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Journal of Chromatography A, 712 (1995) 245–252

JOURNAL OF CHROMATOGRAPHY A

Optimization of conditions for the analysis of a peptide mixture and a tryptic digest of cytochrome *c* by capillary electrophoresis–electrospray-ionization mass spectrometry with an improved liquid-sheath probe

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Abstract

Capillary electrophoresis (CE) has been interfaced with a quadrupole mass spectrometer using electrospray (ES) ionization in order to rapidly analyze a peptide mixture and a tryptic map of cytochrome *c*. A sheath liquid probe has been developed which allows the easy connection of CE with ES and the manipulation of important experimental parameters such as the position of the CE column exit relative to the sheath flow tube exit. In addition, the atmospheric region of the ion source has been modified so that glass windows replace the walls of the chamber, and the electrospray process itself is fully visible during operation. Detection limits for a biologically active peptide, leucine enkephalin, were determined to be 200 fmol in the scan mode of operation and 1.5 fmol in the selected-ion monitoring mode of operation.

1. Introduction

Electrospray (ES) ionization has become widely used as an interface between liquid chromatography (LC) separation techniques and mass spectrometry (MS) detection. The reasons for this success include the ability of ES ionization sources to both ionize and desolvate from solution the analytes of interest. Frequently, these compounds are polar, fragile, thermally-labile species which are subject to decomposition by conventional ionization methods. The "soft" ionization process of ES allows for the creation of ions and successful transfer from the liquid to the gas phase of important biological species, such as proteins, which weigh up to several hundred thousand daltons.

Alternatively, the use of ES ionization for mass detection with capillary electrophoresis (CE) has not yet become well-established even though it has been demonstrated on quadrupole [1–7], magnetic sector [8,9], Fourier transform ion cyclotron resonance (FTICR) [10] and time-of-flight (TOF) machines [11]. The primary reason for this is the additional technical challenges associated with CE–ES coupling. First, the outlet of the CE column is fused-silica, a dielectric material which itself cannot be electrically incorporated into the ES-MS system. Electrical connection with the CE column outlet is

0021-9673/95/$09.50
SSDI 0021-9673(95)00729-6

mandatory for both the CE and the ES processes. Secondly, the buffers most commonly employed with CE separations are aqueous and high in salt concentrations. Both of these solution characteristics are unsuitable for ES operation.

An initial solution for this dilemma was offered by Smith and co-workers [1–3] with the use of a co-axial sheath flow arrangement which provides for both the electrical connection and the addition of a solution more amenable to ES ionization. Since that time, other techniques have been developed which accomplish these goals as well. These arrangements include: (i) the use of a metal-coated, sharpened CE column outlet [4,12], (ii) the use of a gold wire electrode inserted into the CE column outlet [11], and (iii) a liquid-junction interface [6,13]. The metal-coated CE column tip has the advantage that no sheath flow is needed and thus no sample dilution occurs. It has the disadvantages, however, that ES compatible buffers must be used with the CE separation and that specialized CE columns must be prepared. The use of an inserted gold wire electrode requires neither sheath flow nor a specialized CE column. It does, however, imply the somewhat cumbersome and challenging correct placement of the electrode as well as the use of ES compatible buffers with the CE separation. Finally, the liquid-junction technique uses an additional buffer reservoir to electrically isolate the dielectric CE column outlet and a conductive metal ES needle. This method requires the careful alignment of CE column outlet and ES needle.

Because of the inherent advantages and ease of operation of the sheath flow approach, we have developed a convenient probe based on this concept and used it to demonstrate the separation by CE of biologically active peptides with ES-MS detection using a quadrupole instrument. Most importantly, this probe incorporates a translation device which gives the user the ability to easily adjust the CE column exit relative to the sheath flow tube exit so that this crucial parameter can be easily optimized for maximal performance. In addition, the atmospheric chamber where ES actually occurs has been modified so that windows are present, and the ES process can be viewed during operation.

