ELSEVIER

Journal of Chromatography A, 828 (1998) 469–480

JOURNAL OF CHROMATOGRAPHY A

Capillary electrochromatography of biomolecules with on-line electrospray ionization and time-of-flight mass spectrometry

Gargi Choudhary[a,1], Csaba Horváth[a,*], J. Fred Banks[b]

[a]*Department of Chemical Engineering, Yale University, P.O. Box 208286, New Haven, CT 06520-8286, USA*

[b]*Analytica of Branford, Branford, CT 06405, USA*

Abstract

Capillary electrochromatography (CEC) is considered a hybrid of liquid chromatography and capillary electrophoresis. It is expected to combine the high peak efficiency of capillary zone electrophoresis with the versatility and loading capacity of HPLC to bring about another high-performance MS-compatible chromatographic system. This paper explores the potential of CEC coupled with the electrospray ionization and time-of-flight mass spectrometry in biochemical analysis. The packed columns used in this study were tapered at the outlet to retain the packing material, thereby obviating the need for an outlet frit. Electrosmotically driven solvent gradients were employed for the separation of phenylthiohydantoin (PTH)-amino acids by reversed-phase chromatography, and a time-of-flight (TOF) mass spectrometer was employed as the detector for the CEC column effluent. The effect of CEC operating parameters, such as gradient shape, column length, and electric field, on the analytical results from the separation and MS detection of a standard mixture of PTH-amino acids was investigated. Particular attention was paid to the effect of sheath flow-rate, sheath composition and mass spectra acquisition rate on the performance of the electrospray TOF-MS.

Keywords: Electrochromatography–mass spectrometry; Gradient elution; Mass spectrometry; Detection, LC; Electrosmotic flow; Amino acids

1. Introduction

At present there is a great deal of interest in exploring the potential of capillary electrochromatography (CEC) for the analysis of biomolecules in the pharmaceutical industry. If, due to its relatively high separation efficiency and loading capacity, CEC gains importance as a high resolution analytical technique, it likely will be coupled with mass spectrometry (MS), which finds increasing employment as the detection method of choice for liquid-phase separations by virtue of its potential to positively identify all eluting analytes. Since capillary zone electrophoresis (CZE) separates only charged analytes, micellar electrokinetic chromatography (MEKC), has been developed for the separation of neutral analytes. But, being based on the use of high concentrations of surfactants, MEKC has relatively poor selectivity and lacks MS compatibility. In contradistinction, CEC has good MS compatibility, selectivity and general applicability, and thus could fill a gap in modern liquid-phase analytical separation techniques employing fused-silica capillaries.

A capillary electrochromatographic system was first coupled to a mass spectrometer by Verheij et al. [1]. They applied both pressure and voltage gradients across a packed 220-μm I.D. capillary to meet the

*Corresponding author. Tel.: +1-203-4324357; fax: +1-203-4324360; e-mail: csaba.horvath@yale.edu

[1]Present address: Biomeasurements Group, Hewlett-Packard Laboratories, 3500 Deer Creek Road, Palo Alto, CA 94304, USA.

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00817-6

requirements of the fast atom bombardment (FAB) ion source. The coupling of such a pressure assisted electrochromatographic system to the mass spectrometer via electrospray ionization (ES-MS) employing a sheath flow interface was later demonstrated by the same group [2]. Similarly Schmeer et al. [3] have used ES-MS detection with pressure assisted CEC for the separation of a peptide mixture.

An electrochromatographic system without pressure driven flow was coupled to a mass spectrometer with FAB ionization by Gordon et al. [4]. They used a packed capillary column followed by a long open capillary segment for the separation of steroid hormones with modest efficiency. Lane et al. [5,6] have also showed the feasibility of coupling CEC with ES-MS detection. The geometry of the ES source dictated the use of packed capillary columns at least 90 cm long, resulting in long elution times and relatively low separation efficiencies [5]. This system was later modified so that the column length could be reduced to 45 cm [6] resulting in faster separations. Very recently, Lord et al. [7] have separated and identified a mixture of steroid hormones by CEC–ES-MS using a column with a tapered outlet to retain the packing material and serve as the electrospray needle. Also, Ding and Vouros [8] have analyzed polynuclear aromatic hydrocarbon–DNA adducts using CEC coupled with ES-MS.

Electrospray ionization in mass spectrometry has evolved into a powerful tool for the analysis of biological molecules [9–16]. Most mass analyzers are based on the principle of changing magnetic and/or electric fields to stabilize the trajectories of selected ion populations across the m/z range while detection takes place. As a result it takes between 1–10 s to complete a single scan of a predetermined m/z ion population. On the other hand, time-of-flight (TOF) mass spectrometers have the potential to generate and collect very rapidly m/z data. Mass detection by TOF is based on the flight time of the ions from the ionization source to the detector. Since the applied magnetic or electric fields are static, mass spectra can be acquired at frequencies up to 10–20 kHz. The spectra thus obtained can be summed into arbitrary groups to yield as many as 100 complete mass spectra per second. The inherent sensitivity of TOF is relatively high, as the majority of ions survive in the vacuum system and impinge upon the detector. In contrast, the majority of the ions are lost due to the scanning fields [17] in magnetic and quadrupole machines.

MS scan rate has a significant effect on the peak shape in the rapid separation of peptides by CE [18]. Since CEC is also capable of generating peaks which are only a few seconds wide, its coupling to a rapid mass analyzer such as ES-TOF-MS appears to be particularly advantageous. In this study, the potential of this approach was explored and the effects of various CEC parameters such as gradient shape, column length, and electric field on the separation and MS detection of a standard mixture of phenylthiohydantoin (PTH)-amino acids were investigated. The effect of the sheath flow-rate, sheath composition and mass spectra acquisition rate on the performance of the ES-TOF-MS system was also examined.

2. Experimental

2.1. Chemicals

HPLC-grade methanol and acetonitrile were obtained from Mallinckrodt (Paris, KY, USA). Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). Water was purified by a NanoPure unit from Barnstead (Boston, MA, USA). Neat aqueous ammonium acetate buffer was prepared as a 10 m*M*, pH 7.0 stock solution. The solution was filtered through a nylon 66 membrane from Anspec (Ann Arbor, MI, USA). Eluents were prepared by appropriate dilutions of the stock solution with water and acetonitrile without further adjustment of the pH such that the final concentration of ammonium acetate was 2 m*M*. In this text buffer compositions are stated as 2 m*M* ammonium acetate in water–acetonitrile (x:y, v/v) mixture. PTH amino acids were purchased from Sigma (St. Louis, MO, USA).

