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Instrumentation for high-performance capillary electrophoresis–mass spectrometry

RICHARD D. SMITH*, HAROLD R. UDSETH, CHARLES J. BARINAGA and CHARLES G. EDMONDS

Chemical Methods and Separations Group, Chemical Sciences Department, Pacific Northwest Laboratory, Richland, WA 99352 (USA)

ABSTRACT

An automated, commercially available capillary electrophoresis (CE) instrument was modified for interfacing with electrospray ionization mass spectrometry (ESI-MS). A Beckman P/ACE 2000 CE instrument with both electrokinetic and hydrostatic sample injection capabilities and efficient temperature control of the separation conditions was selected on the basis of these characteristics for this project. Modifications to it included extension of the fluid circulation path in an interface block extension and electrical modifications due to changes in electrophoresis current measurement capabilities. The capabilities for complete computer automation, capillary temperature control and on-line UV detection were retained. The minimum capillary length was increased by 35 cm due to the extension to the electrospray ionization–mass spectrometer interface. Initial results employing a laboratory-designed liquid sheath electrospray source are presented which show that very high separation efficiencies can be routinely obtained using the modified instrumentation. Capabilities for combined CE–MS are demonstrated for peptide and protein mixtures.

INTRODUCTION

The applications of capillary electrophoresis (CE) are expanding rapidly and a number of commercial instruments are now available. As applications have developed, the advantages of CE combined with mass spectrometry (MS) have been increasingly recognized and a number of laboratories are now pursuing instrumentation and method development for CE–MS. The first CE–MS was based on an electrospray ionization (ESI) interface developed in our laboratory [1,2]. Following this initial work with capillary zone electrophoresis–MS we reported an improved interface using a flowing sheath liquid electrode [3], the combination of capillary isotachophoresis with MS [4,5] and CE–MS–MS [5,6]. More recently, other researchers have reported on similar interfaces, including Hail *et al.* [7], Moseley *et al.* [8,9] and Lee *et al.* [10]. Alternative CE–MS interface designs have also been reported based on continuous-flow fast atom bombardment (CF-FAB) mass spectrometry [11–14]. However, the ESI methods appear to offer clear advantages in most instances owing to better sensitivity, reduced background and interface designs which do not require long transfer lines or incur a pressure drop across the capillary [15–17]. Per-

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haps the most significant advantage of the ESI interface to CE is the applicability to higher molecular weight compounds which are impractical by CF-FAB methods [18–20].

To date, none of the CE–MS instrumentation developed has effectively utilized the full capabilities of the automated CE instruments now commercially available. Such capabilities include precise electrokinetic or hydrostatic injection, on-column spectrophotometric detection and temperature control of nearly the entire capillary. The advantages of computer-controlled injection are significant for both accuracy and precision, as well as freedom from the frequent artifacts that arise from unreliable manual injection methods [21]. Temperature control has been recognized as important in obtaining good reproducibility for separations. Additionally we have noted the need to cool the ESI source under some operating conditions [17]. This arises from the fact that bubbles generated in either the CE capillary or the ESI sheath electrode (which consists largely of more volatile organic solvent) can create a region of high resistance and effectively terminate a separation. Hence our aim has been to exploit all the advantages of automated CE instrumentation, and particularly provisions for capillary temperature control.

In this paper, we describe the modifications made to a commercially available instrument, a Beckman (Palo Alto, CA, USA) P/ACE 2000 system. This instrument was selected on the basis of its advanced capabilities rather than ease of MS interfacing. Therefore, several hardware modifications were made which reflect the design of this particular CE instrument, as well as the physical constraints imposed by the design of the mass spectrometer used. We describe details of interface and hardware modifications, and report initial results which clearly show that the capabilities for high-performance CE separations are maintained in this CE–MS instrument.

