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COUPLING CAPILLARY ZONE ELECTROPHORESIS AND CONTINUOUS-FLOW FAST ATOM BOMBARDMENT MASS SPECTROMETRY FOR THE ANALYSIS OF PEPTIDE MIXTURES

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SUMMARY

Combined capillary zone electrophoresis (CZE)–continuous-flow fast atom bombardment (CF-FAB) mass spectrometry is described for the analysis of mixtures of peptides. A 90 cm × 50 μm I.D. fused-silica capillary column was used for electrophoretic separations and was connected to the CF-FAB probe via an interface which allows a total flow into the mass spectrometer of about 5 μl/min. Solutions of peptides were pneumatically loaded onto the CZE capillary, providing sample amounts of 0.1–20 pmol. The magnetic mass spectrometer was scanned over the desired mass range, usually between *m/z* 500 and 2500. Results are shown for separation and analysis of mixtures of synthetic peptides and also for protease digests of recombinant human growth hormone and horse heart cytochrome *c*.

INTRODUCTION

Capillary zone electrophoresis (CZE) has been shown to be an extremely effective separation device for compounds of biological origin[1–3]. Its capabilities include the high-resolution separation of charged molecules in open-tubular fused-silica capillaries on amounts in the low femtomole range. These capillaries are generally *ca.* 1 m in length and have inner diameters of 10–100 μm and can achieve separations showing 100 000 or more theoretical plates[1,4].

The primary detection system used in CZE thus far has been a high-sensitivity UV spectrophotometer, although electrochemical and fluorescence detection have also been reported [5–7]. More recently, mass spectrometry (MS) has been coupled to CZE because it provides mass specificity to the detection process, an advantage that is unrivaled by other analytical techniques. Electrospray MS has been coupled to CZE and was used to analyze a variety of compounds of interest to biochemists[4,8]. The combination of CZE with continuous-flow fast atom bombardment (CF-FAB) MS[9,10] was reported by Minard *et al.*[11]. Tomer and co-workers[12,13] described a

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coaxial interface for CF-FAB and capillary separations devices, such as CZE, which provides an efficient means of coupling the low-pressure ionization chamber of the mass spectrometer with liquid flow techniques.

We report here our efforts and results in coupling CZE and CF-FAB MS for the separation and analysis of mixtures of peptides. The interface, conceptually similar to that reported earlier[11,12], was connected to a high-resolution double-focussing mass spectrometer. Data are presented which shows the overall performance of the device at the 50–100-fmol level and that no major peak broadening occurs with this interface. Applications include analysis of mixtures of chemically synthesized peptides and also those obtained from the proteolytic digests of proteins.

EXPERIMENTAL

The CZE–CF-FAB MS experiments were performed using a safety interlock system protected CZE apparatus similar to that described by Jorgenson and Lukacs[1] and constructed at Applied Biosystems (Santa Clara, CA, U.S.A.). Fused-silica capillary (Polymicro Technologies, Tucson, AZ, U.S.A.), 90 cm × 50 μm I.D. × 140 μm O.D., was placed in solution compartments between the high-voltage anode and the electrically grounded cathode present in the CZE–CF-FAB interface. High voltage was applied using a 0–50 kV Bertan Assoc. (Hicksville, NY, U.S.A.) Model 205A-50P power supply run at a constant-voltage setting. Current through the system was measured over a 10-kΩ resistor in the return circuit of the power supply. UV detection was performed through the walls of a short uncoated section of capillary at a point 60 cm from the anode end of the capillary. Absorbance at 210 nm was measured using a modified variable-wavelength HPLC detector (Applied Biosystems Model 773, Foster City, CA, U.S.A).

