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Journal of Chromatography A, 979 (2002) 69–80

JOURNAL OF CHROMATOGRAPHY A

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Sample pre-concentration by isotachophoresis in microfluidic devices

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Abstract

We have designed microfluidic devices with the aim of coupling isotachophoresis (ITP) with zone electrophoresis (ZE) as a method to increase the concentration limit of detection in microfluidic devices. We used plastic multi-channel chips, designed with long sample injection channel segments, to increase the sample loading. The chip was designed to allow stacking of the sample into a narrow band by discontinuous ITP buffers and subsequent separation in the ZE mode. In the ITP–ZE mode, with a 2-cm long sample injection plug, sensitivity was increased by 400-fold over chip ZE and we found that the separation performance after the ITP stacking was comparable to that of regular chip ZE. We report sub-picomolar limits of detection of fluorescently labeled ACLARA eTag reporter molecules electrokinetically injected from cell lysate sample matrixes containing moderate salt concentrations. We evaluated sample injections from buffers with varied ionic strengths and found that efficient stacking and separations were obtained in both low and high conductivity buffers, including physiological buffer with at least 140 m*M* salt. We applied ITP–ZE to the analysis of a cell surface protease (ADAM 17) which used live intact cells in physiological buffers with detection limits below 10 cells/assay.

Keywords: Isotachophoresis–zone electrophoresis; Microfluidics; Sample handling; Peptides

1. Introduction

The use of microfluidic devices for electrophoresis has grown rapidly due to the promising potential of this analysis technology [1–9]. Recent reviews [10–13] emphasize the broad range of analytical operations that can be accomplished within microfluidic structures, such as sample metering, sample mixing, chemical reactions, sample cleanup and sample pre-concentration. The dimensions of micro-channel devices impact the sensitivity of optical detection and it is necessary to increase sample loading capacity to maintain or improve detection sensitivity without loss of peak resolution. Several methods to perform pre-concentration or stacking of the sample in microfluidic devices prior to separation have been reported. Poly(methyl methacrylate) (PMMA) microchips provided with two separation channels in column coupling-configuration, equipped with on-column detection sensors has been previously used for both isotachophoresis (ITP) and ITP–capillary zone electrophoresis (CZE) separations [14–16]. Detection of micro constituents in the complex sample matrixes was achieved by sample pre-concentration by ITP combined with sample clean up by selective injection of the sample into the second separation channel [14–16] as shown for food addi-

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01246-3

tives and waste water analysis. Gated injection [17] by voltage control while utilizing the electroosmotic flow (EOF) in a two step process induces a stacked injection plug or sample stacking can be accomplished in an injection stream of by electrokinetic focusing [18]. Integration of a porous membrane structure [19] into the microfluidic device allowed DNA fragments to be concentrated up to two orders of magnitude. Sample injection from a low conductivity buffer into a high conductivity buffer followed by static sample stacking [20] increased the signal roughly a factor of 100. Field amplification stacking (FAS) and matrix removal by polarity switching prior to separation showed signal enhancement of up to 65-fold [21] and sub-n*M* detection [22]. Transient isotachophoresis (ITP) pre-concentration steps were tested for separation and analysis by mass spectrometry [23]. An integrated sample pre-concentrator for DNA samples using a thin-film electrode at the injection T, which increased the sensitivity of detection by 5-fold [24], has been reported. Detection from isotachophoretic separations on microchips by Raman spectroscopy using directly ITP concentrated samples has also been reported [25].

Recent reviews [26–32] point to frequently used methods to increase the sample loading capacity and thus detection sensitivity in capillary electrophoresis (CE) such as FAS [33–35], pH mediated stacking [36–38] and transient ITP. These methods lend themselves well to the standard capillary format with sequential injections of different buffers. Most reports on ITP pre-concentration utilize coupled capillaries as first proposed by Everaerts et al. [39] and later shown operated in an automated form [40–43]. The use of coupled capillaries take full advantage of the discontinuous buffers used for ITP and the subsequent introduction of the stacked sample into a homogeneous buffer system for separation.

We are developing plastic microfluidic devices for high-throughput genomic and proteomic analysis and drug screening [44–47]. Such a device, the LabCard, can contain many individual separation elements on one card [44,46]. The present microfabrication technology allows easy fabrication of complex patterns of interconnecting channels with densely packed patterns. These patterns can be inexpensively replicated from masters by injection molding. We have applied the concept of ITP pre-concentration, as it has been performed in coupled capillaries, taking advantage of microfluidic replication and fabrication technology to make simple and easy to operate devices.

In many instances biological assay samples contain salts and other ionic compounds, which need to be removed in order to achieve the sensitivity and resolution needed from the electrophoretic separation. Such sample pretreatments are unattractive because it adds time and cost and, in some cases, increases the risk of sample cross contamination. Pre-concentration of samples in high salt prior to CE has been implemented for instance by pH mediated stacking [37,38] many other stacking methods rely on samples in low conductivity buffers [17,20]. In this report we evaluate the performance of ITP coupled with ZE for the analysis of sample in high salt biological buffers.

We have focused on the application of ITP–ZE to the separation of electrophoretic reporter molecules known as eTag reporters, currently under development at ACLARA BioSciences. The eTag reporter technology enables solution-phase, multiplexed assays for gene expression, protein expression, receptor and enzyme profiling, with the same biological sample, directly from cell lysates. eTag reporters are fluorescent labels with unique and well-defined electrophoretic mobilities; each label is coupled to biological or chemical probes via cleavable linkages. When an eTag reporter-labeled probe binds to its target, the coupling linkage is cleaved and the eTag is released. Since each eTag reporter has a well defined electrophoretic mobility, ITP pre-concentration methodologies can be standardized and similar separation protocols can be applied to the separation of eTag reporters generated from protein or nucleic acid assays.

The object of this investigation was to increase the detection sensitivity obtained by CE on plastic microfluidic devices. We therefore designed and evaluated microfluidic devices where large sample plugs could be injected which could then be pre-concentrated by ITP and subsequently separated by ZE. The object was also to design a device that was easy to operate as for example by automated voltage control of the different analysis steps. Since the sample buffer composition (ionic strength) can great-

ly influence the separation sensitivity and resolution in CE we evaluated sample buffers of different ionic strengths. We applied the ITP–ZE separation mode to two types of biological assays which were performed directly from cell lysates. The first assay requires moderate ionic strength in the assay mixtures (12 m*M* $MgCl_2$) for multiplexed assays for gene expression based on the eTag technology, the other assay was a live cell assay in a physiological buffer (>140 m*M* NaCl) of a cell surface protease (ADAM 17) based on the detection of the peptide substrate turnover.

