Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 793 (2003) 151–157
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Automated large-volume sample stacking procedure to detect labeled peptides at picomolar concentration using capillary electrophoresis and laser-induced fluorescence detection

Nathalie Siri[a], Pierre Riolet[a,b], Christophe Bayle[a,b], François Couderc[b,*]

[a]*Picometrics, 10 Avenue de l'Europe, 31520 Ramonville, France*
[b]*IMRCP, Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse, France*

Abstract

We have developed an automated large-volume sample stacking (LVSS) procedure to detect fluorescein isothiocyanate-labeled peptides in the picomolar range. The injection duration is 10 min at 50 mbar to fill 62% of the capillary volume to the detection cell. The calculated limit of detection ($S/N = 3$), filling 1% of the capillary volume, is 74 p*M* for bradykinin and 45 p*M* for L-enkephalin with samples diluted in water and analyzed in a 50 m*M* borate buffer, pH 9.2. With the automated LVSS system, the limits of detection are 7 p*M* for bradykinin, 3 p*M* for L-enkephalin and 2 p*M* for substance P. LVSS is shown to be quantitative from 500 to 10 p*M*.

Keywords: Large-volume sample stacking; Peptides; Fluorescein isothiocyanate

1. Introduction

Large-volume sample stacking (LVSS) procedures are important for increasing the sensitivity. Since Burgi and Chien's work [1], related to the analysis of ions, various researchers have used such methods for separations of drugs [2,3], organic acids [4], plant hormones [5], amino acids [6,7], alkylbenzenes and naphthalenesulfonates [8,9], aromatic amines [10], and metal ions [11,12]. Peptides in body fluids are very important topics for study, because of their involvement in the regulation of physiological processes at very low concentrations. Stacking procedures have been used for the study of peptides or proteins by CE. For instance, de Jong used a miniaturized reversed-phase C_{18} bed connected to a CZE capillary without any dead volume [13]. Glutathione, insulin and other peptides have been analyzed using a conventional or acetonitrile stacking procedure, which increased sensitivity 20-fold [14–16]. Peptide preconcentration has also been performed using immobilized antibodies as on-line preconcentrators and cleaning devices [17]. A few years ago, Tomlinson et al. used a removable membrane preconcentration cartridge to load large sample volumes to study peptides by CE–MS [18]. They succeeded in detecting very low concentrations of peptides that were undetectable by other techniques. Thanks to a betain discontinuous buffer system, Schwer [19] increased the sensitivity of UV detection by a factor of at least 30. More recently, a solid-phase extraction device for discontinuous on-line preconcentration was used for peptide mapping. A preconcentration

*Corresponding author.
E-mail address: couderc@cict.fr (F. Couderc).

1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00372-6

factor of up to 500 was achieved [20]. Waterval et al. attempted the same strategy using octadecyl silica particles inside the capillary. An injection of 25 μL was achieved with preconcentration transient isotachophoresis, yielding an improvement of three to four orders of magnitudes, i.e. a 1 ng/mL solution of model peptides [21]. On-line concentration of peptides or proteins has also been reported using a pH junction between two discontinuous buffers in a coated capillary. Limits of detection of a few nanomolars were achieved with large injection volume [22].

To the best of our knowledge, large-volume sample stacking associated with laser-induced fluorescence has not been used to study labeled peptides. In this work, we present a simple and automated stacking procedure for fluorescein isothiocyanate-labeled peptides to detect picomolar concentrations of analytes by laser-induced fluorescence. The detection of picomolar concentrations of peptides labeled at high concentration was achieved by combining LVSS and LIF.

2. Materials and methods

The CE instrument was an Agilent HP^{3D}CE (Agilent, Walbron, Germany) equipped with a MS cassette. The LIF detector was a Picometrics Zetalif 488 (Picometrics, Ramonville, France) equipped with a 488 nm argon ion laser emitting at 25 mW. 17 mW illuminates the capillary window. The range we used was 2 milli-relative fluorescence units (mRFUs) and a rise time of 0.5 s. The fused-silica capillary (Polymicro Technology, Phoenix, AR, USA) was of 375 μm outer diameter, 50 μm inner diameter, 76 cm total length, and 62 cm effective length. The 0–1 V analog output of the Zetalif detector was connected to the data acquisition software of the CE instrument (ChemStation) through an Agilent analog/digital interface.

