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Journal of Chromatography A, 794 (1998) 377–389

JOURNAL OF CHROMATOGRAPHY A

Review

On-line analysis by capillary separations interfaced to an ion trap storage/reflectron time-of-flight mass spectrometer

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Abstract

The interface of high-resolution capillary separation methods to time-of-flight mass spectrometry (TOF-MS) has generated considerable interest since TOF can provide the rapid and sensitive detection required by high resolution separations. In recent years, our laboratory has developed a variety of high-resolution capillary separation methods interfaced to TOF-MS via an ion trap storage/reflectron time-of-flight (IT/reTOF) instrument. Using this hybrid configuration, detection of fast separations at very low detection levels has been successfully performed for on-line separations of peptides and protein digests using electrospray ionization. In this report, we review the current status in our laboratory of interfacing high-performance liquid chromatography, capillary electrophoresis, and capillary electrochromatography to an IT/reTOF-MS instrument and various applications that have been developed involving this technology.

Keywords: Reviews; Ion trap storage/reflectron time-of-flight mass spectrometer; Interfaces; Peptides

Contents

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0021-9673/98/$19.00
PII S0021-9673(97)00934-5

1. Introduction

Various forms of high-resolution liquid phase capillary separation methods have become indispensable tools in the structural analysis of biological macromolecules [1]. Among these separation methods, reversed-phase high-performance liquid chromatography (RP-HPLC) is the most commonly used technique for the separation and characterization of proteins and peptides due to its high resolution and reproducibility, ease of operation and relatively short analysis time. The separation mechanism in RP-HPLC is based on the difference of the analyte partitioning in the stationary phase and the mobile phase.

Since the late 1980s, an alternative separation method, capillary electrophoresis (CE) has become increasingly popular for the separation of charged species, such as proteins and peptides [2,3]. Unparalleled high separation efficiency and speed have been achieved using extremely small sample volumes. In CE, analytes are separated according to their differences in electrophoretic mobilities.

More recently, capillary electrochromatography (CEC) has drawn considerable attention as a hybrid method since it combines some of the best features from HPLC and CE [4–8]. In CEC, solvent is transported in the column by the electroosmotic flow (EOF) instead of a pressure driven flow. The result is that a nearly flat flow profile and a uniform flow velocity distribution are generated which reduce band broadening. Also, the electrophoretic migrations of charged analytes, e.g., peptides, enhances the separation selectivity, especially under a supplementary pressure which also suppresses bubble formation. This enhanced efficiency and selectivity allow the use of very short columns, resulting in fast separations. In addition, the availability of a variety of HPLC stationary phases provides CEC with more tunable selectivity compared to capillary zone electrophoresis (CZE). Sample stacking, one unique feature of RP-HPLC in the gradient elution mode, is also retained in CEC.

A variety of detection techniques have been developed for these high-resolution capillary separation methods, such as UV absorbance, fluorescence and electrochemical detection. Although they have been shown to be useful for certain applications, these methods can not provide the precise analyte identification and structural elucidation capability as obtained by a mass detector. Indeed, mass spectrometry (MS) as an on-line detector for these high-resolution capillary separations has recently become an extremely important tool for the structural analysis of proteins and other biomolecules. MS allows for differentiation of mixtures eluting from separation methods based not only on their retention times but also on their masses and fragmentation patterns. With the development of electrospray ionization (ESI) [9], and more recently, nanospray ionization [10], a variety of separation methods with different flow-rate requirements have been interfaced directly to MS [11–23].

A mass spectrometer needs to fulfill certain requirements to be used as an on-line detector for high resolution separations. Because of the high resolution and speed achieved in these separations, a rapid full mass range sampling rate is necessary to maintain the high quality of the separation. In CE, for example, the duration of a peak may be on the order of 1 to 2 s. In order to accurately define this peak, a high full mass range sampling speed is necessary. In addition to perform the rapid sampling speed requirement, a high sensitivity is essential for on-line detection. In open tubular column (OTC) CEC experiments, for example, the sample loadability is in the low fmol or even amol range due to the small dimensions of the column, so that a high sensitivity is absolutely necessary to detect those minor components in the sample. To date, quadrupole instruments [12,13,18], ion traps [16], magnetic sectors [15], and Fourier transform mass spectrometers (FT-ICR) [19] have all been used as on-line detectors for these capillary separations.

An alternative means of achieving high sampling speed and sensitivity is the time-of-flight mass spectrometer (TOF-MS) [24]. As a non-scanning device, TOF-MS can measure a complete mass spectrum over an extended mass range following every injection pulse of ions. Thus, this device can provide rapid detection and high duty cycle for high resolution separations, resulting in accurate peak shapes and high sensitivity. In addition, TOF-MS can provide reasonable mass accuracy and mass resolution, especially when used in the reflectron mode.

There have been several recent efforts to interface

ESI and other continuous ion beam sources to TOF-MS. The major difficulty in these efforts is that TOF is intrinsically a pulsed device and requires a pulsed ion source and an accurate start time to achieve time resolution [14,17,25,26]. Therefore, an appropriate method is required to convert a continuous ion beam into pulsed ion packets. In recent work, we have demonstrated the use of a quadrupole ion trap as a front-end storage device prior to TOF analysis [27–29]. The ion trap serves to convert the continuous ion beam for pulsed mass detection by the reflectron TOF-MS. Additional advantages of this ion trap/time-of-flight combination include signal integration in the trap and inherent MS–MS capability as demonstrated in recent work [30–32].

In this review, we describe the results obtained in our laboratory involving the use of an ion trap storage/reflectron time-of-flight mass spectrometer (IT/reTOF-MS) as a rapid and sensitive detector for capillary separations. The use of several high-resolution separation methods, including microbore and capillary HPLC, CE and OTC and packed column CEC as successfully coupled to the IT/reTOF-MS instrument for protein structural analysis are described.

2. Instrumentation

2.1. Mass spectrometer

A schematic diagram of the IT/reTOF-MS instrument is shown in Fig. 1. It consists of a differentially pumped reflectron TOF mass spectrometer (Model D-850) interfaced to a quadrupole ion trap storage device (Model C-1251, R.M. Jordan, Grass Valley, CA, USA). Ion beams generated from the electrospray source were introduced into an atmospheric pressure interface by a 0.5 mm I.D. stainless-steel capillary heated to 120°C. The interface region was evacuated to a pressure of less than 1 Torr (1 Torr=133.322 Pa), where a cylindrical lens and a 325 μm orifice skimmer were aligned co-axially. The cylindrical lens serves to focus the ion beam into the skimmer orifice. The ions traversing through the skimmer were focused into the ion trap by an einzel lens. The ions were stored in the ion trap for a preset period of time (typically from 0.1 to 0.5 s, corresponding to a sampling rate of 2 to 10 Hz) and with a preset rf voltage (typically from 1000 to 1500 Vpp). After the ions were stored for a preset time, a d.c. pulse (−400 V, 2 μs pulse width) was applied to the exit endcap of the ion trap and ions were extracted into the TOF tube for mass analysis.