2.2. Materials

Polyimide-coated fused-silica capillaries of 375

μm O.D.×75 μm I.D. were obtained from Quadrex (New Haven, CT, USA). 3.5 μm Zorbax ODS particles of 80 Å mean pore diameter as well as 6 μm particle and 300 Å mean pore diameter, were a gift from Rockland Technologies (Newport, DE, USA).

2.3. Instrumentation

The electrospray ionization source and the sheath flow CEC probe were from Analytica of Branford (Branford, CT, USA). The sheath probe was constructed in a three-layer coaxial arrangement [19], with the CEC column in the center. The length of the standard probe was 18.2 cm, but a shorter 9 cm long probe was also fabricated. The configuration of the shorter probe was identical to that described by Banks [19] except for the reduced length. Large windows on three sides of the atmospheric region of the source facilitated visual observation of the electrospray process and thus aided the optimization of the conditions. Unless otherwise stated, the applied voltage in the source was −2815 V for the cylindrical electrode, −4406 V for the end plate electrode and −4634 V for the capillary entrance. A dielectric capillary with metal coating on both ends was used to transfer ions generated in the atmospheric region into vacuum and allowed the CEC column exit to be maintained at ground potential [19,20].

The TOF mass spectrometer used here was a short linear instrument of the Wiley/McLaren [21] type developed at Analytica of Branford as a mass detector for high-speed liquid chromatography applications and similar to that described previously [18]. The distance from the center of the extraction volume to the detector surface was 400 mm and the drift energy in the field-free flight tube was 2850 eV for a singly charged ion.

The flight time of the ions was measured with a Model AF820 secondary electron multiplier from ETP (Auburn, MA, USA) at the end of the flight tube. A mass resolving power of 1000 (fwhm) was determined with leucine enkephalin having m/z of 556 by a Tektronix Model TDS 540 digital oscilloscope (Wilsonville, OR, USA), at 500 MHz. For rapid and continuous analog-to-digital conversion and signal averaging, the output signal from the electrically floating ion detector was capacitively coupled into a Model 9825 integrating transient recorder (ITR) data acquisition device from Precision Instruments (Knoxville, TN, USA) operated at 200 MHz. During the experiments described here, the scan repetition rate of the TOF spectrometer was fixed at 8192 Hz.

Total ion chromatograms (TICs) were created by sampling the detector output for each pulse of ions (m/z range from 100 to 2000) with the 200-MHz ITR and then summing these data in real time into individual mass spectra composed of signal intensity versus m/z. The sum of intensities for all m/z values in each mass spectrum was then plotted against time to yield a TIC. Reconstructed ion chromatograms (RICs) were created after data collection was complete, by replotting the signal intensity from only one particular m/z against time.

Gradient CEC was performed using the same scheme as described by Huber et al. [22] and employed a gradient manifold consisting of a polyether ether ketone (PEEK) cross, Model 8125 Rheodyne injector (Cotati, CA, USA) and a three-way valve as illustrated in Fig. 1. A Crystal 310 capillary electrophoresis instrument from Thermoseparations (Franklin, MA, USA) was used for the application of voltage. An ABI Model 140A dual-syringe LC pump from Perkin-Elmer (Foster City, CA, USA) was used as the gradient former.

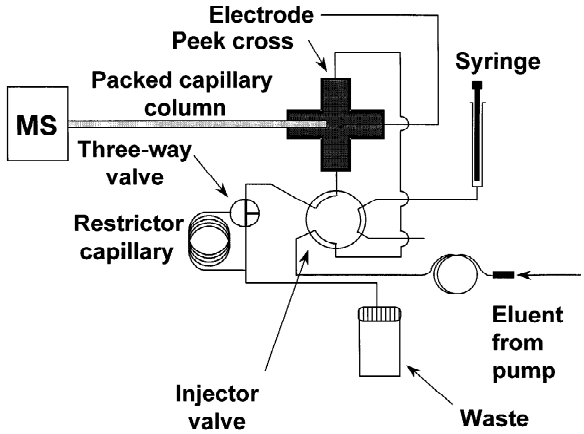

Fig. 1. Schematics of CEC gradient manifold.

3. Results and discussion

3.1. Configuration of column outlet for CEC–ES-MS

In CEC the packed segment of the capillary column is followed by an open segment to facilitate UV detection [23] and the retaining frit between the two segments is believed to act as nucleation site for bubble formation. Grant and Knox [24] recommended the use of pressurized inlet and outlet reservoir to suppress bubble formation and most CEC units embody this feature. However, such a pressurized system is not compatible with the sheath flow interface for ES-MS. Fortunately, in CEC–ES-MS, the column effluent can be electrosprayed directly into the atmospheric region of the ion source provided a suitable termination of the column outlet is available.

Tapered capillaries have been employed as flow restrictors in open tubular supercritical chromatography [25–29] and with metallized tips also for CE–ES-MS in a sheathless configuration [30–34]. Lord et al. [7] demonstrated first the use of packed capillary columns with externally tapered outlet in CEC. A taper of ca 10 μm I.D. at the exit end of the capillary column obviated the need for an outlet end frit to retain 3-μm particles of the stationary phase. Whereas drawing of fused-silica capillaries through a high temperature flame readily yields tapered capillaries with reduced outer and inner diameters, such externally tapered capillary ends were found to be very fragile.

Capillary columns with internal tapers where only the inner diameter is reduced offer an alternative, and we have found such taper to be a suitable restrictor for the CEC column outlet. Both externally and internally tapered packed capillaries are illustrated in Fig. 2. Internal tapers are prepared by melting the tip of the open capillary in a high temperature flame thereby sealing its end. Then, by carefully grinding the sealed end with a ceramic tile an opening of approximately 10 μm is produced. Capillaries with such internal tapers can be prepared reproducibly and they are more robust than capillaries with external tapers. After an initial learning period the internally tapered capillaries could be produced with a failure rate of less than 20%. Upon packing the stationary phase into the capillary tube by using the slurry packing method [22,23], the particles arrange themselves in such a way that a porous plug is formed at the capillary outlet. This plug is sufficiently permeable to pack the column and to be used in CEC as a replacement for the retaining frit. To our knowledge this is the first study employing packed capillary columns with an internal taper for use in CEC–ES-MS with sheath flow.