All reagents were purchased from Sigma (Saint-Quentin Fallavier, France). Bradykinin, angiotensin, substance P, L-enkephalin, and M-enkephalin Trp–Leu were solubilized in a 40 m*M* boric acid buffer at pH 9.2. The resulting solutions contained a 100 μ*M*

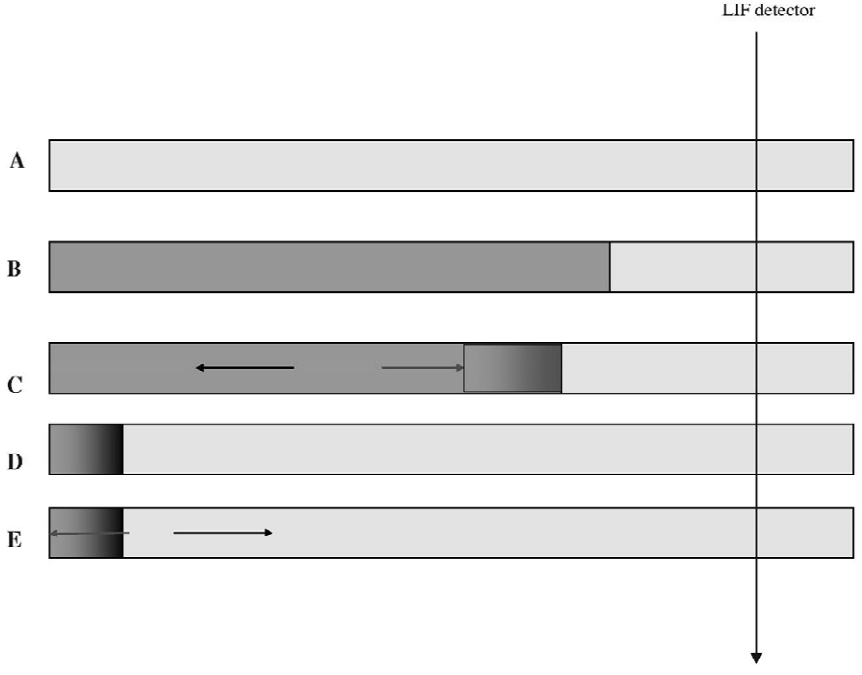

Fig. 1. Principle of the LVSS. (A) The capillary is filled with borate buffer,100 m*M*, pH 9.6. (B) Injection is performed at 50 mbar for several minutes. (C) Reverse polarity is used to empty the injected volume using EOF (black arrow); electromigration (grey arrow) stacks the analyte at the interface between the buffer and the injected volume. (D) After optimization, the injected optimal volume is removed from the capillary. (E) Normal polarity is switched on and the separation of analytes is realized.

concentration of each peptide. To a 500 μL solution of each peptide was added 500 μL of a 1 m*M* fluorescein isothiocyanate solution (water–acetone, 90:10). The labeling reaction was allowed to proceed overnight at ambient temperature in the dark. The samples were then diluted with water to the desired concentration.

2.1. CE buffer

Boric acid and sodium hydroxide were diluted in water to obtain a 0.4 *M* boric acid and 1 *M* NaOH solution. This solution was then diluted 10 times with water and adjusted at pH 9.4 using a 0.1 *M* HCl solution. Ethylene glycol was added to obtain a 10% final concentration. This buffer resulted in a current of 43 μA at 23 kV. Without ethylene glycol, the current was 52 μA and the noise of the baseline was three times higher, reducing the sensitivity of the analysis.

Between runs the capillary was washed with 1 *M* NaOH for 5 min, then 4 min with water and 4 min with the CE buffer. Injection volumes were calculated using CE Expert (Beckman, Fullerton, CA, USA).

2.2. Large-volume sample stacking procedure

The method is derived from the method of Chien and Burgi for cation analysis [1]. Fig. 1 presents the injection principle. First, the capillary was filled with borate buffer, followed by an injection at 50 mbar for several minutes. Then reverse polarity (−23 kV) was applied to empty the injected volume using electroosmotic flow (EOF). Electromigration stacked the analyte at the interface between the buffer and the injected volume. After optimization, an optimal volume of matrix water (in which the peptides are contained) was removed from the capillary. Normal polarity was switched on and the separation of analytes was performed at 23 kV. The entire process was controlled by Chemstation software.

