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Sensitive high-resolution analysis of biological molecules by capillary zone electrophoresis coupled with reflecting time-of-flight mass spectrometry

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Abstract

Off-line and on-line capillary zone electrophoresis–electrospray ionization time-of-flight mass spectrometry (CZE–ESI-TOF-MS) experiments were conducted using uncoated fused-silica capillaries coupled to a reflecting TOF mass spectrometer via a gold-coated sheathless interface. Off-line and on-line experiments were performed on standard mixtures of proteins and peptides. Samples collected off-line electrokinetically in plastic vials were analyzed by standard ESI–TOF-MS at the pmol level. Sheathless CZE–ESI-TOF-MS was first simulated in an off-line experiment, using a test bench, in order to select a suitable running electrolyte, to find the optimal electrospray potential, and also to test the gold-coated capillary tips. This enabled an ease of transition to on-line measurements. On-line CZE–ESI-TOF-MS measurements of the total ion electropherogram (TIE) and of selected ion electropherograms (SIE) on peptide mixtures demonstrated fmol-level sensitivity, with S/N values of 250–400 on raw data (TIE mode) and of 30–760 (SIE mode). The use of reflecting TOF-MS afforded mass resolution values $R>6000$ ($m/\Delta m_{\mathrm{FWHM}}$) and enabled isotopic resolution of peptide components as well as mass accuracy in the 10 ppm range. These results were comparable with values observed with the usual ESI source on the same mass spectrometer, and thus demonstrated no loss in spectral quality from using the sheathless CE interface. On-line CE separation efficiency was equivalent to that obtained off-line for the separation of a peptide mixture, with $N=35\,000$–$87\,000$ theoretical plates. Separations of standard proteins yielded equivalent mass spectral resolution and accuracy with separation efficiencies of $N=2800$–5500 and S/N values of 110–225 on raw data. The gold-coated sheathless CE–ESI interface was found to be relatively easy to prepare with the use of gold vapour deposition. The interface produced a stable electrospray for extended periods of time and was found to be robust and reliable.

Keywords: Capillary electrophoresis–mass spectrometry; Interfaces, CE–MS; Electrospray ionization; Detection, electrophoresis; Peptides; Proteins

1. Introduction

Capillary electrophoresis (CE) constitutes one of the most powerful methods for the separation of biological molecules, including proteins and peptides. Separation efficiencies are typically on the order of 10^5 to 10^6 theoretical plates. The method allows for the analysis of exceedingly small volumes of solution, i.e. on the order of few tens of nanoliters, which is advantageous considering that sample sizes may be limited when dealing with biological materials. The principles of CE are well understood, and

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explained in several review articles and textbooks, with details on various practical aspects (e.g. [1–3]).

Coupling mass spectrometry (MS) with CE yields a very powerful and sensitive means of detection of biologically significant components. A number of different CE–MS approaches have been explored, and as a result, on- and off-line methods have been developed. CE has been coupled off-line with matrix-assisted laser desorption ionization (MALDI)-TOF-MS in order to take advantage of the sensitivity and robustness provided by MALDI. Various methods have been introduced for sample collection prior to MALDI-TOF-MS. These methods differ in their complexity, ease of use and range of application [4–7]. Although CE–MALDI-TOF-MS can be very helpful, sample handling is time-consuming and prevents the method from being used on a routine basis.

Several designs have been introduced for on-line coupling of CE to mass spectrometers. The most common approach involves electrospray ionization (ESI). The use of electrospray is favourable because the flow-rates required by this ionization technique match those generated by CE, i.e. nanoliters per minute. Electrospray is also a soft ionization method which imparts low amounts of energy to the molecules, and thus favours formation of intact molecular ions even for large, labile biomolecules, including non-covalent complexes [8–10]. Multiple protonation of molecules, typical with electrospray, allows the study of high mass molecules, by producing ions at m/z values which fit within the mass range of most commercial instruments.

Several types of CE–MS interface designs have recently been reviewed and compared [11–14]. Two of the most common CE–ESI interfaces are the sheathless and sheath-flow models. The sheath-flow design has been shown to be relatively easy to use and is most popular for commercial applications. Since acidic volatile buffers are used in the sheath liquid, suitable separation buffers for CE can be selected even if they are non-volatile. The major disadvantages of using a sheath liquid are reduction in sensitivity due to higher background, and dilution of the sample as it elutes from the capillary. Another problem is associated with a counterflow of anions from the sheath liquid, which may perturb separation efficiency [15].

A sheathless design has been used by several groups to avoid the above problems [16–22]. Electrical contact at the capillary terminus is provided through a conductive coating, typically gold. Enhanced sensitivity, as well as elimination of mixing problems associated with a sheath liquid, make the sheathless design favourable. Increased sensitivity may also be obtained with the microspray and nanospray designs, for which narrow bore capillaries, and pulled capillary tips, with I.D.s of less than 10 μm, are used [18–20]. However, a gold-coated capillary tip as an electrospray needle requires careful manufacture and is somewhat more difficult and time consuming to make and use compared with the sheath-flow design. The conductive coating used also has a limited lifetime which necessitates its frequent replacement. Sheathless interfaces require that the separation buffers be relatively dilute and volatile in order to maintain compatibility with ESI. This limitation restricts the variety of possible types of separations.

