Journal of Chromatography, 480 (1989) 197–209
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 776

COUPLING OF CAPILLARY ZONE ELECTROPHORESIS AND CAPILLARY LIQUID CHROMATOGRAPHY WITH COAXIAL CONTINUOUS-FLOW FAST ATOM BOMBARDMENT TANDEM SECTOR MASS SPECTROMETRY

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SUMMARY

The coaxial continuous-flow fast atom bombardment (FAB) system has proven to be very useful for interfacing capillary liquid chromatography and capillary zone electrophoresis (CZE) with sector mass spectrometry (MS). The interface can be used for the acquisition of both MS and MS–MS spectra from femtomole levels of non-volatile and/or thermally labile analytes while maintaining separation efficiencies of hundreds of thousands of plates. The use of coaxial fused-silica capillary columns to independently deliver the microcolumn effluent and the FAB matrix to the tip of the FAB probe offers the following advantages: the composition and flow-rates of the two liquid streams can be independently optimized; the FAB matrix does not effect the microcolumn separation process; peak broadening is minimized since the two liquid streams do not mix until they reach the tip of the FAB probe where ion desorption occurs; and, with CZE, active electrophoretic transport delivers the analytes directly to the FAB probe tip. These features combine to make this coaxial continuous flow fast atom bombardment interface particularly well suited for use with microcolumn separation methods.

INTRODUCTION

Capillary zone electrophoresis (CZE) and capillary liquid chromatography (LC) have proven to be extremely useful methods for the analysis of non-volatile and/or thermally labile compounds such as biomolecules. The high separation efficiencies that can be obtained with these microcolumns allow the separation of the components of very complex mixtures, such as biological extracts. While LC remains the most widely

0021-9673/89/$03.50

used separation method for the analysis of biomolecules, the distinctly superior separation efficiency of CZE over LC is attracting the attention of an increasingly large number of chemists. CZE has proven to generate in excess of 10^6 theoretical plates in less than 20 min[1,2] and greater than 10^5 theoretical plates in less than 1 min[3].

The first report of the coupling of CZE with mass spectrometry (MS) was made by Olivares *et al.*[4] who used an electrospray ionization (ESI) interface at atmospheric pressure in conjunction with a quadrupole mass spectrometer. Using quaternary ammonium salts, this system demonstrated separation efficiencies up to 140 000 theoretical plates and detection limits down to 0.7 fmol. Two additional papers on CZE–ESI–MS were published in 1988 by Smith and co-workers[5,6] reporting improvements in the interface design and the analysis of peptides and neurotransmitters. Also in 1988, Lee *et al.*[7] reported the successful coupling of CZE with MS using an ion-spray interface and a triple quadrupole mass spectrometer. Detection limits of 24 fmol and separation efficiencies of 250 000 theoretical plates were obtained using peptides. The suitability of this system for the acquisition of MS–MS spectra in conjunction with CZE–MS was also demonstrated. The extension of this interface to the analysis of negative ions was demonstrated in 1989[8] by the CZE–MS analysis of sulfonated azo dyes.

We have successfully interfaced open tubular LC with electron impact and chemical ionization MS[9–11]. To date, however, the MS interface used with these methods has limited utility for biomolecules. The development of fast atom bombardment (FAB)–MS interfaces with flowing liquid streams by Caprioli *et al.*[12] as "continuous-flow FAB" and Ito *et al.*[13] as "frit FAB" have provided an MS interface particularly well suited for the analysis of non-volatile and/or thermally labile analytes. Therefore, our research groups have been actively pursuing the coupling of capillary LC (both open tubular LC and packed microcapillary LC) with tandem sector MS using a coaxial continuous-flow (CF)-FAB interface[14–16]. As an extension of this work we have successfully applied our interface to the coupling of CZE with tandem sector MS[17]. The very low flow-rates of these microcolumns (25–50 nl/min) place stringent requirements on the nature of the CF-FAB–MS interface, and precludes the use of "traditional" methods of interfacing LC with CF-FAB. Therefore, an interface was designed which uses a pair of coaxial fused-silica capillary columns to independently deliver the microculumn effluent and the FAB matrix directly to the FAB probe tip face. This coaxial CF-FAB system offers the following advantages: independent optimization of the composition and flow rates of the two liquid streams; minimal band broadening; and preclusion of any deleterious effects of the polar, viscous FAB matrix upon the separation process.

