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Reversed-phase capillary high-performance liquid chromatography with on-line UV, fluorescence and electrospray ionization mass spectrometric detection in the analysis of peptides and proteins

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ABSTRACT

Analysis of peptide mixtures by reversed-phase capillary HPLC with gradient elution using three detectors in series: UV (214 nm), fluorescence ($\lambda_{exc.} = 280$ nm, $\lambda_{emiss.} = 356$ nm), and electrospray ionization mass spectrometry (ES-MS) is reported. The chromatographic integrity of the system and the detection limits were evaluated. The effect of the mass spectrometer's acquisition rate on the total ion current (TIC) profile was also examined. The utility of fluorescence monitoring with UV and ES-MS detection was demonstrated in the analysis of proteolytic digests of proteins. The native fluorescence character of tryptophan-containing peptides provides selectivity in peptide mapping, while monitoring UV absorption at 214 nm affords detection of the peptide bond. Three tryptophan-containing tryptic peptides of bovine serum albumin were immediately located by fluorescence among many UV peaks and ES-MS provided molecular masses allowing the peptides to be identified.

INTRODUCTION

The combination of mass spectrometry (MS) and microbore or capillary reversed-phase high-performance liquid chromatography (RP-HPLC) has emerged as a powerful technique for the analysis of complex proteolytic digests of proteins [1–23]. Peptide mapping with mass spectrometric detection is used to validate or confirm deduced protein sequences [2,4]. This strategy relies on comparing measured molecular masses of proteolytic fragments determined by mass spectrometry with theoretical molecular masses of peptides anticipated from the chemical reaction of the enzyme and protein [2].

Electrospray ionization (ES) [1–9] and continuous flow fast atom bombardment (CF-FAB) [1,2,10–23] are two ionization methods which enable a mass spectrometer to be interfaced with HPLC for generating gas-phase ions from peptides in solution. For RP-HPLC, solvents usually consist of aqueous mixtures of acetonitrile or methanol with $\leq 0.1\%$ addition of an ion-pairing agent. A commonly used ion-pairing agent in the analysis of peptides is trifluoroacetic acid (TFA) which is compatible with both ES-MS and CF-FAB-MS. Electrospray ionization mass spectrometry offers several significant advantages over CF-FAB-MS as a detection method for liquid chromatography. One is that mobile phase compositions of this sort work well for ES-MS and do not require modification of the mobile

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phase prior to mass spectrometric detection. (Involatile buffers, or those with electrolyte content of equivalent conductivity higher than that of 10^{-3} *M* NaCl result in reduced sensitivity and spray instability [24].) However, CF-FAB-MS requires a viscous matrix, usually glycerol, at 1–5% in the mobile phase [10–12]. If a viscous matrix is added prior to the column, additional band broadening occurs in the chromatographic separation. Although not as simple to implement, post-column addition of the matrix immediately preceding the CF-FAB probe tip or a coaxial design circumvents this problem [10–12]. However, there still may be memory effects which degrade the total ion current (TIC) profile obtained from the mass spectrometer. The effect that viscous-matrix addition to the mobile phase has on chromatographic integrity has been examined by other investigators [10–12,25,26].

Electrospray ionization mass spectrometry also offers advantages over CF-FAB-MS in the analysis of large peptides and glycopeptides. Detection limits with CF-FAB-MS decrease for peptides with M_r greater than *ca.* 3000 [1,27]. For example, Hemling *et al.* [1] reported that following HPLC separation, glycopeptides with M_r *ca.* 3000–4000 at the 50 pmol level were readily detected by ES-MS whereas CF-FAB failed to detect them. However, CF-FAB-MS with a coaxial interface design apparently overcomes this limitation in the analysis of high mass peptides [23]. Because CF-FAB generates singly and perhaps doubly protonated molecules, the limiting factor in CF-FAB-MS analysis of large peptides can be the mass-to-charge (m/z) range of the instrument. In contrast to CF-FAB, electrospray ionization generates multiply protonated molecules [24,28–32] which enables conventional mass spectrometers (quadrupoles, sectors) with limited m/z ranges (*e.g.* 4000 or less) to measure molecular masses of molecules with $M_r > 100\,000$ [31,32]. The ability to analyze peptides and proteins at the low pmol level [1–9], along with the ease with which electrospray ionization mass spectrometry can be interfaced to capillary and microbore HPLC make it an attractive alternative to CF-FAB-MS detection.

Typically in HPLC–MS analyses, on-line UV detection at 214 nm is incorporated for monitoring absorption by the peptide bond. Background ion current may obscure minor components in the TIC profile from mass spectrometric detection. The UV chromatographic trace gives a fingerprint of the peptide map when analyzing complex mixtures. This aids in determining which regions of the chromatogram to examine for the mass spectral information. The native fluorescence emission at $\lambda_{emiss.} = 356$ nm of tryptophan residues following excitation at $\lambda_{exc.} = 280$ nm provides a means to further characterize the peptides in mixtures such as in proteolytic digests [33,34]. Monitoring fluorescence as well as UV absorption reveals which peptides in mixtures contain tryptophan residues while the mass spectrometer measures the molecular masses.