2. Experimental

2.1. CE

The CE instrument used was an ATI (Boston, MA, USA) crystal 300 model with a four-position sampler. The CE column was fabricated from fused-silica, 75 μm I.D. × 365 μm O.D. from Polymicro Technologies (Phoenix AZ, USA) and was 1 meter in length. A potential of 30 kV was used for all separations except for the tryptic map where 15 kV was used. The sheath flow was delivered with a Harvard Apparatus Model 11 syringe pump (South Natick, MA, USA) and was pure methanol in all cases.

2.2. ES

The electrospray ionization source (Fig. 1) was from Analytica (Branford, CT, USA). This system was unaltered from its original form except for the use of a new CE–ES probe. Large windows on three sides of the atmospheric region of the source (P1) allowed for the visualization of the electrospray process itself and greatly aided in the optimization of instrumental conditions necessary to obtain good results. The source was used with the CE column exit at ground potential, the cylinder electrode (Vcyl) at −2500 volts (V), the endplate electrode (Vend) at −3700 V and the capillary entrance (Vcap) at −4500 V. The use of a dielectric capillary having metal coating on both ends to transfer the ions generated from atmosphere into vacuum is important in this case as it allows for the CE column exit to be maintained at ground potential. ES source designs which depend on the application of a positive voltage to the spray needle itself have two disadvantages when CE is used. First, the CE column exit must be floated at the required ES potential (several kV) causing the resulting electric field applied to the CE capillary to be the difference between the applied CE voltage and the ES voltages. Secondly,

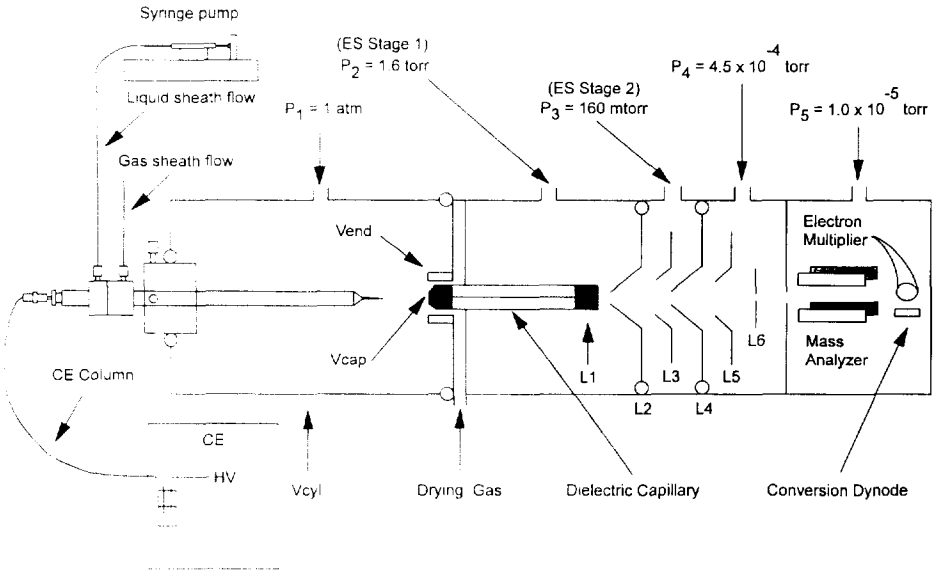

Fig. 1. Schematic of CE–ES-MS system.

if the ES source is operational during the injection phase and subsequent vial movements, the sample loaded on the capillary will have a tendency to migrate back out of the capillary entrance and be diluted in the final run buffer before the voltage can be applied to the run buffer. This phenomena has the potential to greatly reduce sensitivity, make quantitation difficult and degrade the separation quality.

The sheath flow CE probe which was constructed consisted of a 3-layer coaxial arrangement where the CE column exit was the center or first layer. The second layer or sheath liquid flow needle was a piece of stainless-steel tubing, 0.0160 in. I.D. × 0.0280 in. O.D., and was just large enough to allow passage of the CE fused-silica capillary. The third layer or gas flow needle was another piece of stainless-steel tubing, 0.0325 in. I.D. × 0.0425 in. O.D. The modest gas flow used (50 ml/min) served only to cool the needle and did not affect the electrospray process itself. This gas flow may find additional use with negative ion ES where a bath gas of SF_6 or O_2 is helpful to suppress the occurrence of a corona discharge in the source. Most importantly, this probe allows for the easy manipulation of the relative distance between the CE column exit and the sheath flow tube exit through the use of an incorporated translation device which does not apply torsional stress to the CE column during adjustment.