The coaxial CF-FAB system has been used to interface both CZE and capillary LC with a VG ZAB 4F four-sector mass spectrometer. The combined systems have been used to analyze several classes of biomolecules, including peptides. They have been successfully used to acquire on-the-fly FAB–MS spectra, and, with capillary LC, on-the-fly MS detection limits of 500 amol have been obtained. The capabilities of the ZAB 4F have been exploited to obtain on-the-fly collisionally activated decomposition (CAD) MS–MS spectra of pmol to fmol levels of biomolecules, which yield abundant structural information[16]. With CZE this interface has proven to be capable of acquiring MS and MS–MS spectra from low femtomole amounts of peptides while maintaining high (hundreds of thousands of plates) electrophoretic separation efficiencies[17].

In this paper we present results which demonstrate the utility of this coaxial CF-FAB–MS interface with both capillary LC and CZE.

EXPERIMENTAL

Capillary liquid chromatography instrumentation

The fundamentals of our capillary LC–coaxial CF-FAB–MS interface have been previously described[14–16]. Briefly, the fused-silica capillary LC column is inserted into the fused-silica sheath column through which the FAB matrix solution is pumped via a syringe pump (μLC-500; Isco, Lincoln, NE, U.S.A.). For open tubular work the typical dimensions of the columns are an analytical capillary column of 10 μm I.D. and 150 μm O.D., and a sheath capillary column of 200 μm I.D. and 350 μm O.D. (Polymicro Technologies, Phoenix, AZ, U.S.A.). Evaluations of a variety of different FAB matrices has revealed[15] the most suitable FAB matrix with capillary LC to be glycerol–water (25:75) at a flow-rate of approximately 1 μl/min.

Both the analytical capillary and the sheath capillary terminate at the tip of the FAB probe (Fig. 1). Thus, the two flow streams do not merge until they arrive at the face of the FAB probe, minimizing the loss of separation efficiency due to mixing effects.

The apparatus used for capillary LC is shown in Fig. 2. Helium gas pressure is used to pump the mobile phase through the system in a pulse-free manner. Stainless-steel tubing is used to deliver the mobile phase to the injection valve. To preclude the absorption of polar biomolecules onto metal tubing, polyether ether ketone tubing (Alltech, Deerfield, IL, U.S.A.) is used between the injection valve and the waste valve. For the same reason a metal free in-line 2-μm filter (Alltech) is used to prevent the plugging of the capillary column with particulates.

The injection procedure utilized with this sytem is notably different from LC "loop" injections, and is more similar to the "splitless/split" injection method used in capillary gas chromatograhy[9,15]. This injection system allows the injection of as little as 10 pl with minimal chromatographic band broadening. Typical injection volumes used in this work range from 2 to 10 nl.

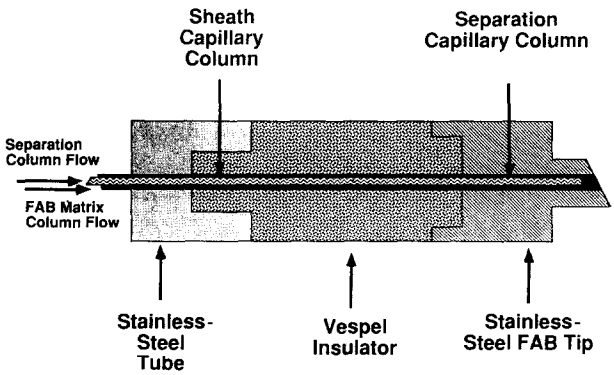

Fig. 1. Schematic of coaxial CF-FAB tip.

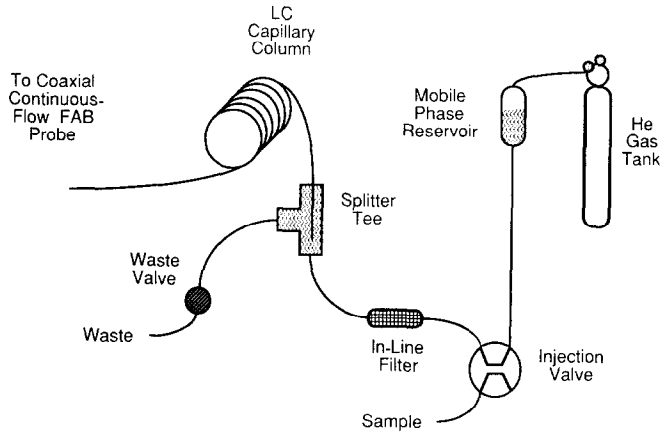

Fig. 2. Schematic of open tubular liquid chromatography system.