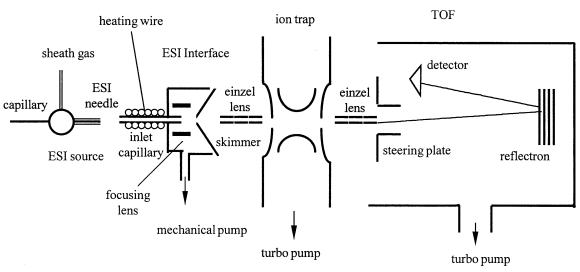

Fig. 1. A schematic digram of the IT/reTOF MS.

2.2. ESI/nanospray

In the HPLC and packed CEC experiments, the column effluents were transferred by a 75 μm I.D. fused-silica capillary to a 0.004 in. (1 in.=2.54 cm) stainless-steel electrospray needle which was floated at about 4 kV relative to the inlet capillary to the mass spectrometer. For CE and open tubular column (OTC)-CEC experiments, a nanospray source was constructed in-laboratory in which the outlet end of the capillary was directly used as the electrospray needle. The polymer coating of the capillary tip was first burnt off, then the tip was etched by concentrated hydrofluoric acid, and finally electroless plated with silver [33].

2.3. Data system

The data system for these experiments was based on a 200 MHz high-speed transient recorder (Model 9845, Precision Instruments, Knoxville, TN, USA) embedded in a Pentium PC computer, and data processing was also performed on this computer with a user-written program [34]. In this program, data reduction was accomplished by setting a threshold slightly above the background level and only signals with intensities higher than the threshold were saved with their corresponding masses as indexed flight-time/intensity pairs producing a typical reduction ratio of 30:1 in data set size. As a result, this system

is capable of recording, at rates of over 10 Hz, an individual mass spectrum with 16 000 data points and 10 ns resolution for a minimum length of 30 min. In addition, the data system is capable of displaying, for real-time evaluation of the analysis, each individual mass spectrum and the total ion chromatogram. Furthermore, the selected ion chromatograms of given masses and a 3-dimensional topographic map describing a separation process can be rapidly generated from the collected data for the unambiguous and high fidelity identification of target analytes in a complex mixture. A sampling time window width of 150 μs, which corresponds to a m/z range from 0 to ~1500, was used for most of the studies in this work. Mass calibration was carried out by measuring the flight time (T) of a few model peptides to determine the constants a and b in the equation by linear regression analysis, $(m/z)^{1/2} = aT + b$.

2.4. HPLC

Both microbore and capillary HPLC methods have been interfaced to the IT/reTOF-MS instrument in our laboratory [35,36]. Microbore HPLC was performed with a 15×1.0 mm C_{18} column from Alltech Associates (Deerfield, IL, USA). A Star 9012 solvent delivery system (Varian Associates, Walnut Creek, CA, USA) was operated at a constant flow-rate of 200 μl/min. The solvent split was performed by a prime/purge valve located immediately before the injection valve with a ratio of 3:1. Capillary HPLC was performed with a 150×0.25 mm C_{18} column packed in-laboratory using a slurry packing method. A higher split ratio (39:1) resulted in a flow-rate of 5 μl/min for the capillary separations. For all LC–MS experiments, a Star 9050 (Varian) variable-wavelength UV detector with a microflow cell (Varian) was used to monitor the effluents from the column at 214 nm. The effluents from the UV detector were directly transferred to the ESI source. In the gradient elution mode, 0.1% trifluoroacetic acid (TFA) in water was used as solvent A and 0.09% TFA in acetonitrile–water (90:10) as solvent B. Sample stacking was performed by running solvent A for 2 min before the start of the gradient.

2.5. Capillary electrophoresis

The CE apparatus used in our laboratory was built in-laboratory [33,37]. Fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) were used as separation capillaries, whose inner surface were modified by either 3-aminopropyltrimethoxysilane (APS) or polybrene coating, so that the adsorption of peptides with the capillary inner wall was minimized. A high voltage power supply (Model CZE 1000R, Spellman, Plainview, NY, USA) operated in the negative mode was used to provide the separation voltage. The typical electric field used was about 300 V/cm capillary. 10 mM ammonium acetate was used as the running buffer for the APS coated capillary, while 100 mM formic acid supplemented with 5 mM ammonium acetate was used as the running buffer for the polybrene coated capillaries. On-column detection was performed using a variable-wavelength UV detector (Model SC 100, ThermoSeparation Products, Fremont, CA, USA) at 198 nm.

2.6. OTC-CEC

Open tubular CEC columns were prepared from 9 μm I.D. fused-silica capillaries (Polymicro Technologies). The stationary phase (C_8) was coated onto the inner surface of the capillary by the sol-gel method. After preparing the stationary phase, the inner capillary surface was further modified with APS. This coating has been proven in our experiments to effectively enhance EOF and reduce nonspecific adsorption.

2.7. Packed column CEC

Packed CEC columns were prepared by slurry packing of a 180 μm I.D. fused-silica capillary with 3 μm C_{18} silica gel (courtesy of Vydac, Hesperia, CA, USA). The use of a relatively large diameter of the column allowed enhanced loadability and easy preparation of end- column frits. Each column end was assembled in a Valco micro-column end-fitting with a small amount of glass wool as the frit. Electrical contact was made at the column end-

fittings. A Star 9012 solvent delivery system (Varian) was used to provide the supplementary pressure.

3. Results and discussion

A major goal of this work has been to explore the possibility of using the hybrid IT/reTOF-MS combination as a rapid and sensitive detector for high-resolution capillary separations. Efforts have also been made to develop novel separation techniques with enhanced performance for protein digest analysis. In addition, these on-line separation–MS methods have been used to solve real biomedical problems.