The CZE–CF-FAB interface was a 1 in. × 1 in. × 3/8 in. plexiglass block consisting of two intersecting passageways (1/16 in. I.D.) oriented 90° to each other, as shown in Fig. 1. The effluent or cathode end of the CZE capillary meets the intake end of the CF-FAB capillary in a short segment of PTFE tubing of 0.5 mm I.D. × 1/16 in. O.D. placed in the left horizontal passageway of the block. In the upper vertical passageway there is a "flow-through" electrode, 4.5 cm × 1/16 in. O.D. × 0.03 in. I.D. stainless-steel tubing attached to a 10-ml syringe. In the lower vertical passageway there is a 1/16 in. O.D. × 1/32 in. I.D. PTFE inlet tube to allow for the introduction of CF-FAB solvent from a reservoir to the cathode compartment. The "flow-through" electrode permitted periodic flushing of the compartment with CF-FAB solvent to remove bubbles formed in the interface. The 10-ml syringe attached to the interface also provided a means of introducing a sample at the anode end of the CZE capillary. Using methylene blue dye as a visual indicator, 2 ml draws on this syringe reproducibly introduced a 15 mm length slug of dye representing a sample injection volume of 30 nl. After introducing a sample in this manner, the anodic end of the capillary is transferred from a sample vial to the electrophoresis buffer and high voltage is applied. The temperature of the CZE capillary was maintained at 30°C for all experiments. The flow-rate differential between that in the CF-FAB capillary (75 μm I.D. × 280 μm O.D.) created by atmospheric pressure (about 5 μl/min) and the electrophoretic flow-rate in the CZE capillary (about 0.1 μl/min) permits efficient transfer of CZE eluate to the mass spectrometer. The compositions of the CZE buffer

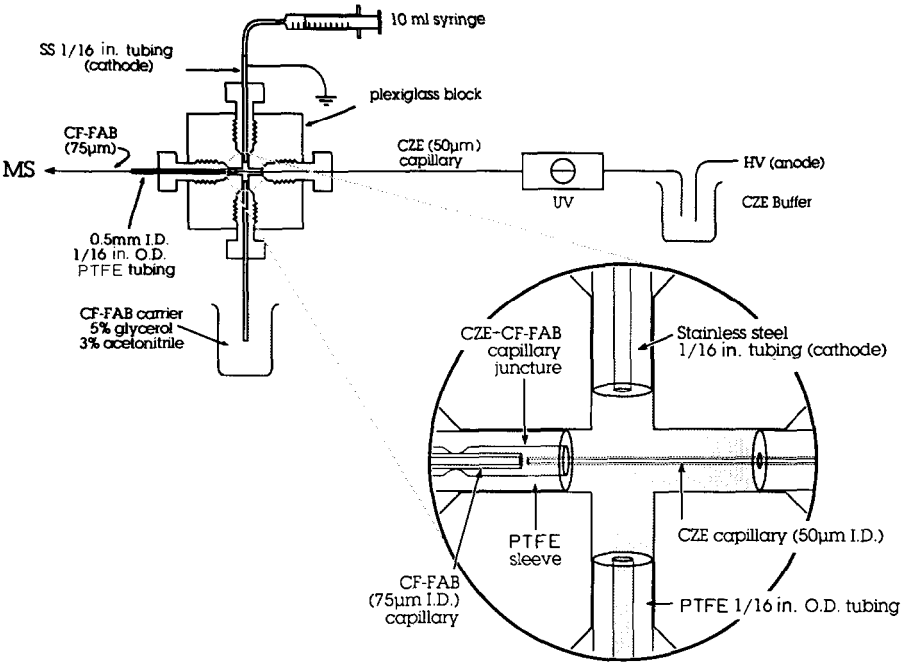

Fig. 1. Schematic representation of the CZE–CF-FAB instrument arrangement and interface. See text for details. SS = stainless steel; HV = high voltage.

and CF-FAB solvent varied depending on the samples analyzed and are indicated in the respective figure legends.

CF-FAB MS was performed with a Finnigan MAT 90 high-resolution instrument equipped with a saddle field ion gun using Xe to create energetic atoms. The CF-FAB probe used was the Finnigan-MAT Bioprobe. The mass spectrometer was generally operated at a resolving power of 1500 and an accelerating voltage of 4.7 kV. The magnet was scanned at 10 s/decade. CsI was used to calibrate the instrument.