2. Experimental

2.1. Instrumentation

The instrumentation used comprised a confocal microscope system (Nikon Eclipse TE300; Melville, NY, USA) equipped with an Ar^+ laser (JDS Uniphase, San Jose, CA, USA) for excitation. The fluorescence signal was collected with a 10× long microscope objective (NA=0.55) and imaged through a 0.5-mm pinhole onto the photomultiplier tube. A dichroic cube with 460–490 nm excitation filter and 520–550 nm emission filter was used for the optical setup in analyzing fluorescein-based samples. Data acquisition and electronics control for the instrument was accomplished on a Model 2837 combination A/D digital I/O board and a Model 2815 D/A board with LabView software (National Instruments, Austin, TX, USA) on a Compaq personal computer, the data collection rate was 100 Hz with the cutoff frequency set at 10 Hz. A laboratory-built power supply with independently controlled voltages (0–5 kV) operated the injection, stacking and separation processes.

2.2. The LabCard

LabCard microfluidic devices of two different designs, designated chip A and B were used for ITP–ZE (Figure1). The ITP stacking step of the sample uses a discontinuous buffer system, consisting of a leading electrolyte (LE) and a terminating electrolyte (TE). The sample is introduced as a plug between these electrolytes. In chip A, voltages

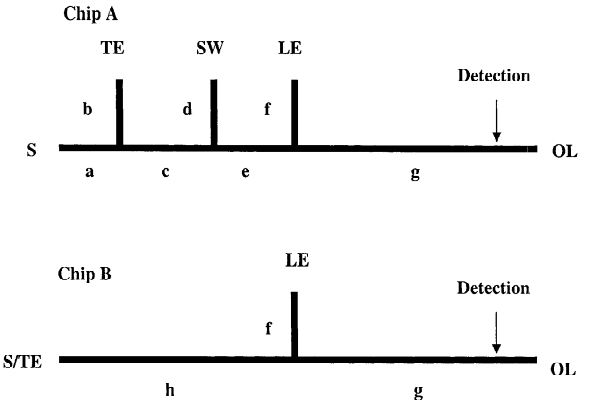

Fig. 1. Schematic drawings of a single separation element on a LabCard designed for ITP–ZE. Chip A: Channel segments are denoted a–g. Buffer wells are: sample (S), terminating electrolyte (TE), sample waste (SW), leading electrolyte (LE) and outlet (OL). The channels and wells SW, LE and OL are filled with LE buffer; S and TE are filled with sample and TE buffer, respectively. The analysis steps, accomplished by voltage application to electrodes in the wells, are as follows: (a) sample injection—S at ground, SW at high voltage (hv); (b) TE migration to sample—TE at ground, S at hv; (c) stacking—TE at ground, OL at hv; (d) separation—LE at ground, OL at hv. Chip B: Channels and wells are filled with LE buffer and sample is placed in S/TE. Sample is injected into channel segment h by applied voltage from S/TE to OL, then sample is manually replaced with TE buffer. Voltage is applied from S/TE to OL until the stacked sample migrates past channel segment f, voltage is then applied from LE to OL. The electrodes not in use float.

required for the sample injection, TE buffer migration, stacking and separation were automatically sequentially applied. The sample injection plug, segment c, was 2 cm and the detection point was 5 cm from the LE electrolyte channel unless otherwise noted. Channel segments a, b, d, and f varied in length from 5 to 15 mm. The chip B design used manual replacement of sample by the TE buffer in well S/TE (Figure 1, chip B). Chip B had an injection/stacking channel (h) of 16 mm and a separation channel (g) of 47 mm. Comparative separations in ZE mode were performed on LabCards designed with a channel opposite channel f (Figure 1, chip B) and injecting sample in standard ZE chip operating mode (42,44). In the comparative experiments, the ZE chips had the same cross-sectional areas as the ITP–ZE chips. The channel cross sections were 30 μm deep×80 μm wide or 50 μm deep and 120 μm wide. Electrolyte wells were 3 mm

in diameter and 1.5 mm deep. The LabCards were injection molded PMMA and bonded with a PMMA cover film as previously described [41,42]. The movable microscope stage allowed detection on any point on the LabCard.

2.3. Separation conditions

Chemicals for the separation and sample buffers were from Sigma (St. Louis, MO, USA) unless noted and all the buffers were prepared with Milli-Q water (18.2 MΩ/cm).

The ITP–ZE separations were done with a leading electrolyte (LE) of 20 m*M* HCl–25 m*M* imidazole with 1% poly(ethylene oxide) (PEO) (M_r 600 000, Aldrich, Milwaukee, WI, USA), pH 6.5. The PEO was added to increase the viscosity of the buffer to counteract hydrostatic flow and reduce EOF by coating the channel walls. The TE was 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–10 m*M* imidazole, pH 6.7 or 40 m*M* HEPES–160 m*M* imidazole, pH 7.7. Control experiments in the ZE mode were done in the LE buffer. The LabCard channels and wells were initially filled with LE buffer. The LE buffer was thereafter replaced by sample and TE buffer in the assigned wells (Fig. 1). The voltages applied in the ITP–ZE separations has one common feature, only one electrode was at ''high'' voltage and only one electrode was set to ground at each process step, while the other electrodes were floating. The field strengths varied from 300 to 700 V/cm depending on which chip was used. Control experiments in ZE mode used the standard injection scheme with sample ''pullback'' with voltages applied to four electrodes simultaneously. The buffer and sample volumes in the wells were 8–10 μl. Unless otherwise noted the recorded analysis time include sample injection, stacking and separation.

2.4. Samples and sample buffers

The eTags reporter molecules were synthesized in the laboratory, all the reporters were fluorescein labeled. The ACLARA eTag reporter synthesis strategy was to generate sets of eTag reporters separable by electrophoresis for multiplexed sample detection. Sample A contained 13 eTag reporters (ACLA 163, 156, 33, 26, 25, 174, 1, 187, 188, 189, 190,191 and 192) and sample B contained five eTag reporters (ACLA 33, 26, 174, 1 and 187). The mobilities of the eTag reporters are listed in Table 1.