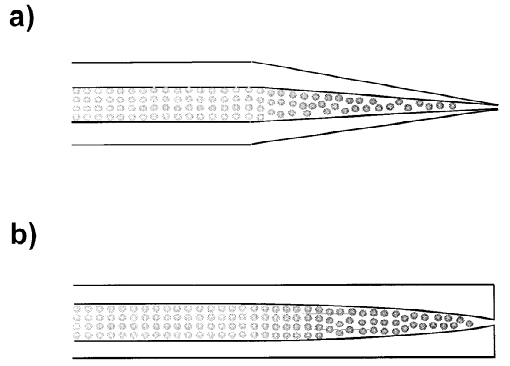

Fig. 2. Illustration of (a) external and (b) internal taper at the outlet of the CEC columns. The lengths of the actual tapered ends vary between 1 and 3 mm.

3.2. Effect of TOF-MS integration time

The time-of-flight mass spectrometer employed in this study acquires mass spectra at the nominal rate of 8 kHz and the individual spectra can be compiled in electronic data registers for nearly any arbitrary length of time until eventually a single spectrum is recorded. Here, ‘integration time’ is the length of time over which the pulses are summed. The effect of integration time on observed peak shape is shown in the chromatograms of Fig. 3, obtained by a 25-cm column packed with 3.5-μm octadecylated particles at an applied voltage of 30 kV with gradient elution. The base width of the PTH-asparagine peak as determined from the RIC is about 6 s. At integration time of 2 s/spectrum only three data points were acquired under the peak, and the peak shape was distorted due to artificial broadening. Upon decreasing the integration time to 0.25 s/spectrum, however, 24 data points could be acquired for the same PTH-asparagine peak. Thus, as the integration time is reduced, decreasing the number of scans summed

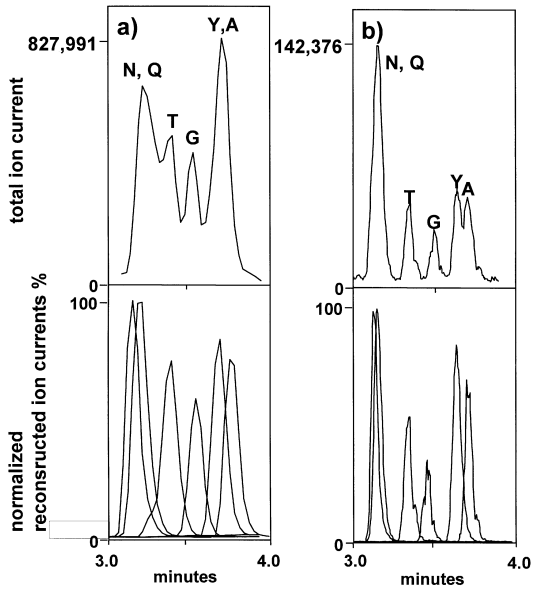

Fig. 3. Effect of the integration time on the total and reconstructed ion chromatograms of six PTH-amino acids. Column, 25 cm×75 μm packed with 3.5-μm Zorbax ODS particles having a mean pore size of 80 Å; starting eluent (A), 2 m*M* aqueous ammonium acetate, pH 7.0; gradient former (B), 2 m*M* ammonium acetate in water:acetonitrile (1:9, v/v); flow-rate of the mobile phase through the column inlet reservoir, 100 μl/min; gradient, 30–80% B in 5 min, 80% B for 5 min, 80–30% B in 1 min; applied voltage, 30 kV; electrokinetic injection, 2 kV, 2 s. Integration time: (a) 2 s/spectrum; (b) 0.25 s/spectrum. Sheath liquid, 0.2 m*M* ammonium acetate in water:methanol (1:9, v/v); flow-rate, 3 μl/min. Peaks in order of elution: PTH-asparagine, PTH-gutamine, PTH-threonine, PTH-glycine, PTH-tyrosine, PTH-alanine.

together to produce the final recorded spectrum, the total ion chromatogram (TIC) began to accurately portray the characteristics of the actual separation. At 0.25 s/spectrum the time-of-flight mass spectrometer was recording data at a rate eight times faster than achievable with a quadrupole instrument at the highest speed (2 s/spectrum). This observation stresses the need for fast mass analyzers with fast data acquisition capabilities for use with CEC that is evolving as an efficient, rapid chromatographic technique.

3.3. Effect of sheath liquid composition

In addition to assisting in formation of the electrospray itself and completing the electrical circuits for both CEC and the electrospray, the sheath liquid also acts as the outlet buffer reservoir. Electrospray functions best with solutions having high organic strength and low salt concentration. Thus, the optimal composition of the sheath liquid is frequently different from the mobile phase in CEC. The composition of the sheath liquid which replaces the buffer reservoir at the column outlet has a significant effect on the migration times of analytes [35]. The effect of the sheath liquid composition on the separation of PTH-amino acids by CEC is illustrated in Fig. 4. In this set of experiments a 25-cm long capillary column packed with 3.5-μm Zorbax ODS particles was used again, but with an applied potential of 25 kV. With neat methanol as the sheath liquid, the migration times of the analytes are quite long, as seen in Fig. 4a. Possible explanation for this behavior rests with the absence of electrolyte, e.g. ammonium acetate, in the sheath liquid and the concomitant low conductivity of neat methanol. This is supported by the observation that upon including

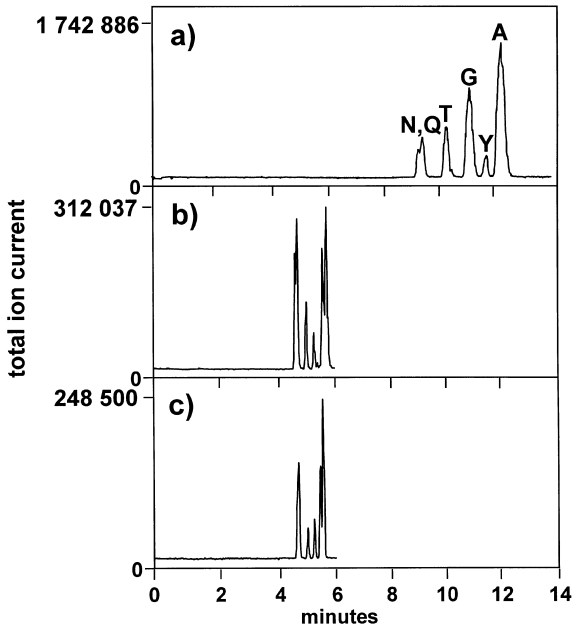

Fig. 4. Total ion chromatograms illustrating the effect of sheath liquid composition on the separation of PTH-amino acids. Applied voltage, 20 kV; integration time, 0.5 s/spectrum. Sheath liquid: (a) neat methanol; (b) 0.2 m*M* ammonium acetate in water:methanol (1:9, v/v); (c) 2 m*M* ammonium acetate in water:methanol (1:9, v/v). Other conditions as in Fig. 3.