The synthetic peptide mixture was derived from a preparation that was synthesized on a Model 430A automated peptide synthesizer using the solid phase *t*-Boc (*tert.*-butyloxycarbonyl) methodology (Applied Biosystems, Foster City). The tryptic digest of β-lactoglobulin A and cytochrome *c* were obtained by treating the proteins at a 1:50 enzyme-to-substrate ratio (w/w) in ammonium bicarbonate for 20 h. The tryptic digests of recombinant human growth hormone was provided by Dr. Gerald Becker of Lilly Research Laboratories (Indianapolis, IN, U.S.A.). β-Lactoglobulin A, cytochrome *c* and angiotensin II were obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

CZE–MS interface

The basic need for an interface results from the incompatibility of the CF-FAB process and CZE for liquid flow. The CF-FAB source requires a solvent, usually

water – glycerol (95:5), which is maintained at a steady flow-rate in the range of 2–15 μl/min. Optimal performance of the CZE process, in contrast, requires no mechanical flow at all, with only a slight flow-rate in the nl/min range resulting from electroosmotic flow. The interface shown in Fig. 1 satisfies both these conditions by allowing the low pressure in the mass spectrometer to pull the carrier solution from the interface block and thereby permits the CF-FAB probe to be stabilized. At the same

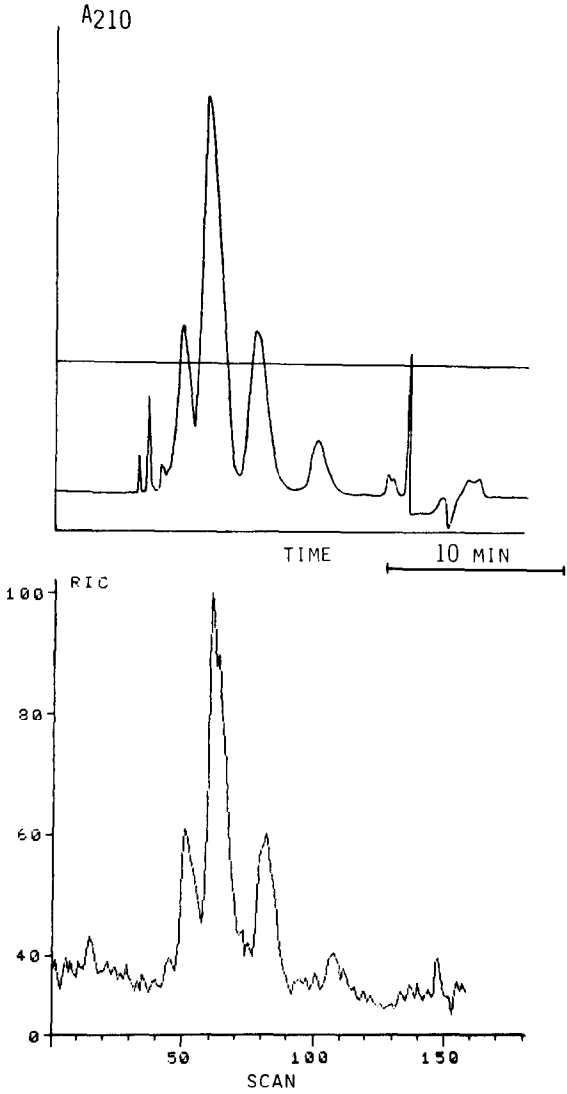

Fig. 2. Separation of synthetic peptides from a crude reaction mixture for the purpose of evaluating performance of the interface. (Top) The UV detector trace, showing the current stability (horizontal line); (bottom) the total ion chromatogram from the mass spectrometer detector. The CZE apparatus was run at 20 kV and 13 μA with 40 m*M* citric acid adjusted to pH 3.6 with ammonium hydroxide. The CF-FAB carrier solvent was 5% glycerol and 3% acetonitrile in water containing 2.36 m*M* acetic acid.

time, compounds eluting from the end of the CZE capillary are pulled into the probe capillary and are subsequently analyzed.