EXPERIMENTAL

P/ACE 2000 system modifications

Physical constraints dictated some modifications. The Beckman P/ACE 2000 system as marketed is too large to be mounted directly in front of our mass spectrometers and still maintain the solution vials at the same height as the capillary outlet and inlet to the mass spectrometer. Height differences between the inlet and outlet lead to pressure driven flow (siphoning), which disturbs the flat flow profile of electrophoresis and degrades the separation efficiency. We opted to separate the P/ACE 2000 system into two subunits according to their practical and logical functions.

The autosampler, interface block, optics module and detector were removed from the mainframe and mounted in a box as a separate "experimental module". The system power supplies (high and low voltage), controller, keyboard, temperature and pressure controls were left on the mainframe as a "control module" (Fig. 1). All connecting electrical leads and cooling fluid and pressure lines were lengthened. The system was tested before and after modification to assure that no degradation of CE separation performance resulted from these changes.

One of the attractive features of the Beckman P/ACE 2000 system is the capability for temperature regulation of the capillary by means of fluid (a mixture of C_5–C_{18} perfluoro compounds containing sodium) circulation around the capillary. Our aim was to retain and enhance this feature when the system was adapted to use

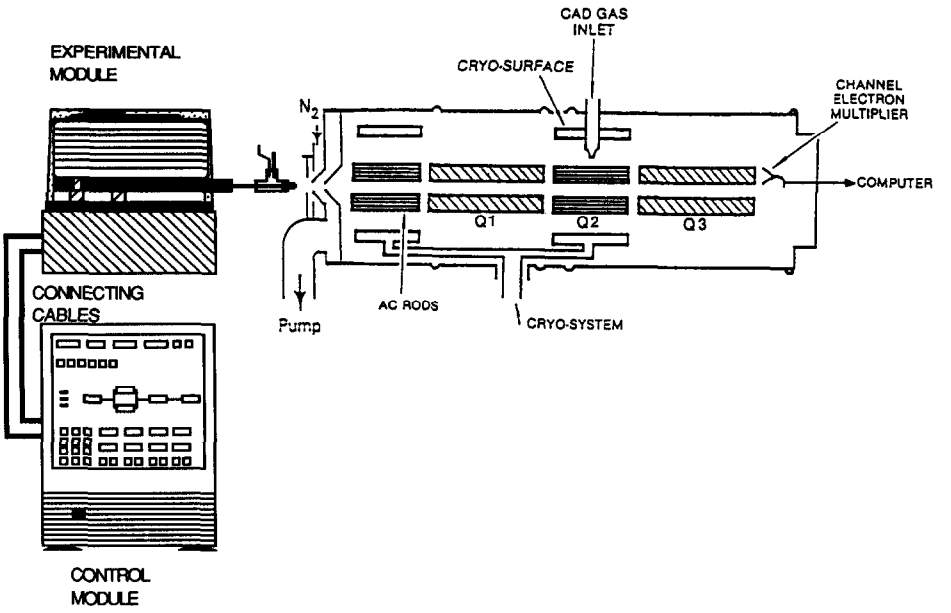

Fig. 1. Overall schematic illustration of the instrumentation for combined CE–MS showing the separated experimental and control modules of the Beckman P/ACE 2000 system.

on a mass spectrometer so as to retain temperature control over as much of the capillary as possible. In order to accomplish this, the interface block of the instrument (which holds the capillary cartridge) was modified by the addition of an extension. This extension attaches to the existing block and houses most of the added capillary. The extension is sealed by septa at each end, has separate inlet and outlet lines for the temperature regulation fluid and bolts onto the block immediately below the normal capillary exit. With the downstream septa removed the capillary can be threaded through the extension without removing it from the instrument. In this manner only 1 cm of the capillary between the cartridge and the extension housing is not temperature regulated by the fluid. Fluid is circulated in series through the capillary cartridge and the extension to the electrospray interface.

Relatively few changes to the instrument electronics were needed. The interlock to the high-voltage access panel of the mainframe was disabled. The instrument also checks to see that the current entering the capillary is the same as that leaving the capillary. When an electrospray source is coupled to the end of the column this condition could not be effectively fulfilled as the electrospray removes *ca.* 0.1 μA. This feature was disabled and the system no longer reported the column current. However, the output current of the high-voltage supply can be externally monitored, and this was done in our experiments.