0.2 m*M* ammonium acetate in the sheath liquid the components elute in less than half of the time as before. As seen in Fig. 4b some resolution is sacrificed and the signal intensity is six times lower than that obtained with neat methanol as sheath. This is attributed to the so-called charge competition between the ions in the sheath liquid and the PTH-amino acids. In Fig. 4c it is seen that with further increase in the ammonium acetate concentration to 2 m*M*, the signal intensity further decreased, although the retention times of the six PTH-amino acids were about the same as in Fig. 4b. Moreover, PTH-asparagine and -glutamine that were partially resolved in Fig. 4b now coelute. The above results serve as a caveat that the make-up of sheath liquid be carefully selected in order to obtain adequate signal intensity, resolution of sample components and the time of analysis at the same time.

3.4. Effect of column length

Review of the pertinent literature shows that the minimum CEC column length that could be effectively coupled to a mass spectrometer is 45 cm. This is stated in an earlier report by Lane et al. [6] who discuss electrospray source modification to lower the column length from 90 to 45 cm. However, with the geometry of the first electrospray source described above, CEC columns as short as 25 cm in length could be easily employed as shown in this study. Nonetheless, to explore the possibility of further decreasing the CEC column length, the dimensions of the ES source cover and the sheath probe were further reduced. The respective lengths of the source cover and the sheath probe were 14 and 18 cm originally, but shortened to 7 and 9 cm upon modification.

The effect of column length on the separation of the six PTH-amino acids is shown in Fig. 5. In Fig. 5a the chromatogram of the mixture obtained with a 25-cm long packed column at an applied voltage of 20 kV is depicted. The components were separated within 6 min by using an electrosmotically drawn acetonitrile gradient generated by the LC pump in 5 min with acetonitrile concentration increasing from 30 to 90% (v/v) in the gradient former. Fig. 5b illustrates the separation of the mixture on a 15-cm long column with the same gradient and applied

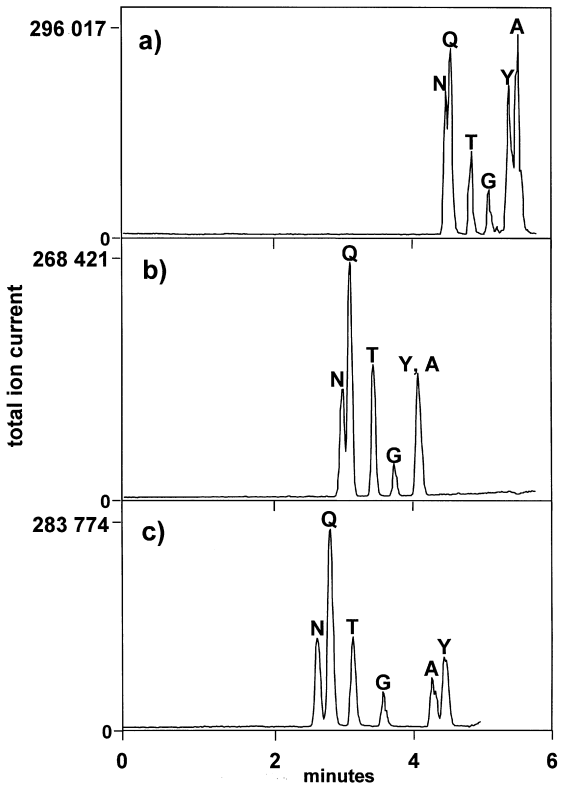

Fig. 5. Total ion chromatograms illustrating the effect of column length on the separation of PTH-amino acids. Applied voltage, 20 kV; integration time, 0.5 s/spectrum. Column length and gradient: (a) 25 cm, 30–80% B in 5 min, 80% B for 5 min 80–30% B in 1 min; (b) 15 cm, 30–80% B in 5 min, 80% B for 5 min 80–30% B in 1 min; (c) 15 cm, 30–60% B in 5 min, 60% B for 5 min 60–30% B in 1 min. Other conditions as in Fig. 3.

voltage, and it is seen that the separation was completed in 4 min, but the last two components, PTH-alanine and PTH-tyrosine, were no longer resolved at all. However, by decreasing the slope of the gradient, all six PTH-amino acids were separated within 5 min as shown in Fig. 5c with a higher resolution. Thus, short columns with gradient elution can provide for fast separations not only in HPLC but also in reversed-phase CEC. It should be noted that the elution order of PTH-alanine and PTH tyrosine is reversed upon changing the conditions used for the separation shown in Fig. 5a to those shown in Fig. 5c. The selectivity reversal would not have been seen without using the mass spectrometer

as the detector for positive identification of peaks by their molecular mass.

3.5. Isocratic and gradient elution of PTH-amino acids

For the first set of experiments the gradient manifold depicted in Fig. 1 was operated isocratically, i.e., mobile phase of constant composition was pumped continuously to flush the 17-μl cavity of the PEEK cross. In order to determine the magnitude of the pressure-driven flow, if any, through the packed capillary column, the effect of the LC pump flow-rate on the separation of six PTH-amino acids was investigated by isocratic elution. The mobile phase delivered by the LC pump contained 2 m*M* ammonium acetate in a water–acetonitrile mixture (7:3, v/v). The sheath liquid contained 2 m*M* aqueous ammonium acetate in a water–methanol (1:9, v/v) mixture, and its flow-rate was 3 μl/min.

