Journal of Chromatography A, 659 (1994) 217–222
Elsevier Science B.V., Amsterdam

CHROM. 25 575

Short Communication

Sheathless capillary electrophoresis–electrospray ionization mass spectrometry using 10 μm I.D. capillaries: analyses of tryptic digests of cytochrome *c*

Jon H. Wahl, David C. Gale and Richard D. Smith*

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

(First received July 22nd, 1993)

ABSTRACT

The analyses of tryptic digest of proteins present a difficult challenge to the analytical chemist due to the wide range of molecular masses and hydrophobicities of the peptides produced. In this study, we demonstrate the separation of tryptic digests of bovine, *Candida krusei* and equine cytochrome *c* using a new electrospray ionization (ESI) interface for CE–MS that does not require additional sheath make-up fluid or mechanical assistance to aid the ESI process. The utility of this new CE–ESI-MS interface is demonstrated using a 10 μm I.D. CE capillary where the injected sample amounts are in the 30 femtomole (of protein) region. The CE electroosmotic flow rates when aminopropylamine treated capillaries are utilized are in the 10 nl/min region for a relatively conductive buffer system (0.01 *M* ammonium acetate–acetic acid buffer system, pH 4.4 and a 300 V/cm field strength).

INTRODUCTION

In the development of analytical techniques for biochemical identification and quantitation, intense effort has been directed to increased solute detectability and sensitivity, because sample size is often limited or obtained at great effort. The application of capillary electrophoresis (CE) to biochemical analyses has led to increasing demand for detection methods that are more sensitive and that provide component information. In principle, mass spectrometry (MS) is recognized as a nearly ideal detector for analytical separations; however, in practice it has not generally been considered adequately sensitive when compared with techniques such as laser induced-fluorescence [1–3] or electrochemical detection [4]. For CE, these methods have demonstrated detection limits at the zeptomole (10^{-21} mole) level; however, compound identification is generally determined by comparison between standard and unknown solute electrophoretic mobilities, which requires a high degree of precision. Consequently, a major advantage of MS detection for CE is that component identification can readily be achieved without the need for precise mobility measurements.

Presently, the technique that is used to interface CE with MS is often a critical feature for the success or failure of a CE–MS experiment. Electrospray ionization (ESI) has gained accept-

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(93)E1005-K

ance as the interface method of choice to combine CE with MS. This acceptance is due to the variety of approaches that have been developed for CE-ESI–MS interfaces, which allow the CE separation to occur relatively unperturbed by the method of establishing and maintaining electrical contact at the CE capillary terminus. In the first CE–MS interface reported electrical contact was made by the use of silver metal deposition onto the fused-silica capillary terminus [5]. The metalized capillary terminus served to define the CE field strength along the capillary and also as the electrospray electric field by having the terminus at a voltage of 3 to 5 kV relative to the mass spectrometer inlet. In a subsequent design, a coaxial sheath liquid was introduced; this served to establish the electrical contact with the CE effluent and facilitate the electrospray process [6]. Another variation on the CE–MS interface was introduced by Henion and co-workers [7], in which a liquid-junction was used to establish electrical contact with the analytical capillary and provide an additional make-up flow to the buffer. A disadvantage of both the latter two approaches is that they depend upon an additional flow of liquid that contains charge-carrying species, which will generally degrade solute detectability and sensitivity [8]. The relative contributions to the electrospray ion current arising from the sheath flow become greater as the capillary I.D. is decreased. Because improving CE–MS sensitivity is generally recognized as an important goal to the further utility of CE–MS in biological analyses, interest remains in the development of more sensitive CE–MS instrumentation based upon alternative interface designs that are simple, versatile and more reliable. In this report we demonstrate the utility of CE–ESI-MS using a chemically modified 10 μm I.D. capillary for the analysis of tryptic digests of three proteins using a new ESI interface that does not use a liquid sheath, but has similarities to the first CE–MS interface design.

EXPERIMENTAL

In this work, a fused-silica capillary of 10 μm I.D., *ca.* 150 μm O.D., and approximately 50 cm in length was utilized (Polymicro Technologies, Phoenix, AZ, USA). The inner wall of the capillary was chemically treated with 3-aminopropyltrimethoxysilane (Aldrich, Milwaukee, WI, USA) [8,9], in a manner similar to that described by Bruin *et al.* [10], and previously reported by Lukacs [11]. To taper the outlet of the analytical capillary, the polyimide coating was removed from approximately one centimeter of the capillary terminus, and the exposed fused silica was etched with a 40% hydrofluoric acid solution (Aldrich). The buffer system used in the experiments was a 0.01 *M* ammonium acetate–acetic acid solution, pH 4.4. The CE electrical field strength used for all experiments is -300 V cm^{-1}.

