A 777 (1997) 31.
[26] C. Tragas, J. Pawliszyn, Electrophoresis 21 (2000) 227.
[27] S. Hjerten, M.D. Zhu, J. Chromatogr. 346 (1985) 265.
[28] Y. Ghaemi, R.A. Wall, J. Chromatogr. 174 (1979) 51.
[29] C. Cifuentes, M.A. Rodríguez, F.J. García-Montelongo, J. Chromatogr. A 742 (1996) 257.
[30] W. Ding, J.S. Fritz, J. High Resolut. Chromatogr. 20 (1997) 575.