All peptides used in this study were negatively

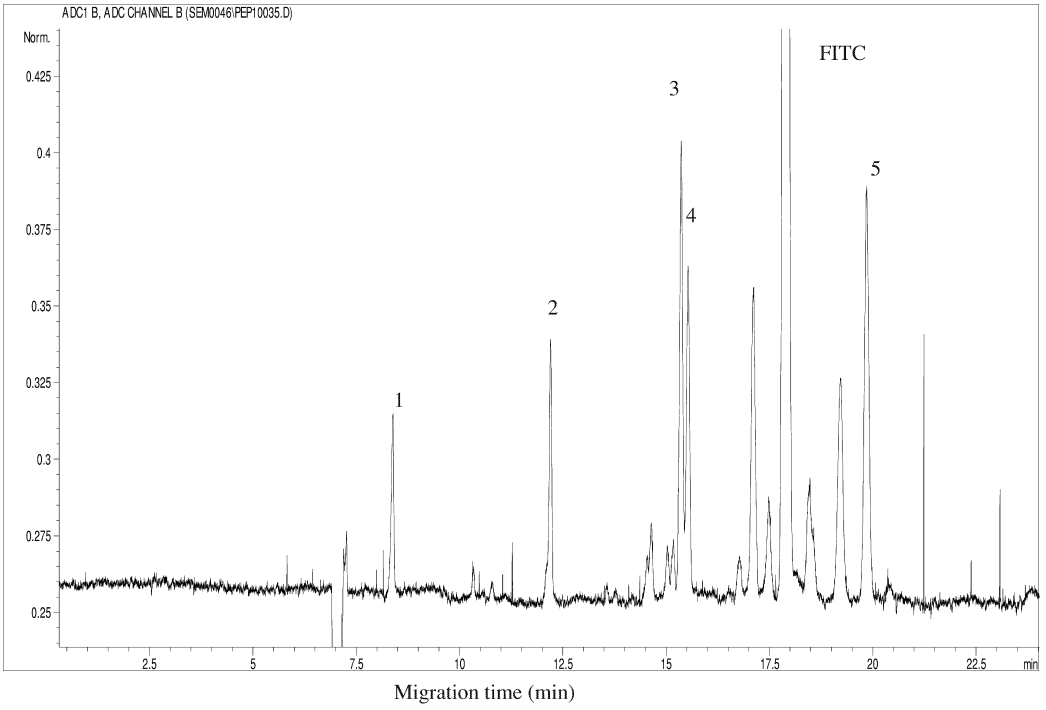

Fig. 2. Separation of 13 nL of a solution of five peptides labeled with FITC, bradykinin (1), angiotensin (2), leucine–enkephalin (3), methionine–enkephalin (4) and tryptophan–leucine dipeptide (5), at a concentration of 500 p*M* each diluted in water. The CE buffer was as described in the Materials and methods section.

charged by fluorescein nucleus and C-terminus carboxylate functions.

3. Results and discussion

In this work we attempted to develop an automated procedure to analyze a mixture of labeled peptides in the picomolar range using a stacking step.

Fig. 2 shows the separation of five peptides labeled with fluorescein isothiocyanate (FITC), i.e. bradykinin, angiotensin, substance P, leucine–enkephalin and tryptophan–leucine dipeptide, at a concentration of 500 p*M* each diluted in water. The injection was performed without stacking for 10 s at 50 mbar. Thirteen nanoliters was injected, i.e. 1% of the capillary length to the window. The signal-to-noise ratio varied from 15 for bradykinin to 73 for leucine–enkephalin.

3.1. Optimization of the stacking procedure

Fig. 3 shows the variation of the current following the reverse polarity of a blank sample injected for 10 min at 50 mbar, 758 nL injected, i.e. 62% of the capillary length to the window. The current increased slowly (more and more negative) and then remained constant, mimicking the output of the sample plug driven out of the capillary by the EOF while the FITC stacked at the sample buffer interface. The best results for peptide peak height were obtained after stopping the reverse polarity mode at 85% of the current Ia at the time the plug came out. With a coefficient of variation of 6.3% at 3 min and 7.5% at 1 min, the reproducibility of the current is very good.