2.3. MS

The mass spectrometer used was an HP 5989A MS Engine from Hewlett-Packard (Palo Alto, CA, USA). ES control was accomplished through the use of a digital interface. In the scan mode of operation, the m/z range was scanned from 100–1000 m/z units at the rate of 1000 m/z per second (s). One sample was collected at each 0.1 m/z point with an integration time of 100 μs. In the selected-ion monitoring (SIM) mode of operation, the m/z value selected was used with a dwell time of 100 ms.

2.4. Materials

Buffers were prepared from distilled water obtained from a Barnstead NANOpure II (Boston, MA, USA) system. Acetic acid, ULTREX II Ultrapure Reagent Grade, was purchased from J. T. Baker (Phillipsburg, NJ, USA). Methanol was obtained from Mallinckrodt

(Paris, KY, USA). All solvents were filtered through Nylon 66 membranes from the Anspec (Ann Arbor, MI, USA). Peptide and protein samples were purchased from Sigma (St. Louis, MO, USA) as was trypsin and ammonium carbonate.

2.5. Methods

The cytochrome *c* tryptic map was prepared by dissolving 1 mg or 80 nmoles of horse heart cytochrome *c* in 200 μl of a 50 mmol solution of ammonium carbonate buffer (pH = 8.1 by ammonium hydroxide addition). Then 2 μl of a 1 mg/ml solution of trypsin (in the same buffer as above) was added to the solution. The mixture was incubated for 14 h at 37°C. The reaction was halted by the addition of 1 μl of trifuoroacetic acid and the resultant mixture lyophilized and reconstituted in the same volume of water.

3. Results and discussion

3.1. CE buffer composition

The first experimental variable examined was the effect of CE buffer salt concentration. In all cases, acetic acid was used as a CE buffer due to its volatility. Fig. 2 shows the effect in the MS total ion current (TIC) of a separation of peptides when using 0.010, 0.050 and 0.100 *M* acetic acid in water as the CE buffer. Here, 500 fmol of each peptide were applied to the CE column while the mass analyzer was scanned from 100–1000 *m/z* units. Several observations on this data are noteworthy. As the concentration of acetic acid was increased, the overall migration times for the peptides increased slightly, due to an associated decrease in the electro-osmotic flow through the column. Also, although the peak widths did not change, the migration times of the analytes were differentially affected by the increasing buffer concentration so that varying

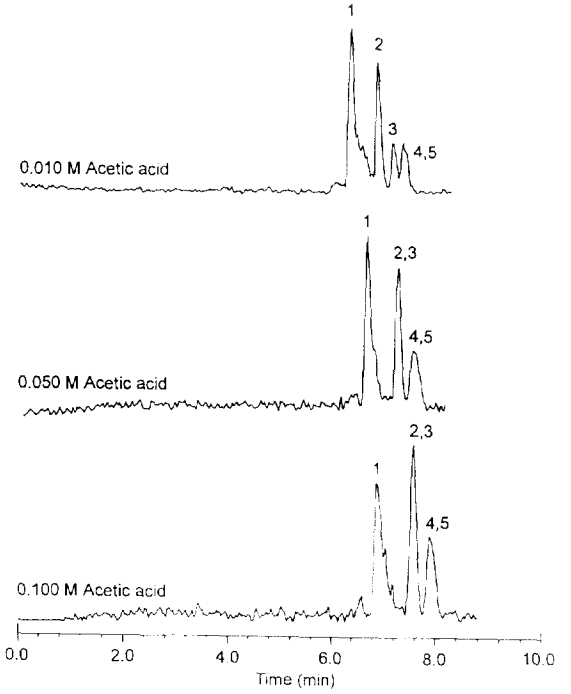

Fig. 2. Effect of acetic acid concentration in the CE buffer on MS TIC from a peptide separation. Peaks: 1 = angiotensin II; 2 = Val–Tyr–Val; 3 = leucine enkephalin; 4 = Gly–Tyr; and 5 = methionine enkephalin.