With this arrangement, a capillary of *ca.* 35 cm additional length is required. A portion of this length remains in the air in order to facilitate connection to the current ESI source. However, this length can be reduced in future modifications by direct incorporation of the electrospray interface at the end of the extension.

Electrospray interface

The electrospray interface used is similar to that reported previously [3–6, 15–21] and is shown in Fig. 2. It incorporates a flowing sheath of liquid which allows the composition and flow-rate of the electrosprayed solution to be different than those of the electrophoresis system, a desirable feature when working with aqueous solutions. The electrical contact at the terminus of the capillary is also established through the liquid sheath (typically composed of methanol, acetonitrile, acetone or isopropanol with the addition of acetic acid and in some instances a small percentage of water). The electrical contact is made remotely to eliminate metal parts near the end of the capillary. In this interface design the sheath liquid and the electrospray/terminating electrode are introduced through on the same arm of a tee (see Fig. 2).

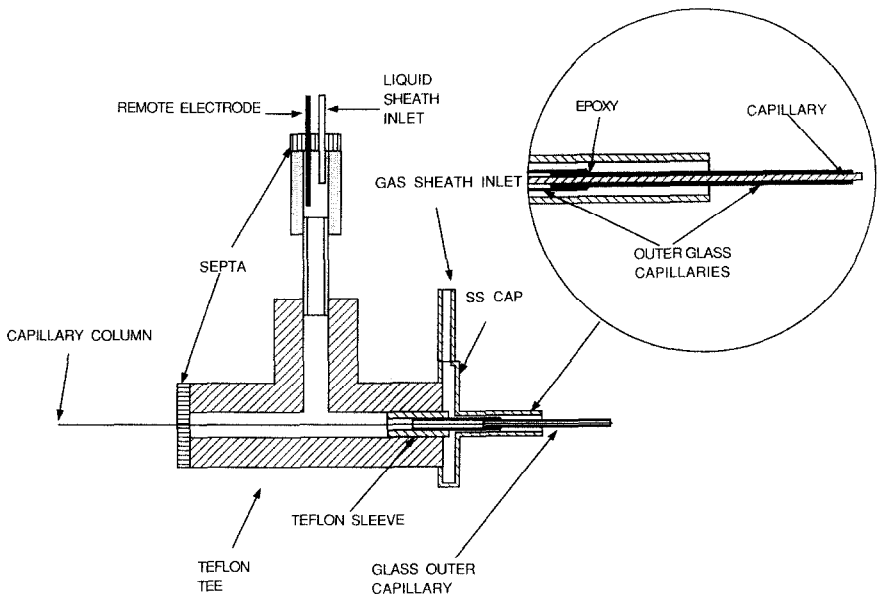

Fig. 2. Detailed schematic illustration of the electrospray ionization interface for combined CE–MS. The inset shows details of the ESI source "tip" region.

The ESI source consists of a 50 μm I.D. uncoated fused-silica capillary used for CE that protrudes 0.2–0.4 mm from a concentric fused-silica capillary. The continuously flowing liquid sheath provides a conducting path to the terminating electrode. This electrode is mounted in the adjacent arm of a 1/16-in. PTFE tee. High voltage, generally +4 to +6 kV for positive ions or −5 kV for negative ions, is applied to this electrode. The ESI source (CE capillary) tip is mounted *ca.* 1.5 cm from the ion sampling nozzle of the sampling orifice (nozzle) inlet to the quadrupole mass spectrometer. The mass spectrometer used for all reported results was a Sciex TAGA 6000E triple quadrupole mass spectrometer modified in our laboratory by the addition of a stage of differential pumping to improve sensitivity (Fig. 1).

A 3–6 l/min counter-current flow of nitrogen is introduced between the nozzle and ESI source to aid desolvation of the highly charged electrospray droplets and to minimize any solvent cluster formation during expansion into the vacuum chamber.