Fig. 6 shows the effect of the LC pump flow-rate on the separation of PTH-amino acids. It is seen that the retention times of PTH-amino acids slightly increase upon increasing the LC flow-rate from 50 to 200 μl/min. This would suggest at first glance (Fig. 6a–c) that there is some pressure driven flow through the CEC column. In order to choose an appropriate flow-rate for the LC pump the inlet end of the column was placed in a 4-ml vial containing 2 m*M* ammonium acetate, in a water–acetonitrile mixture (7:3, v/v). Upon comparing Fig. 6b and d it is seen that the migration times of the PTH-amino acids when an LC pump flow-rate of 100 μl/min was continuously flushing the cavity at the column inlet were identical to that obtained with the vial present at the column inlet. The result suggests that a flow-rate of 100 μl/min through the cross does not produce a viscous flow through the packed CEC capillary column. The increased elution time for the 50 μl/min (Fig. 6a) flow-rate is most likely the result of electrolysis of buffer at the column inlet [36]. Finally, at 200 μl/min (Fig. 6c), the elution times are decreased as a result of some viscous flow. Based on these results, LC pump flow-rate of 100 μl/min was used in all remaining experiments.

The chromatogram in Fig. 7 shows the separation of the six PTH-amino acids by isocratic and gradient elution with a 25-cm long column at an applied

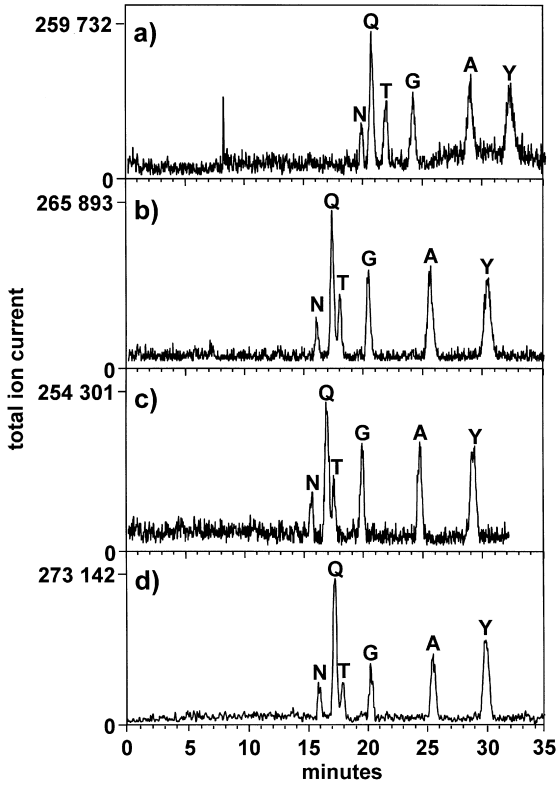

Fig. 6. Total ion chromatograms illustrating the effect of the mobile phase flow-rate delivered by the LC pumps on the isocratic separation of PTH-amino acids. Column, 28 cm×75 μm packed with 3.5-μm Zorbax ODS particles having a mean pore size of 80 Å; mobile phase, 2 m*M* ammonium acetate in water:acetonitrile (7:3, v/v); flow-rate of the mobile phase through the column inlet reservoir: (a) 50 μl/min, (b) 100 μl/min, (c) 200 μl/min, (d) vial; applied voltage, 15 kV; integration time, 0.5 s/spectrum. Other conditions as in Fig. 3.

voltage of 15 kV. Under isocratic conditions, at an electrosmotic flow velocity of 0.6 mm/s, as determined by measuring the migration time of thiourea as the non-retained tracer, only four PTH-amino acids eluted within 30 min as seen in Fig. 7a. However, by employing a 5-min gradient from 30 to 80% (v/v) gradient former, the six sample components were separated within 7 min as shown in Fig. 7b. The data illustrates that also in CEC the time of analysis can be greatly reduced by using gradient elution and that CEC–ES-MS can be operated with gradient elution without untoward effects on the analytical results.

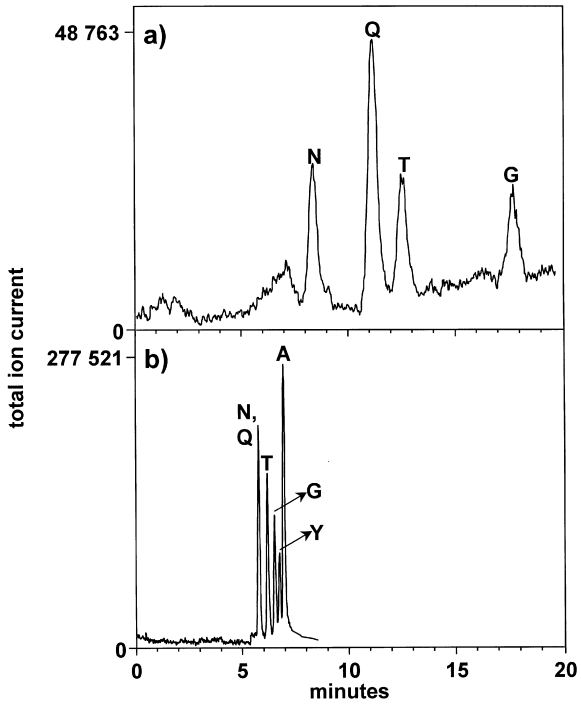

Fig. 7. Total ion chromatograms illustrating the separation of PTH-amino acids by isocratic and gradient elution. Applied voltage, 15 kV; integration time, 0.5 s/spectrum. Elution: (a) isocratic 30% B; (b) gradient, 30–80% B in 5 min, 80% B for 5 min, 80–30% B in 1 min. Other conditions as in Fig. 3.