3.1. Interface of HPLC to the IT/reTOF-MS instrument

The capabilities of the IT/reTOF-MS instrument as an on-line detector are demonstrated in the analysis of a tryptic digest of bovine β-casein separated by microbore HPLC as shown in Fig. 2, where the UV trace and the total ion chromatogram (TIC) are compared [33]. Bovine β-casein is a relatively large protein with 209 amino acid residues and an averaged mass of 23583.4 u. The TIC shown in Fig. 2b was qualitatively similar to the UV trace in Fig. 2a. There are some differences in the relative peak heights of the corresponding peaks in the UV trace and TIC. This results from the differences in UV absorption efficiency versus the ionization and detection efficiency of the mass spectrometer. The TIC shown in Fig. 2b was obtained by monitoring ions of all masses over a broad mass range. However, the data can be reprocessed by our computer software to provide the ion chromatogram for selected masses only. This procedure provides a selected ion chromatogram (SIC) for each mass in the spectrum detected. The use of the SIC mode allows the correlation of each chromatographic peak with one or more particular masses, so that unresolved chromatographic components can be unambiguously identified. In addition, since the background ions at other masses have been eliminated from the chromatogram, the S/N is greatly improved. One example of the capabilities of the SICs is shown in Fig. 3,

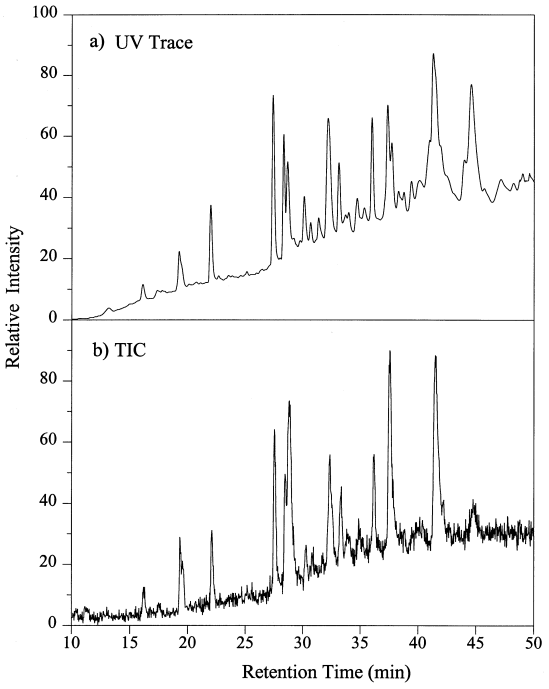

Fig. 2. (a) UV trace and (b) TIC of the microbore HPLC separation of a tryptic digest of bovine β-casein. Reprinted with permission from Ref. [35].

where the SIC profiles corresponding to the unresolved peak eluting at ~27.7 min in Fig. 2b are shown. From these SICs, it can be concluded that this peak contains three closely eluting components with retention times of 27.6 min, 27.7 min and 27.7 min, respectively. Their corresponding mass spectra are shown in Fig. 4.

In recent work, capillary HPLC has been interfaced to an IT/reTOF-MS instrument for structural confirmation of recombinant protein isoforms [36]. Nucleoside diphosphate kinase (NDPK) is a family of proteins functioning as a catalyst for conversion of diphosphate to its triphosphate counterpart. Fig. 5 shows the UV traces and TICs of CNBr/V8 digests of two NDPK isomers, NDPK A and NDPK B. As shown in Fig. 5, a large number of fragments result from the V8 digests of the protein isoforms, which is attributed to the relatively high abundance of glutamic and aspartic amino acid residues and efficient separation using a C_{18} capillary column. From the CNBr/V8 digestion, 86% and 98% of the

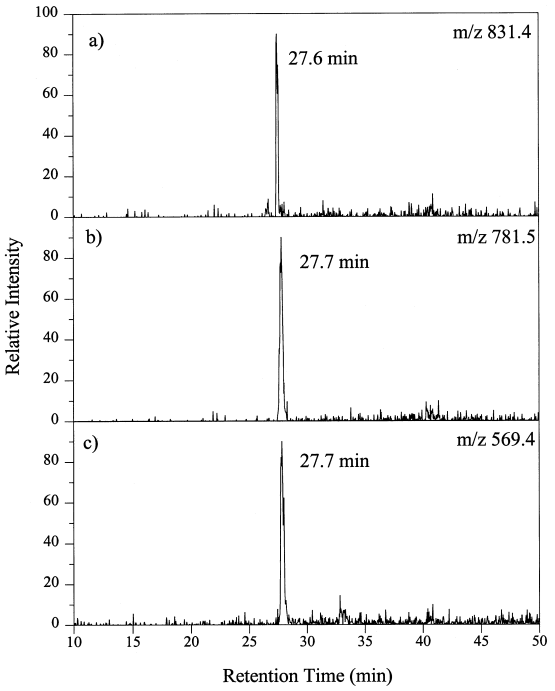

Fig. 3. SICs of the separation of bovine β-casein tryptic peptides shown in Fig. 1. Reprinted with permission from Ref. [35].

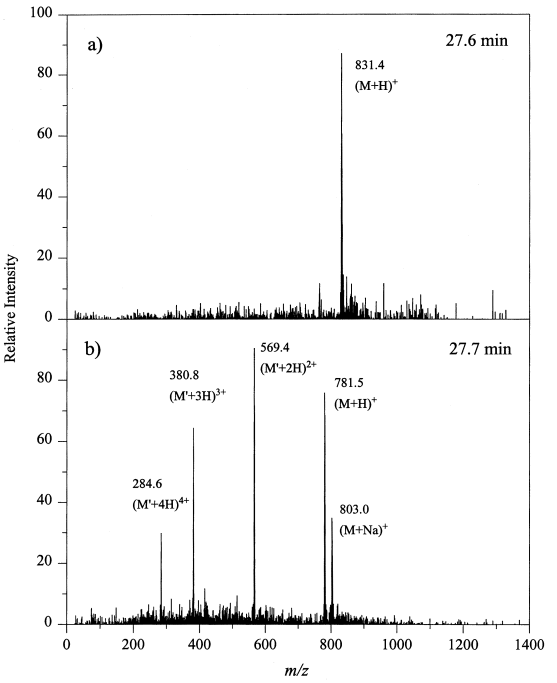

Fig. 4. Mass spectra corresponding to the SICs shown in Fig. 3. Reprinted with permission from Ref. [35].

sequences have been verified for NDPK A and B, respectively. The specific digest fragment regions obtained by CNBr/V8 digestion are summarized in Table 1. The calculated and actual measured masses are within ±1 Da.

3.2. Interface of CE to the IT/reTOF-MS instrument

As a fast and highly efficient separation technique, CE separations are usually accomplished within 10 to 15 min with over 100 000 theoretical plates. The result is that the peaks eluted are extremely narrow, with a peak width on the order of a second or even subsecond. In order to detect these sharp peaks, the detector must be sampling at a sufficiently fast rate. In addition, the sample loadability of CE is much lower than that in HPLC, mainly due to the small dimensions of the capillary. Therefore, a highly sensitive detector is required for CE experiments. The IT/reTOF-MS instrument meets the required speed and sensitivity [33]. Fig. 6 shows the total ion electropherograms (TIEs) of the separation of a synthetic peptide mixture at a sampling rate of 0.5 Hz, 2 Hz and 10 Hz, respectively. These separations clearly demonstrate that a high sampling rate allows for a more accurate representation of the true CE profiles and the IT/reTOF-MS instrument has the capability to preserve the high separation quality for even the narrowest peaks. These TIEs also appear to be retaining similar signal-to-noise ratios. Although the number of ions stored and detected per pulse decreases as the repetition rate increases, the number of ions detected per unit time is relatively the same.