Analyte clustering of charged species is precluded by the mutual repulsion of highly charged ions and droplets. Clustering of any remaining solvent with analyte ions, and removal of residual solvent association, is effected by collisional activation in the interface [22]. A lens placed in front of the sampling nozzle is used to help focus the ions (or electrosprayed droplets) to the sampling orifice. Ions enter through a 1-mm diamter orifice and are focused efficiently into a 2-mm diameter skimmer orifice directly in front of the radiofrequency focusing quadrupole lens.

Materials

Biochemical samples were purchased from Sigma (St. Louis, MO, USA) and were used without further purification. Tryptic digests utilized an enzyme-to-substrate ratio of 1:25 in 50 m*M* ammonium hydrogen carbonate solution (pH 8.3) at 37°C for 18 h. The capillaries used in these studies are 50 μm I.D. × 185 μm O.D. uncoated fused silica with a polyimide sheath. It was purchased in bulk from Polymicro Technologies (Phoenix, AZ, USA) and cut to length. The acetonitrile was of UV grade from Burdick & Jackson Labs. (Muskegon, MI, USA). The water used was distilled and then processed in a Barnstead (Boston, MA, USA) Nanopure II reverse osmosis and ion-exchange system. As a final step before use, the water was filtered to remove particulates larger than 0.2 μm.

RESULTS AND DISCUSSION

Instrumental design considerations

In addition to maintaining the desired capabilities of the commercial CE instrumentation, *i.e.*, automation, precision injection, ancillary on-column UV detection and capillary temperature regulation, part of our efforts were dictated by a set of dimensional constraints. We wished to minimize any increase in the capillary length and minimize height differences between capillary termini. It was also desired that this could be accomplished with any of three ESI-MS instruments used in our laboratory. We decided to modify the CE instrument by separating the instrument into two modules consisting of the CE experimental module (automation, injection and detection hardware) and control module (power supplies, programming interface and ancillary equipment). Although physical separation of these modules was necessarily limited, these modifications removed the major constraints on obtaining the desired proximity to the ESI source.

Figure 1 gives a schematic illustration of the arrangement, showing the separated CE experimental and control modules of the Beckman P/ACE 2000 and the triple quadrupole mass spectrometer, a Sciex 6000 E modified as described previously. The separated modules allow the height of the experimental module to be adjusted for the separate demands of various mass spectrometers. The separation capillary extends *ca.* 10 cm beyond the temperature-regulated region of the interface block extension and forms the central capillary of the ESI source region (shown in detail in Fig. 2). In this region, the temperature is separately controlled by temperature control of the sheath liquid and surrounding gas flow (air). In these studies all experiments were conducted at 25°C, close to ambient temperature, and no additional temperature regulation of the ESI source was needed. Thus uniform temperature was well maintained over most of the capillary length. The minimum column length for the Beckman P/ACE 2000

with the conventional cartridge design was increased by *ca.* 35 cm, increasing the minimum column length to *ca.* 60 cm. Incorporation of the ESI interface on the end of the interface block extension, and minimizing the size of this extension, would reduce this length by *ca.* 10 cm.

CE–MS operation

Prior to and between each experiment, the capillary column was flushed and rinsed on a fixed schedule. Approximately four column volumes of 0.1 *M* sodium hydroxide solution were used to flush the column. This was followed by *ca.* eight column volumes of doubly distilled water and finally by 24 column volumes of the running buffer solution. Samples were injected for 10 s in the low-pressure injection mode. UV detection was monitored at 214 nm and on-line with the mass spectrometry in all instances. The UV detection window was approximately in the middle of the capillary column and thus analyte signals in the UV trace could be directly compared with the MS signals by approximately doubling the time of detection.