An important operational aspect of an interface is the amount of band broadening produced from the mixing of the two solutions. In this case, one is flowing at approximately 5 μl/min and the other at 100 nl/min or less. Fig. 2 shows both the UV detector trace and the MS total ion chromatogram obtained from the same analysis of a mixture of peptides obtained from a synthetic reaction designed to produce a 15 residue peptide having the sequence Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn-Ala(NH_2). The UV cell was located near the cathode end of the capillary as described in the experimental section, *i.e.*, before the interface, and the mass spectrometer after the interface. Comparison of the chromatograms indicate that the separation of the peaks is nearly identical and that little band broadening occurs as a result of dilution in the interface. This result is somewhat fortuitous, since band broadening must occur under these conditions. Most probably, peaks recorded on the UV trace are actually narrower than recorded and appear broader because of cell geometry and dimensions. Nevertheless, the result shows resolution loss in the interface is not a major problem. For example, the asymmetry of the trailing edge of the major peak can be seen in both, although more clearly for the MS data. The mass spectrum of the second component showed it to be a methionyl sulfoxide analogue of the peptide.

Achieving the very high number of theoretical plates for high-resolution separations by CZE is important and remains an asset of the technique. However, in this regard, there is also some incompatibility with the mass spectrometer as a detector. Most mass spectrometers are scanning instruments that take generally 2–20 s to scan a mass range, depending on the m/z values to be covered. Thus, a typical magnetic instrument with a fast scanning laminated magnet would require approximately 10 s or more to cover a mass range of m/z 300–3000 in order to achieve good ion statistics in the acquisition process. If a minimum of 3–4 scans across an eluting peak is desired, then the eluting peak width at half-height would need to be approximately 30 s wide. The situation is not unlike that encountered with gas chromatography–MS applications where high-resolution capillary columns are employed. Of course, the narrower the scan range, the greater the number of scans per unit time and the shorter in time the peak width needs to become to meet this criteria. Instruments which have integrating detectors, such as those equipped with the new array detectors, will improve the situation markedly even if they scan over several groups of masses.

Limit of detection

The limit of detection of the CZE–CF-FAB method was determined for angiotensin II, an octapeptide of molecular weight 1045. The mass spectrometer was scanned over a 4-mass-unit-wide window centered on m/z 1046, the $(M+H)^+$ ion. As shown in Fig. 3, on injection of 368 fmol of the peptide, a peak having a signal-to-noise ratio of 12:1 was recorded, and for 75 fmol, a ratio of 2.5:1 was recorded. Such a measurement determines the relative sensitivity of the instrument under specific operating conditions. One factor affecting this sensitivity is the mass range scanned. To a first approximation, the relative sensitivity is inversely proportional to the number of masses scanned in a given application. Thus, if one is analyzing tryptic peptides where it is desirable to scan a mass range of m/z 500–3000, then the amount of sample