In order to exploit the full potential of MS as a detection method for CE, a complete mass spectrum should be recorded at suitable intervals. To accurately represent the analyte peaks, which are typically on the order of 1 s in width, several mass spectra should be recorded per second. This presents a problem for most of the types of mass spectrometers which have been used as detectors for CE–ESI, such as quadrupoles, ion traps, and ion cyclotron resonance cells [3,11–14]. In these instruments, particularly in scanning instruments such as quadrupole mass filters and ion traps, rapid data acquisition can only be accomplished at the expense of mass resolution ($m/\Delta m_{\mathrm{FWHM}}$) or sensitivity, or both. The performance limits of conventional scanning mass spectrometers generally limit the spectral range to a few hundred daltons when the scan rate exceeds one spectrum/s. These instruments are therefore often used to acquire the total ion electropherogram (TIE) and selected ion electropherogram (SIE) for one or a few ions at a time.

Time-of-flight mass spectrometry has a distinct advantage as a detector for CE because it uses parallel detection and is capable of high repetition rates (up to several kHz). The rate at which useful spectra can be recorded is determined by the rate at which ions can be injected into the mass spectrome-

ter, i.e. by the available sample and the instrument sensitivity, and not by characteristics of the measurement itself. Therefore, recording the complete mass spectrum at each selected interval rather than the intensities of only a few ions does not compromise the performance of the instrument. Total and selected ion electropherograms can then be extracted from the single recorded data set. Reflecting ESI-TOF-MS for peptides and proteins up to molecular mass of 10 000, with mass resolution higher than 5000, mass accuracy in the range of 10 ppm, and fmol sensitivity, is well suited to CE [8]. Moreover, TOF instruments have an unlimited m/z range such that high mass ions with lower charge-states (such as non-covalent complexes) can be analyzed.

The use of orthogonal injection ESI-TOF as a detector for CE has recently been reported by a limited number of groups [21–25]. Both sheath-flow and sheathless designs have been adopted. The mass analyser modes used in these studies included linear TOF [23,24], inverted perfectron TOF [22], and ion-trap reflectron TOF [21,25]. Early work by Fang et al. employed a sheathless interface which used a 25-μm gold wire for electrical contact placed inside the terminus of a fused-silica capillary [23]. Spectra from a separation of peptides were acquired at 10 kHz on a linear TOF spectrometer with a resolution of ca. 100. A detection limit of ca. 40–80 fmol was reported. Banks and Dresch demonstrated fast CE separations of peptides and proteins with coated capillaries using a co-axial sheathflow ESI interface coupled with a linear TOF mass spectrometer [24]. TIEs were integrated at rate up to 8 Hz with a scan rate of 8192 Hz. A resolution of 1000 was obtained and a sensitivity limit of 8 fmol shown for leucine enkephalin. An inverted perfectron TOF mass spectrometer was employed by Muddiman et al. for the separation of three peptides [22]. Coated capillaries were used and coupled via a gold-coated sheathless interface. Mass spectra were generated at a rate of 3500 Hz, but due to limited signal averaging capabilities only one out of 70 spectra acquired were recorded with ca. 2 Da bin width. TIEs were obtained with an S/N of 6 on raw data. An ion-trap storage/reflectron TOF mass spectrometer has recently been interfaced as a detector for CE [21,25]. Separations of peptides from proteolytic digests of proteins has been performed using coated capillaries coupled via a sheathless interface. TIEs were generated with an integration rate of 0.5–4 Hz on fmol amounts of material, resulting in a resolution of ca. 1500 (10 ns bin width) and a S/N of 17.2 on raw data. Detection limits of 2–5 fmol were reported.

In this paper, we demonstrate off-line coupling of capillary zone electrophoresis (CZE) with TOF-MS via fraction collection followed by ESI-TOF analysis, and the feasibility of on-line coupling using a sheathless interface on the Manitoba reflecting ESI-TOF instrument. In contrast to previous work, this instrument uses an ion mirror and single ion counting with a time-to-digital converter (TDC). The instrument performance is better than the TOF instruments mentioned above and our goal was to maintain this high degree of performance when coupled with an on-line separation method such as CZE. CZE is performed on peptide and protein standard mixtures using uncoated fused-silica capillaries. These experiments demonstrate that adequate separations with good CZE and mass resolution may be performed as efficiently as when using coated capillaries. The manufacture of a sheathless interface for this specific system, using gold vapour deposition on etched fused-silica capillaries, is discussed. Off-line optimization of the CZE and ESI conditions using a test bench is described. During on-line experiments, various CZE and ESI parameters were optimized in order to obtain a system that is relatively easy to use, that offers good CZE separation efficiency, and that yields good quality mass spectra. The use of an ion mirror and of single ion counting afforded high mass resolution >6000 and mass accuracy at the 10 ppm level. With the high repetition rate of the instrument, exceptional S/N was observed on raw data, demonstrating that fmol sensitivity is easily achieved. Such S/N values have not been reported before on raw data and are a direct result of the high quality performance of the Manitoba ESI-TOF mass spectrometer [8,26]. In these measurements, a complete mass spectrum was acquired but not recorded in full at each time interval, because storage and software facilities are not yet implemented to record the large data sets associated with CE–MS [22,25]. To demonstrate the potential of CZE–ESI-TOF-MS, TIEs and SIEs were recorded on separate runs. The technology necessary to attain the full potential of this approach is currently being installed.