Because of the ion storage capability of the ion trap and the non-scanning property of the TOF mass spectrometer, high sensitivity could be achieved with the IT/reTOF-MS configuration. In Fig. 7 are shown the TIEs of a CE–MS separation of a tryptic digest of bovine cytochrome *c* with sample injection of 50, 12 and 3 f*M*, respectively. A 4 Hz sampling rate was used in these experiments. At 12 f*M* sample injection, the TIE is still reproducible, and all the peaks that appear in the 50 f*M* TIE can be observed with a high S/N ratio. At 3 f*M* injection, though, only four of the major peaks can be observed, two of

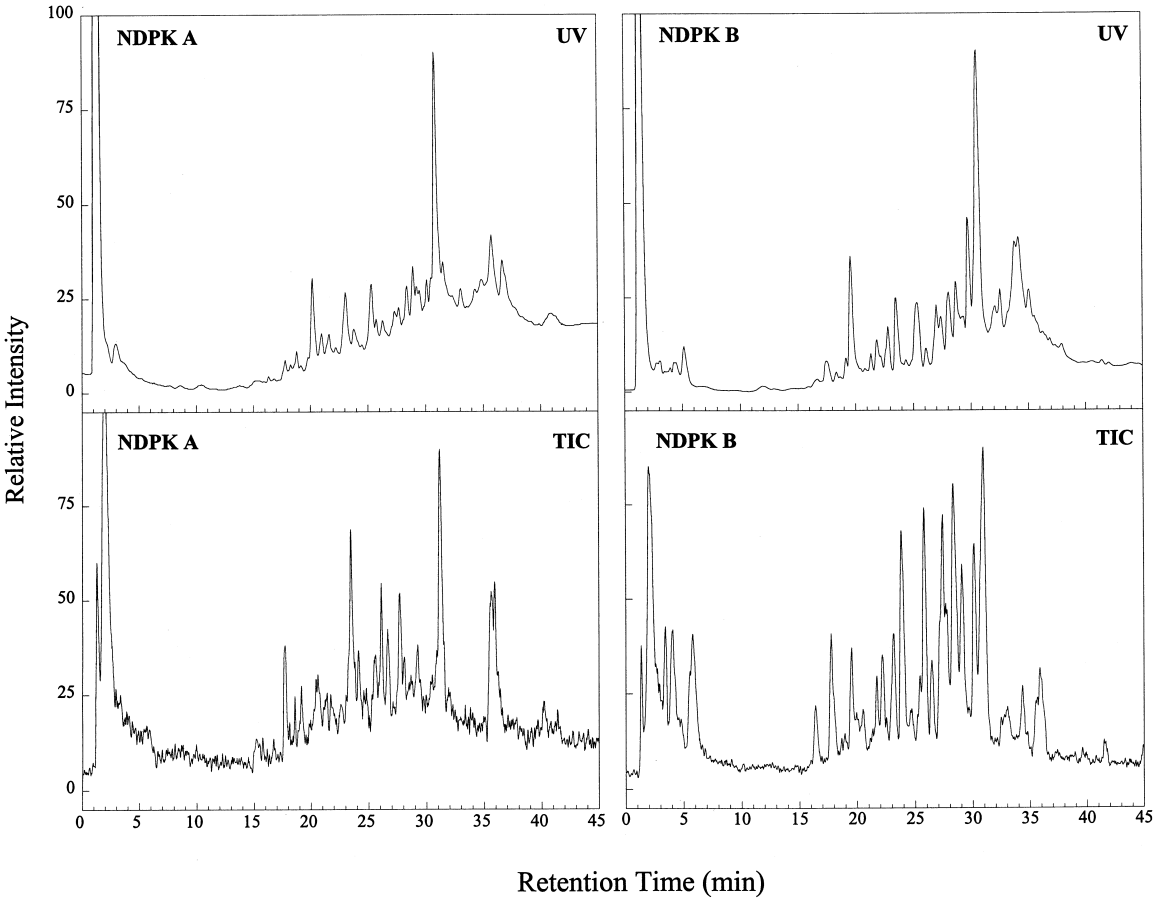

Fig. 5. UV traces and TICs of the capillary HPLC separation of the CNBr/V8 digests of NDPK A and B. Reprinted with permission from Ref. [36].

them with low S/N ratios. However, under the selected ion electropherogram (SIE) mode, these two peaks can still be identified with a high S/N as is shown in Fig. 8. It is noteworthy that all these results were obtained with a 41 μm I.D. capillary, which is in the range of typical capillary dimensions for conventional CE experiments. Wahl et al. [39] reported amol level detection of proteins using a 5 μm I.D. capillary for the CE–MS experiment and found that there was a sensitivity boost of 25–50 fold compared to the results obtained with 50 μm I.D. capillaries. Currently the detection limit of our CE–MS experiments is still not as low as that reported by Wahl et al. with the 5 μm I.D. capillary, but the potential for this instrument to reach a comparable detection limit is realistic if the dimensions of the capillaries used in our experiment could be reduced sufficiently. Also, if the ion transmission efficiency of our current instrument could be improved by using an rf only multipole ion guide and various dynamic trapping methods to improve trapping efficiency [40], then a significant improvement in sensitivity might be expected.