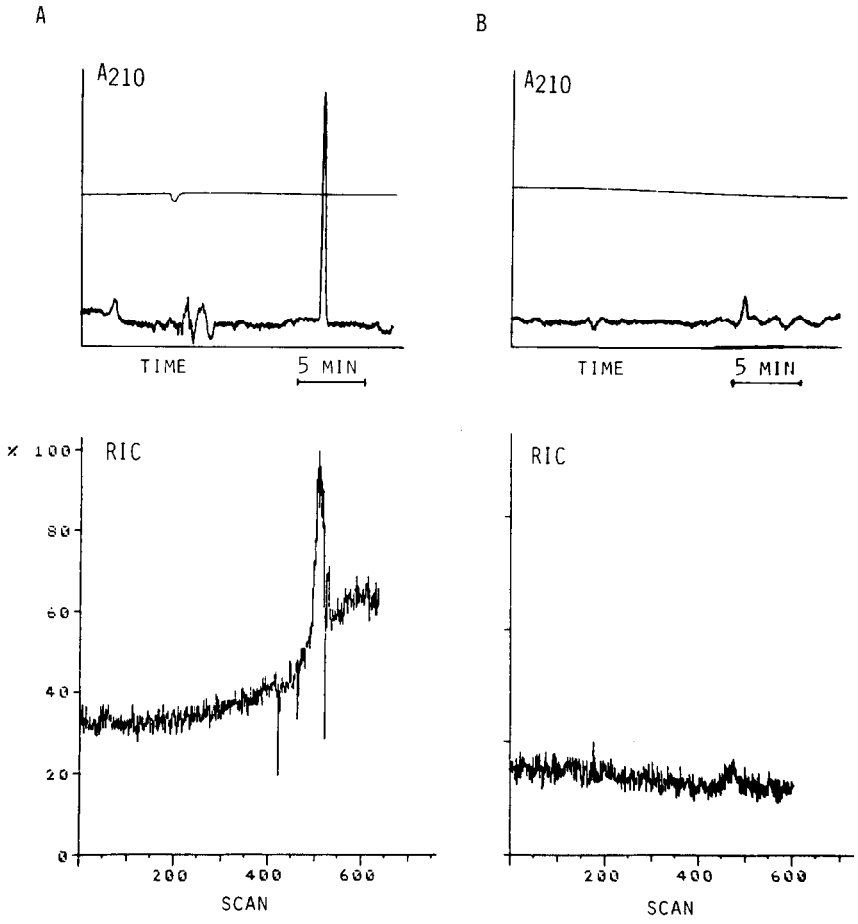

Fig. 3. Limit of detection study of the CZE–CF-FAB instrument for angiotensin II (mol. wt. 1045). Panel A shows the UV (top) and MS (bottom) electropherograms for the injection of 368 fmol of the peptide, and panel B that for the injection of 75 fmol of the peptide. The mass spectrometer was scanned over a 4-mass-unit range centered at the $(M+H)^+$ species.

needed to give reasonable signal-to-noise measurements is more likely to be in the high fmol or low pmol range. This is shown later for the analysis of the proteolytic digests of several proteins.

Effect of alkali salt

Many investigators have pointed out the advantage of having relatively high concentrations of salt in the CZE buffer in that one can achieve high separation efficiencies. Thus, 50–150 m*M* sodium chloride or sodium phosphate is often employed in these buffers[1,14]. Unfortunately, the FAB process does not tolerate these salt concentrations well. Generally, the sensitivity is decreased relative to a sample containing no salt and the FAB spectra are characterized by sodium adduct ions at 22, 44, 66, etc., mass units above the $(M+H)^+$ molecular species of the compound,