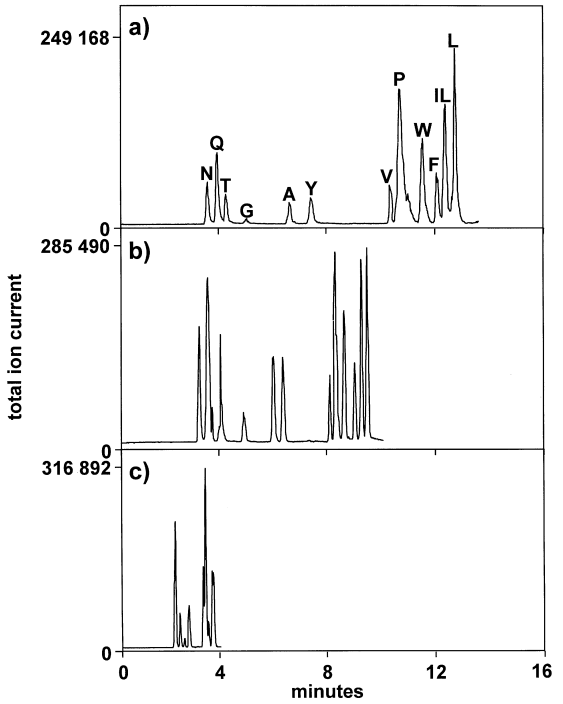

Fig. 8. Effect of gradient slope on the separation of 12 PTH-amino acids. Column, 15 cm×75 μm packed with 3.5-μm Zorbax ODS particles having a mean pore size of 80 Å; starting eluent (A), 2 m*M* aqueous ammonium acetate, pH 7.0; gradient former (B), 2 m*M* ammonium acetate in water:acetonitrile (1:9, v/v); flow-rate of the mobile phase through the column inlet reservoir, 100 μl/min; applied voltage, 20 kV; electrokinetic injection, 2 kV, 2 s; integration time, 0.5 s/spectrum. Sheath liquid, 0.2 m*M* ammonium acetate in water:methanol (1:9, v/v); flow-rate, 3 μl/min. Gradient: (a) 30–90% B in 25 min, 90% B for 1 min, 90–30% B in 1 min; (b) 30–90% B in 15 min, 90% B for 1 min, 90–30% B in 1 min; (c) 30–90% B in 5 min, 90% B for 1 min, 90–30% B in 1 min. Peaks in order of elution: PTH-asparagine, PTH-gutamine, PTH-threonine, PTH-glycine, PTH-alanine, PTH-tyrosine, PTH-valine, PTH-proline, PTH-tryptophan, PTH-phenylalanine, PTH-isoleucine, PTH-leucine.

A mixture of 12 PTH-amino acids was separated on a 15-cm long column and the effect of changing the gradient slope was investigated. In the experiments the starting eluent was 2 m*M* aqueous ammonium acetate, whereas the gradient former was 2 m*M* ammonium acetate, in a water–acetonitrile mixture (1:9, v/v). The sheath liquid containing 0.2 m*M* ammonium acetate, in a water–methanol mixture (1:9, v/v) was delivered at a flow-rate of 3 μl/min. At an applied voltage of 20 kV, a 25-min linear gradient from 30 to 90% (v/v) gradient former sufficed to completely resolve the 12 PTH-amino acids in 14 min as shown in Fig. 8a. In order to decrease the overall analysis time a slightly steeper slope from 30 to 90% (v/v) gradient former in 15 min was used and the resulting chromatogram is depicted in Fig. 8b. It is seen that the 12 PTH amino acids were completely resolved and the analysis time is reduced to less than 10 min. Fig. 8c shows that upon further increasing the gradient slope from 30 to 90% (v/v) gradient former in 5 min all sample components elute in less than 4 min, but the overall resolution of the components deteriorates significantly. The results strongly indicate that the effect of gradient slope on the separation in RP-CEC is similar to that in RP-HPLC [37].

3.6. Effect of applied voltage

Fig. 9 illustrates the effect of applied voltage on

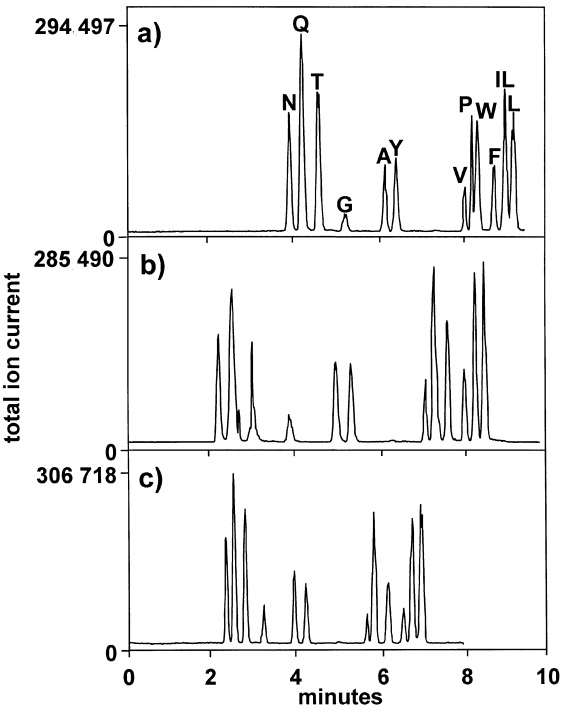

Fig. 9. Effect of applied voltage on the separation of 12 PTH-amino acids. Gradient: 30–90% B in 15 min, 90% B for 1 min, 90–30% B in 1 min. Applied voltage: (a) 15 kV, (b) 20 kV, (c) 25 kV. Other conditions as in Fig. 8.

chromatograms of the 12 PTH-amino acids obtained by ES-TOF-MS using a 75-μm×15 cm fused-silica capillary column packed with 3.5-μm Zorbax ODS particles and gradient elution under conditions shown in Fig. 8b. The results presented in Fig. 9 demonstrate that the applied voltage has a significant effect on the separation. At 20 kV, all sample components, vide Fig. 9b, were resolved, whereas at higher and lower voltage no complete resolution was obtained. As seen in Fig. 9a PTH-proline and PTH-tryptophan co-eluted and the resolution of PTH-leucine and isoleucine was rather poor at 15 kV applied voltage. At 25 kV PTH-valine and proline coeluted and leucine and isoleucine were not resolved, as shown in Fig. 9c. Also, upon increasing the applied voltage from 15 to 25 kV the time required to complete the separation was reduced by about 3 min.

In a simple chromatographic system, retention factor and selectivity are independent of flow-rate. Our system is far more complex, since the electrosmotic flow-rate across the column is generated by the applied voltage, whereas the LC gradient is formed by the pumps independently of the electrosmotic flow. The column can be thought of 'sampling' the mobile phase composition in the cavity of the PEEK cross at the rate dependent on the electrosmotic flow (EOF), and this can give rise to changes in the selectivity upon changing the EOF rate.

Following a change in the eluent composition in the cavity, the magnitude of EOF is likely to change, e.g. due to varying viscosity and dielectric constant, with concomitant changes in the rate at which the mobile phase is drawn into the CEC column. However, the flow-rate of the pumped mobile phase is not affected, and the pumps continue forming the gradient according to the preset program. As the rate of the electrosmotic flow, but not that of the pumped flow, changes with the eluent composition, the column experiences an eluent gradient different from that generated by the pumps (indigenous gradient divergence). By the same token, an exogenous gradient divergence arises when the EOF rate changes in the column upon altering the applied voltage. The chromatograms in Fig. 9 illustrate the effect of such exogenous gradient divergence on the selectivity of the separation. Of course, with isocratic elution, the selectivity is expected to be invariant upon changing the applied voltage. The gradient divergence described above has to be considered when gradient elution is used in a CEC unit similar to that employed here [22]. Changing the applied voltage and/or the gradient generated by the pumps is likely to change not only the migration rates but also the relative retention of the sample components.