The capillary electrophoresis instrument used for this work was constructed at our laboratory and has been discussed previously [8,9]. The CE system is interfaced to a modified Sciex TAGA 6000E triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) through an electrospray ionization interface as previously described [12]. The mass spectrometer was scanned from m/z 600–1200 in 2-m/z steps at 0.6 s/scan in all experiments. The electrospray interface was constructed by applying a silver conductive coating (Epoxy Technology, Billerica, MA, USA) to the analytical capillary terminus. In addition, a gold conductive coating has also been successfully demonstrated for use in the electrospray interface [13]. The electrospray is produced using a potential difference of approximately +2.5 kV between the capillary terminus and the MS sampling orifice. Coaxial to the analytical capillary, a sheath gas of sulfur hexafluoride (SF_6) was used to suppress corona discharge. The CE–ESI-MS interface region is schematically illustrated in Fig. 1.

The samples chosen for this work were tryptic digests of bovine, *Candida krusei* and equine cytochrome *c*. The proteins were used as purchased (Sigma, St. Louis, MO, USA) without further purification. The tryptic digests utilized an enzyme-to-substrate ratio of approximately 1:25 in 0.05 *M* ammonium bicarbonate (pH 8.3) solution at 37°C for 18 h. Electrokinetic injection at -20 V cm^{-1} for approximately 5 s for each digest was utilized for the separations, which consumed approximately 30 femtomoles of original protein.

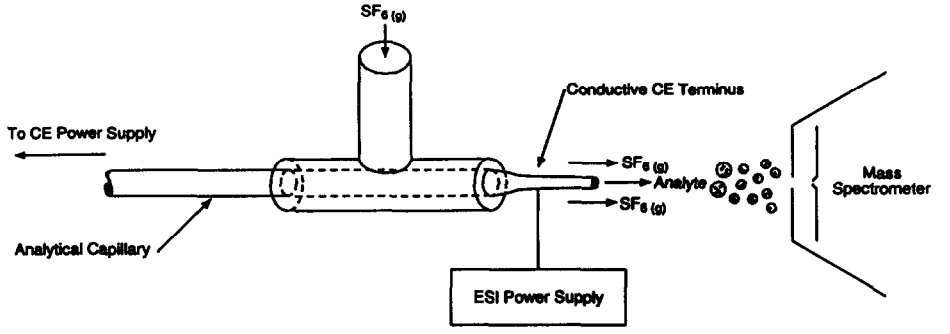

Fig. 1. Schematic illustration of the electrospray ionization interface used for the CE–ESI-MS experiments. See text for further discussion.

RESULTS AND DISCUSSION

The complex mixture of peptides produced from the tryptic digestion of proteins presents a difficult challenge to the analytical chemist due to the wide range of solute molecular masses and hydrophobicities encountered. The proper choice of a buffer system is essential to achieve a successful separation. Moreover, the success of the CE–ESI-MS analysis depends upon many factors, including the electrospray interface, buffer composition, and solute interaction with the inner surface of the analytical capillary. Specifically in CE–ESI-MS, analyte detectability and sensitivity can vary significantly with buffer composition and concentration. In general, the optimum analyte signal obtained for CE–ESI-MS occurs when volatile buffer systems are utilized at the lowest practical concentration. Conversely, when large quantities of ionic buffer species are delivered to the electrospray source, the analyte is not efficiently ionized by the electrospray process, and analyte detectability is greatly reduced. As a result, the ideal ionization process for a CE–ESI-MS system occurs when the number of charge carrying species to the ESI source is supplied at a rate that is equal to or less than that necessary to maintain the maximum electrospray current normally achievable. Previously, we have demonstrated that the use of small I.D. capillaries offers a simple method to control the number of ionic species entering the electrospray source during a CE–ESI-MS experiment [8,9].