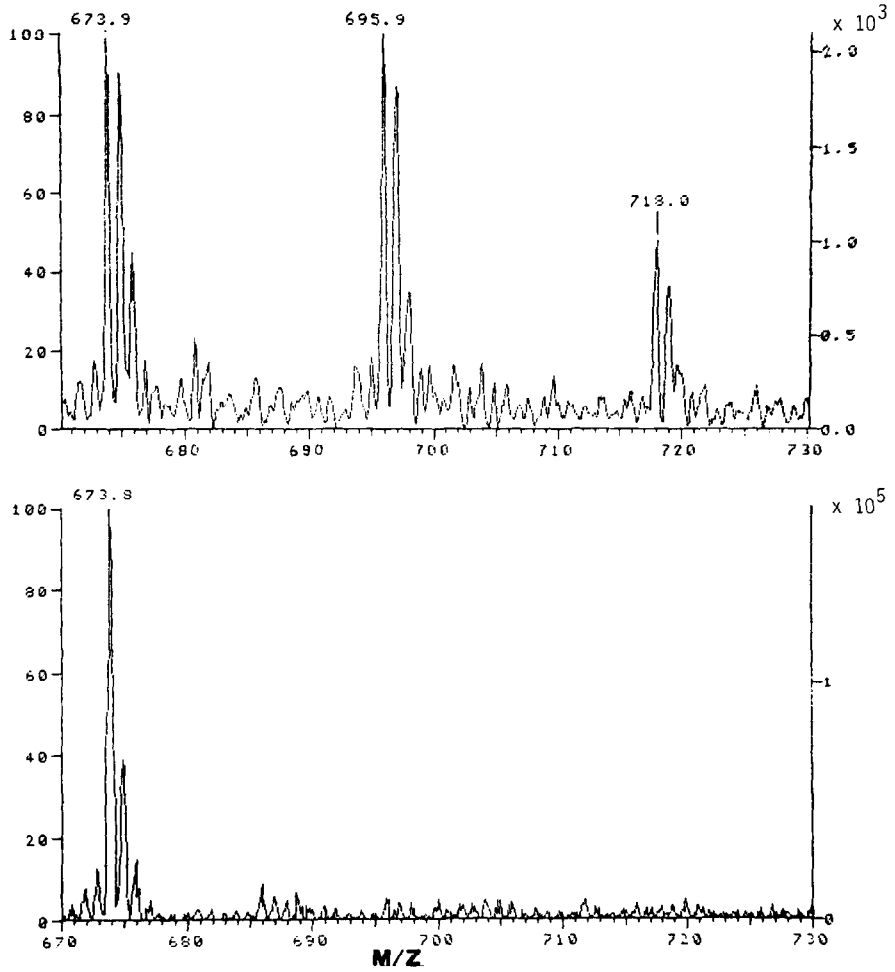

Fig. 4. The mass spectra of a single peptide, m/z 673.9, from the analysis of the tryptic digest of β-lactoglobulin A. (Top) use of a continuous buffer system containing 50 mM NaCl in the CZE device and also in the MS interface in addition to the normal carrier solvent. (Bottom) use of a discontinuous buffer system where 40 mM NaCl was used in the CZE device alone. The MS interface contained the normal carrier solvent of 5% glycerol and 3% acetonitrile in water containing 2.3 mM acetic acid.

corresponding to $(M+Na)^+$, $(M+2Na)^+$, $(M+3Na)^+$, etc., respectively. Furthermore, the limit of detection of a given compound is poorer because the molecular species are distributed into several molecular masses. This is shown in Fig. 4 for a peptide at $(M+H)^+$ 673.9 identified in the analysis of the tryptic hydrolysis of β-lactoglobulin A. The top of the figure shows the CZE–FAB spectrum of the molecular species obtained from separations performed in a "continuous" CZE–CF-FAB buffer system containing 50 mM NaCl (50 mM NaCl, 5% glycerol, 3% acetonitrile, 0.02% TFA), whereas the bottom spectrum shows that taken in a "discontinuous" buffer system containing no alkali salt at set-up in the cathodic compartment and 40 mM NaCl in the anodic compartment and the CZE capillary. One buffer system was

denoted a "continuous" system since the same solvent was present in both the cathodic and anodic compartments and in the CZE capillary. The other buffer system was denoted "discontinuous" since the cathodic compartment contained the CF-FAB solvent: 5% glycerol, 3% acetonitrile, 2.36 m*M* acetic acid, 5 m*M* ammonium hydroxide and the anodic compartment and the CZE capillary contained 40 m*M* citric acid and 40 m*M* NaCl. This comparison indicates that using the interface permits the inclusion of alkali salts in the CZE buffers without compromising CF-FAB detection.