Fig. 10 shows that MS detection can convey information that is of different nature than that obtained by the conventional detectors. The data was obtained by averaging the area under the PTH-alanine peak in the total ion chromatogram shown in Fig. 9b. As seen in Fig. 10 there are four dominant peaks at m/z values of 207, 224, 413 and 430. They correspond to the protonated PTH-alanine, $(M+H)^+$, PTH-alanine with an ammonium adduct, $(M+NH_4)^+$, the protonated dimer, $(M_2+H)^+$, and the dimer with the ammonium adduct, $(M_2+NH_4)^+$. Formation of such dimers in the electrospray process has also been illustrated previously [38,39]. Further,

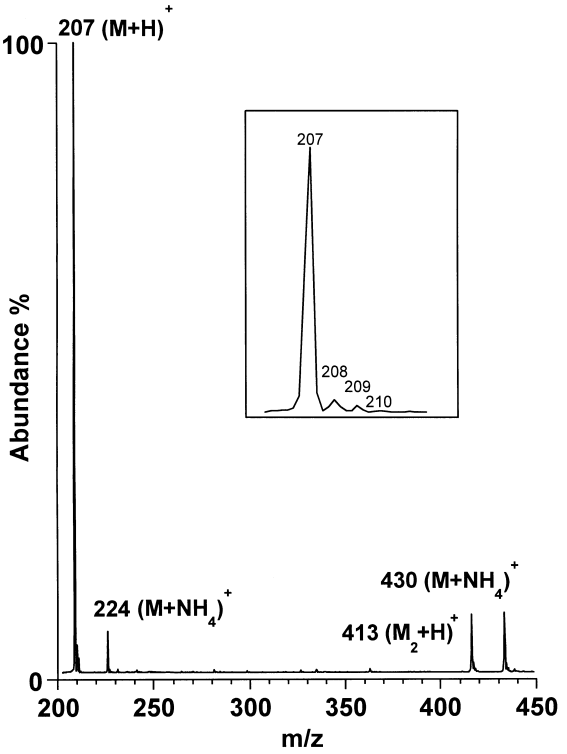

Fig. 10. Mass spectra obtained by averaging the area under the total ion chromatogram of the PTH-alanine peak. Conditions as in Fig. 9b.

the cluster of peaks near the peak of PTH-alanine at $m/z=207$ is shown magnified in the insert of Fig. 10. The peak represents PTH-alanine with lesser carbon and sulfur isotopes and they can aid in the identification of the analyte proper.

3.7. Detection sensitivity

The ion signal of the mass spectrometer obtained from an ES source depends mainly on the ionic concentration in the sample solution, and it is only weakly dependent on the flow-rate of the column effluent entering the ionization source [20,40–45]. In order to evaluate the sensitivity of the on-line system, a series of samples having different concentrations of PTH-glutamine were injected into the CEC–time-of-flight mass spectrometer unit. The mobile phase was 2 m*M* ammonium acetate in a water–acetonitrile (1:9, v/v) mixture in which PTH-glutamine eluted in the dead volume of the column. The applied voltage was 20 kV and the samples were injected at 2 kV for 6 s. The intensity of the RIC signal thus obtained was plotted against the concentration of PTH-glutamine in the sample and the results are shown in Fig. 11. It is seen that first the signal intensity increases linearly with the sample concentration, but when it reaches about 200 μg/ml deviation from linearity occurs due probably to column and electrospray overloading. The lowest concentration of sample that could be detected on the linear portion of the curve in Fig. 11 was 0.3 μg/ml. It has already been reported [38] that there is a critical concentration of analyte above which it precipitates in the electrospray droplets and forms clusters, such as the dimers described above so that the dependence of the signal on the sample concentration is no longer linear. When this point is reached with further increase in the analyte concentration the ion signal decreases.

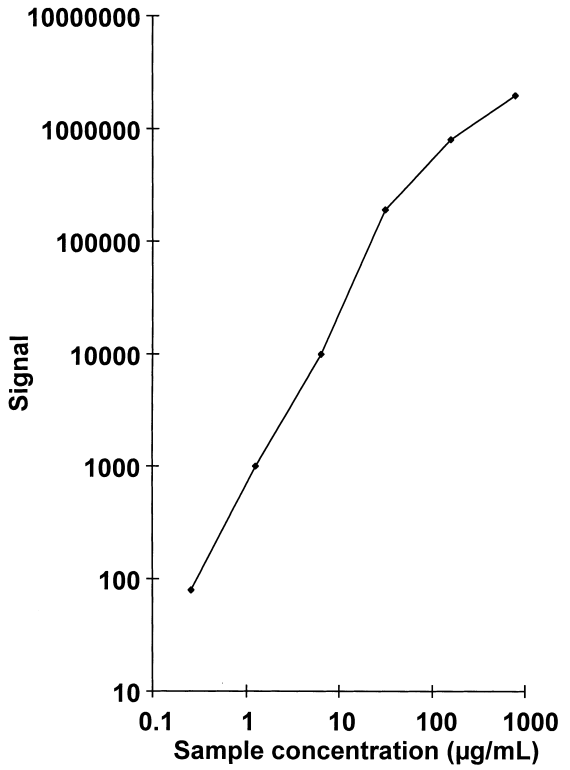

Fig. 11. Plot of RIC intensity versus concentration of PTH-glutamine in the sample. Isocratic elution, 90% B; applied voltage, 20 kV. Other conditions as in Fig. 8.

4. Conclusions

Recent advances increasingly foster the use of the mass spectrometer as an on-line detector for liquid-phase analytical separation methods. In turn, MS-compatibility has become a major consideration in the design of such separation techniques as far as the dimensions of the column and the nature and rate of consumption of the mobile phase are concerned. For this reason, MS-compatibility prominently features in the development of capillary electrochromatography, a new high-performance analytical separation tool. The present study illustrates the potential of interfacing CEC with ES-TOF-MS, with sheath liquid replacing the outlet buffer reservoir, and with packed capillary columns having internally tapered outlet end. The results show that internal taper of the column end offer a viable alternative to frits in meeting the particularly demanding specifications of CEC–MS systems. In fact, they indicate that internal taper may be a useful approach to column preparation in CEC at large. This is the first study, to our best knowledge, which demonstrates the feasibility of using columns as short as 15 cm in length for CEC with the time-of-flight mass spectrometer as the detector. It is also shown that gradient elution facilitates rapid separations by CEC–ES-MS without compromising the resolution. Time-of-flight mass spectrometers with high resolution and fast scan rates are uniquely suited to obtain MS data without peak distortion due to temporal under sampling of eluting peaks.