2. Experimental

2.1. Chemicals

Solutions of protein and peptide standards were prepared in water at concentrations of 10^{-6} to 10^{-3} *M*. Angiotensinogen fragment 11–14 (M_r 481.6), bradykinin fragment 1–6 (M_r 659.7), luteinizing hormone releasing hormone (LHRH) (M_r 1182.5), renin substrate (M_r 1759.0), substance P (M_r 1347.6), bovine insulin (M_r 5734), myoglobin (M_r 16951) and cytochrome *c* (M_r 12359) were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Acetic acid (99.9985%) was used to make up solutions of the running electrolyte and was obtained from Alfa Aesar (Wordhill, MA, USA). Hydrofluoric acid (HF) was obtained from Fisher (Nepean, Canada).

2.2. Capillary zone electrophoresis

Fused-silica capillaries of 350 μm O.D. and 50 μm I.D. were supplied by Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillaries was 50 cm, with a window located at 40 cm for UV–visible detection. Fraction collection and ESI detection were both performed at the end of the 50-cm capillaries, whereas UV–visible detection was performed at 40 cm. On-capillary UV–visible detection was performed by burning a 0.5-cm window in the polyimide coating of the capillary and passing the capillary through a Linear Instruments M-200 HPLC UV–visible detector (Fremont, CA, USA), modified in the laboratory. CE was performed using a Spellman CZE 1000R 30-kV power supply (Plainview, NY, USA) operated in the positive polarity mode. The electrospray potential at the cathodic end of the capillary was generated using an EG&G Ortec No. 459 5 kV bias supply (Oak Ridge, TN, USA) operated in the positive polarity mode. Acetic acid (1–10% in water) was used as the running electrolyte.

2.3. Off-line fraction collection

Fraction collection for off-line CZE–ESI-TOF-MS was performed by placing the end of the capillary into 50-μl conical plastic vials. Electrical connection was made with a 28-gauge stainless steel wire placed along the side of the capillary. Samples were eluted electro-kinetically into 5 μl of running electrolyte and subsequently analysed by ESI-TOF-MS.

2.4. Preparation of gold-coated capillaries

The capillaries used for on-line CZE–TOF-MS were prepared based on the sheathless ESI approach [16,19]. Approximately 1 cm of the capillary terminus was etched in 40% HF to make a conical end. During the process, the capillary was flushed with water to prevent etching of the inner wall. The capillary was then washed with water and methanol prior to drying with hot air. A coating of gold was deposited on the etched end by vapour deposition at 10^{-5} Torr using an apparatus constructed in the laboratory (1 Torr=133.322 Pa). The coating process involved ca. 60 s of deposition on one side of the capillary placed horizontally over a gold source, after which the capillary was inverted and the other side was coated, again for ca. 60 s. A 3-cm length of stainless steel tubing, 26 gauge, was slid over the capillary near the base of the tapered end to provide structural support and to provide electrical contact with the ESI interface. Two-part silver epoxy, Circuit Works (Kennesaw, GA, USA), was used to provide electrical connection between the stainless steel sleeve and the gold-coated end of the capillary.

2.5. Off-line optimization of the CE–MS interface

Optimization of the CE conditions were performed off-line using a test bench consisting of an ESI interface mounted opposite a grounded 1.5-cm diameter steel plate which served as a counter electrode and simulated the entrance to the mass spectrometer. The sheathless ESI interface was mounted on a *xyz* stage to allow for mobility. A Wild M7A binocular microscope, equipped with a 35-mm camera mount (Wild Leitze Canada, Willowdale, Canada), enabled visualization of the capillaries and of the effluent being electrosprayed. The separation potentials investigated ranged from 5 to 15 kV and the electrospray potential from ca. 3.0 to 3.8 kV, with 1–10% solutions of acetic acid as the running electrolytes.