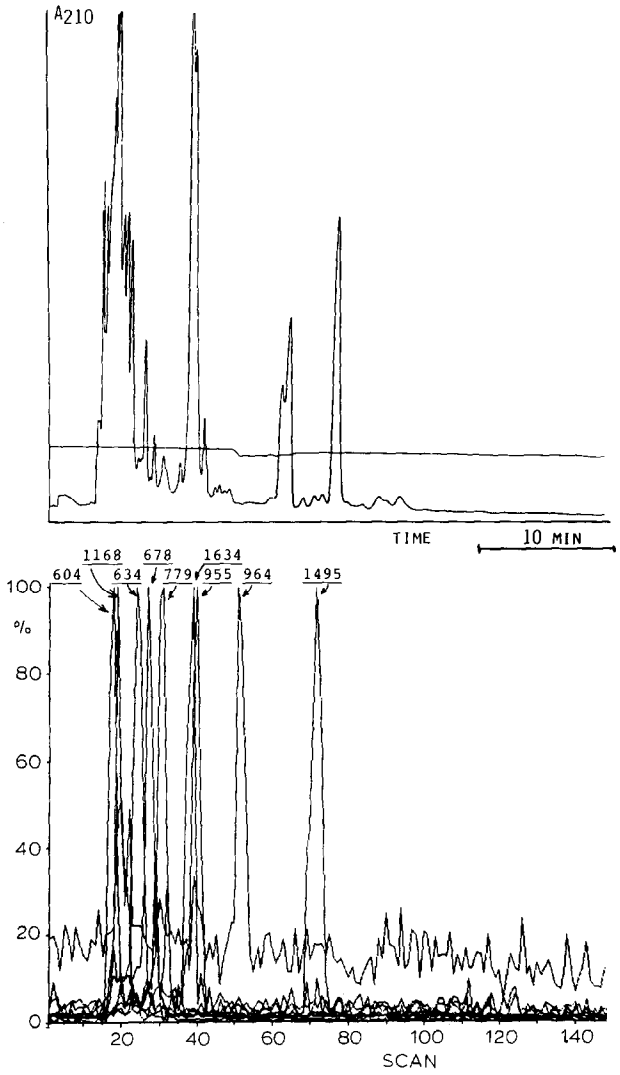

Fig. 5. Analysis of the tryptic digest of cytochrome *c*. (Top) UV detector trace obtained using 15 kV and 8 μA current (horizontal line shows current stability), and (bottom) selected ion chromatograms for individual peptides obtained from the MS data. The major peak in each selected ion chromatogram is marked with the *m/z* value for that chromatogram. See text for details.

Presumably sodium ions migrate to the interface during a run but are diluted by the differential CZE–CF-FAB flow configuration in the interface and therefore do not result in sodium adduct molecular ion formation.

Analysis of tryptic digests

The analysis of the tryptic digest of horse heart cytochrome *c*, a protein of about 12 200 molecular weight, was performed to determine the efficacy of the CZE–CF-FAB technique on the separation and identification of the peptides in the mixture. An amount of the proteolytic reaction mixture equivalent to 60 pmol of the intact protein was loaded. Electrophoresis was performed at 15 kV (8 μA) with 40 m*M* sodium citrate and 40 m*M* NaCl, pH 2.5, as the buffer. The CF-FAB carrier solvent was 92% water, 5% glycerol and 3% acetonitrile containing 2.3 m*M* acetic acid and 1 m*M* ammonium hydroxide. Fig. 5 (top) shows the UV detector trace of the separated components and (bottom) the selected ion chromatogram from the mass spectrometer detector. The latter is a mass-specific electropherogram which was ob-

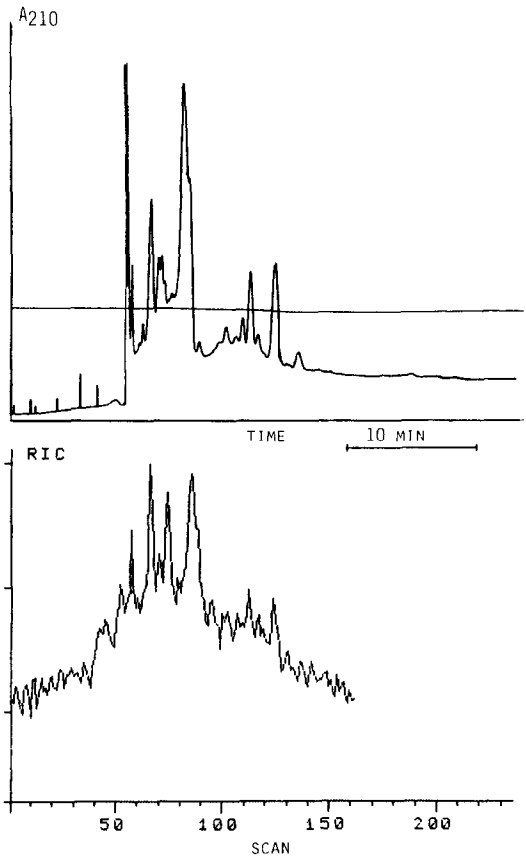

Fig. 6. Analysis of the tryptic digest of recombinant human growth hormone. (Top) UV detector trace obtained using 15 kV and 8 μA current, and (bottom) total ion chromatogram obained from the MS data. See text for details.

tained from the superimposed plots, independently normalized, of selected ion chromatograms for peptides known to be present. Such a presentation provides an easy and clear method of recording the $(M+H)^+$ ions of the peptides and simplifies data analysis for studies involving structure modification or sequence verification of proteins. For this example, ten peptides were expected within the mass range scanned and nine were identified in this single analysis.