The effect of siphoning on the performance of the system was investigated in a series of experiments in which the height difference between the inlet reservoir and the electrospray tip was changed from 0 to 2.5 cm. This resulted in a 4% decrease in the elution time and no significant change in the widths of the peaks or the resolution of the separation. It was found, however, that the reliability of the ESI source was improved with the 2.5-cm height difference. The background noise in the spectra was reduced and the signal levels were improved. Therefore, in these preliminary studies a 2.5-cm height difference was maintained.

The mass spectrometer was tuned by injecting a standard solution through the electrophoresis capillary by using the high-pressure rinse mode of the P/ACE system to force the sample through the column. Hence neither the physical layout of the system components nor the operating parameters are changed when switching from MS tuning to CE–MS operation. The full specificity in mass spectrometric analysis in combination with analytical separations is obtained in general by the acquisition of the full m/z range over the time course of the separation. With the present quadrupole instrumentation, the time required for acquisition of the full mass spectrum at useful ratios signal-to-noise ratios for large molecules exceeds the typical peak width in capillary electrophoresis. These limitations could be largely removed by the development of more efficient ion transport methods from the ESI source, or the use of an ion trap mass spectrometer. In our experiments we have monitored selected m/z values specific to the known constituents of our samples determined by prior injection of the unseparated sample. For the present experiments this multiple ion monitoring method permits the evaluation of our interface development. Additionally, practical applications based on the analysis of known targeted compounds is well suited to current capabilities. However, the full realization of the specificity for scanning mass spectrometry in combination with capillary electrophoresis for the classes of analyte chosen here (particularly proteins) will benefit significantly from further advances in the sampling, analysis and detection of electrospray ions.

Peptide and protein CE–MS separations

The performance of the combined CE–MS system was evaluated in these preliminary studies with a mixture of myoglobin proteins ($M_r \approx 17$ kilodalton). The

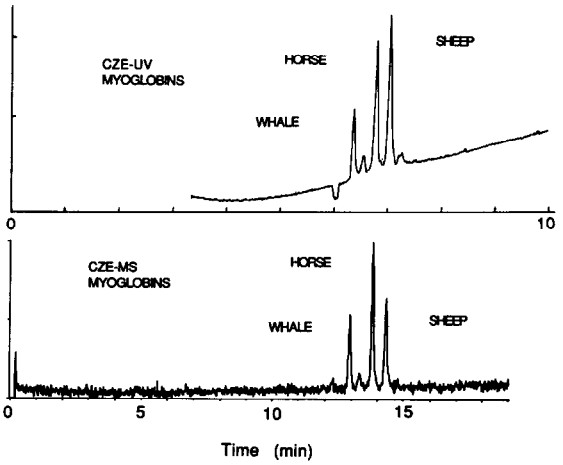

Fig. 3. CE–MS separation of a mixture of whale (M_r 17 199.1), horse (M_r 16 950.7) and sheep (M_r 16 923.3) myoglobins. The on-line UV response and later MS reconstructed ion signal have been superimposed with adjusted time scales. The separation was done in 10 m*M* Tris (pH 8.3) at 120 V/m. Approximately 100 fmol per component were injected.

separation was done in 10 m*M* Tris buffer (pH 8.3) with an electric field strength of 120 V/cm. Approximately 100 fmol per component were injected onto the capillary column. The results are shown in Fig. 3. The top half of the figure shows the UV trace for the separation and the bottom half is the reconstructed ion electropherogram (RIE) from MS detection for the same separation. MS detection is accomplished at about twice the separation time for the on-column UV detection. It is clear that the ESI–MS interface has not degraded the separation by broadening of the peaks in the electrospray source. We estimate that the additional volume of mixing at our interface is less than 10 nl (composed primarily of sheath liquid) and therefore one would not expect significant peak broadening. Measurements of the peak widths in this experiment show that peaks in the UV trace are 3.5 s wide at half-height and those in the RIE are 4.5 s wide. These measurements show that the combined electrophoretic and detector contributions to broadening are modest and the major source of peak broadening in this experiment results from the injection step. More efficient injection methods designed to concentrate the analyte at the head of the column would reduce the peak width in these separations to *ca.* 1 s at the UV detector. As the widths of the peaks decrease, the effect of the width of the detector window becomes more important. Finite width windows in optical systems will inevitably broaden very narrow peaks. The mass spectrometer with this ESI interface offers a detector where the only possible peak broadening is mixing in the small volume at the ESI source [2,4], which is negligible for conventional CE separation times. In fact, these peak widths are sufficiently narrow to exceed current capabilities when signal intensities are low and slower scan speed required. For targeted compound analysis, where a single ion could be monitored continuously, detecting peaks of 0.1 s in width could be done without detector broadening.