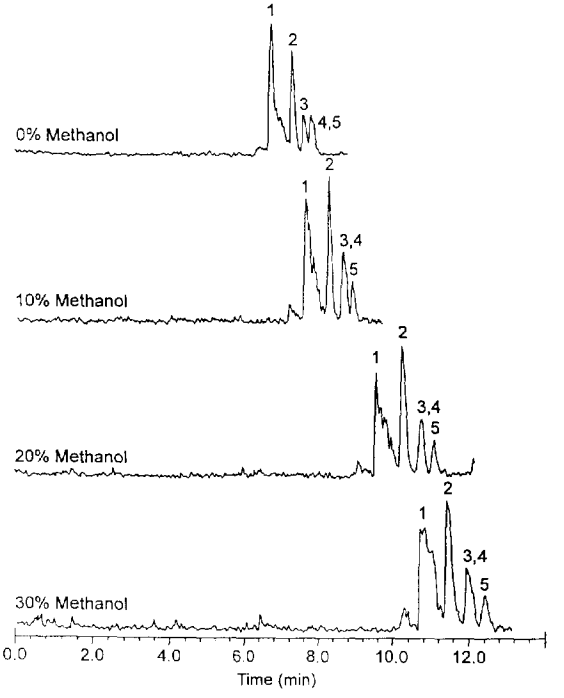

Fig. 3. Effect of methanol content in 0.010 *M* acetic acid buffer on MS TIC from peptide separation. Peaks as in Fig. 2.

instances of co-migration of peaks occurred. This might be used to an advantage in certain situations, but it was unable to improve the quality of the separation in this case. The results shown here are essentially in agreement with those of Moseley et al. [7] where the use of 0.010 *M* acetic acid was found to be optimum as well.

Fig. 3 shows the TIC for the separation of the same peptide mixture from above but with varying amounts of methanol in a 0.010 *M* acetic acid buffer. Again, a number of observations are noteworthy. First, the use of increasing amounts of methanol clearly increased the migration times of the analytes. This is the result of zeta potential alterations and viscosity increase due to the presence of methanol and has been previously demonstrated by others [14]. Also, the peak widths steadily broadened with increasing amounts of methanol. In conclusion, the addition of methanol had only negative effects on the resolution of the peaks or the quality of the separation, thus further supporting the use of a sheath flow probe which does not require the addition of methanol to the CE buffer directly.

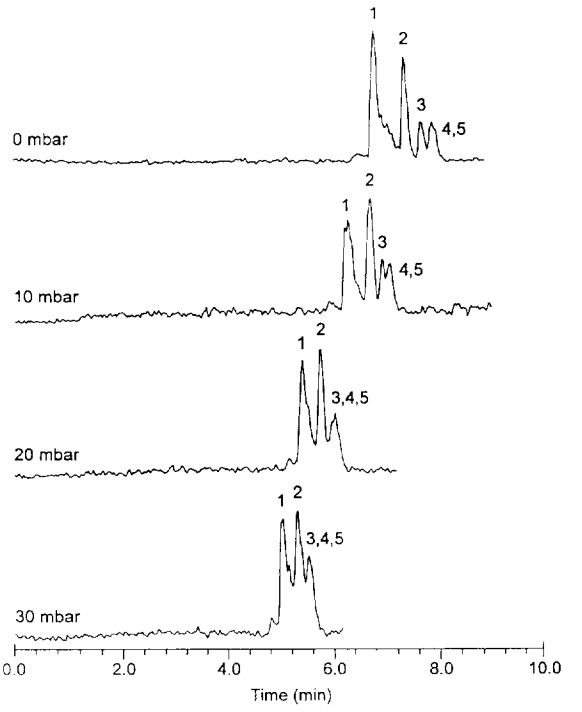

Fig. 4. Effect of applied back pressure on MS TIC from peptide separation. Peaks as in Fig. 2.