Capillary zone electrophoresis instrumentation

The CZE–coaxial CF-FAB–MS interface is very similar to the capillary LC interface, and is shown schematically in Fig. 3. The CZE fused-silica capillary column (1 m × 13 μm I.D.) is inserted into the sheath fused-silica capillary column, again using a 1/16-in. stainless-steel tee to mate the two columns. The two coaxial capillary columns terminate at the FAB probe tip (Fig. 1), which is electrically insulated from the probe shaft with a Vespel insulator. A very important feature of this CZE interface is that the +8 kV FAB probe tip is used as the electrical "ground" of the CZE system. Therefore, active electrophoretic transport drives the analytes through the CZE column to the FAB probe tip where ion desorption takes place, obviating the use of a transfer line from the end of the CZE capillary to the FAB probe tip. This precludes the zone broadening that will occur within the transfer line due to the parabolic flow profile of the pressure driven flow used to transport the CZE separated analytes through the transfer line, and in the connections between the CZE column and the transfer line.

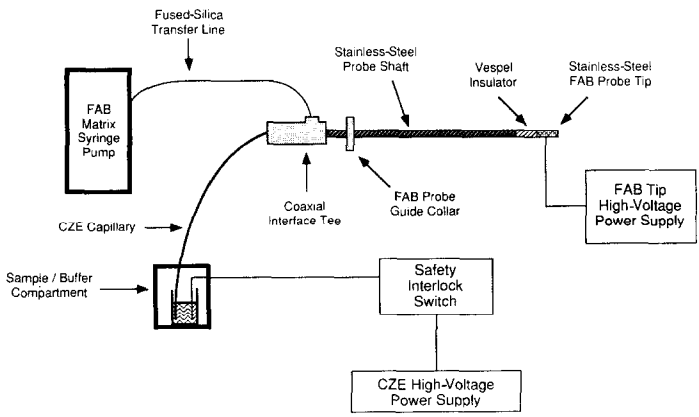

Fig. 3. Schematic of on-line CZE–coaxial CF-FAB–MS system.

The CZE high-voltage power supply (Glassman High Voltage, Princeton, NJ, U.S.A.) was maintained at 30 kV, and the FAB probe tip is maintained at a potential of 8 kV, creating a 22-kV potential drop across the length of the CZE column. A safety interlock system incorporating a high-voltage relay (Kilovac, Santa Barbara, CA, U.S.A.) was used for operator safety[17].

The FAB matrix used with the CZE–FAB–MS system was glycerol–water (25:75) with 0.0005 *M* heptafluorobutyric acid. The heptafluorobutyric acid served both to provide ions for electrical contact between the FAB tip and the CZE column effluent, and to acidify the solution on the FAB probe tip, increasing the production of protonated molecular ions.

Sample introduction onto the CZE capillary column has been accomplished by two methods. The combination of electroosmotic flow and electrophoretic migration has been used to "inject" the sample solution onto the CZE capillary, referred to as an electromigration sample introduction. Electromigration sample introduction is performed by replacement of the buffer reservoir at the high voltage end of the CZE capillary with a vial of sample solution, followed by the application of high voltage for the desired time interval. The application of high voltage causes sample to enter the CZE column by both electrophoretic migration of sample ions and the electroosmotic flow of the sample solution. Electromigration sample introduction, at a 22 kV potential drop in our system, yields an "injection" of approximately 250 pl/s into the CZE column. One disadvantage of electromigration sample introductions is a (relatively minor) discrimination between cationic and anionic analytes due to the opposite direction of electromigration of the two species.

The alternate sample introduction method used in CZE is referred to as hydrostatic sample introduction. This method uses pressure induced flow to introduce sample onto the column while the high voltage is turned off. This method, therefore, does not discriminate between cationic and anionic analytes. In our system hydrostatic sample introductions are accomplished by utilizing the hydrostatic flow in the CZE column induced by the vacuum of the mass spectrometer ion source.

It is interesting to note that this hydrostatic sample introduction utilizes what is otherwise an undesirable consequence of mating CZE with high-vacuum mass spectrometer systems—vacuum-induced flow. The electroosmotic flow profile in CZE is flat and piston-like across the capillary tube cross-secton, decreasing only at the walls of the capillary[18]. On the other hand, pressure driven flow or vacuum induced flow results in the formation of a parabolic flow profile across the capillary tube cross-section. Electroosmotically driven flow does not contribute to zone broadening, whereas pressure driven parabolic flow is a substantial cause of zone broadening. It is this difference in flow profiles which is responsible for a significant part of the superior separation efficiency of CZE over LC. Therefore any pressure driven flow in the CZE system will lead to zone broadening and a loss of CZE separation efficiency.