2.6. Reflecting time-of-flight mass spectrometry

Off-line and on-line ESI measurements were made on the Manitoba reflecting ESI-TOF instrument, modified in the laboratory to accept the sheathless CE–ESI interface [8,26]. In this setup, the electrospray needle/capillary interface is mounted on an *xyz* stage near the sampling orifice of the instrument and is held at the electrospray potential relative to ground. A gentle counter flow of N_2 heated to 70°C is used as a curtain gas. The aerosol enters the mass spectrometer through a heated stainless steel capillary (~120°C) held near ground. Ions are focused using an r.f.-only quadrupole ion guide which also provides collisional cooling of the ions before they enter the mass spectrometer. Ions are injected orthogonally into the flight tube of the mass spectrometer (2.8 m effective path length) by a 4-kV pulse with a repetition rate of 3400 Hz to give a duty cycle of about 20%. A single-stage electrostatic mirror is used to correct for velocity distribution of the ions and increases the resolving power to >5000. Ions are detected using two 40 mm diameter microchannel plates in a chevron geometry. Mass spectra were recorded using single-ion counting with a multiple-stop time-to-digital converter (Orsay model CTN-M2), resulting in a dynamic range $>10^3$ and a sensitivity in the low fmol range. The resolution of the TDC is 0.5 ns, but for these measurements spectra were recorded in 2-ns channels. TIEs and SIEs were recorded at 1, 2 and 5 Hz. Although possible, no attempt was made to record the TIEs or SIEs at higher rates for these initial results. The reflecting TOF mass spectrometer and the acquisition and analysis software (TOFMA) were developed in the laboratory. The instrument has been described in greater detail elsewhere [8,26].

3. Results and discussion

3.1. Off-line CZE–MS

Off-line experiments were initially conducted in order to optimize CZE separation conditions and ESI parameters, and also to ascertain stability of the system prior to on-line use with MS. Fig. 1a shows the electropherograms corresponding to separation of three proteins, with UV–visible detection. The injection volume was ca. 40 nl (by gravity), and the protein concentration was 10^{-3} *M*. A relatively concentrated solution of electrolyte (10% acetic acid) was used in order to minimize interactions of the protein molecules with the negatively charged silanol groups on the inner walls of the fused-silica capillary. The concentration and low pH of the electrolyte solution allowed for complete separation of the three proteins with minimal adsorption. With a lower concentration of electrolyte (5% acetic acid) and the high concentrations loaded, the protein peaks could not be resolved. High protein concentration was required to compensate for the small injection volume and for dilution in the receiving vial upon collection. Also, the loading volume is limited when no pre-concentration technique is used. The final protein concentration in the collection vial was 80 μ*M*, sufficient for recording TOF mass spectra.

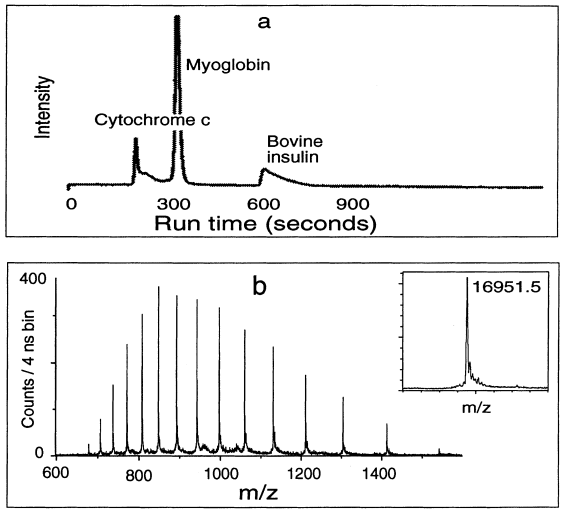

Fig. 1. Results from off-line CE–TOF-MS experiments. (a) UV-electropherogram (214 nm) of cytochrome *c*, myoglobin and bovine insulin with 10% acetic acid as the electrolyte and a 15-kV separation potential. (b) ESI-TOF-MS of fraction 2 (myoglobin), 12 pmol, collected in 5 μl of 5% acetic acid.

The ESI time-of-flight spectra obtained for the myoglobin fraction is shown in Fig. 1b. The ESI spectrum obtained was identical to that of a standard myoglobin sample prepared for ESI (R>5000, 10 ppm accuracy) which was predictable since CE conditions used for collection were compatible with

ESI. Results similar to those of Fig. 1b were obtained for the two other proteins, bovine insulin and cytochrome *c*.

3.2. Preparation and testing of the gold-coated capillaries

Optimization of CZE and ESI conditions was conducted using the test bench off-line. We chose to use single-piece, gold-coated, etched capillaries since they were simpler to manufacture than the dual-piece capillaries used by others [20]. The method for coating the capillaries was reliable and allowed for preparation of robust and reproducible gold coating. Each capillary manufactured was inspected off-line using the test bench described above. Approximately 65% of the capillaries tested were found to produce adequate electrospray without clogging. These were then rinsed with water, dried and stored for future use. These same capillaries, when used on-line, typically performed well for a minimum of 2 h to a maximum of 8 h. These conditions enabled us to study the effect of varying the CE and ESI potentials, which were optimized on a range of 5–15 kV (CZE) and 3.0–3.8 kV (ESI). Acetic acid solutions (1–5%, aqueous) yielded stable electrospray and provided suitable conditions for the separation of our standard mixtures of peptides and proteins. Better separation conditions were obtained with higher concentrations of acetic acid in the running electrolyte. At 10% acetic acid, separation of the three proteins was feasible. At lower acetic acid concentrations, the separation was not possible due to the high concentrations of protein employed (10^{-3} *M*). Electrospray conditions favoured the reverse: lower concentrations of acetic acid yielded stable electrospray at lower potentials (1–5%). Higher concentrations of acetic acid (10%) required electrospray potentials greater than 4.0 kV, at which corona discharge occurred. The final conditions chosen were 9 kV separation potential with a 3.2–3.8-kV electrospray potential. A photograph of one of the capillaries showing the gold coating and stable electrospray is given in Fig. 2.