For CE–MS experiments using conventional scanning mass spectrometers, mass resolution is usually sacrificed for sensitivity. In the case of the IT/reTOF MS instrument, mass resolution is not related to sensitivity since it is a non-scanning instrument. High sensitivity is achieved with a reasonably high resolution, which makes it possible to solve many real problems, such as rapid analysis of mutation sites in human hemoglobin [37,38]. The detection

Table 1
Selected Fragments of NDPK A and B from the CNBr/V8 digestion

No.	Fragment	Retention time (min)	$[M+H]^+$		Δm	Sequence
			Calculated[a]	Measured[b]		
NDPK A						
14	77–79	18.57	433.2	433.2	0.0	VWE
15	80–90	26.10	1125.7	1124.7	−1.0	GLNVVKTGRVM
16	91–93	19.77	318.2	317.9	−0.3	LGE
16, 17	91–98	36.33	816.4	816.0	−0.4	LGETNPAD
17	94–98	18.97	517.2	517.7	0.5	TNPAD
18	99–107	21.37	930.5	931.0	0.5	SKPGTIRGD
21	125–127	17.67	306.1	306.2	0.1	SAE
21, 22	125–129	25.53	563.3	563.4	0.1	SAEKE
23, 24	130–138	26.03	1127.6	1128.7	1.1	IGLWFHPEE
24, 25	138–141	26.20	475.2	475.1	−0.1	ELVD
NDPK B						
6	30–38	25.87	1001.6	1000.7	−0.9	QKGFRLVAM
7	39–45	31.00	850.5	850.8	0.3	KFLRASE
7, 8	39–46	30.83	979.5	979.7	0.2	KFLRASEE
12	69–76	28.37	726.4	727.2	0.8	NSGPVVAM
12, 13	69–79	27.47	1188.6	1186.9	−1.7	NSGPVVAMVWE
13	77–79	19.93	433.2	433.3	0.1	VWE
14	80–90	25.70	1125.7	1125.7	0.0	GLNVVKTGRVM
19	122–127	21.67	620.3	619.9	−0.4	SVKSAE
19, 20	122–129	25.43	877.5	877.4	−0.1	SVKSAEKE
20, 21	128–137	17.70	1276.7	1277.9	1.2	KEISLWFKPE
23	139–141	19.19	346.2	346.5	0.3	LVD
24	142–148	27.73	880.4	880.9	0.5	YKSCAHD
25	149–152	26.30	596.3	596.9	0.6	WVYE

[a] Assuming conversion of methionine to homoserine lactone and alkylation of cysteine by iodoacetamide.
[b] Average mass of all charge states of the fragment observed.
Reprinted with permission from Ref. [36].

and identification of hemoglobin variants has become an important area in clinical practice. More than 700 structural variants of hemoglobin are known to date, some of which may be responsible for severe diseases such as sickle cell anemia. Fig. 9 shows the TIEs of the separation of tryptic digests of a normal hemoglobin (HbA, Fig. 9a) and a variant hemoglobin (HbS, Fig. 9b). HbS is responsible for sickle cell anemia. There is an additional peak in the TIE of HbS compared to normal hemoglobin as marked by an arrow in Fig. 9b. The mutation in HbS represented by this peak, corresponds to a substitution of a glutamic acid group by a valine at the β6 position, where the variation of the charge altered the elution time under the electric field for this specific fragment. This was confirmed by the mass spectrum corresponding to this additional peak in the TIE as shown in Fig. 10. In Fig. 10 are shown the mass spectra for βT1 fragment in normal hemoglobin (Fig. 10a) and in HbS (Fig. 10b). The relatively high mass resolution in these mass spectra clearly shows that there is a 15 Da mass difference between these two doubly charged ions, corresponding to a mass difference of 30 Da for singly-charged ions, which is expected for the substitution of a glutamic acid by a valine.

3.3. Interface of CEC to the IT/reTOF-MS instrument

CEC is a novel liquid chromatographic separation method that uses EOF to transport solvent in a reversed-phase column. The use of EOF generates a nearly flat flow profile and a uniform flow velocity

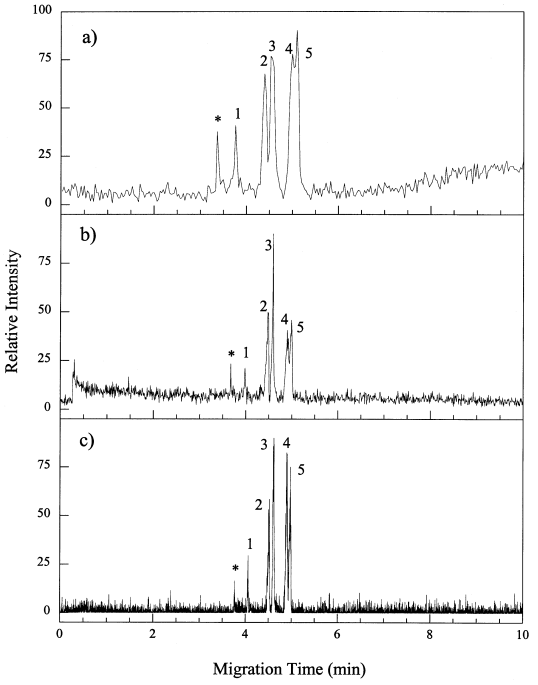

Fig. 6. TIEs of the CE separation of a five peptide mixture at three different sampling speeds of (a) 0.5 Hz, (b) 2 Hz and (c) 10 Hz. The five peptides are: (1) Met-enkephalin, (2) Leu-enkephalin-Arg, (3) neurotensin, (4) bradykinin and (5) angiotensin III. Reprinted with permission from Ref. [34].

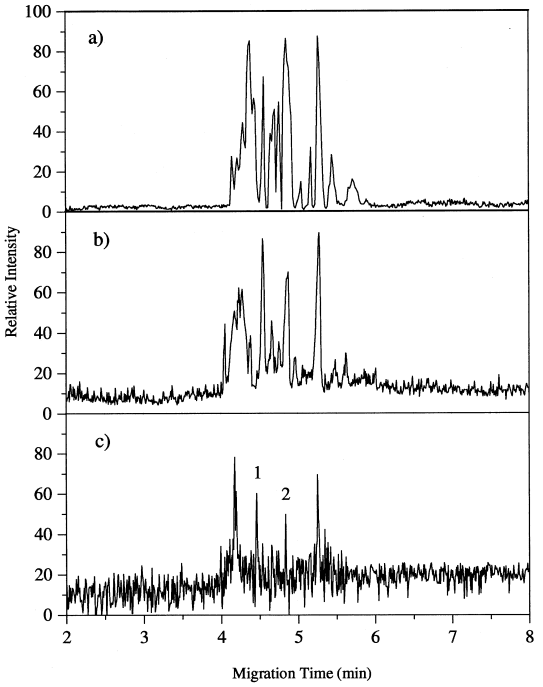

Fig. 7. TIEs of the CE separation of bovine cytochrome *c* digest with sample injection amount of (a) 50 f*M*, (b) 12 f*M* and (c) 3 f*M*. Reprinted with permission from Ref. [33].

distribution, which result in high separation efficiency. In our laboratory, both OTC and packed-column CEC have been developed and successfully coupled to the IT/reTOF-MS instrument [41,42].