Acknowledgements

This work was supported by grants GM 53886 (to J.F.B). and GM 20993 (to Cs.H.) by the National Institutes of Health, US Public Health Service.

References

[1] E.R. Verheij, U.R. Tjaden, M.A. Niessen, J. van der Greef, J. Chromatogr. 554 (1991) 339.
[2] M. Hugener, A.P. Tinke, W.M.A. Niessen, U.R. Tjaden, J. van der Greef, J. Chromatogr. 647 (1993) 375.
[3] K. Schmeer, B. Behnke, E. Bayer, Anal. Chem. 67 (1995) 3656.
[4] D.B. Gordon, G.A. Lord, D.S. Jones, Rapid Commun. Mass Spectrom. 8 (1994) 544.
[5] S.J. Lane, R. Boughtflower, C. Paterson, T. Underwood, Rapid Commun. Mass Spectrom. 9 (1995) 1283.
[6] S.J. Lane, R. Boughtflower, C. Paterson, M. Morris, Rapid Commun. Mass Spectrom. 10 (1996) 733.
[7] G.A. Lord, D.B. Gordon, P. Myers, B.W. King, J. Chromatogr. A 768 (1997) 9.
[8] J. Ding, P. Vouros, Anal. Chem. 69 (1997) 379.
[9] C.M. Whitehouse, R.N. Dryer, M. Yamashita, J.B. Fenn, Anal. Chem. 57 (1985) 675.
[10] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[11] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Mass Spectrom. Rev. 9 (1990) 37.
[12] K.L. Duffin, J.K. Welply, E. Huang, J.D. Henion, Anal. Chem. 64 (1992) 1440.
[13] T. Covey, R.F. Bonner, B.I. Shushan, J.D. Henion, Rapid Commun. Mass Spectrom. 2 (1988) 249.
[14] E. Huang, J.D. Henion, J. Am. Soc. Mass Spectrom. 1 (1990) 158.
[15] D.F. Hunt, R.A.H. Shabanowitz, K. Sakaguchi, M. Hanspeter, N. Sevilir, A.L. Cox, Science 255 (1992) 1261.
[16] R.D. Smith, J.A. Loo, R.R.O. Loo, M. Buhman, H.R. Udseth, Mass Spectrom. Rev. 10 (1991) 359.
[17] J.F. Banks, Electrophoresis 18 (1997) 2255.
[18] J.F. Banks, T. Dresch, Anal. Chem. 68 (1996) 1480.
[19] J.F. Banks, J. Chromatogr. A 712 (1995) 245.
[20] J.F. Banks, C.M. Whitehouse, Electrospray ionization mass spectrometry, Methods Enzymol. 270A (1996) 486.
[21] W.C. Wiley, I.H. McLaren, Rev. Sci. Instrum. 26 (1955) 1150.
[22] C.H. Huber, G. Choudhary, Cs. Horváth, Anal. Chem. 69 (1997) 4429.
[23] G. Choudhary, Cs. Horváth, J. Chromatogr. A 781 (1997) 161.
[24] I.H. Grant, J.H. Knox, Chromatographia 32 (1991) 317.
[25] M. Novotny, S.R. Springston, P.A. Peaden, J.C. Fjeldsted, M.L. Lee, Anal. Chem. 53 (1981) 407.
[26] E.J. Guthrie, H.E. Schwartz, J. Chromatogr. Sci. 24 (1986) 236.
[27] T.L. Chester, J. Chromatogr. 299 (1984) 424.
[28] T.L. Chester, D.P. Innis, G.D. Owens, Anal. Chem. 57 (1985) 2243.
[29] J.C. Fjeldsted, R.C. Kong, M.L. Lee, J. Chromatogr. 279 (1983) 449.
[30] M.S. Wilm, M. Mann, Anal. Chem. 68 (1995) 1.
[31] G.A. Valaskovic, N.L. Kelleher, D.P. Little, D.J. Aaserud, F.W. McLafferty, Anal. Chem. 67 (1995) 3802.
[32] S.A. Hofstadler, F.D. Swanek, D.C. Gale, A.G. Ewing, R.D. Smith, Anal. Chem. 67 (1995) 1477.
[33] M.S. Kriger, K.D. Cook, R.S. Ramsey, Anal. Chem. 67 (1995) 385.
[34] J.H. Wahl, D.C. Gale, R.D. Smith, J. Chromatogr. A 659 (1994) 217.

[35] F. Foret, T.J. Thompson, P. Vouros, B.L. Karger, P. Gebauer, P. Bocek, Anal. Chem. 66 (1994) 4450.
[36] R.P. Oda, J.P. Landers, in: J.P. Landers (Ed.), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1996, p. 37.
[37] Cs. Horváth, W. Melander, in E. Heftmann (Ed.), Chromatography, Elsevier, Amsterdam, 1983, p. 117.
[38] J.F. Banks, C.M. Whitehouse, Int. J. Mass Spectrom. Ion Processes 162 (1997) 163.
[39] J.F. Banks, S. Shen, C.M. Whitehouse, J.B. Fenn, Anal. Chem. 66 (1994) 406.
[40] R.D. Smith, J.H. Wahl, D.R. Goodlett, S.A. Hofstadler, Anal. Chem. 65 (1993) 574.
[41] D.R. Goodlett, J.H. Wahl, H.R. Udseth, R.D. Smith, J. Microcol. Sep. 5 (1993) 57.
[42] H.K. Jones, N.T. Nguyen, R.D. Smith, J. Chromatogr. 504 (1990) 1.
[43] J.H. Wahl, D.R. Goodlett, H.R. Udseth, R.D. Smith, Electrophoresis 14 (1993) 448.
[44] J.F. Banks, J.P. Quinn, C.M. Whitehouse, Anal. Chem. 66 (1994) 3688.
[45] J.F. Banks, J. Chromatogr. A 743 (1996) 99.