The total ion electropherograms obtained from the tryptic digests of bovine, *Candida krusei* and equine cytochrome *c* and using this new CE–ESI-MS interface are shown in Fig. 2. Each separation utilized a 0.01 *M* ammonium acetate–acetic acid buffer system (pH 4.4) and a 10 μm I.D. analytical capillary. From Fig. 2, each analysis was complete within six minutes, although greater separation resolution could have been obtained using longer capillaries. From the mass spectrometric data, however, peaks corresponding to singly or multiply protonated molecular ions of the individual tryptic fragments can be isolated. For example, shown in Fig. 3 are examples of extracted ion electropherograms for the individual tryptic fragments YIPGTK (m/z 678), which is a tryptic fragment common to all three proteins, EDLIAYLK (m/z 964), which is common to bovine and equine cytochrome *c*, and MAFGGLK (m/z 723), which is specific to *Candida krusei* cytochrome *c*. The three extracted ion electropherograms show additional solute zones of the same m/z as the tryptic fragments. These additional zones are likely due to collisional dissociation of other tryptic fragments or intact protein during transport into the MS.

An important characteristic of ESI is that under appropriate conditions intact molecular ions are formed with little dissociation, unless created during transport into the MS. As a result, molecular mass measurements for large biomolecules that exceed the instrumental MS mass range can be obtained because their ESI mass spectra generally consist of a distribution of molecular ion charge states that are in the MS

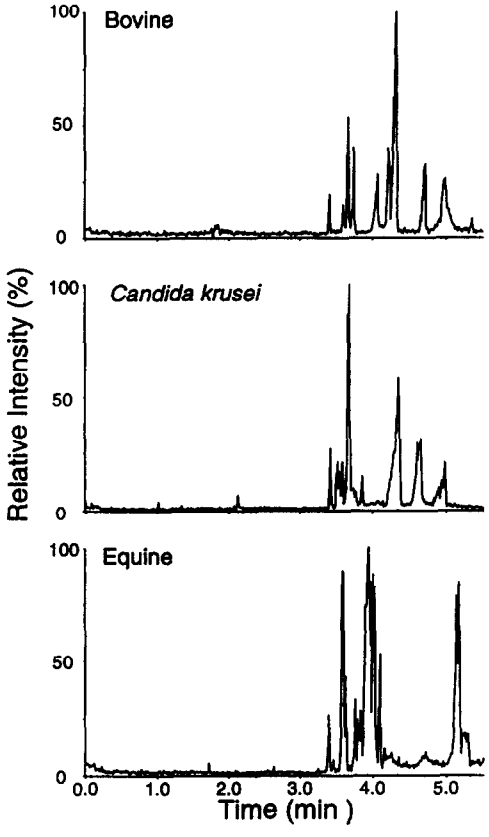

Fig. 2. Total ion electropherograms obtained from tryptic digests of bovine, *Candida krusei* and equine cytochrome *c* . Separation conditions: 0.01 *M* ammonium acetate–acetic acid buffer system, pH 4.4; capillary: 10 μm I.D., 50 cm in length and chemically modified with 3-aminopropyltrimethoxysilane; electric field, -300 V cm^{-1}; injection: 5 s at -1 kV; concentration of original proteins: *ca.* 750 μg/ml; mass spectrometer: scanning from *m/z* 600–1200, 2-*m/z* steps, 0.6 s/scan.