OTCs have many distinct advantages compared to packed columns. OTCs with inner diameters around 10 μm have been found to have a smaller plate height compared to packed columns due to the lack of band broadening effects associated with the existence of packing particles and end column frits. High concentration sensitivity is also another advantage of OTCs since columns with extremely small dimensions are used. Also, the small diameters of the capillaries allow the use of a higher voltage in CEC without significant Joule heating. Furthermore, OTCs provide more rapid separations than packed columns by eliminating intraparticulate diffusion, which is the dominant limitation for ultrafast separations in packed columns. It is therefore a unique tool for ultrafast reversed-phase separations.

In spite of these advantages, OTCs are not widely used in HPLC separations mainly because of the difficulties associated with sample injection and detection. The injection volume for OTCs is in the low nl or even pl range. For such a small injection volume, a split device must be used, which results in a waste of sample and complexity of instrumentation. In the CEC mode, however, samples can be injected electrokinetically, thus dramatically decreasing sample consumption and avoiding the complexity of split injection required for OTC-HPLC. Although the extremely small inner diameters of OTCs make optical detection methods difficult, they are compatible with a concentration sensitive detection method such as electrospray ionization-mass spectrometry (ESI-MS), whose response is independent of the optical path length of the column. Thus, the major disadvantages of OTCs may be overcome in a CEC–ESI-MS configuration.

Because of the characteristics of the capacity factors of peptides, small changes in isocratic concentrations of the organic component in the mobile

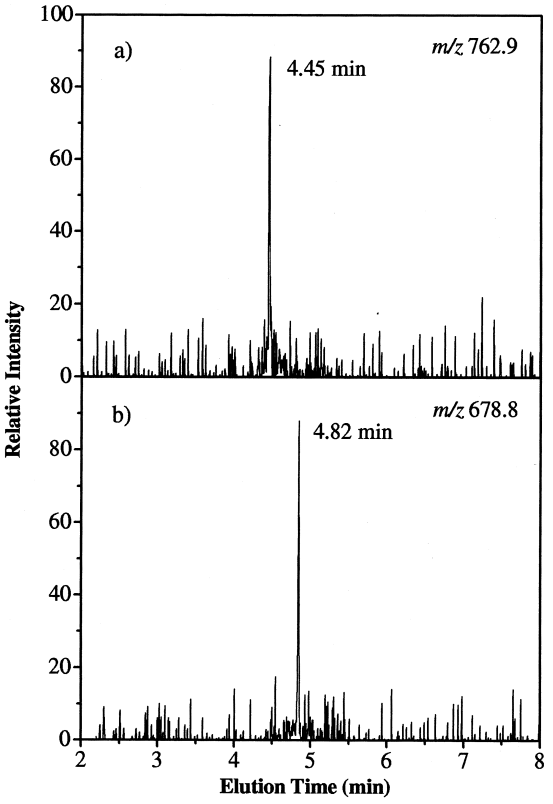

Fig. 8. SIEs corresponding to the two peaks marked by ''1'' and ''2'' in Fig. 7c. Reprinted with permission from Ref. [33].

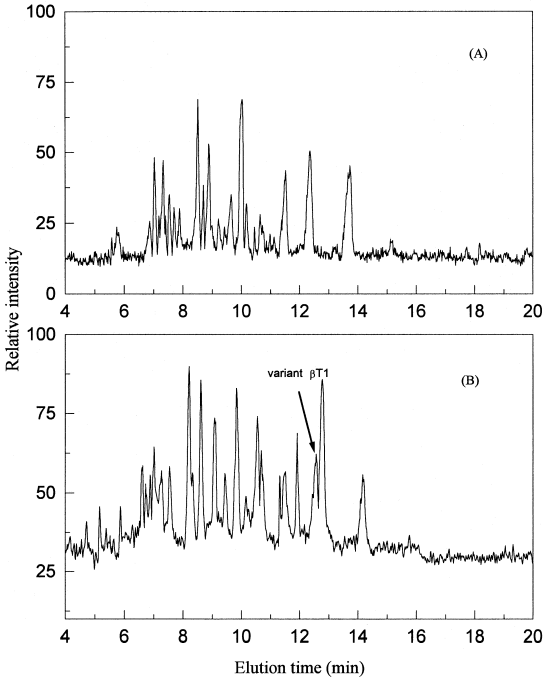

Fig. 9. TIEs of the CE separation of tryptic digests of (a) HbA and (b) HbS. Reprinted with permission from Ref. [38].

phase result in dramatic changes in peptide retention times [43]. It is thus difficult to separate a complex peptide mixture such as a protein digest using the isocratic mode. Gradient CEC without a pressure driven flow has been reported recently by Yan et al. [44], in which two separate high voltage power supplies were used to generate the EOFs in two solvent delivery capillaries to form the gradient. In our experiments, a more straightforward setup was used to perform a gradient CEC separation. For gradient experiments, solvent B [5 m*M* ammonium acetate in acetonitrile–water (80:20) with 0.05% TFA] was delivered at a rate predetermined by the gradient into the inlet buffer reservoir which contained a certain amount of solvent A (5 m*M* ammonium acetate in water with 0.05% TFA) and was stirred on a magnetic ministirrer. Fig. 11 shows the UV trace and the TIC of a gradient OTC-CEC separation of a tryptic digest of horse heart myoglobin. Taking advantage of the gradient elution, over 10 peaks were resolved in both the UV and TIC within 6 min. Among the peaks shown in the TIC, at least 15 (including co-eluting components) could be assigned to the expected fragments covering about 90% percent of the protein sequence. The corresponding mass spectra were collected over a full mass range at a sampling rate of 8 Hz and the mass resolution was ~1500.

In recent work, packed-column CEC has been interfaced to the IT/reTOF instrument for the analysis of protein digests. The use of a supplementary pressure appears to suppress bubble formation in packed CEC. More importantly, though, it may provide a unique advantage for the separation of charged species in the CEC mode. The separation mechanism in CEC for neutral analytes is the solute partition between the stationary phase and the mobile phase as in conventional HPLC. The mechanism for charged species, however, is more complicated, since both partition and electrophoresis will contribute to the separation. Generally, the extent of partitioning

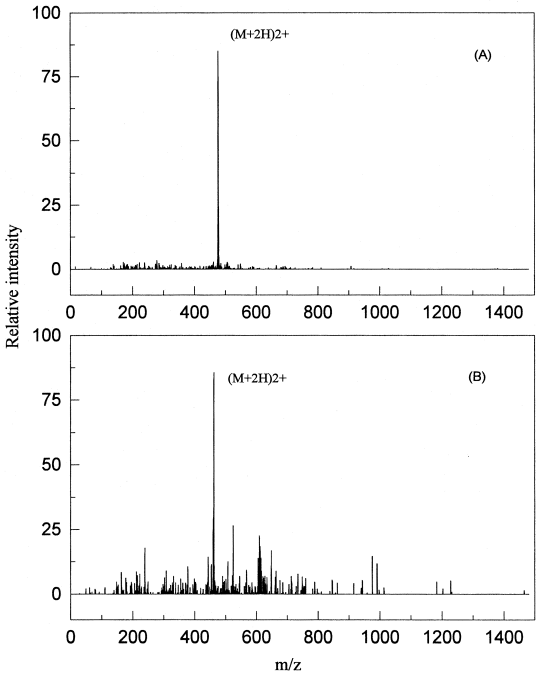

Fig. 10. Mass spectra of βT1 fragment in (a) HbA and (b) HbS. Reprinted with permission from Ref. [38].