3.3. On-line CE–ESI-TOF-MS

Initial on-line testing of the capillaries was performed using a constant infusion of a solution of substance P (3×10^{-5} *M* in 1% aqueous acetic acid). A 9-kV separation potential was applied and flow-rates of 100–300 nl/min were generated, making the CE system compatible with the ESI-TOF instrument. The spectrum shown in Fig. 3 was obtained during this constant infusion experiment. Performance was typical of the instrument [8,26], with mass resolution >6000, enabling baseline isotopic resolution as shown in the inset, and mass accuracy <10 ppm.

The amount of sample consumed was ca. 50 pmol over 4 min and the electrospray produced by constant infusion was stable for several hours. Under these conditions, an average of 1.25 ions were detected for each injection pulse which, for a pulse repetition-rate of 3400 Hz, corresponds to about 4000 counts/s. In contrast, the background signal for constant infusion of a 1% solution of acetic acid produced less than 175 counts/s with a variance of $(175)^{1/2}=13$. These values give an indication of the performance that can be expected of CZE–MS. If peaks are of the order of 1 s in width, then the S/N in the TIE should be about 300. Even if spectra are recorded and integrated at 10/s, the ratio of peak height to background variance will be about 100. When spectra are recorded for a shorter time during CE–MS, no sacrifice in instrument performance is necessary. Only the number of counts/spectrum is reduced which limits the dynamic range of individual spectra.

TIEs obtained for on-line CZE–MS separation of a mixture of five peptides is shown in Fig. 4. The concentration of the peptides in 1% acetic acid was 10^{-5} *M*, and approximately 230 fmol of sample were loaded onto the capillary. Separation proceeded with 5% acetic acid as the running electrolyte. In order to generate the TIE trace, a mass spectrum was acquired once every second and the total counts in a mass window (m/z 400–2500) were integrated and recorded. During the elution of the analytes, approximately 5000 counts/s were measured, corresponding to 5000 ions summed over the 1-s recording interval. It was not necessary to integrate more than one spectrum per second in this case to represent the peaks, which are typically 3–5 s in width with a $S/N=250$–400. The high ion current during elution (giving a $S/N>300$) indicates that considerably higher integration rates can be used and that analytes