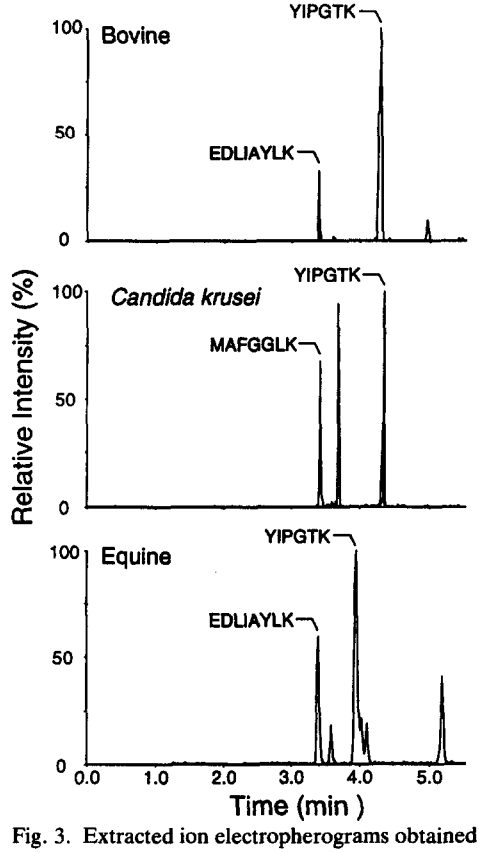

Fig. 3. Extracted ion electropherograms obtained from Fig. 2 for the individual tryptic fragments YIPGTK (*m/z* 678), which is a tryptic fragment common to all three proteins, EDLIAYLK (*m/z* 964), which is common to bovine and equine cytochrome *c*, and MAFGGLK (*m/z* 723), which is specific to *Candida krusei* cytochrome *c*. The additional solute zones of the same *m/z* as the tryptic fragments are likely due to incomplete digestion and fragmentation of other tryptic fragments occurring during transport to the mass spectrometer.

mass range [14,15]. This distribution of molecular ion charge states for peptides and proteins, which generally arises from protonation of basic residue sites for positive ion ESI, yields a distinctive pattern of peaks due to the discrete nature of the electronic charge, where adjacent peaks vary by one charge. Generally, the peptides that result from a tryptic digest produce singly and doubly charged molecular ions during the ESI process. To determine the molecular mass of a specific solute zone in a separation, this extremely small charge state distribution must first be recognized, which may be difficult because the distribution may not be within the instrumental mass range or because additional mass spectra peaks, which exist due to dissociation in the MS interface, may interfere. This lack of a definable charge distribution can make mass spectra assignment and component identification ambiguous unless higher MS resolution is used to determine exact molecular ion charge states. Fortuitously, the use of the silver conductive CE terminus can produce silver adducts (addition of *ca.* 108 u) with each molecular species. These adducts are formed at the electrospray tip where the oxidation of silver occurs in a manner de-

scribed previously [16]. As a result, the charge state for a known or unknown MS peak may be determined when silver adducts are present without the necessity of greatly degrading sensitivity to obtain resolution sufficient for molecular ion charge state determination. From the charge state information the actual molecular mass of the tryptic fragment can be determined within the precision of the MS measurement and with greater accuracy than with contaminant adduction such as sodium, which may not be resolved under all CE–ESI-MS conditions. For example, the mass spectra obtained from the bovine tryptic fragments of TGQAPGFSYTDANK (M_r 1457), which is observed at 3.7 min in the electropherogram, and of KTGQAPGFSYTDANK (M_r 1585), which is observed at 4.1 min, are illustrated in Fig. 4. The

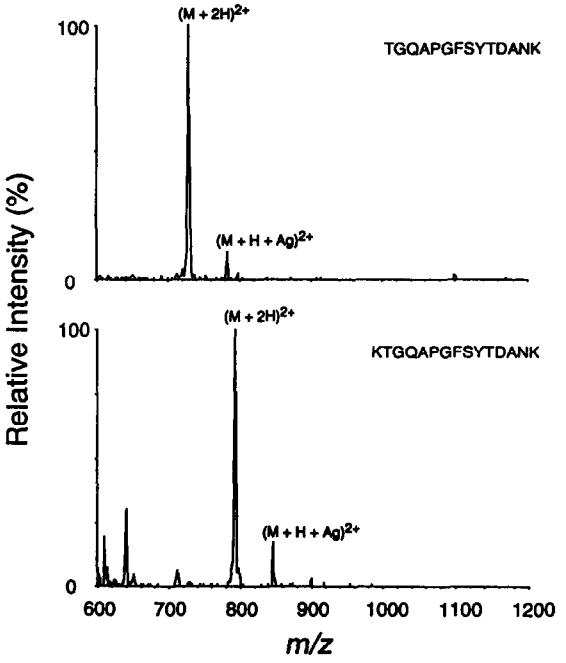

Fig. 4. Mass spectra obtained from the bovine tryptic fragments of TGQAPGFSYTDANK (M_r 1457, observed at 3.7 min) and of KTGQAPGFSYTDANK (M_r 1585, observed at 4.1 min). The singly charged species of both solutes is greater than can be observed with the MS instrument used However, because of the silver capillary terminus, both mass spectra show an additional peak that is approximately 54 greater than the base peak. Consequently, the base peak in each mass spectra is presumed to be the doubly charged molecular ion, $(M + H + Ag)^{2+}$.

molecular mass of both solutes is greater than can be observed with the MS instrument used; consequently, the singly charged species can not be detected, but the doubly charged species is observed. Moreover, because of the silver capillary terminus, both mass spectra show an additional peak that is approximately 54 m/z units greater than the molecular ion. As a result, the base peak in each mass spectrum is presumed to be the doubly charged molecular ion because this second peak is a multiple of two greater relative to silver (*ca.* 108 u) addition. Accordingly, the use of a silver-metalized capillary terminus has the advantage that exact charge states may be determined for solutes under certain conditions.