We have previously noted that, for the coaxial continuous flow fast atom bombardment interface, the flow of FAB matrix around and over the end of the separation capillary minimized the effect of the ion source vacuum on the separation column flow, and it was indicated at the time that this would be an advantage of the coaxial interface with CZE[14]. Due in part to this effect of the sheath flow, the vacuum-induced flow in our CZE column has been found to be approximately 50 pl/s, which is sufficiently low so as to allow high-resolution separations to be acquired with

the system. It is this 50 pl/s vacuum induced flow that is exploited to perform hydrostatic sample introductions into the CZE column.

Mass spectrometry

The mass spectrometer used in this work was a VG ZAB-4F (VG Analytical, Manchster, U.K.) tandem four-sector mass spectrometer of B1-E1-E2-B2 geometry[19]. An Ion Tech FAB gun was used with xenon as the FAB gas (8 kV at 1 mA). The desorbed ions were accelerated to 8 kV for analysis. Mass spectra were acquired by scanning MS-I (B1-E1) and detecting the ions with a photomultiplier tube-based detector located in the third field-free region. MS–MS spectra were acquired by using MS-I to select the parent ions and direct them into the collision cell located in the third field free region. Helium was used as the collision gas (50% parent ion beam supression). Daughter ion spectra were acquired by a linked scan of E2-B2 and detection in the fifth field-free region with a photomultiplier tube-based detector.

Chemicals

All analytes were obtained from Sigma (St. Louis, MO, U.S.A.) and were used as delivered.

RESULTS AND DISCUSSION

Capillary LC–coaxial CF-FAB–MS

Previous work[14,15] has utilized the capillary LC–coaxial CF-FAB–MS system to acquire full-scan mass spectra and single-ion mass chromatograms from several classes of biomolecules, including peptides, phospholipids, steroids and carbohydrates. Peptides have been the most widely studied class of analytes. Full-scan peak centroided mass spectra (mol.wt. 1500 to 150) have been obtained from as little as 54 fmol of the tripeptide Met-Leu-Phe, and using the multichannel data acquisition mode in conjunction with a narrow mass range scan, detection limits of 500 amol of Met-Leu-Phe have been obtained[15]. The full-scan mass spectrum of N-acetylangiotensin I resulting from the injection of 3 pmol in 4.4 nl is given in Fig. 4. The detection limits are significantly reduced if only the region of the mass spectrum containing the

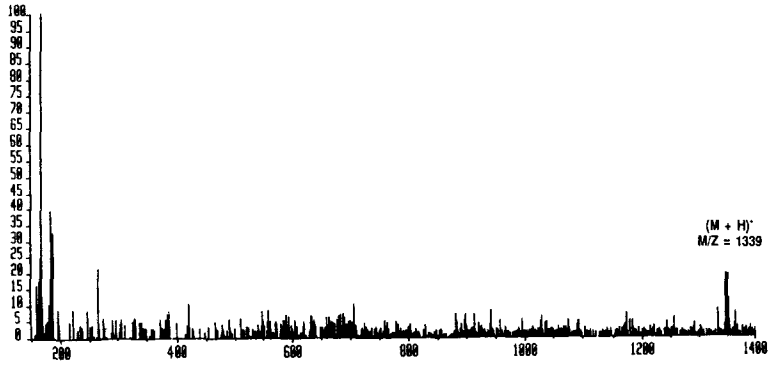

Fig. 4. Open tubular LC–coaxial CF-FAB–mass spectrum of 3 pmol of N-acetylangiotensin I acquired from a 4.4-nl injection.

protonated molecular ions is scanned. This is illustrated by the analysis (Fig. 5) of triplicate injections of a $4 \cdot 10^{-6}$ *M* solution of N-acetylangiotensin I (16 fmol per injection), giving an average signal to peak-to-peak noise ratio of approximately 6:1.

Bradykinin has arginine terminal residues as both the N-terminal and C-terminal amino acids. Chromatographic peak tailing has been found to occur unless the mobile phase is acidified, presumably due to the adsorption of these terminal residues onto the walls of the fused silica tubing. Therefore, 0.1% trifluoroacetic acid was added to the analyte solution and the mobile phase for the analysis of bradykinin. Previous work[15] has produced a full-scan mass spectrum from the injection of 900 fmol of bradykinin. The single-ion mass chromatogram obtained from triplicate injections of 4.4 nl of a $2 \cdot 10^{-6}$ *M* solution of bradykinin (8 fmol per injection) is shown in Fig. 6.