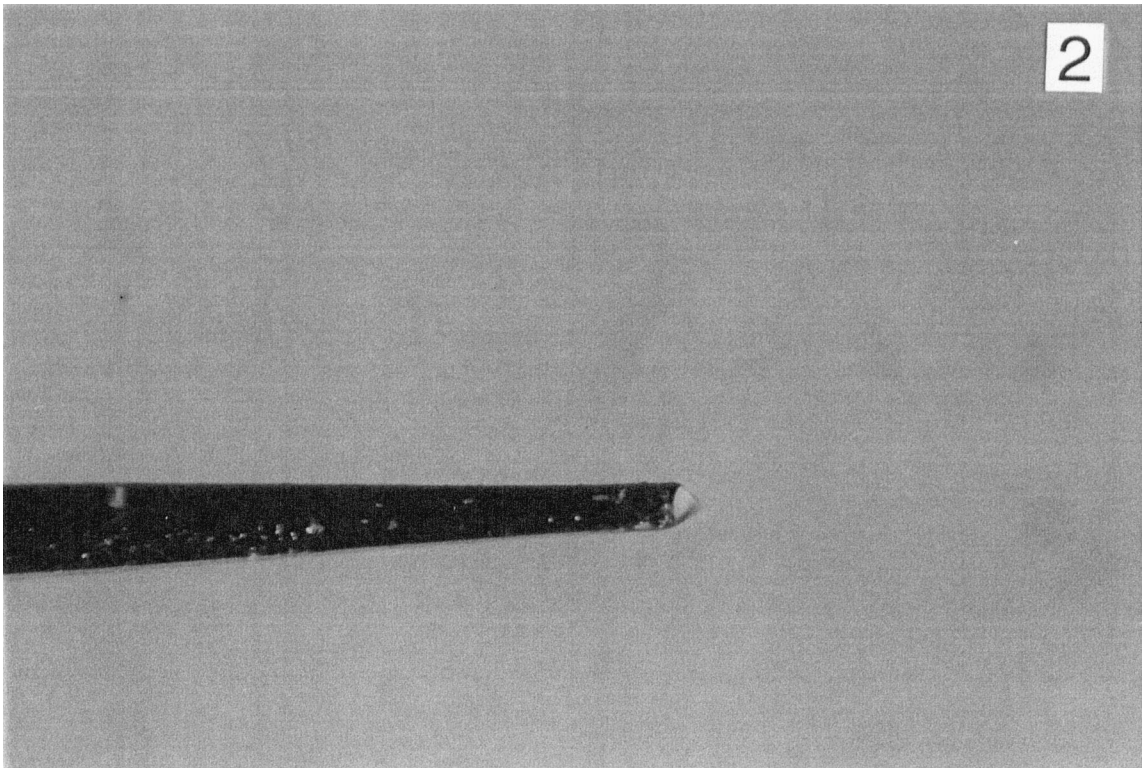

Fig. 2. Photograph of a capillary being tested, showing the gold-coated etched tip and stable electrospray. Magnification 30×.

in lower concentrations may be detected using this on-line system. Trace amounts of salts present in the original peptide sample eluted at ca. 380 and 410 s. The electrophoretic resolution was between 3.3 and 18.7, with theoretical plate counts of 35 000–87 000. The broad base of the cluster of peaks at around 700 s is due to intermittent perturbations in the spray as components eluted from the capillary.

Fig. 5 shows the cumulative mass spectrum

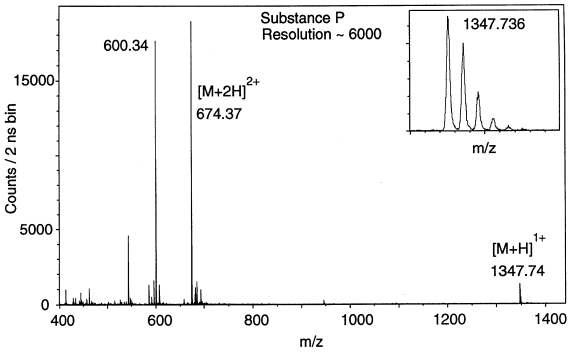

Fig. 3. On-line CE–ESI-TOF-MS mass spectrum recorded of a constant infusion of a 30-μ*M* solution of substance P. Electrolyte, 1% acetic acid, 9 kV separation potential, 3.2 kV ESI potential. Amount consumed, ~50 pmol.

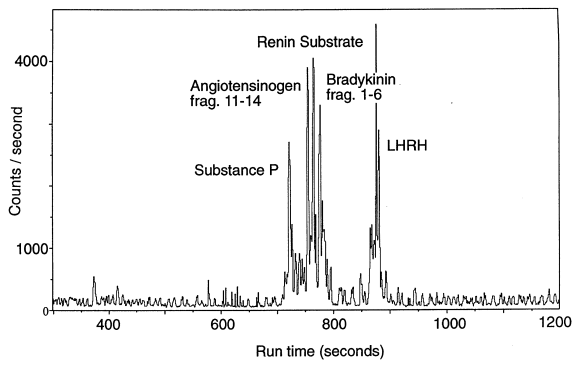

Fig. 4. On-line CE–ESI-TOF-MS total ion electropherogram of a mixture of five peptides, 230 fmol injected. Electrolyte, 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential.

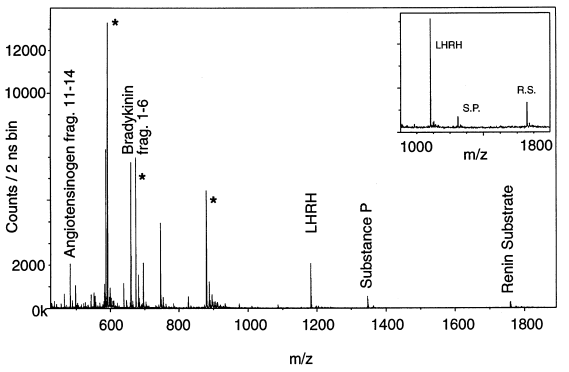

Fig. 5. On-line CE–ESI-TOF-MS mass spectrum recorded during the separation of five peptides. *Indicates multiple charged species.

obtained once the CZE–ESI-TOF-MS experiment was completed. This spectrum contains ions produced by the five peptides, some of which are doubly or triply charged in the case of larger peptides. Very low background signal appears in this spectrum. Resolution >6000 and ppm accuracy facilitated identification of multiple charge states for each of the peptides.