The specificity of mass spectrometric detection can provide useful additional information even when only a few ions are monitored, as in this example. Three ions

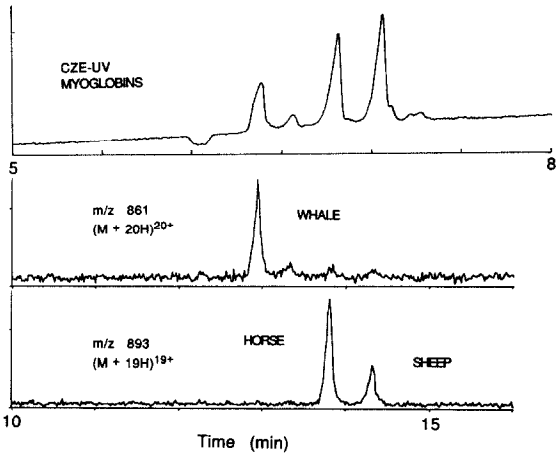

Fig. 4. Expanded view and single-ion electropherograms for the separation shown in Fig. 3. The molecular ion at m/z 861 is characteristic of whale myoglobin and shows that this molecule elutes first. The ions from horse and sheep myoglobin could not be resolved in these experiments, and a common response is seen at m/z 893. The excellent reproducibility of the electropherograms allowed retention time indices from the injection of pure compounds to determine the order of elution.

were monitored at m/z 893, 861 and 617. The resolution setting of the mass spectrometer was too low to distinguish the 19^+ molecular ion of horse myoglobin (M_r 16 950.7) from the same ion of sheep myoglobin (M_r 16 923.3) and both species were detected at m/z 893. Whale myoglobin (M_r 17 199.1) was detected at m/z 861 and carried 20^+ charges. The ion at m/z 617 is due to the heme group of the myoglobins. Fig. 4 gives an expanded view of the UV and selected m/z electropherogram for the molecular ions. As the heme is not covalently bound to these proteins it is labile under typical ESI conditions (*i.e.*, with organic solvents and/or acidic pH). In these experi-

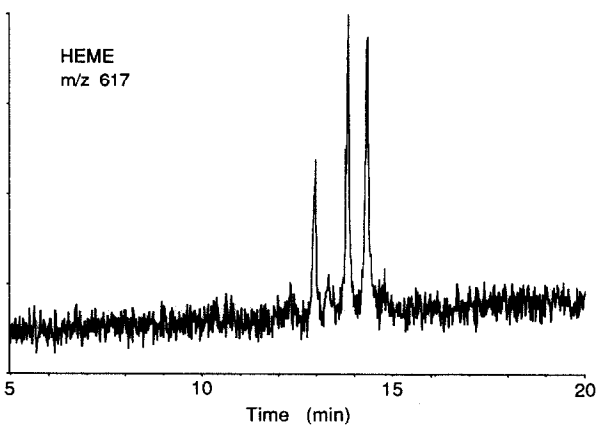

Fig. 5. Single-ion electropherogram of the ion of m/z 617 from the separation shown in Fig. 3. This signal derives from the non-covalently bonded heme group, which is labile (detached) under the ESI source conditions. The peak between the whale and horse myoglobins indicates that the heme moiety is also present in the contaminant and detached during ESI-MS analysis.