The injection time was examined. Fig. 4 shows a comparison between injection times of 1, 5, and 10 min at 50 mbar, corresponding to 76 nL (6% of the capillary length to the window), 379 nL (31% of the capillary length to the window) and 758 nL (62% of the capillary length to the window), respectively. An

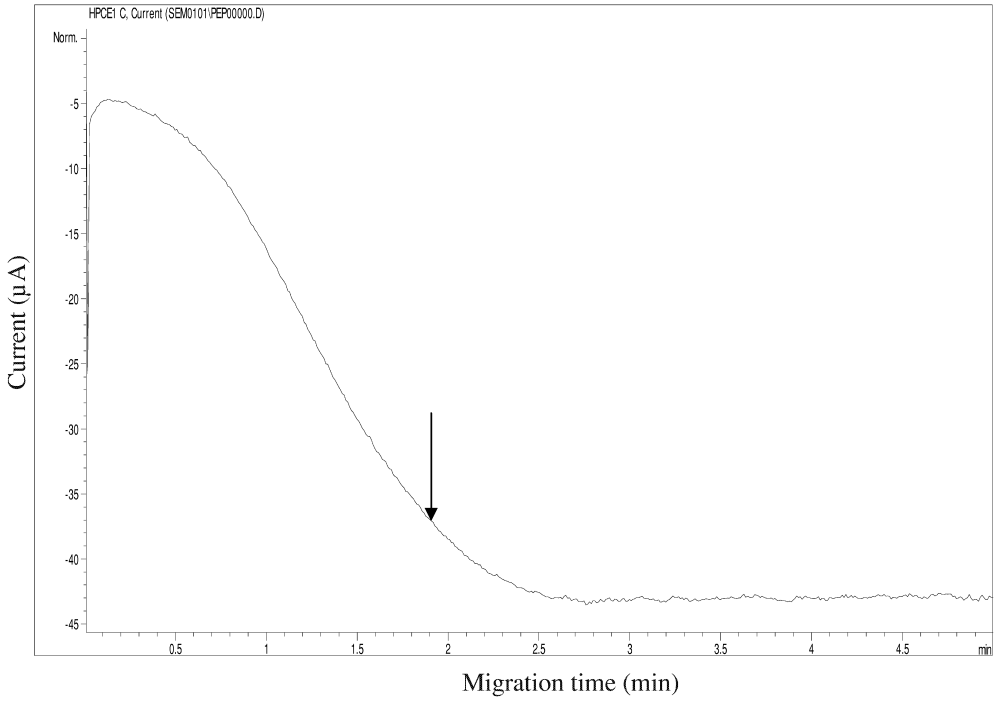

Fig. 3. Plot of the current during the reverse polarity phase at −23 kV after a 10 min injection at 50 mbar. In this case, after 2.5 min the current remains constant at −43 μA and the entire injected plug has flowed out of the capillary. The arrow indicates the time at which the reverse polarity is stopped and normal polarity is switch on to obtain the LVSS.