3.2. Applied pressure

Fig. 4 shows the effect on the TIC of the same peptide mixture when additional pressure was applied on the CE column during the separation. The CE buffer used was 0.010 *M* acetic acid in water with no methanol. This effect was investigated since the ES has the potential to apply a slight vacuum on the CE column exit resulting in a net forward flow. Also, the CE column inlet must be aligned to be level with the CE column outlet and the ES system to prevent siphoning. This is sometimes difficult, although not in this case, due to the constraints of the CE system and the result may be that the particular CE unit cannot be made level with the ES system. This effect can be duplicated by the application of pressure as is done here. The results show that as the pressure was increased, the migration times of all the species decreased as expected. Also, the resolution and quality of the separation decreased as well. These results are not unexpected since the application of pressure induces the LC-type parabolic flow profile that CE normally avoids.

3.3. Sheath flow-rate

The sheath flow is expected to dilute the CE sample zone as it passes concentrically around the CE column effluent and mixes with it. This effect was investigated by varying the sheath flow (100% methanol) rate and measuring the signal in the TIC as the leucine enkephalin ion (m/z = 556) eluted. This signal was plotted against the sheath flow-rate in Fig. 5 and shows that increasing the sheath flow definitely decreased the ion signal measured. Because only sheath flow-rate values of 2–8 μl/min could produce a stable electrospray, measured data were limited to this range. Comparing the ion signal at the flow-rate of 2 μl/min and 8 μl/min (4 μl/min being the optimum in terms of ease of use and stability), suggested that the signal loss due to dilution of the sample zone by the sheath flow was just

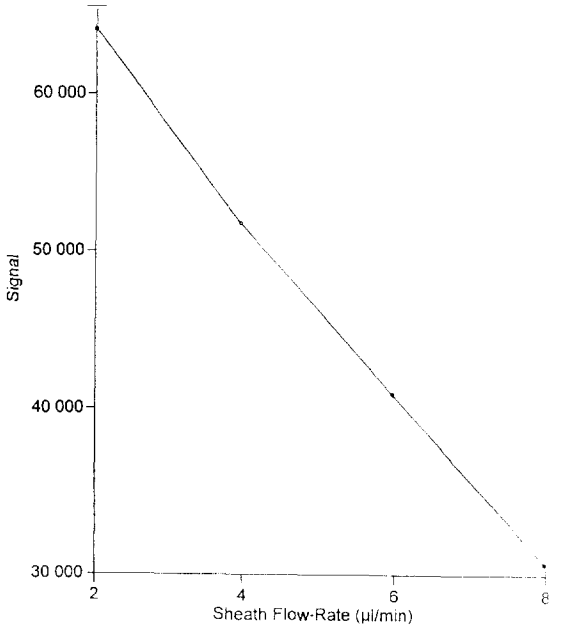

Fig. 5. Effect of sheath flow-rate (100% methanol) on leucine enkephalin ion signal. Peaks as in Fig. 2.

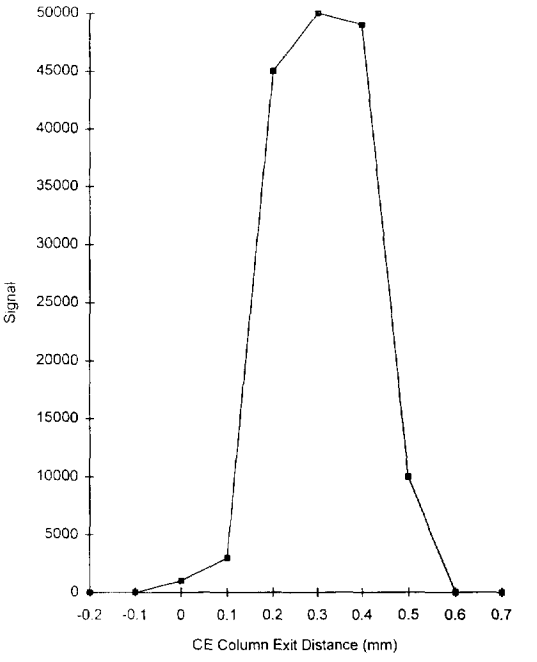

Fig. 6. Effect of distance between CE column and sheath flow tube exits. "+" values indicate that the CE column is protruding beyond the sheath tube and "−" values indicate that the CE column is retracted inside the sheath tube.

greater than a factor of two over the range studied.