The ITP–ZE separations were performed in the following samples buffers with eTag reporters to evaluate detection sensitivity and separation resolution in both low and high ionic strength buffers:

(1) eTag assay buffer (10 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS)–12.5 m*M* $MgCl_2$–0.05% Triton X-100)

(2) eTag assay buffer, diluted 1:10 (1 m*M* MOPS–1.25 m*M* $MgCl_2$–0.005% Triton X100)

(3) Formamide–H_2O (60:40, v/v) buffer

(4) Hanks' buffer with 0.1% BSA (138 m*M* NaCl, 5.3 m*M* KCl, 5.6 m*M* glucose, 4 m*M* $NaHCO_3$, 1.3 m*M* $CaCl_2$ with other salts less than 1 m*M*) (GIBCO/BRL/Life Sciences, Rockville, MD, USA)

In addition eTag reporters were spiked into mock reaction mixtures consisting of lysed cells and eTag assay reagents.

2.5. Reagents for the live cell ADAM-17 assay

THP-1 cells (human monocytic cells) were grown in the laboratory using standard procedures. Just prior to use, the cells were collected by centrifugation, washed and counted on a hemacytometer. A dilution series of the cells (1–1000 cells/10 μl) was

Table 1
Mobility range of ACLARA eTag reporters measured in the leading electrolyte buffer at pH 6.5

eTag reporter	Mobility (10^5 cm^2/V/s)
ACLA 163	34.0
ACLA 156	33.1
ACLA 33	32.0
ACLA 26	29.1
ACLA 25	27.8
ACLA 174	26.5
ACLA 1	23.9
ACLA 187	21.5
ACLA 188	19.1
ACLA 189	17.5
ACLA 190	16.4
ACLA 191	15.5
ACLA 192	14.9

performed on ice with Hanks' buffer supplemented with 10 m*M* HEPES, 1% BSA and serine and thiol protease inhibitors (Complete-EDTA free antiprotease cocktail tablets, Roche Diagnostics). A fluorescently labeled ADAM-17 specific peptide substrate (5 μ*M*) was then added to the cells, again on ice. Incubation proceeded at 37 °C for 1 h in a water bath, whereupon the samples were returned to ice to stop the reaction. The samples, containing all reagents including the cells, were analyzed directly without any pre-treatments by ITP–ZE.

2.6. Data processing and calculations

The Ceasar computer program (Analytical Devices, Alameda, CA, USA) was used to display the collected electrophoregrams. The DAx software (Data Acquisition and Data Analysis by Van Mierlo Software Consultancy, http://www.dax.nl) was used to calculate the peak heights to compare signal intensities under different separation condition (but with the same detector settings) and to determine signal/noise (*S*/*N*) to define limits of detection (LODs). Calculations of the terminating ion mobilities in the TE buffers (Fig. 7) used the Peakmaster software (http://www.natur.cuni.cz/~gas) by Bob Gas and Michal Janos, the mathematical model of this free software is described in Ref. [48].

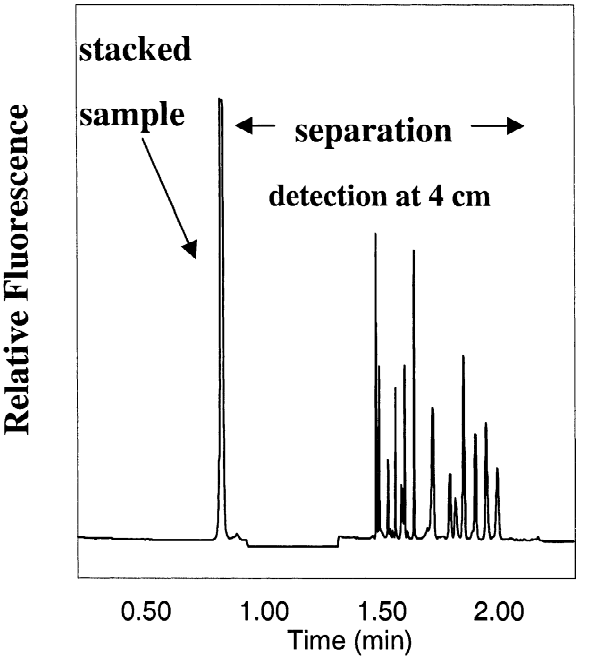

Fig. 2. Detection of the ITP stacked sample and separation by ZE of the same sample in chip A (Fig. 1). The stacking was detected in channel segment e at the intersection of channel section f and in channel segment g. The separation was detected in channel segment g, 4 cm from the intersection of channel section f. The injected sample plug was 2 cm (channel segment c). Sample: 13 eTag reporters mix in eTag assay buffer.

3. Results and discussion

3.1. Comparison of the separation of eTags reporters by ITP–ZE and ZE: ITP–ZE buffer optimization

Initial stacking conditions were established for the negatively charged eTag reporters, listed in Table 1, with chloride as the leading ion and HEPES as the terminating ion. Imidazole was the buffering counter-ion in both the LE and TE buffers. The pH for the leading electrolyte was 6.5 and the terminating electrolyte was 6.7. Sample stacking and subsequent sample separation (Fig. 2) is shown in one single run by detecting the same sample in two different locations on the chip A (Fig. 1). Sample concentrations ranged from 200 to 400 p*M* for the individual eTag reporters and the sample contained 13 different eTag reporters. The experiment confirmed that the LE and TE buffers did adequately stack the sample components and that the subsequent switch to the LE buffer did de-stack and separate the sample components. The non fluorescent sample anionic buffer components also stacked during ITP, thus the chloride ions in the sample migrate ahead of the eTag reporters, efficiently desalting the sample. Stacking of the eTag reporter samples was achieved with MOPS in the sample buffer indicating that the MOPS anions stacked after the eTag reporters or migrates in the terminating electrolyte buffer.

To further evaluate the ITP–ZE separation mode it was compared directly to standard ZE methods of separations in microfluidic devices with channel configuration as previously described [45,46]. The sample, containing the 13 eTag reporter molecules in both cases diluted into the same buffer, was injected in a 50-fold lower concentration in the ITP–ZE

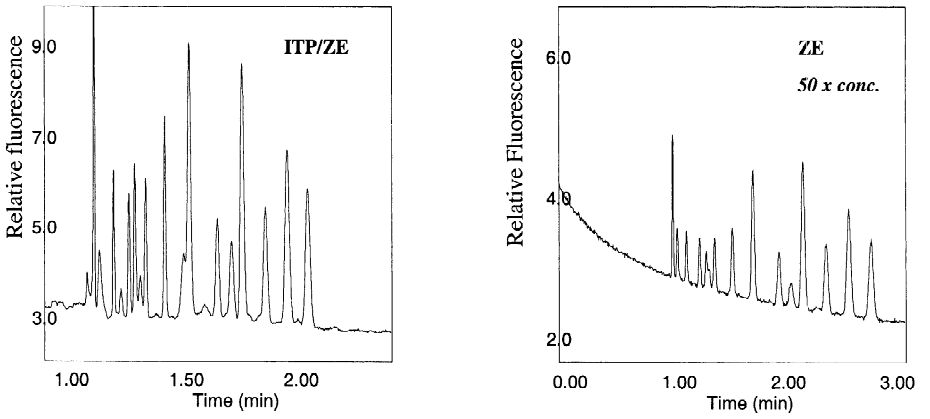

Fig. 3. Comparison of signal response in ITP–ZE and ZE. Separation of 13 eTag reporters with the same detector setting and separation channel dimensions. The concentration of injected sample for ITP–ZE was 40–80 p*M* and for ZE 2–4 n*M*, both samples were diluted in the eTag assay buffer. Estimated sample plug length of the eTag reporters in chip B was 3–9 mm depending on analyte mobility. Both separations were detected at 4 cm from the stacking or injection point, respectively.