The capabilities of the ZAB-4F mass spectrometer have been exploited to acquire "on-the-fly" MS–MS spectra of the protonated molecular ion of representative compounds of a variety of analyte classes[16]. MS–MS spectra have been acquired from as little as 54 fmol of the tripeptide Met-Leu-Phe. The MS–MS spectrum of the antibiotic dihydrostreptomycin acquired with this system (Fig. 7) clearly shows the formation of daughter ions corresponding to the glucosamine subunits of the molecule, which can readily be used for structural identification of the parent compound.

On-line CZE–coaxial CF-FAB–MS

The buffers used to control analyte pH in CZE systems can be problematic when used with FAB–MS. Perhaps the most significant problem arising from these buffers is

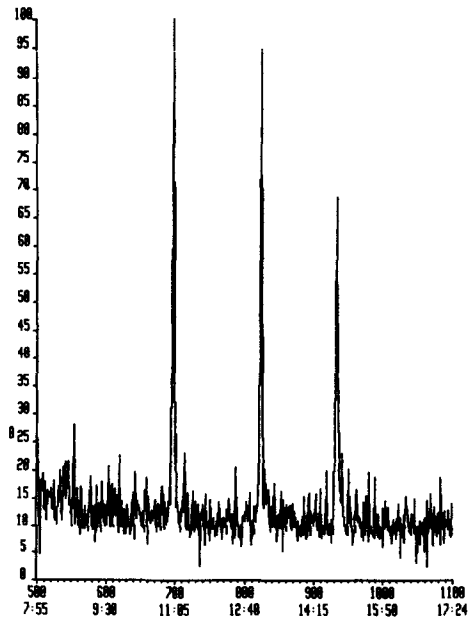

Fig. 5. Single-ion mass chromatogram of the $(M + H)^+$ ion of N-acetylangiotensin I resulting from triplicate injections of 16 fmol of the decapeptide.

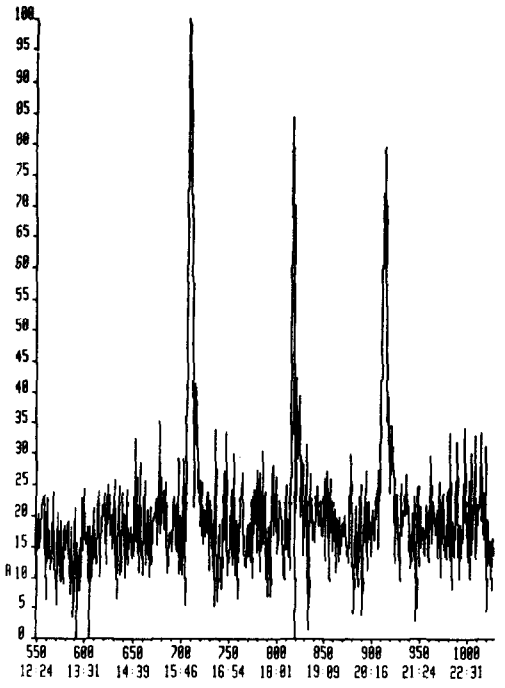

Fig. 6. Single-ion mass chromatogram of the $(M + H)^+$ ion of bradykinin resulting from triplicate injections of 8 fmol of the decapeptide.

that the ions of the buffer can compete with protons in the formation of adducts with the charged molecular ions of the analytes. This can lead to the division of the analyte signal into several different ion types, therefore, lowering the observed signal-to-noise ratio of the protonated molecular ions in the MS spectra, and lowering the signal-to-noise ratios of the daughter ions in MS–MS spectra.

Therefore, a series of experiments have been conducted to ascertain the effect of the CZE buffer on the FAB–MS spectrum. There are two classes of buffers of interest: non-volatile buffers such as the "traditional" potassium phosphate buffer, and volatile buffers such as ammonium acetate.

The effect of potassium phosphate buffers over a concentration range of 0.05 to 0.01 *M* at pH 7 has been evaluated by studying the mass spectrum of the tripeptide Met-Leu-Phe, measuring the intensity of the protonated molecular ion $(M + H)^+$ at *m/z* 410 and the potassium adduct of the molecular ion $(M + K)^+$ at *m/z* 448 as a function of buffer concentration. The data show that the intensity of the $(M + H)^+$ ion decreases as the potassium phosphate buffer concentration is increased, commensurate with an increase in the $(M + K)^+$ ion intensity (Fig. 8). Prolonged use of potassium containing buffers has been observed to lead to the formation of not only $(M + K)^+$ ions, but also $(M - H + 2K)^+$ ions. The formation of both proton and potassium adducts from the peptide divides the analyte signal into several different signals, reducing the signal-to-noise ratio of the molecular ion data. The use of ammonium acetate buffers has been observed to yield only protonated molecular ions,