ments it accounts for approximately half the signal in each of the large peaks. Two other peaks are clearly evident in the UV trace. The peak eluting after the three myoglobins does not appear in the RIE and is not closely related to the myoblobins. The peak appearing between the whale and horse myoglobins does respond to the mass spectrometer. An examination of the single-ion plot for m/z 617 (Fig. 5) shows a corresponding response indicating that the peak is in response to an ion containing the heme moiety. As the heme appears in the mass spectrum at its characteristic m/z value, it is bound by hydrogen bonds to the protein during electrophoretic separation in the column. This is consistent with the known structure of myoglobin under the separation conditions. A response at m/z 861 but not at m/z 893 allows us to conclude that the polypeptide is similar in molecular weight to the whale myoglobin, and therefore that the small peak is probably due to a variant of whale myoglobin with a non-covalenly bound heme group. These results were obtained from the injection of 100 fmol per component.

With the ability of ESI to produce molecular ions with more than one charge, the idenfication of a compound from its response at a single m/z in the spectrum is risky. Multiple ion detection of two or more of these allows for more positive identification. The analysis of a sample of B-chain insulin provides an example. Fig. 6 shows the single-ion electropherograms for two molecular ions found in the ESI mass spectra of B-chain insulin. The separation was done in 10 m*M* Tris (pH 8.3) at an electric field strength of 150 V/cm on 0.7 pmol of sample. UV detection showed a second peak eluting after the main peak. The simultaneous MS detection of a corresponding peak in both single-ion plots suggests that the contaminant is probably a dimer, or possibly a variant of the peptide differing only slightly in mass. The dimer is indeed a significant contributor to the ESI mass spectra of this species, supporting this origin.

As an example of a separation of a more complex mixture, a tryptic digest of tuna cytochrome *c* was also examined. A sample of the digest was initially infused into the mass spectrometer and an ESI mass spectrum recorded. The most prominent

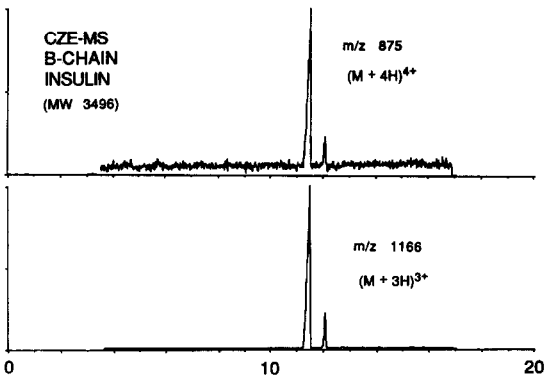

Fig. 6. Multiple-ion detection of the two ions characteristic of B-chain insulin in ESI. The electrophoresis was done in 10 m*M* Tris (pH 8.3) at 150 V/m. The response at both values of m/z suggests that the second peak is likely a dimer, or alternatively is a digopeptide variant of similar molecular weight to B-chain insulin.