separation mode (Fig. 3). The average calculated signal, based on peak height measurements, was 100-fold enhanced in the ITP–ZE separation compared with ZE. The estimated sample injection plug lengths in ITP/CE mode on chip B were 3–9 mm, with 3 mm for lowest mobility eTag reporter A192 and with 9 mm for the highest mobility eTag reporter A163. The ITP–ZE separation on the chip B design exhibits the same mobility injection bias as regular electrophoretic injection in CE [49], reflecting moving boundary migration of the sample constituent [50], whereas the injection from steady sample concentrations in the injection channel in chip ZE has a much smaller injection bias [51,52]. Thus signal enhancement for the ITP–ZE separation chip B design favored the fastest eTag reporter molecules. A good correlation of migration times between the individual eTag reporters was obtained for the two separation modes (Fig. 4), confirming that the on line ITP stacking process did not compromise the ZE separation.

To further optimize the ITP–ZE separation we evaluated different TE buffers. We noticed that the migration time to a fixed detection point during the stacking phase could vary considerably with the same terminating ion (HEPES) depending of the pH of the TE buffer. A higher pH resulted in a more rapid stacking of the sample plug. We also investigated other terminating ions as TE buffers of different pH values, buffered by an imidazole counter-ion. The average velocity of the sample plug during the stacking phase of the separation was plotted versus the calculated mobilities of the anionic TE ions (Fig. 5). Thus the average velocity of the sample plug during the stacking phase was dominated by the composition of the TE buffer (and thus pH) and not by the steady state condition in ITP. We are attributing the varied average velocities to a fixed stacking point to that initial stacking phase before the ITP equilibrium has been reached. In the steady state condition in ITP, the terminating ion concentration and the pH in the capillary is regulated by the mobility, concentration and pH of the LE [50]. Thus at steady state the mobility of the terminating ions do not depend on the TE buffer pH, whereas our data indicated TE buffer pH dependence. We found that is was an advantage to use a possibly fast stacking process to increase the resolution of the separation. A separation with HEPES as the terminating ion in the TE buffer at pH 7.7 is shown as an example of a separation with the optimized TE buffer (Fig. 6).

A final comparison of detection sensitivity of ITP–ZE and ZE was performed on the chip A (Fig. 1) with a 2-cm long injection plug (Fig. 7). The ITP stacking started after the steady state sample concentration had been reached in the injection channel. The optimal TE buffer (pH 7.7) was used for the ITP stacking. The ZE separation was performed in the

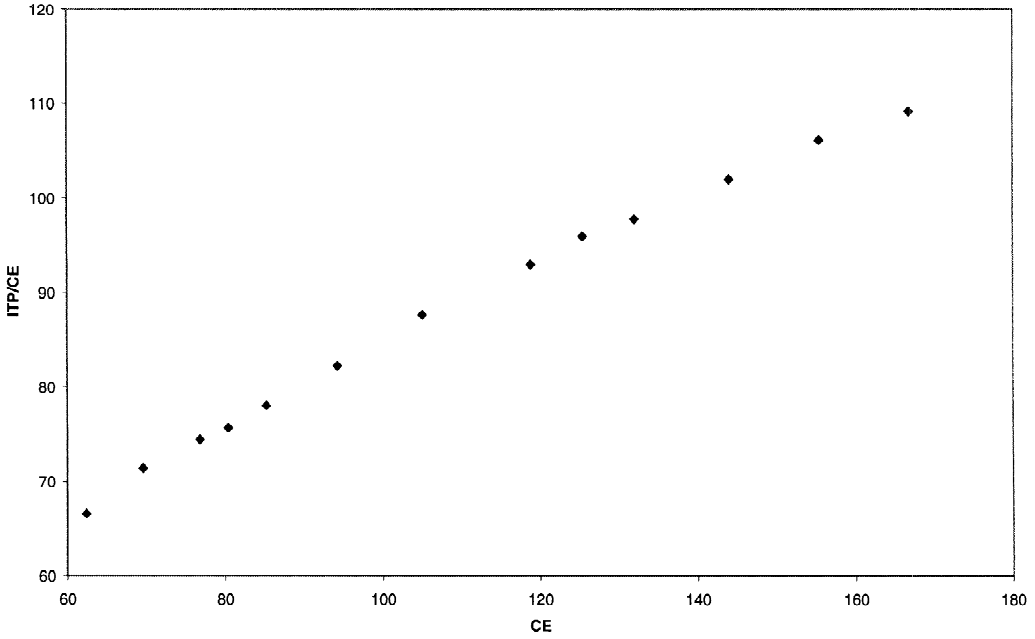

Fig. 4. Separation time (s) in ITP–ZE versus ZE for eTag reporters (A163–A192).

LE buffer with channel configurations as previously described [45,46]. A comparison of peak heights showed an average sensitivity increase of 400-fold by ITP–ZE compared to ZE. The injection mode in chip A reduced the electrophoretic injection bias seen on chip B and the signal sensitivity increase was independent of the eTag mobility (Table 1). The resolution between peaks (at half height) was marginally higher for the ZE separation (Table 2).

3.2. Sample buffer composition

Since ACLARA is developing multiplexed assays

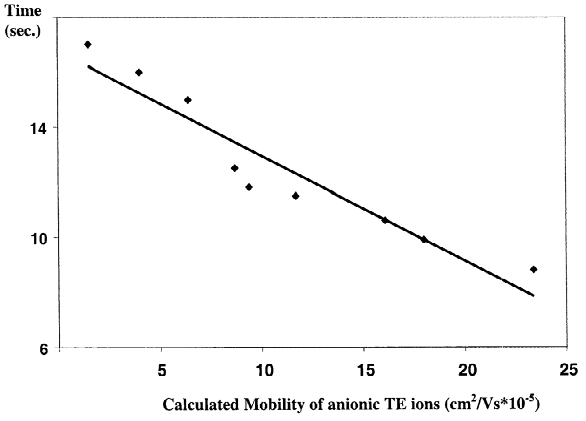

Fig. 5. Migration time of the sample plug to reach the intersection of channel segments f and h of in chip B (Fig. 1) as function of the calculated mobility of the terminating ion in the TE buffer. Terminating ions were HEPES, MOPS and ACES with buffer pH adjusted with imidazole.