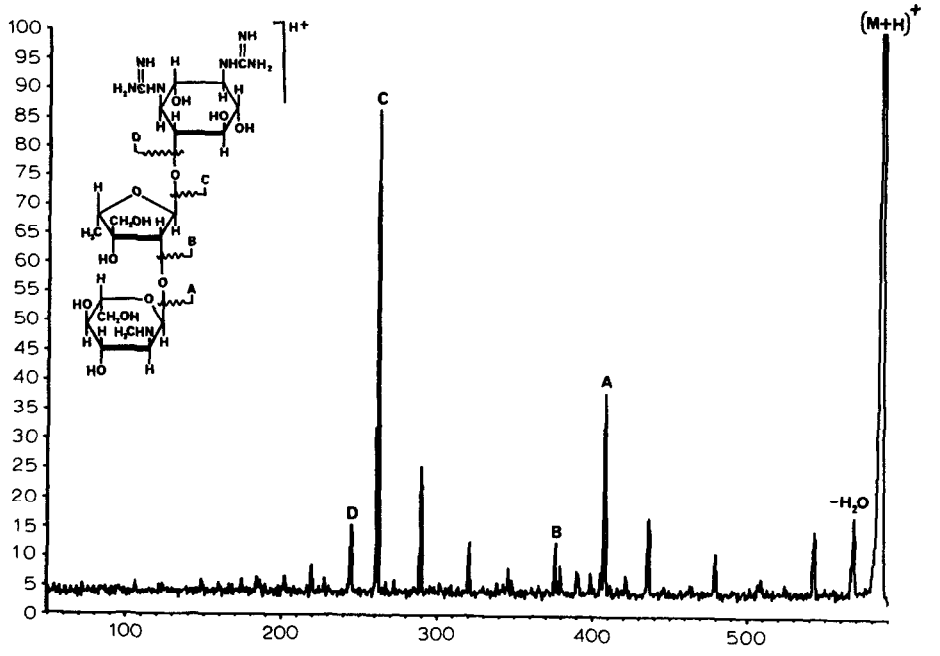

Fig. 7. Coaxial CF-FAB–MS–MS spectrum of the $(M + H)^+$ ion (m/z 584) of 230 pmol of the antibiotic dihydrostreptomycin.

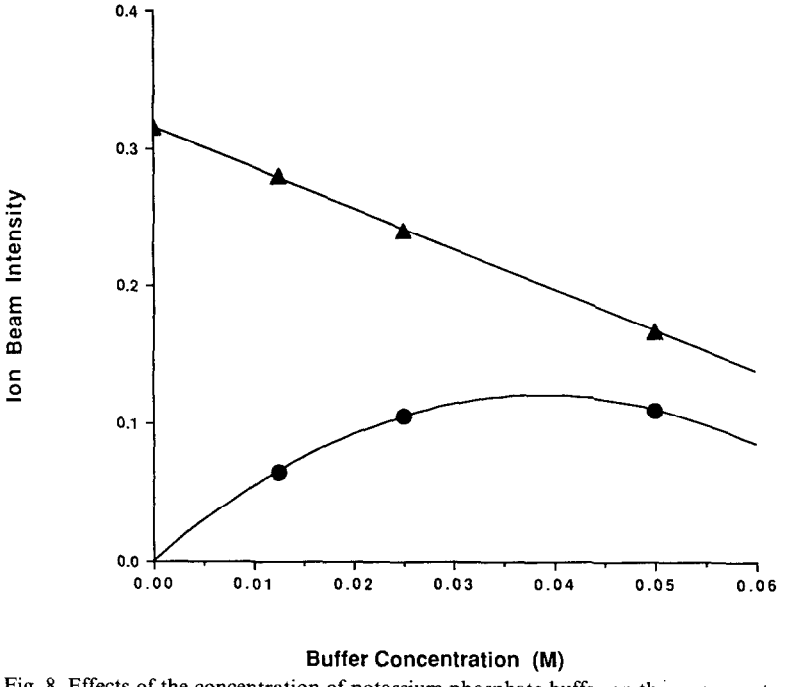

Fig. 8. Effects of the concentration of potassium phosphate buffer on the mass spectrum of the tripeptide Met-Leu-Phe. ▲ = $(Met\text{-}Leu\text{-}Phe + H)^+$, $y = 0.31570 - 2.9863x$, $R^2 = 1.000$; ● = $(Met\text{-}Leu\text{-}Phe + K)^+$, $y = 2.8460 \cdot 10^{-19} + 6.2000x - 80.000x^2$, $R^2 = 1.000$.