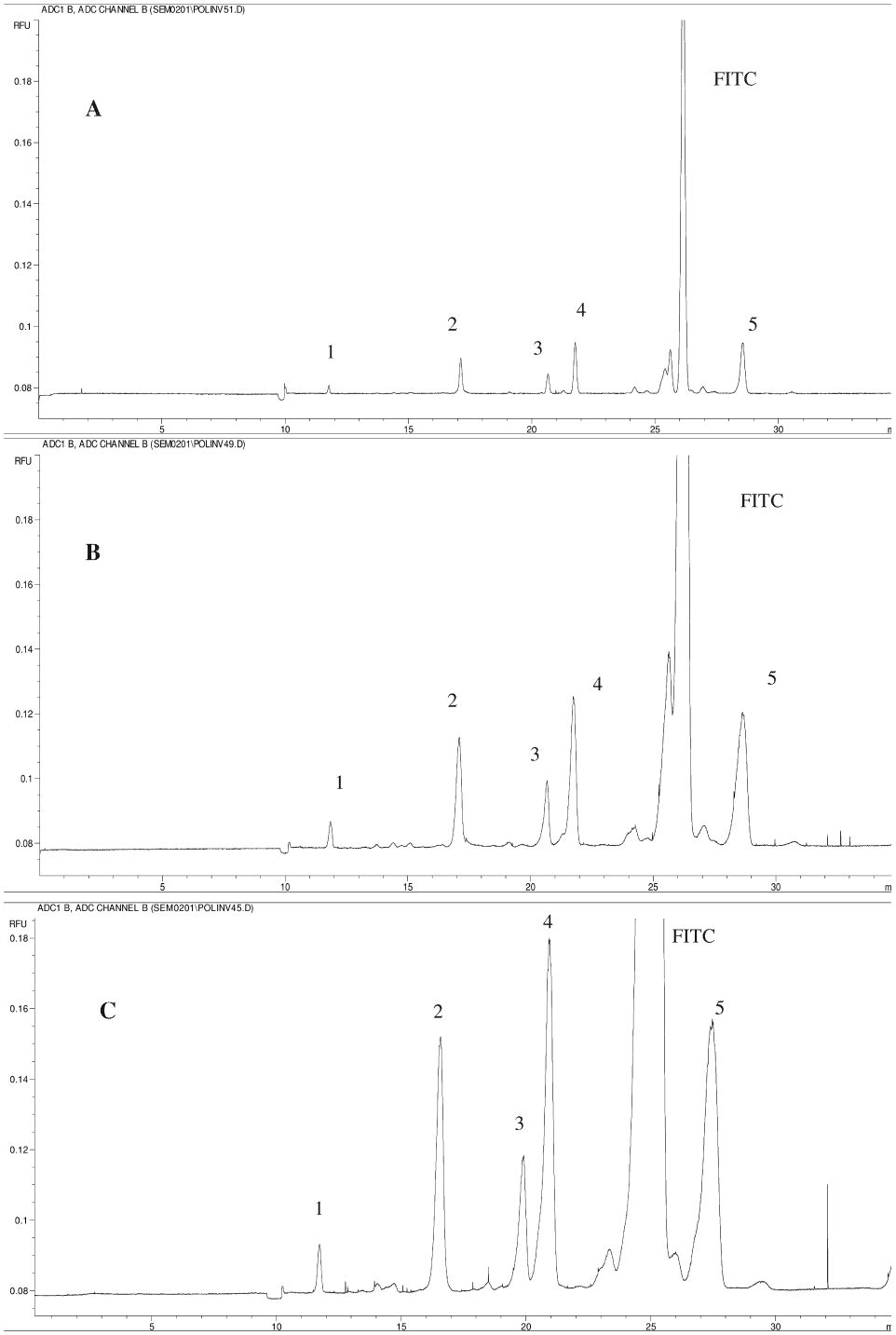

Fig. 4. Separation of bradykinin (1), angiotensin (2), substance P (3), leucine–enkephaline (4) and tryptophan–leucine dipeptide (5) at a concentration of 500 p*M* each diluted in water and for 1 min (A), 5 min (B) and 10 min (C) hydrodynamic injection according to the LVSS method. The separation conditions are shown in Fig. 2.

injection time of 10 min is the maximum time we tried because of the decreasing resolution between peaks. With a 10 min injection time the resolution between substance P and leucine–enkephalin is 0.95. This decreases to 0.75 at 11 min. Values for the peak height, width and migration time of the analysis following hydrodynamic injection are summarized in Table 1. This table shows that the migration times are only slightly affected by the stacking time. The peak width increases slightly when the injection time increases, whereas the peak height increases proportionally.

3.2. Quantitation results

Measurements of the concentrations of derivatized samples in the range from 500 to 10 p*M* are shown in Table 2 with the lowest calculated concentration detected for each peptide. For the experiments at 10 min injection, the coefficients of variation (C.V.) ($n=3$) for the surface area are in the range from 6.2% for Trp–Leu to 1.8% for bradykinin, which is acceptable for the quantitation of so small a concentration. For the same experiments, the C.V. values ($n=3$) for the migration times are in the range from 0.3% for bradykinin to 5.6% for Trp–Leu. The limits of detection ($S/N=3$) are in the range from 2 to 0.3 p*M*. To the best of our knowledge, the results obtained in this study are among the most sensitive for derivatized peptides detected by laser-induced fluorescence.

This study indicates that the detection of derivatized peptides in the picomolar range using an automated large-volume sample stacking procedure is possible. Fluorescein-based dyes are well adapted to LIF detection and peptide stacking. The next step in this study is to obtain a purer FITC to derivatize the samples at very low concentration. Dye purity is a major limiting factor for the successful derivatization of samples at very low concentrations.