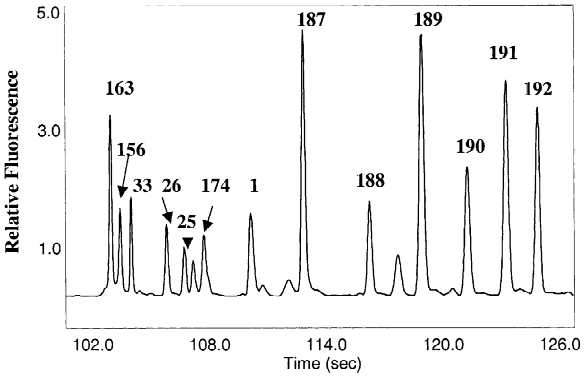

Fig. 6. ITP–ZE separation of the 13 eTags reporters in eTag assay buffer with the optimized TE buffer (pH 7.7) in chip A. The sample injection plug was 2 cm and the combined stacking and separation distance was 5 cm.

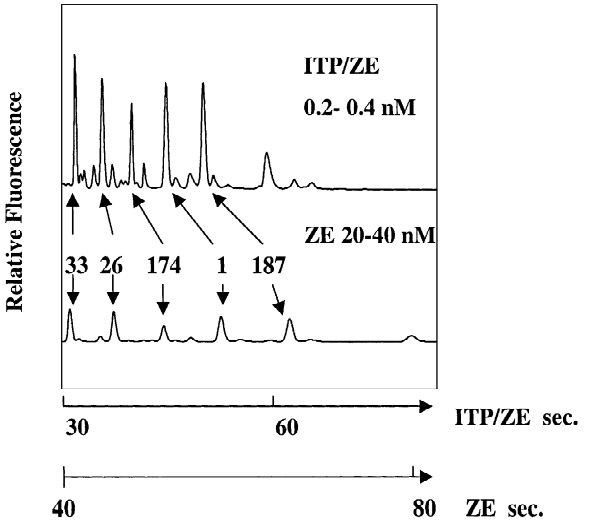

Fig. 7. Comparison of the signal response, at the same detector setting, from ITP–ZE and ZE. The ITP–ZE injection plug was 2 cm; in ZE the sample was injected from a 80-μm wide channel. Both channels had a depth of 30 μm. Both separations were detected at 4 cm from the stacking or injection point, respectively. In order to compare peak heights for the two separations in the same graph different time scales were used to represent for the two separations.

for gene expression, the ITP–ZE separation mode was first evaluated with samples compatible with these assays, that is the eTag gene expression assay buffer where the main salt is $MgCl_2$ at 12.5 m*M*. We also evaluated other sample buffers and their impact on sensitivity and resolution in the ITP–ZE separation mode. Frequently documented in CE literature is the electrophoretic sample injection's dependence of the conductivity of both the sample buffer and the electrophoresis buffer as has been theoretically explained [49,53]. In the condition that apply for the ITP–ZE (that is no EOF), the amount of a electrophoreticly injected analyte is inversely proportional to the sample buffer conductivity [49]. In the first set of samples the comparison was between the eTag assay buffer and a 60% formamide buffer which had a low conductivity compared to the eTag assay buffer (Fig. 8A). The data indicated that a 10-fold sensitivity increase could be obtained in this low conductivity formamide buffer at comparable resolution. The next sensitivity comparison was between the high ionic strength Hanks' buffer and the eTag assay buffer (Fig. 8B). There was a clear loss of detection sensitivity in the high ionic strength sample buffer compared to the eTag assay buffer, however the resolution was maintained or even improved. In separate experiments the LOD ($S/N=3$) in Hank's buffer was determined to be 3–4 p*M* for a fluorescein derivative. The injection phase of the samples are shown in Fig. 9A. In the eTag assay buffer there was some initial stacking of the sample during injection, whereas with the high conductivity sample no such stacking occurred. For low conductivity sample buffers the stacking is even more pronounced than with the eTag assay buffer. By proper timing of the stack migration, the second ITP step could be started when the sample has its maximal concentration thus enhancing the detection limit further. In this investigation the sample has always been injected from the steady state concentration marked with an arrow in Fig. 9A. With identical separation conditions but with an analyte dissolved in different conductivity buffers, the signal (as peak height) from the analyte (ACLA 189) was measured and plotted against the inversed buffer conductivity (Fig. 9B). This data verifies the predicted injection efficiency of sample related to the conductivity of the sample buffers.

Table 2
Resolution at half height of ITP–ZE and ZE for various eTag reporters

eTag reporter	Signal enhancement	Resolution at half height	
		ITP–ZE	ZE
ACLA 33	430	5.4	5.8
ACLA 26	250	5.6	5.9
ACLA 174	530	6.1	5.9
ACLA 1	420	5.2	6.2
ACLA 187	430		

ITP–ZE separations of several eTag reporter samples in the low p*M* range were used to determine the LOD for the eTag reporters in their assay buffer. As an example of such a separation, eTag reporters of known concentration (5–7 p*M*) are shown in Fig. 10. At these detection levels the purity of buffer reagents can be critical, specially since the ITP stacking step will not only stack the sample components but also other ionic species of appropriate mobilities in the LE, TE or sample buffers. We have used commercially available buffer reagent without further purification and been able to perform sepa-