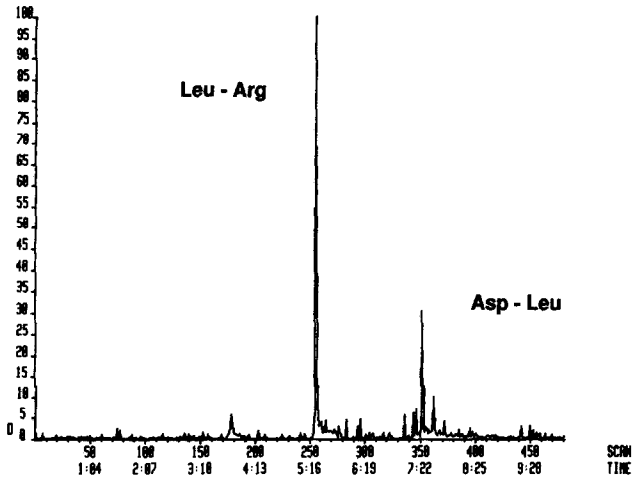

Fig. 9. Summed ion electropherogram of the $(M + H)^+$ ions of Leu-Arg and Asp-Leu at pH 8.0. Leu-Arg: 450 fmol, 290 000 plates; Asp-Leu: 530 fmol, 550 000 plates.

with no evidence of the formation of any ammonium adducts with peptides. Therefore, ammonium acetate buffers have been exclusively used in all of our CZE–FAB–MS separations.

Initial separations using the on-line CZE–coaxial CF-FAB–MS system have focused on the analysis of peptide mixtures. All separations have used ammonium acetate buffers (0.005 *M*) over a pH range of 6.6 to 8.5 (adjusted to the desired pH using ammonium hydroxide). The separation of a 0.1-ng/nl (10^{-4} *M*) solution (pH 8.0) of the dipeptides Leu-Arg and Asp-Leu (Fig. 9) was accomplished using a 5-s electromigration sample introduction at 22 kV, corresponding to an "injection" of approximately 450 fmol of Leu-Arg and 530 fmol of Asp-Leu. This separation shows

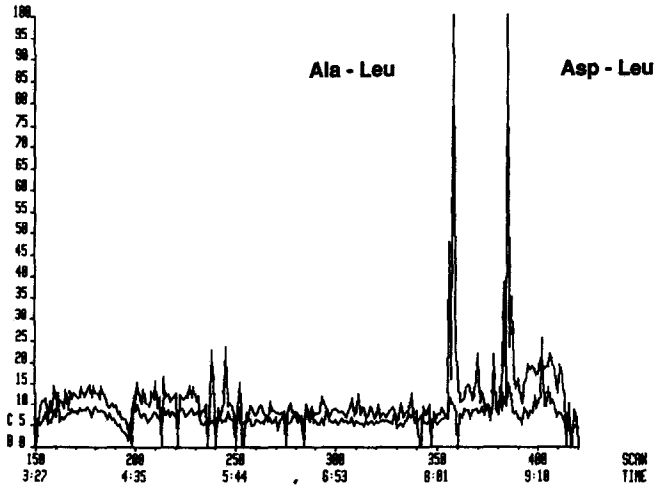

Fig. 10. Overlaid ion electropherograms of the $(M + H)^+$ ions of Ala-Leu and Asp-Leu at pH 8.5. Ala-Leu: 250 fmol, 590 000 plates; Asp-Leu: 210 fmol, 600 000 plates.

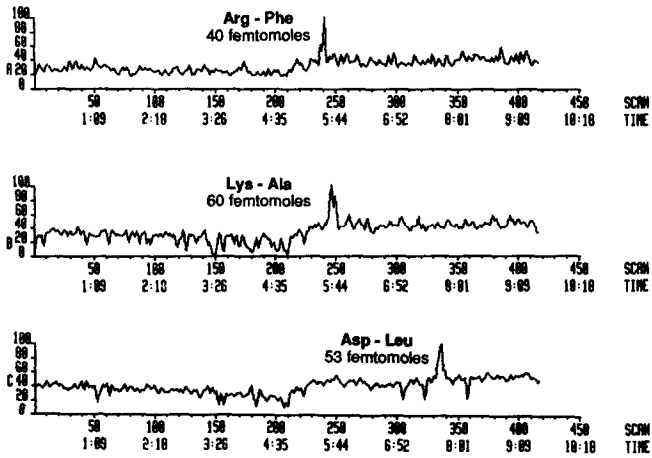

Fig. 11. Single-ion electropherograms of the $(M + H)^+$ ions of Arg-Phe, Lys-Ala and Asp-Leu at pH 8.5.