A selected ion monitoring (SIM) experiment was performed on the peptide mixture, using sampling windows of ca. 5 Da for each peptide and integrated at a rate of 2 Hz. The SIEs displayed in Fig. 6 were obtained from injecting ca. 350 fmol of each peptide. As mentioned in Section 1, the data for all SIEs are available simultaneously but because of limitations in our present data storage and handling, separate runs were necessary to record and store them. The peaks were between 4 and 6 s wide and signal intensity was in excess of 8000 counts/s for each peptide eluted. This corresponds to >4000 counts integrated at each half-second interval. Some peptides produced peaks which coincided with the selected window of other peptides, due to multiple charging or fragmentation, and this caused some electrophoretic traces in Fig. 6 to contain more than one peak. For example, the m/z windows monitored for substance P and LHRH contained peaks due to other mixture components which produced ions of m/z within the recorded window. Due to higher loading, peptide peaks could not be resolved in the TIEs (not shown) but are resolved in the series of SIEs shown here. Another significant advantage of

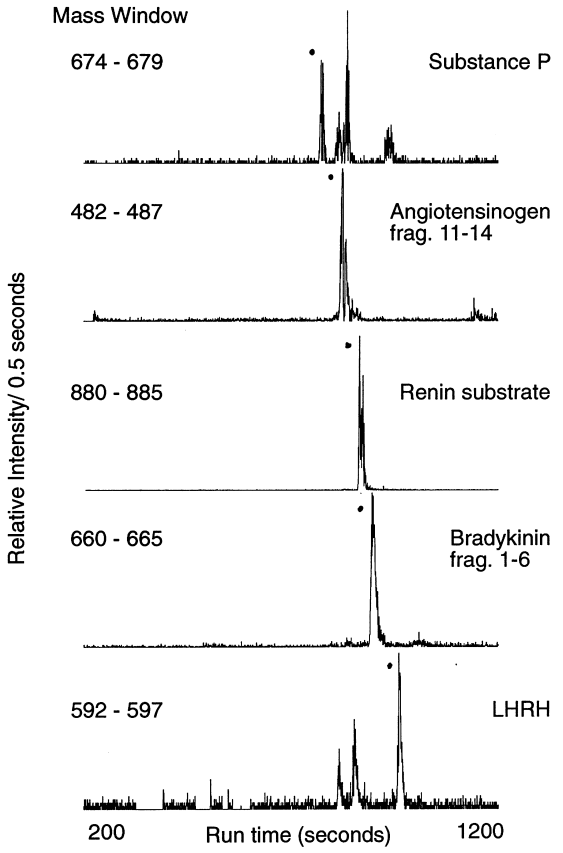

Fig. 6. On-line CE–ESI-TOF-MS selected ion monitoring of a mixture of five peptides, 350 fmol injected. Electrolyte, 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential. Scan windows were 5 Da in width.

recording SIEs is the increase in S/N observed over a small mass window, due to lower background noise present in different regions of the mass spectrum. As an example, a S/N value of ca. 760 was observed for the renin substrate peak, whereas the LHRH peak was recorded with slightly higher background noise and thus a S/N value of only ca. 30.

An example of what is observed during the elution of a compound is shown in Fig. 7 for the $[M+H]^+$ peak of LHRH. Before and after elution of the peptide, very low background noise, with count rates less than 200 counts/s, is recorded in the mass window shown here (Fig. 7a). As the component eluted, the mass spectrum was acquired (Fig. 7b). The mass spectrum of LHRH was recorded during

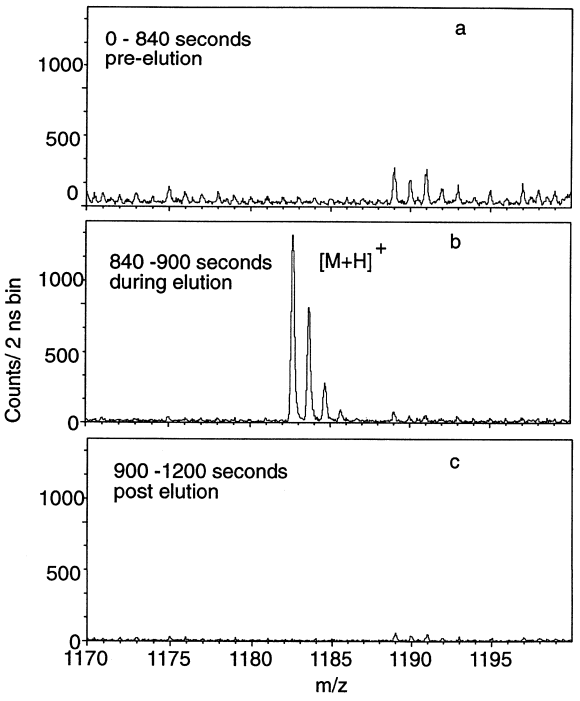

Fig. 7. Mass spectra recorded (a) before (b) during and (c) after elution of LHRH. Electrolyte, 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential.

60 s with the peak width of LHRH of ca. 5 s. The spectrum was produced by ca. 350 fmol of peptide. Resolution was about 5000 allowing baseline resolution of the isotopic pattern. The total number of ions contributing to the $[M+H]^+$ peak is about 10 000 indicating that if the spectra were acquired at 10/s there would still be >200 ions in the molecular ion peak. As mentioned above, this higher spectrum rate does not sacrifice performance of the instrument; only the dynamic range is reduced in shorter acquisition times. The mass spectrum in Fig. 7 which represents about 5 s of elution has an excellent S/N ca. 500 and dynamic range indicating good sensitivity at 350 fmol. Spectra may thus be obtained with much lower amounts of material.