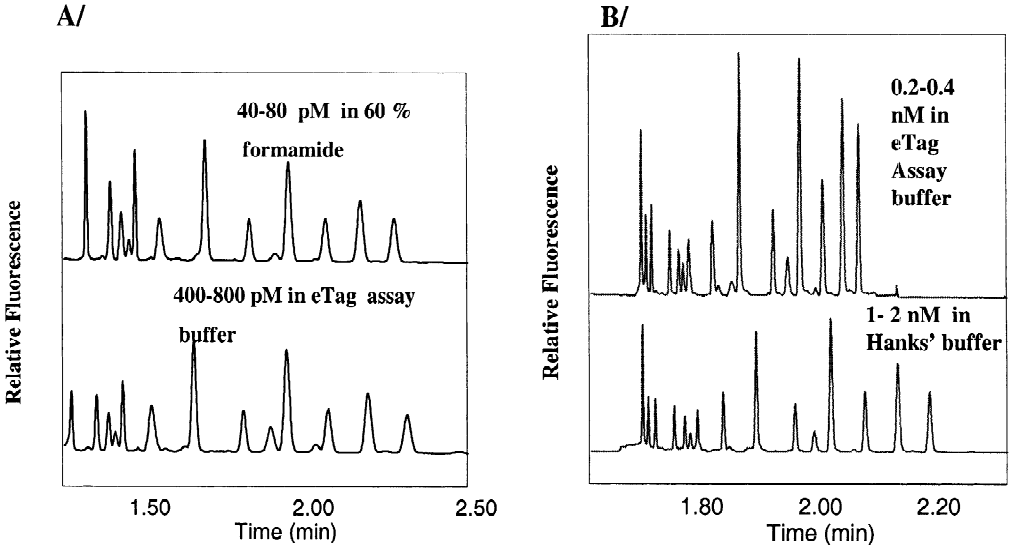

Fig. 8. ITP–ZE of 13 eTag reporters (A163–A192) diluted into different buffers: (A) on chip B: 40–80 p*M* in 60% formamide (top) and 400–800 p*M* in eTag assay buffer (below); (B) on chip A: in eTag assay buffer (top) and Hanks' buffer (below). The injection plug length was 2 cm and the separation distance was 5 cm.

rations in the low p*M* range. From the electrophoresis buffers or the sample buffer only one fluorescent component, present in low p*M* concentrations, was stacked by ITP as seen both in the buffer blank and the sample. We have not been able to remove this impurity, however the impurity peak was pH sensitive and could thus be manipulated by buffer pH changes. We determined the LOD (with

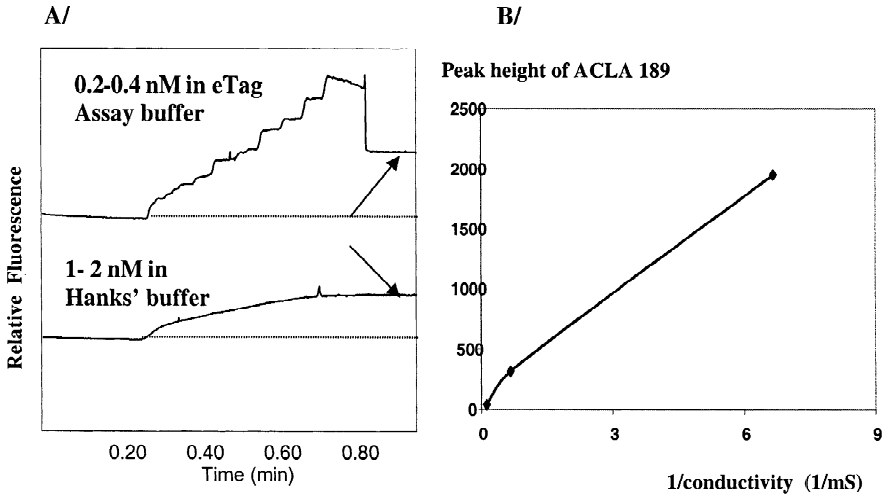

Fig. 9. (A) Injection phase of eTag reporters dissolved in different buffers detected in channel segment c of chip A. Sample in eTag assay buffer (top) and Hanks' buffer (below). Arrows indicates relative injection concentrations. (B) Injection efficiency in different sample buffers as related to the peak height of ACLA 189 plotted against inverse of the sample buffer conductivity. Buffer 1: eTag assay buffer; 2: eTag assay buffer, diluted 1:10; 4: Hanks' buffer.

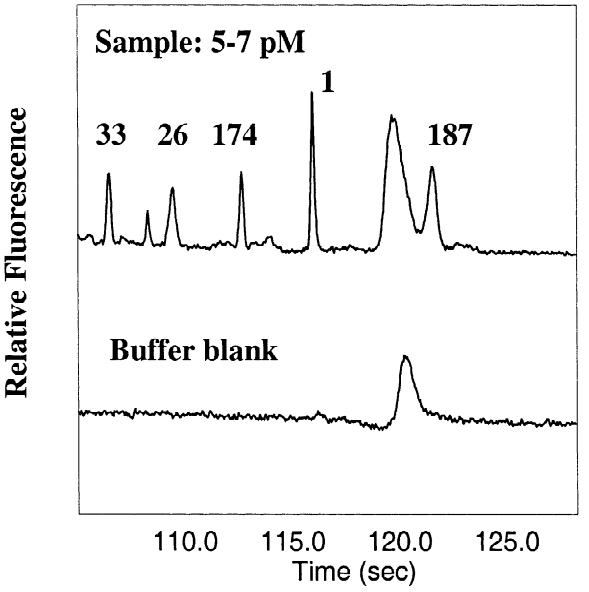

Fig. 10. Separation of eTag reporters in low p*M* concentrations in eTag assay buffer by ITP–ZE with a 2-cm sample injection plug. LOD is 0.4 p*M* ($S/N=3$).

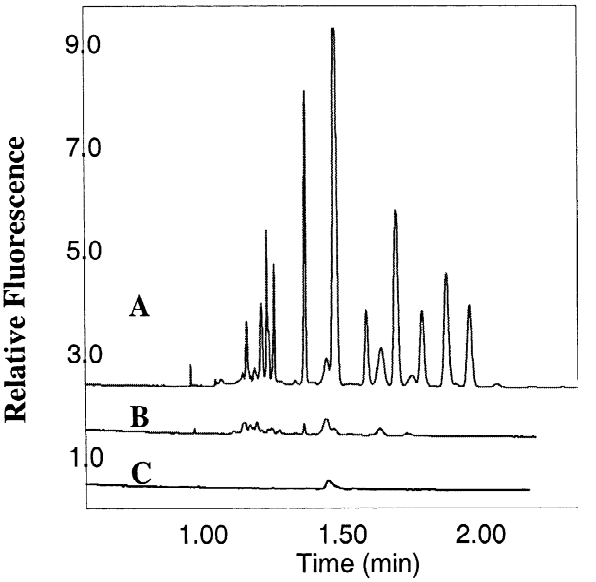

Fig. 11. ITP–ZE in cell lysate samples: (A) eTags reporters in cell lysate 200–400 p*M*. (B) Cell lysate blank (with assay reagents). (C) Buffer blank (eTag assay buffer).

$S/N=3$) to be 0.4 p*M* for eTag reporters in the eTag assay buffer.