the electromigration order expected on the basis of the nature of the amino acid residues of these small dipeptides, with a time separation of approximately 2 min and a total required analysis time of approximately 8 min. The peak width at half height of these two peaks corresponds to separation efficiencies in excess of 250 000 and 500 000 plates, respectively. It should be noted that an accurate measure of separation efficiency is best performed using the method of statistical moments with at least 20 data points collected across the electrophoretic peak. The width at half height of these peaks is so small that the useful scanning rates available with the ZAB-4F mass spectrometer preclude the acquisition of the desired number of data points across the CZE peak. Therefore, the plate counts given for the CZE separations in the work are approximations of the true separation efficiency, which is certainly in the hundreds of thousands.

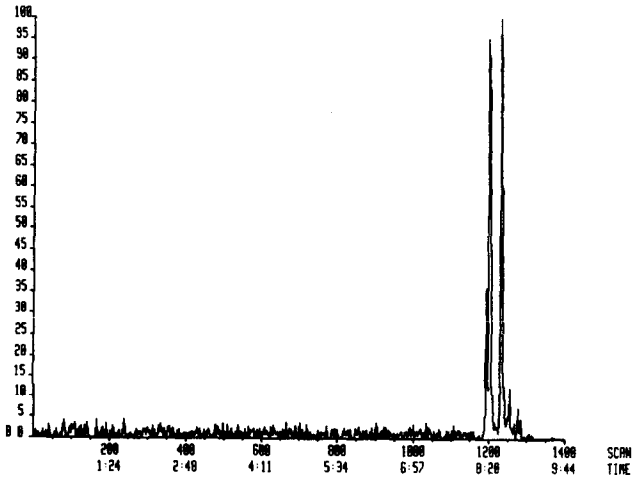

Fig. 12. Single-ion electropherogram of the $(M + H)^+$ ion of Met-Leu-Phe resulting from duplicate electromigration sample introductions; 32 fmol, 455 000 plates (average).

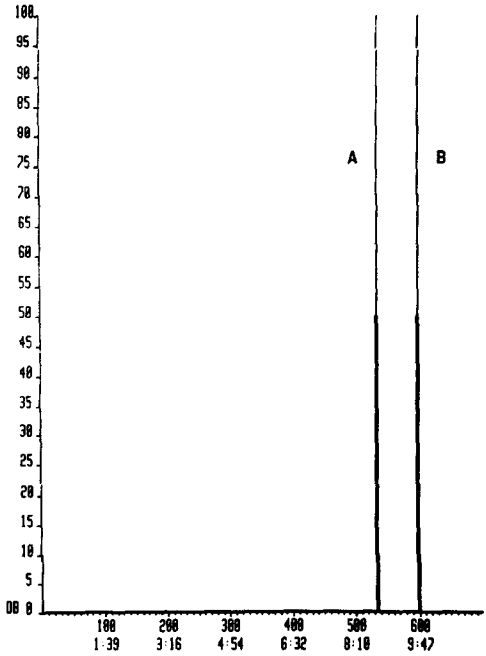

Fig. 13. Single-ion electropherograms of the $(M + H)^+$ ions of N-acetylangiotensin I (peak A, 10 fmol) and angiotensin I (peak B, 20 fmol) resulting from the analysis of a 10^{-6} *M* solution of the decapeptides.

The separation of 250 fmol of Ala-Leu from 210 fmol of Asp-Leu from 10^{-4} *M* solutions (pH 8.5) is shown in Fig. 10. Calculated separation efficiencies for these two peaks are in excess of 550 000 plates. The analysis of a 10^{-5} *M* solution (pH 8.5) of Arg-Phe, Lys-Ala, and Asp-Leu is given in Fig. 11. The electrophoretic peaks correspond to 40, 60, and 50 fmol of the dipeptides, respectively.

The reproducibility of the electromigration sample introduction procedure with our system is illustrated via the duplicate "injections" of 32 fmol of the tripeptide Met-Leu-Phe (Fig. 12). The peak heights show good reproducibility, and the average separation efficiency was calculated to be in excess of 450 000 plates.

The suitability of the system for the analysis of larger peptides is illustrated by the analysis (Fig. 13) of a 10^{-6} *M* solution of the decapeptides angiotensin I (20 fmol) and N-acetylangiotensin I (10 fmol). The high signal-to-noise ratio of this separation is a consequence of the low levels of FAB matrix background ions in the mass region being scanned. The very narrow electrophoretic peak widths resulted in the acquistion of only ona data point across the electrophoretic peak.

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