The TIE obtained from the separation of a mixture of three proteins, myoglobin, bovine insulin and cytochrome *c*, is shown in Fig. 8. The amount injected was ca. 3.5 pmol of each protein, i.e. larger than in the case of the peptide mixture. A sampling rate of 5 Hz was used. The peaks obtained were

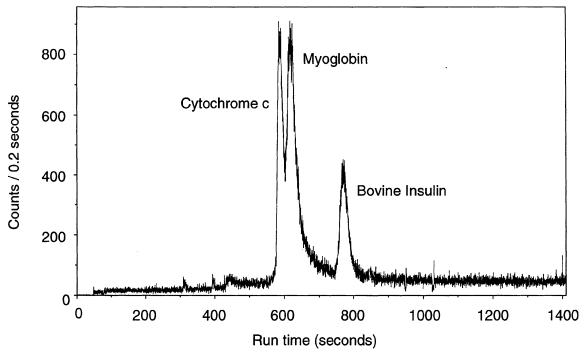

Fig. 8. On-line CE–ESI-TOF-MS total ion electropherogram of a mixture of three proteins, 3.5 pmol injected. Electrolyte, 5% acetic acid, 9 kV separation potential, 3.6 kV ESI potential.

broad and not well resolved between the first two eluted components. Resolution values for the CZE separation ranged from 0.8 to 6.5, and the theoretical plate counts were from 2800 to 5500 due presumably to the large sample size and interaction of the sample with the capillary walls. Perturbations in the baseline at ca. 300 and 400 s were caused by salts present in the sample. The ion count rate was between 2200 and 4000 counts/s during elution of the proteins with low background signal of ca. 200 counts/s. The S/N value was between 110 and 225 on raw data recorded at 5 Hz. The large peak widths (>10 s) enabled acquisition of a good quality mass spectrum for each protein. The spectra, recorded for 4–5 s for each component, are shown in Fig. 9. Multiply charged ions were observed and the deconvoluted spectra are presented in the insets. The high resolving power of the TOF mass analyzer made it possible to resolve peaks of oxidation products of bovine insulin at m/z intervals of 16 (Fig. 9c). In the deconvoluted spectrum of myoglobin (Fig. 9b) it is possible to observe molecular ions of the protein with and without adduction of acetate moieties from the electrolyte. In all cases, the mass spectra obtained were essentially identical to those observed during the off-line experiments performed earlier.

4. Conclusions

Off-line and on-line CZE–ESI-TOF-MS were investigated and found to be useful and produce good results for the separation and analysis of

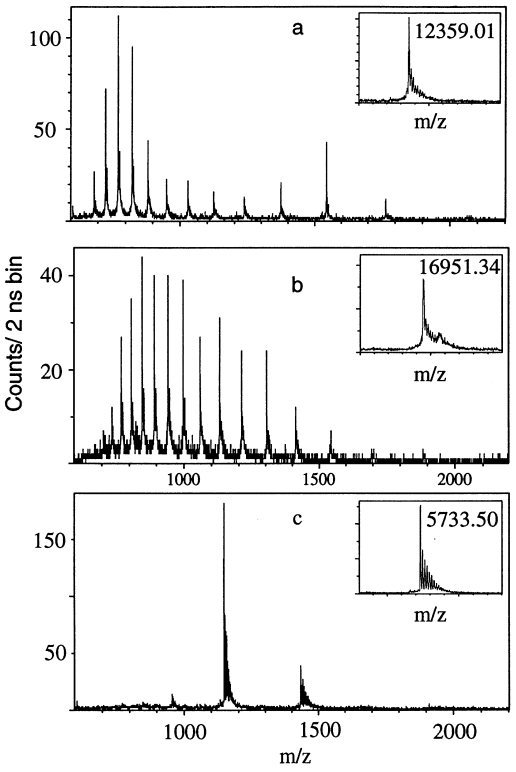

Fig. 9. CE–ESI-TOF-MS mass spectra recorded during the separation of three proteins: (a) cytochrome *c*, (b) myoglobin, and (c) bovine insulin. Deconvoluted spectra are shown in the inset.

peptides and proteins. The use of a test bench enabled optimization of ESI and CZE conditions prior to performing on-line experiments. The gold-coated capillaries used as the sheathless ESI interface produced a stable electrospray for extended periods of time. On-line CE–ESI-TOF-MS produced typical separations with 35 000–87 000 theoretical plates and fmol-level sensitivity. Electrophoretic separations were not compromized due to coupling of the mass spectrometer, nor were the mass spectra obtained. Reflecting TOF mass spectra were recorded with mass resolution and accuracy typical for the instrument used (R>6000, 10 ppm). Single ion counting resulted in good S/N values obtained for TIEs and SIEs indicating fmol sensitivity. We are currently developing software to take full advantage of the capabilities of our instrument and are currently investigating on-line CE–MS analysis of proteolytic digests of proteins.

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