3.3. ITP–ZE applied to cell based assays

The feasibility of multiplexed gene expression assay without any sample pre-treatment was evaluated by adding the 13 eTag reporters mixture to lysed cell samples which contained all reagent used in a multiplexed gene expression assay. This sample was analysed and compared to the cell lysate control and to the assay buffer blank (Fig. 11). All eTags were resolved in the cell lysis buffer and for samples in the high p*M* (200–400) range the background was negligible. This data also indicated that sensitivities comparable to those obtained for eTags in their assay buffer can be achieved for the low mobility eTags since there are no background peaks in that mobility area.

The next assay was developed to measure protein activity in live cells with the ultimate goal of being able to develop methods to analyse protein activity in single cells. As an example of live cell analysis we have evaluated an assay for a cell surface protease (ADAM 17) in the ITP–ZE format (Fig. 12). The enzymatic assay is based on the cleavage of the fluorescently labeled peptide substrate. The substrate itself can not be detected, however the cleaved fluorescently labeled peptide is negatively charged and can be detected by ITP–ZE. Samples with whole live cells in a physiological buffer, incubated with the substrate, were analyzed without any pre-treatment by ITP–ZE. The cleaved peptide product was easily detected and there was a linear signal response with cell concentrations (Fig. 12). The extra peaks in the electropherograms were attributed to impurities mostly in the substrate peptide, which was added to each sample to 5 μ*M* final concentration. The extended view of the samples with low cell concentrations (Fig. 12b) including a control of known concentration showed clearly the detect ability of this assay down to less than 10 cells/μl corresponding to high p*M* detection of an enzymatic product.

4. Conclusion

We have designed microfluidic devices which were amenable to ITP–ZE separations and evaluated these devices for the purpose of increasing concentration limit of detection over what is obtainable

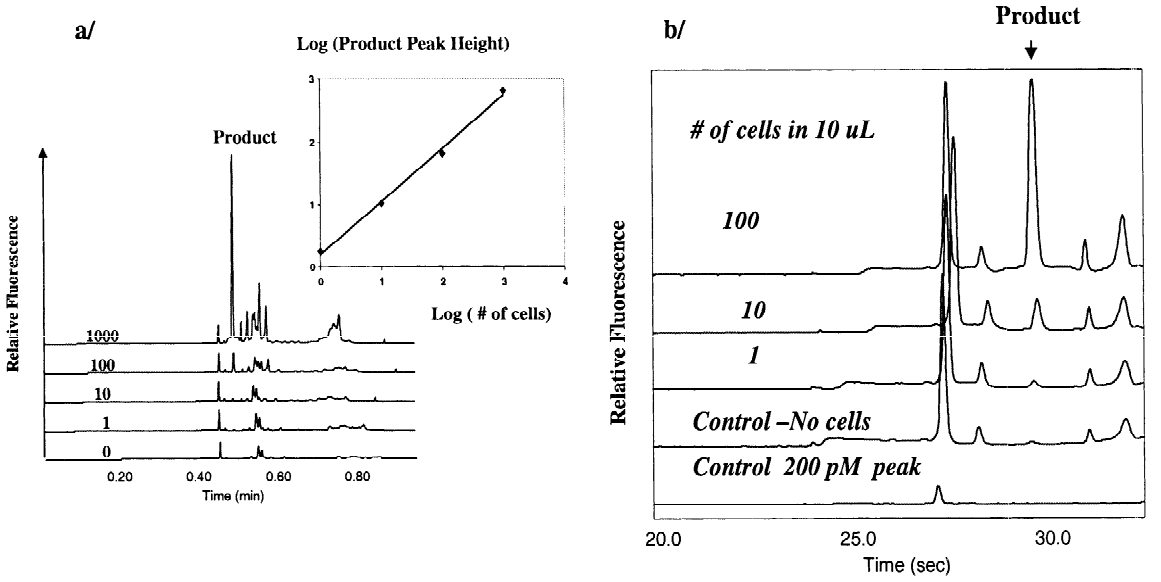

Fig. 12. Detection of peptide cleavage by a cell surface protease (ADAM 17) by ITP–ZE in chip A. Separations show the peptide product as function of cell concentration (number of cells in 10 μl). Samples were injected directly from physiological buffers (Hanks' buffer). The separation started at 0 s. (b) Enlarged view of the low cell concentration samples.

by ZE alone. By injection of a long sample plug of up to 2 cm and stacking this sample plug into a narrow band by ITP discontinuous buffers the limit of detection was increased by 400-fold over ZE. We found that the separation performance after the ITP stacking was comparable to that of regular chip ZE. The control of voltages could be simplified since each step (injection, TE buffer introduction, stacking and separation) only required that one high voltage be applied in each step with one electrode at ground and the other electrodes floating. The different steps can be performed by switching the voltages between the electrodes.

We evaluated sample injections from buffers with varied ionic strengths and found that efficient stacking and separations was obtained in both low and high conductivity buffer, including physiological buffer with at least 140 m*M* salt. The LOD was dependent of the sample buffer ionic strength. The LOD for eTag reporter molecules was 0.4 p*M* measured in the eTag assay buffer (12.5 m*M* $MgCl_2$–10 m*M* MOPS) with a 2-cm long injection segment. The estimated LOD for a low conductivity buffer such as a10-fold diluted eTag assay buffer is 0.04 pM.

We can conclude that the stacking process by ITP was compatible with cell based assays such as those with lysed cells in multiplexed gene expression assays as well as in assays for cell surface proteins with live intact cells.

Acknowledgements

The authors are grateful to Tony Ricco, Travis Boone, Uyen Nguyen, Yi-Je Juang, Charles Espejo, Say Yang, Audra Meng, Frank Sit, Sharat Singh, Tracy Matray, Liching Cao, Lili Chen and Tor Bjornson for prototype LabCards, eTag assay reagents and instrumentation advice.

References

[1] A. Manz, D.J. Harrison, E.M.J. Verpoorte, J.C. Fettinger, A. Paulus, H. Lüdi, H.M. Widmer, J. Chromatogr. 593 (1992) 253.
[2] D.J. Harrison, A. Manz, Z. Fan, H. Lüdi, H. Widmer, Anal. Chem. 64 (1992) 1926.
[3] D.J. Harrison, K. Fluri, K. Seiler, Z. Fan, C.S. Effenhauser, A. Manz, Science 261 (1993) 895.