References

[1] R.L. Chien, D.S. Burgi, Anal. Chem. 64 (1992) 1046.
[2] H.C. Vo, P.A. Henning, D.T. Leung, S.L. Sacks, J. Chromatogr. B 772 (2002) 291.
[3] S. Palmarsdottir, L. Mathiasson, J.A. Jonsson, L.E. Edholm, J. Chromatogr. B 688 (1997) 127.

Table 1
Signal-to-noise ratio, half-height peak width (in parentheses, min) and migration time (bold, min) of the labeled peptides following different injection times at $2.5\cdot10^{-10}$ *M* concentration (the coefficient of variation of the peak width was below 6.2 and 5.6% for the migration time, $n=3$)

Hydrodynamic injection time (min)	Bradykinin	Angiotensin	Substance P	L-Enkephalin	Trp–Leu peptide
1	9 (0.07) **11.8**	41 (0.11) **17.1**	23 (0.11) **20.6**	60 (0.17) **21.7**	59 (0.22) **28.6**
5	27 (0.14) **11.8**	147 (0.20) **17.1**	86 (0.19) **20.7**	176 (0.25) **21.8**	218 (0.45) **28.6**
10	46 (0.15) **11.7**	289 (0.17) **16.5**	181 (0.22) **19.7**	396 (0.25) **21.0**	345 (0.6) **27.4**

Table 2
Calibration curves for FITC-labeled peptides with a 10 min injection (quantification range 25–500 p*M*)

Peptide	Slope (RFU/n*M*) ($\cdot10^{9}$)	Intercept (RFU) ($\cdot10^{-3}$)	R^2	LOD (p*M*)
Substance P	2.3	0.2	0.9920	0.8
Angiotensin	3.1	191.6	0.9720	0.5
Bradykinin	2.4	16.7	0.9948	2
Trp–Leu peptide	10.1	48.4	0.9826	0.35
L-Enkephalin	5.0	50.8	0.9950	0.3

[4] B. Kim, D.S. Chung, Electrophoresis 23 (2002) 49.
[5] B.F. Liu, X.H. Zhong, Y.T. Lu, J. Chromatogr. A 945 (2002) 257.
[6] R.M. Latorre, J. Saurina, S. Hernandez-Cassou, Electrophoresis 22 (2001) 4355.
[7] J.H. Lee, O.K. Choi, H.S. Jung, K.R. Kim, D.S. Chung, Electrophoresis 21 (2000) 930.
[8] W.H. Ding, C.H. Liu, J. Chromatogr. A 929 (2001) 143.
[9] M.J. Cugat, F. Borrull, M. Calull, Analyst 126 (2001) 1312.
[10] J.P. Quirino, S. Terabe, Electrophoresis 21 (2000) 355.
[11] S. McClean, E. O'Kane, D.J. Coulter, S. McLean, W.F. Smyth, Electrophoresis 19 (1998) 11.
[12] P. Zhang, G. Xu, J. Xiong, Y. Zheng, O. Yang, F. Wei, Electrophoresis 22 (2001) 3567.
[13] M. Begona Barroso, A.P. de Jong, J. Capillary Electrophor. 5 (1998) 1.
[14] Z.K. Shihabi, M.E. Hinsdale, C.P. Cheng, Electrophoresis 22 (2001) 2351.
[15] Z.K. Shihabi, J. Chromatogr. A 744 (1996) 231.
[16] Z.K. Shihabi, M. Friedberg, J. Chromatogr. A 807 (1998) 129.
[17] N. Guzman, LC·GC 17 (1999) 7.
[18] A.J. Tomlinson, L.M. Benson, S. Jameson, S. Naylor, Electrophoresis 17 (1996) 1801.
[19] C. Schwer, F. Lottspeich, J. Chromatogr. 623 (1992) 345.
[20] E. Bonneil, K.C. Waldron, J. Chromatogr. B 736 (1999) 273.
[21] J.C. Waterval, G. Hommels, P. Bestebreurtje, C. Versluis, A.J. Heck, A. Bult, H. Lingeman, W.J. Underberg, Electrophoresis 22 (2001) 2709.
[22] S.J. Wang, W.L. Tseng, Y.W. Lin, H.T. Chang, J. Chromatogr. A 979 (2002) 261.