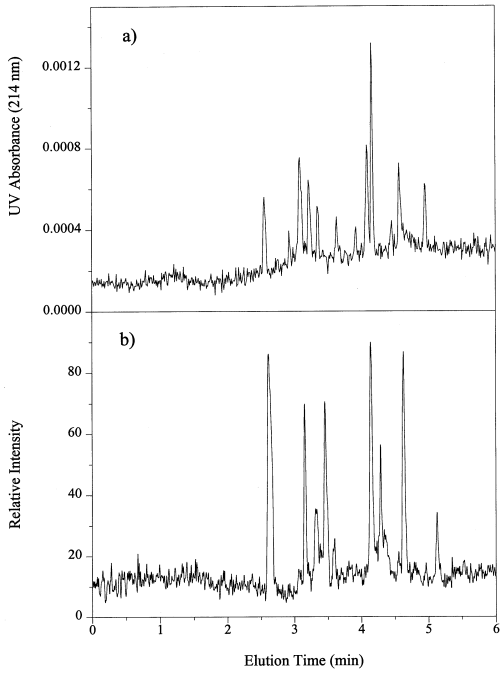

Fig. 11. (a) UV trace and (b) TIC of the OTC CEC separation of tryptic digest of horse heart myoglobin. Reprinted with permission from Ref. [41].

and the electrophoretic mobility are two orthogonal properties of a peptide. There might be cases in which the electrophoretic migration could adversely affect a reversed-phase separation. Therefore, it is usually necessary to optimize the electrophoretic migration of peptides in a CEC separation. In CEC experiments without a supplementary pressure, the solvent flow results only from the EOF, which is proportional to the applied voltage. A change in the electrophoretic migration rate is always accompanied by a change in the mobile phase flow-rate. Our laboratory has demonstrated theoretically that the capacity factors of peptides in an unpressurized CEC system are independent of the applied electric field. Thus, it may be difficult to optimize the selectivity of peptides using the applied field in CEC without a supplementary pressure. In a pressurized CEC system, however, the electrophoretic migration rate and the mobile phase flow-rate can be optimized independently. The tuning of the selectivity of peptides becomes possible since the applied voltage and the supplementary pressure are available as two separate tunable parameters that can be optimized for achieving higher selectivity than using either the HPLC or CE mode alone.

The concept of tunable selectivity in pressurized packed CEC has been demonstrated in Fig. 12, which shows the TICs of a tryptic digest of bovine cytochrome *c* using the packed CEC separation. A 6 cm long column with gradient elution was used in this experiment. In Fig. 12a, no separation voltage was applied and the separation was performed in the normal HPLC mode with a backpressure of 90 bar. The use of a short column in the HPLC mode made it difficult to resolve all the components in the digest as indicated by the peak marked by an arrow, which contains two co-eluting components. In Fig. 12b, a 1000 V voltage was applied on the column and the backpressure was reduced to 50 bar. Even with a reduced backpressure, all the components eluted faster in Fig. 12b than in Fig. 12a, due to the contribution from EOF and electrophoretic migration. The peaks are sharper in Fig. 12b, indicating an increase in separation efficiency. The two co-eluting

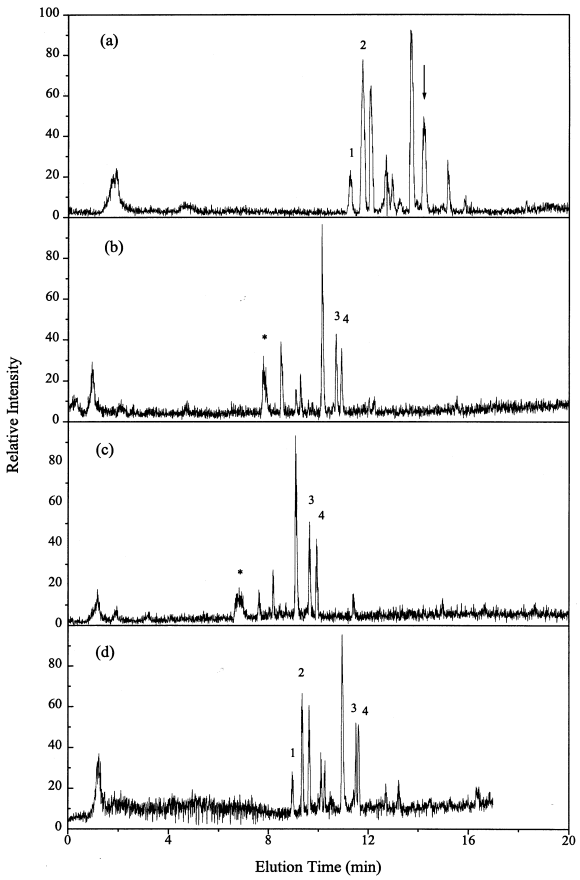

Fig. 12. TICs of pressurized packed CEC separation of bovine cytochrome *c* digest. Column length 6 cm. Column operation conditions are: (a) HPLC mode with a backpressure of 90 bar; (b) 1000 V applied voltage with 50 bar supplementary pressure; (c) 1400 V applied voltage with 50 bar supplementary pressure; (d) 600 V applied voltage with 70 bar supplementary pressure. Reprinted with permission from Ref. [42].

components in the peak marked by an arrow in Fig. 12a were separated to the baseline in Fig. 12b (marked by ''3'' and ''4'') mainly due to the electrophoretic separation of these two components. However, the first two peaks in Fig. 12a (marked by ''1'' and ''2'') migrated into one peak in Fig. 12b (marked by an asterisk), since apparently for these two particular fragments, the electrophoretic separation is in the opposite direction as the separation resulting from partition. A further increase in the applied voltage resulted in even faster separations (1400 V, Fig. 12c), but the peak marked by the asterisk is still unresolved. This indicates that it might be possible to resolve all the components by optimizing the electrophoretic migrations of these peptides, so that they are large enough to resolve peaks 3 and 4, but small enough not to result in co-eluting of peaks 1 and 2. With an adjustable supplementary pressure in CEC, the applied voltage can be tuned to optimize the selectivity over a wide range as shown in Fig. 12d, where the separation was performed at a relatively low voltage of 600 V with a supplementary pressure of 70 bar. At this specific pressure and voltage, all the four peaks were clearly resolved. It should also be noted that without this supplementary pressure, the EOF generated by this relatively low voltage would be too low to perform a chromatographic separation in a reasonable time frame.