CONCLUSIONS

The results shown in this report demonstrate several important findings. First, this new CE–ESI–MS interface can produce and maintain a stable electrospray signal during a CE–ESI–MS experiment without additional sheath or make-up fluid or mechanical assistance to aid the ESI process. The absence of these additional liquids should offer improved solute detectability and sensitivity in CE–ESI–MS because of improved solute ionization efficiency. Second, the use of a silver conductive CE terminus can allow the exact determination of a charge state for a peak in a mass spectra; however, these additional peaks in the mass spectra may make mixture determination more problematic. Finally, the combination of this new ESI interface with small I.D. capillaries (10 μm) in CE–ESI–MS analyses illustrates that low nl/min effluent flow rates can be electrosprayed directly from a conductive CE capillary terminus. These low CE flow rates permit the ESI–MS to function as a mass sensitive detector, where the ESI current is limited by the rate of delivery of charge carrying species in solution to the ESI source. Consequently, a constant analyte sensitivity is to be expected within the linear dynamic range of the MS detector [8]. These initial results demonstrate a simple and versatile electrospray interface design, which appears to offer increased solute detectability and sensitivity [13], for the use in ultrasensitive peptide and protein analyses.

ACKNOWLEDGMENT

This research is supported by internal exploratory research and the Director, Office of Health and Environmental Research, U.S. Department of Energy. Pacific Northwest Laboratory is operated by Battelle Memorial Institute for the US Department of Energy, through contract DE-AC06-76RLO 1830.

REFERENCES

1 T.F. Cheng and N.J. Dovichi, *Science*, 242 (1988) 562.
2 J.V. Sweedler, J.B. Shear, H.A. Fishman and R.N. Zare, *Anal. Chem.*, 63 (1991) 496.
3 L. Hernandez, J. Escalana, N. Joshi and N. Guzman, *J. Chromatogr.*, 559 (1991) 183.
4 R.A. Wallingford and A.G. Ewing, *Anal. Chem.*, 61 (1989) 98.
5 J.A. Olivares, N.T. Nguyen, C.R. Yonker and R.D. Smith, *Anal. Chem.*, 59 (1987) 1230.
6 R.D. Smith, C.J. Barinaga and H.R. Udseth, *Anal. Chem.*, 60 (1988) 1948.
7 M.D. Lee, W. Mück, J.D. Henion and T.R. Covey, *J. Chromatogr.*, 458 (1988) 313.
8 J.H. Wahl, D.R. Goodlett, H.R. Udseth and R.D. Smith, *Electrophoresis*, 14 (1993) 448.
9 J.H. Wahl, D.R. Goodlett, H.R. Udseth and R.D. Smith, *Anal. Chem.*, 64 (1992) 3194.
10 G.L.M. Bruin, R. Huisden, J.C. Kraak and H. Poppe, *J. Chromatogr.*, 480 (1989) 339.
11 K.D. Lukacs, *Diss. Abstr. Int.*, 44 (1983) 3766.
12 R.D. Smith, H.R. Udseth, C.J. Barinaga and C.G. Edmonds, *J. Chromatogr.*, 559 (1991) 197.
13 J.H. Wahl, D.C. Gale, S.A. Hofstadler, H.R. Udseth and R.D. Smith, presented at the *41st Conference on Mass Spectrometry and Allied Topics, San Francisco, CA, May 1993*; paper ThP 226.
14 J.B. Fenn, M. Mann, C.K. Meng and S.F. Wong, *Mass Spectrom. Rev.*, 9 (1990) 37.
15 R.D. Smith, J.A. Loo, R.R. Ogorzalek Loo, M. Busman and H.R. Udseth, *Mass Spectrom. Rev.*, 10 (1991) 359.
16 A.T. Blades, M.G. Ikonomou and P. Kebarle, *Anal. Chem.*, 63 (1991) 2109.