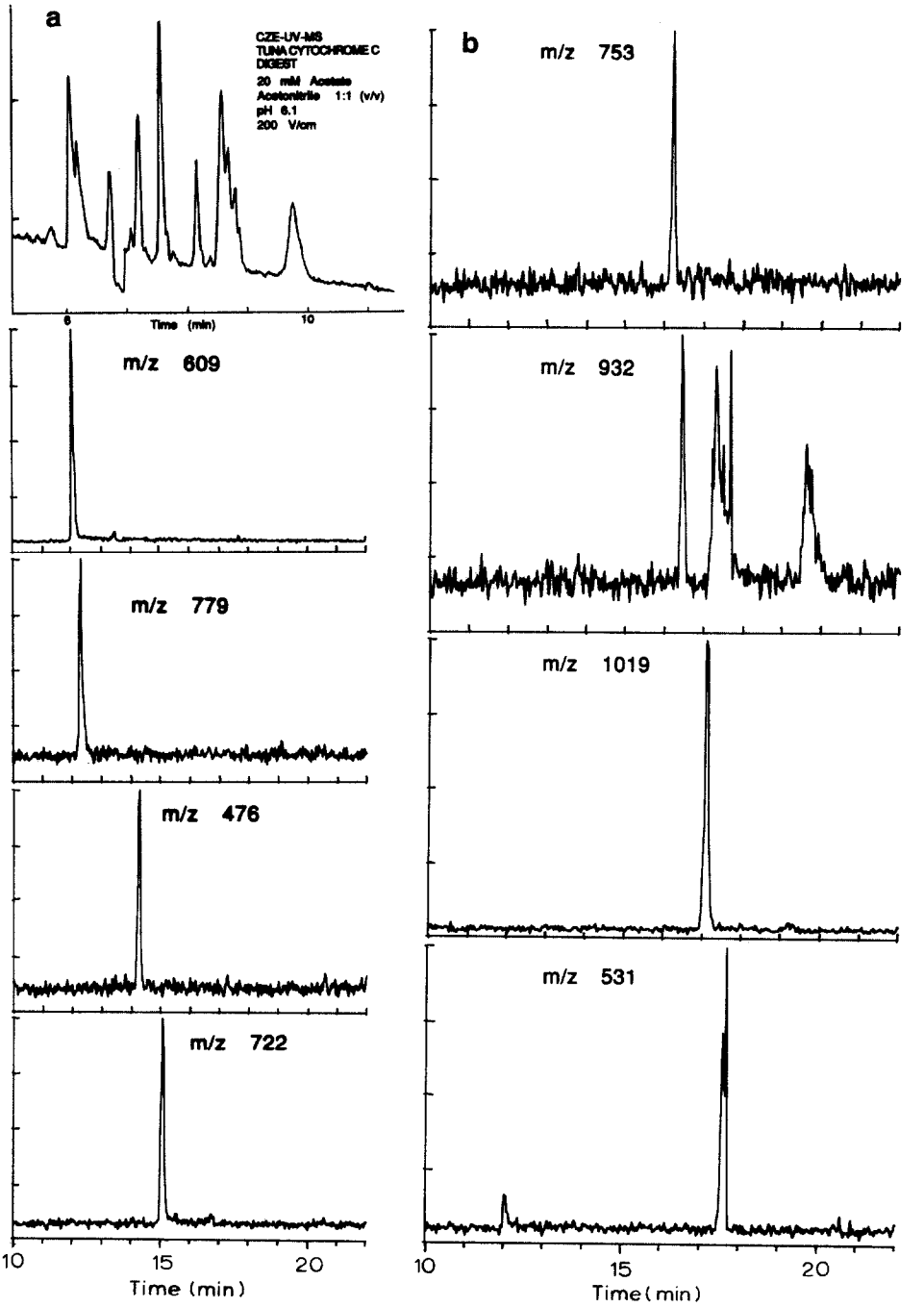

Fig. 7. CE–MS separation with on-line UV detection of a tryptic digest of tuna cytochrome *c*. The UV trace and several selected ion electropherograms are given in (a), superimposed as in Fig. 3. In (b) four later eluting ions are shown. Most of the peaks in the UV signal correlate with strong signals in mass spectrometry. Approximately 0.6 pmol per component was injected.

ions in the spectra were identified and these were monitored in a multiple-ion detection CE–MS experiment. The separation was carried out in 20 m*M* acetate buffer (pH 6.1) mixed with an equal volume of acetonitrile. The field strength used for the separation was raised to 200 V/cm to compensate for the reduced electroosmosis and the resulting longer analysis times. The results are shown in Fig. 7. Again, the time to the mass spectrometer is approximately twice the time of detection at the UV monitor. Most of the major features of the UV trace are reproduced by the ions monitored. These ions correlate with expected tryptic fragments. It is again apparent that the ESI interface has not degraded the separation.

CONCLUSION

A commercially available, automated electrophoresis instrument can be successfully adapted to ESI-MS. The interface provides a very low dead volume detector that does not compromise the performance of the system. This low dead volume is of interest in the detection of very narrow peaks, where detector broadening is intolerable, and optimum separation efficiency is desired.

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