4. Conclusions

A variety of capillary separation methods have been developed in our laboratory, which exhibit enhanced separation efficiency and speed. These separation methods have been successfully interfaced to the IT/reTOF-MS instrument for the characterization of proteins and peptides. The ion storage capability of the ion trap and the non-scanning property of the TOF-MS make it possible to detect low fmol levels of peptides while maintaining the high separation quality. The high efficiency of these separation methods, combined with the rapid and sensitive IT/reTOF mass detector, are very useful tools in solving real biomedical problems.

Acknowledgements

We gratefully acknowledge support of this work by the National Institutes of Health under Grant No. 1R01GM49500 and the National Science Foundation under Grants No. BIR-9223677 and BIR-9513878.

References

[1] B.L. Karger, W.S. Hancock, Methods Enzymol. 270 (1996).
[2] J.W. Jorgenson, K.D. Lukacs, Science 222 (1983) 266.

[3] M.J. Gordon, X. Huang, S.L. Pentoney Jr., R.N. Zare, Science 242 (1988) 224.
[4] V. Pretorius, B.J. Hopkins, J.D. Schieke, J. Chromatogr. 99 (1974) 23.
[5] J.W. Jorgenson, K.D. Lukacs, J. Chromatogr. 218 (1981) 209.
[6] J.H. Knox, I.H. Grant, Chromatographia 32 (1991) 317.
[7] B. Behnke, E. Bayer, J. Chromatogr. 680 (1994) 93.
[8] C. Yan, R. Dadoo, H. Zhao, R.N. Zare, D.J. Rakestraw, Anal. Chem. 67 (1995) 2026.
[9] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[10] M.S. Wilm, M. Mann, Int. J. Mass Spectrom. Ion Processes 136 (1994) 167.
[11] M.R. Emmett, R.M. Caprioli, J. Am. Soc. Mass Spectrom. 5 (1994) 605.
[12] T.R. Covey, E.C. Huang, J.D. Henion, Anal. Chem. 63 (1991) 1193.
[13] R.D. Smith, J.H. Wahl, D.R. Goodlett, S.A. Hofstadler, Anal. Chem. 65 (1993) 574A.
[14] L. Fang, R. Zhang, E.R. Williams, R.N. Zare, Anal. Chem. 66 (1994) 3696.
[15] J.B. Perkins, K.B. Tomer, Anal. Chem. 66 (1994) 2835.
[16] R.S. Ramsey, D.E. Goeringer, S.A. McLuckey, Anal. Chem. 65 (1993) 3521.
[17] J.F. Banks, T. Dresch, Anal. Chem. 68 (1996) 1480.
[18] D. Figeys, I. van Oostveen, A. Ducret, R. Aebersold, Anal. Chem. 68 (1996) 1822.
[19] S.A. Hofstadler, J.H. Wahl, J.E. Bruce, R.D. Smith, J. Am. Chem. Soc. 115 (1993) 6983.
[20] K. Schmeer, B. Behnke, E. Bayer, Anal. Chem. 67 (1995) 3656.
[21] S.J. Lane, R. Boughtflower, C. Paterson, M. Morris, Rapid Commun. Mass Spectrom. 10 (1996) 733.
[22] S.E.G. Dekkers, U.R. Tjaden, J. van der Greef, J. Chromatogr. 712 (1995) 201.
[23] D.B. Gordon, G.A. Lord, D.S. Jones, Rapid Commun. Mass Spectrom. 8 (1994) 544.
[24] R.J. Cotter, Anal. Chem. 64 (1992) 1027A.
[25] O.A. Mirgorodskaya, A.A. Shevchenko, I.V. Chernushevich, A.F. Dodonov, A.I. Miroshnikov, Anal. Chem. 66 (1994) 99.
[26] A.N. Verentchikov, W. Ens, K.G. Standing, Anal. Chem. 66 (1994) 126.
[27] S.M. Michael, B.M. Chien, D.M. Lubman, Anal. Chem. 65 (1993) 2614.
[28] B.M. Chien, S.M. Michael, D.M. Lubman, Anal. Chem. 65 (1993) 1916.
[29] M.G. Qian, D.M. Lubman, Anal. Chem. 67 (1995) 234A.
[30] M.G. Qian, D.M. Lubman, Rapid Commun. Mass Spectrom. 9 (1995) 1275.
[31] L. He, J.-T. Wu, S. Parus and D.M. Lubman, Rapid Commun. Mass Spectrom., (1997) in press.
[32] J.-T. Wu, L. He, M.X. Li, S. Parus and D.M. Lubman, J. Am. Soc. Mass Spectrom., (1997) in press.
[33] J.-T. Wu, M.G. Qian, M.X. Li, L. Liu, D.M. Lubman, Anal. Chem. 68 (1996) 3388.
[34] M.G. Qian, J.-T. Wu, S. Parus, D.M. Lubman, Rapid Commun. Mass Spectrom. 10 (1996) 1209.
[35] M.G. Qian, D.M. Lubman, Anal. Chem. 67 (1995) 2870.
[36] M.G. Qian, K. Zheng, Y. Chen, C.L. Chang, S.M. Hanash, D.M. Lubman, Rapid Commun. Mass Spectrom. 10 (1996) 1079.
[37] M.X. Li, J.-T. Wu, L. Liu, D.M. Lubman, Rapid Commun. Mass Spectrom. 11 (1997) 99.
[38] M.X. Li, L. Liu, J.-T. Wu, D.M. Lubman, Anal. Chem. 69 (1997) 2451.
[39] J.H. Wahl, D.R. Goodlett, H.R. Udseth, R.D. Smith, Electrophoresis 14 (1993) 448.
[40] J. Qin, B.T. Chait, Anal. Chem. 68 (1996) 2102.
[41] J.-T. Wu, P. Huang, M.X. Li, M.G. Qian, D.M. Lubman, Anal. Chem. 69 (1997) 320.
[42] J.-T. Wu, P. Huang, M.X. Li, D.M. Lubman, Anal. Chem. 69 (1997) 2908.
[43] C.T. Mant, T.W. Lorne Burke, R.S. Hodges, Chromatographia 24 (1987) 565.
[44] C. Yan, R. Dadoo, R.N. Zare, D. Rakestraw, D.S. Anex, Anal. Chem. 68 (1996) 2726.