[4] S.C. Jacobson, R. Hergenröder, L.B. Koutny, J.M. Ramsey, Anal. Chem. 66 (1994) 1114.
[5] S.C. Jacobson, R. Hergenröder, L.B. Koutny, R.J. Warmack, J.M. Ramsey, Anal. Chem. 66 (1994) 1107.
[6] S.C. Jacobson, R. Hergenröder, L.B. Koutny, J.M. Ramsey, Anal. Chem. 66 (1994) 2369.
[7] S.C. Jacobson, R. Hergenröder, A.W. Moore Jr., J.M. Ramsey, Anal. Chem. 66 (1994) 4127.
[8] A.T. Woolley, R.A. Mathies, Proc. Natl. Acad. Sci. USA 91 (1994) 11348.
[9] A.T. Woolley, R.A. Mathies, Anal. Chem. 67 (1995) 3676.
[10] S.C. Jacobson, J.M. Ramsey, in: J.P. Landers (Ed.), Handbook of Capillary Electrophoresis, 2nd ed, CRC Press, Boca Raton, FL, 1997, p. 827.
[11] C.S. Effenhauser, G.J.M. Bruin, A. Paulus, Electrophoresis 18 (1997) 2203.
[12] C.S. Effenhauser, in: A. Manz, H. Becker (Eds.), Microsystem Technology in Chemistry and Life Science, Springer, Berlin, 1998, p. 51.
[13] G.J.M. Bruin, Electrophoresis 21 (2000) 3931.
[14] D. Kaniansky, M. Masár, J. Bieliková, F. Iványi, F. Eisenbeiss, B. Stanislawski, B. Grass, A. Neyer, M. Jöhnck, Anal. Chem. 72 (15) (2000) 3596.
[15] R. Bodar, V. Majajova, D. Kaniansky, M. Masár, M. Jöhnck, B. Stanislawski, J. Chromatogr. A 916 (2001) 155.
[16] R. Bodar, M. Zuborova, E. Olvecka, V. Majajova, M. Masár, D. Kaniansky, B. Stanislawski, J. Sep. Sci. 24 (2001) 802.
[17] S.C. Jacobson, J.M. Ramsey, Electrophoresis 16 (1995) 481.
[18] S.C. Jacobson, J.M. Ramsey, Anal. Chem. 69 (1997) 3212.
[19] J. Khandurina, S.C. Jacobson, L.C. Waters, R.S. Foote, J.M. Ramsey, Anal. Chem. 71 (1999) 1815.
[20] H. Yang, R.-L. Chien, J. Chromatogr. A 924 (2001) 155.
[21] J. Lichtenberg, E. Verpoorte, N.F. de Rooij, Electrophoresis 22 (2001) 28.
[22] J. Li, C. Wang, J.F. Kelly, D.J. Harrison, P. Thibault, Electrophoresis 21 (2000) 198.
[23] B. Zhang, H. Liu, B.L. Karger, F. Foret, Anal. Chem. 71 (1999) 3258.
[24] Y.-C. Lin, H.-C. Ho, C.-K. Tseng, S.-Q. Hou, J. Micromech. Microeng. 11 (2001) 189.
[25] P.A. Walker, M.D. Morris, M.A. Burns, B.N. Johnson, Anal. Chem. 70 (1998) 3766.
[26] J.L. Beckers, P. Bocek, Electrophoresis 21 (2000) 2747.
[27] D.M. Osbourn, D.J. Weiss, C.E. Lunte, Electrophoresis 21 (2000) 2768.
[28] J.P. Quirino, S. Terabe, J. Chromatogr. A 902 (2000) 119.
[29] G. Hempel, Electrophoresis 21 (2000) 691.
[30] Z.K. Shihabi, J. Chromatogr. A 902 (2000) 107.
[31] T. Stroink, E. Paarlberg, J.C.M. Waterval, A. Bult, W.J.M. Underberg, Electrophoresis 22 (2001) 2374.
[32] M. Vacarcel, L. Arce, A. Rios, J. Chromatogr. A 924 (2001) 3.
[33] F.E.P. Mikkers, F.M. Everaerts, T.P.E.M. Verheggen, J. Chromatogr. 169 (1979) 11.
[34] D.S. Burgi, R.-L. Chien, Anal. Chem. 63 (1991) 2042.
[35] R.-L. Chien, D.S. Burgi, Anal. Chem. 64 (1992) 1046.
[36] R. Aebersold, H.D. Morrison, J. Chromatogr. 516 (1990) 79.
[37] Y. Zhao, C.E. Lunte, Anal. Chem. 71 (1999) 3985.
[38] D.J. Weiss, K. Saunders, C.E. Lunte, Electrophoresis 22 (2001) 59.
[39] F.M. Everaerts, Th.P.E.M. Verheggen, F.E.P. Mikkers, J. Chromatogr. 169 (1979) 21.
[40] D. Kaniansky, J. Marak, J. Chromatogr. 498 (1990) 191.
[41] F. Foret, V. Sustacek, P. Bocek, J. Microcol. Sep. 2 (1990) 229.
[42] T.J. Thompson, F. Foret, P. Vouros, B.L. Karger, Anal. Chem. 65 (1993) 900.
[43] S. Chen, M.L. Lee, Anal. Chem. 72 (2000) 816.
[44] M.T. Cronin, T. Boone, A.P. Sassi, H. Tan, Q. Xue, S.J. Williams, A.J. Ricco, H.H. Hooper, J. Assoc. Lab. Autom. 6 (1) (2001) 74.
[45] R.M. McCormick, N.J. Nelson, M.G. Alonso-Amigo, D.J. Benvegnu, H.H. Hooper, Anal. Chem. 69 (1997) 2626.
[46] A. Sassi, I. Cruzado, T. Björnson, H. Hooper, A. Paulus, J. Chromatogr. A 894 (2000) 203.
[47] Q. Xue, A. Wainright, S. Gangakhedkar, I. Gibbons, Electrophoresis 22 (2001) 4000.
[48] B. Gas, P. Coufal, M. Jaros, J. Muzikar, I. Jelinek, J. Chromatogr. A 905 (2001) 269.
[49] D.J. Rose, J.W. Jorgenson, Anal. Chem. 60 (1988) 375.
[50] F.M. Everaerts, J.L. Beckers, T.P.E.M. Verheggen, Isotachophoresis. Theory, Instrumentation and Applications, Elsevier, Amsterdam, 1976.
[51] J.P. Alarie, S.C. Jacobson, J.M. Ramsey, Electrophoresis 22 (2001) 312.
[52] J.P. Alarie, S.C. Jacobson, C.T. Culbertson, J.M. Ramsey, Electrophoresis 21 (2000) 100.
[53] J.D. Olechno, J.A. Nolan, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996, Chapter 3.