3.4. Relative position of CE column exit

The only major physical adjustment which substantially affected performance when using the sheath flow system was the position of the CE column exit relative to the sheath flow exit. The electrospray is in fact defined by the physical dimensions of the sheath layer tube exit (diameter and edge thickness), and the CE column exit must be positioned so as to not interfere with the spray formation process. If the CE column is positioned too far out of the sheath flow tube then a stable spray cannot be maintained. Similarly, if the CE column exit is positioned too far towards the inside of the sheath flow tube, then again a stable spray cannot be maintained and additionally, the CE effluent is diluted greatly in the sheath flow and may even undergo some electrochemical degradation [3]. At this point the ion signal falls to zero. Fig. 6 shows the signal obtained from triplicate applications of leucine enkephalin to the CE column (optimum buffer conditions used) with varying distances between the CE column exit and the sheath flow tube exit. The best position for the CE column exit was found to be 0.30 mm protruding from the sheath tube. This result is supported by the work of others [3,5,7,9] who have found similar optimal distances ranging from 0.20 mm to 1.0 mm. While the previous investigators have found slight differences in the optimal position, it is important to note that all agree that the determination and maintenance of the correct relative position of the CE column exit is important to achieving satisfactory results.

3.5. Sensitivity

In order to determine the sensitivity of this system, leucine enkephalin was applied to the column in varying amounts while the mass spectrometer was operated in both the SIM and scan modes of operation. In the SIM mode (100 μs dwell time), the smallest amount of sample

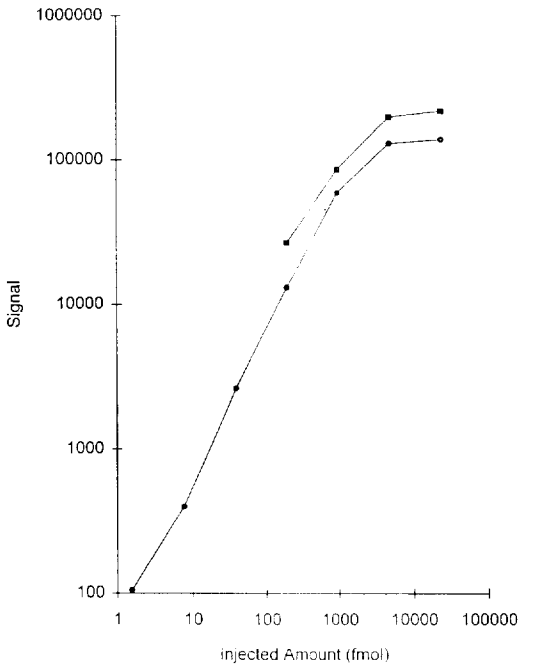

Fig. 7. Ion signal from leucine enkephalin in scan (■) and SIM (●) mode vs. amount injected.

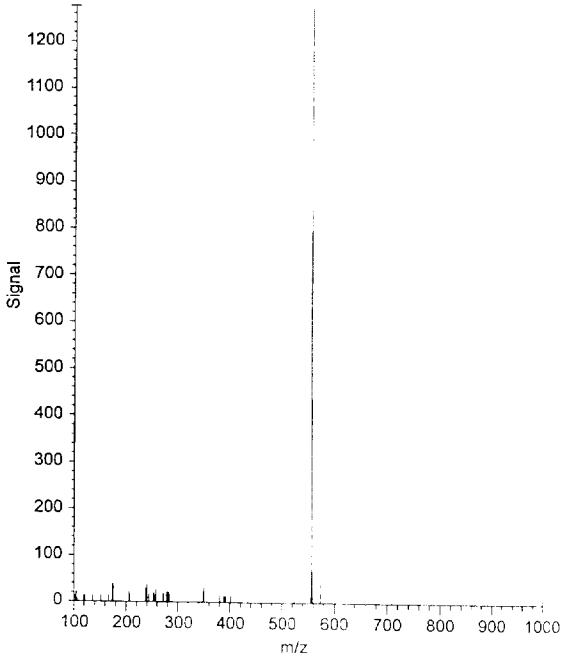

Fig. 8. Average of scans acquired during elution of 200 fmol injection of leucine enkephalin.