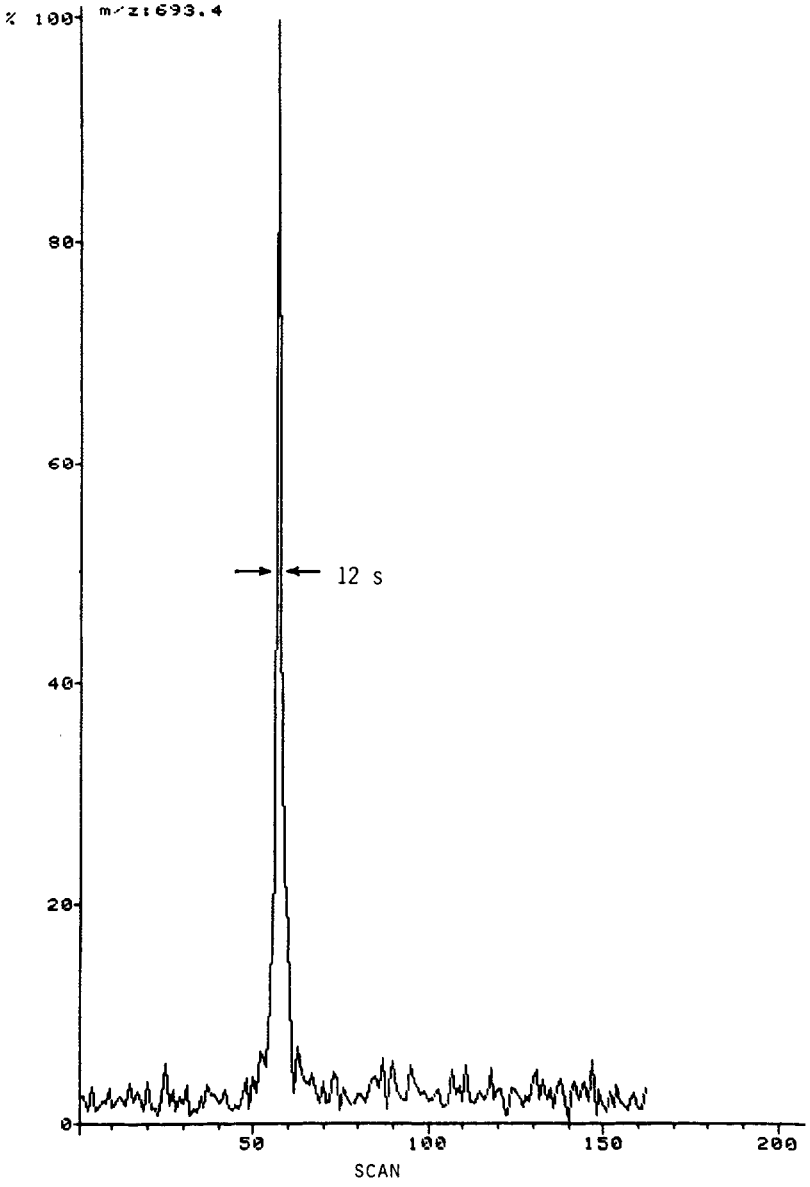

Fig. 7. The selected ion chromatogram for *m/z* 693.4 from the analysis of the growth hormone shown in Fig. 6.

The analysis of the tryptic digest of 40 pmol of recombinant human growth hormone is shown in Fig. 6. The UV trace, top panel, and the MS total ion chromatogram, bottom panel, show very similar profiles. The instrumental conditions were the same as those described above for the cytochrome *c* digest analysis. In this example, the mass spectrometer was scanned from *m/z* 600–2000 at about 10 s/scan. Specific mass analysis of these data was used to identify molecular species of the individual peptides. The resolution of an individual peptide in such a mixture is shown in Fig. 7 for the peptide having an $(M+H)^+$ of 693.4. At half-height, the width of this peak is approximately 12 s. The column resolution is excellent despite the relatively large sample load applied to the column.

CONCLUSIONS

The combination of CZE and MS provides an excellent analytical tool for the separation and identification of mixtures of organic compounds. The high resolving power of CZE is enhanced by the mass specific capability of MS. CF-FAB MS has been shown to be an effective interface for the two instrumental methods for a variety of types of samples.

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