which was detectable above the background noise at $S/N = 3$ was 1.5 fmol. In the scan mode (100–1000 m/z units at 0.1 m/z intervals) the smallest amount of sample which gave rise to a visible peak in the TIC was 200 fmol. These data are plotted together in Fig. 7. Fig. 8 shows an

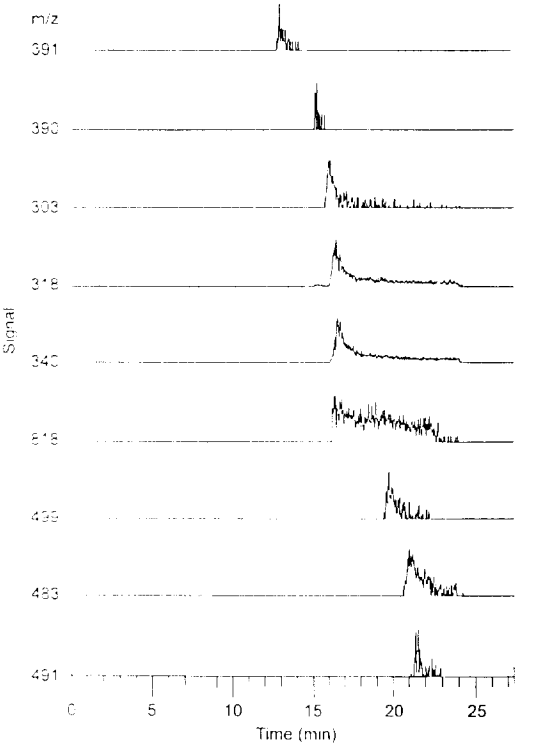

Fig. 9. Reconstructed ion currents from selected m/z values of a typtic digest of cytochrome c separated by CE.

Table 1
Relationship between molecular mass, charge state and observed m/z peak

Observed m/z peak	Charge state	Molecular mass
391	+2	779
390	+3	1168
303	+2	604
318	+2	634
340	+2	678
818	+2	1635
499	+3	1496
483	+2	964
491	+3	1471

average of scans which were acquired during the elution of the 200 fmol application of leucine enkephalin. The molecular ion at 556 m/z units is clearly visible.

3.6. MS analysis of a tryptic map

In order to demonstrate the use of this system with a likely application, cytochrome *c* was digested with trypsin and the resultant products subjected to analysis by CE–ES-MS. This tryptic map is represented in Fig. 9, where the reconstructed ion currents (RIC) from selected m/z values are shown for the major peptides present in the cytochrome *c* (horse) trypsin digest. All the expected peptide fragments as seen by others [15] were present. The amount of material applied to the CE column was equivalent to 1 pmol of the original protein and the relationship between the observed m/z peaks and the molecular mass of the fragments is shown in Table 1.

4. Conclusions

In these studies, it has been shown that an improved sheath liquid probe can provide for the easy and convenient interfacing of CE with ES-MS. The effects of dilution of the sample zone by the sheath liquid were shown to be minimal over the range studied. Furthermore, the addition of an organic modifier to the CE buffer, which is required with the use of most non-sheath CE–ES-MS probes, has proven to degrade the separation, as has the addition of back pressure on the CE column. This type of pressure can be unintentionally applied if the CE column entrance is higher than the CE column exit due to instrumental constraints. Using the SIM mode of operation, as little as 1.5 fmol of leucine enkephalin could be detected by CE–ES-MS. Finally, this system was used to detect the peptide fragments from 1 pmol of a tryptic digest of cytochrome *c* when separated by CE.

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