The performance of a system consisting of this combination of three detectors: UV, fluorescence and ES-MS, following gradient separation with a 0.500 mm I.D. reversed-phase capillary C_{18} column is evaluated with respect to chromatographic integrity and detection. The utility of this combination of detectors is demonstrated in the analysis of proteolytic digests of bovine serum albumin (BSA) and stromelysin catalytic domain (SCD) protein expressed in *E. coli*.

EXPERIMENTAL

Instrumentation

Chromatographic separations were performed using a Michrom BioResources Ultrafast Microprotein Analyzer (Michrom BioResources, Pleasanton, CA, USA) which included a Valco 10-port valve with a 20-μl injection loop. The solvents are pumped through heated (30°C) precolumns and combined at a micro mixing tee. After mixing, the solvent at a flow of 70 μl/min, passes through a stainless steel fixed precolumn splitter which directs approximately 14 μl/min flow to the capillary C_{18} HPLC column (Reliasil C_{18}, 5 μm, 300 Å, 150 mm × 0.5 mm I.D.) and the remainder to waste through a 1 mm I.D. balance column [20,35]. The chromatograph is interfaced to a modified Linear UVis 200 Detector (Linear Instruments, Reno, NV, USA) equipped with a 0.8-μl, 2-mm pathlength flow cell. Absorbance is monitored at 214 nm. The

outflow from the UV detector is connected by fused-silica capillary tubing (75 μm I.D/150 μm O.D.) to a Gilson 121 Filter Fluorometer (Gilson, Middleton, WI, USA) equipped with a 0.6-μl flow cell. The fluorometer is fitted with a mercury lamp with $\lambda_{exc.} = 280$ nm. A glass filter with wavelength maximum transmission at 356 nm is used in monitoring fluorescence emission. The total dead volume between the UV and fluorescence detector is 5 μl. The sampling interval for both the UV and fluorescent detectors is 0.1 s.

A 1:1 split of the eluent following the fluorescent detector is accomplished by using a PEEK tubing tee and fused-silica capillary (50 μm I.D/150 μm O.D. × 1.25 m; dead volume, 2.5 μl). Flow is delivered at *ca.* 7 μl/min to a Vestec (Houston, TX, USA) electrospray interface fitted to a Finnigan MAT (San Jose, CA, USA) TSQ 70 triple stage quadrupole mass spectrometer (Fig. 1). The Vestec interface uses a heated source block, which was held at 225°C, for desolvation. Prior to performing a gradient HPLC–ES-MS analysis, the needle voltage was set to 2.2 kV, and the solvent composition to 60% A. Then, the distance between the ES needle tip and nozzle aperture of the interface was adjusted until a spray current of *ca.* 0.25 μA was achieved. (A spray current of *ca.* 0.25 μA was found to be optimum in terms of sensitivity). The electrical characteristics of the mobile phase change over the course of the gradient separation due to changes in solvent composition so the needle voltage was adjusted manually during the HPLC runs in order to maintain the spray current. Typically, the needle voltage ranged from *ca.* 2.3 kV at 90% A to *ca.* 1.9 kV at 10% A. The repeller voltage was held at 15–20 V. These interface conditions minimized fragment ion formation. Mass spectra were acquired by scanning the third quadrupole (Q3) from m/z 500–2000 at a rate of 300 (m/z) s^{-1} unless otherwise indicated.

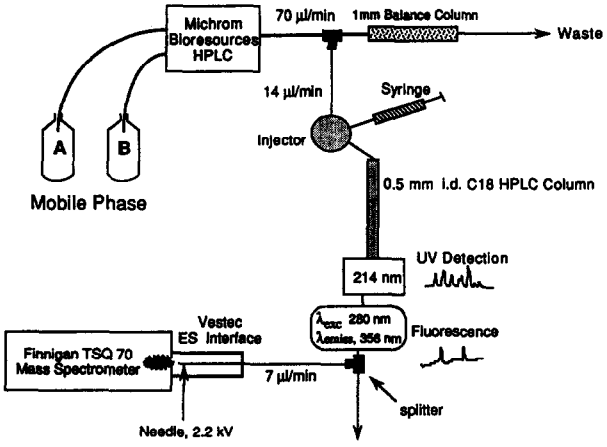

Fig. 1. Capillary HPLC–MS interface.

Mobile phase

The mobile phase was prepared from HPLC grade acetonitrile (Mallinckrodt, St. Louis, MO, USA), distilled, deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA) and protein sequencing grade trifluoroacetic acid (TFA) (Applied Biosystems, Foster City, CA, USA). Mobile phase A was prepared with a composition of water–acetonitrile (98:2) with 0.1% TFA. Mobile phase B was acetonitrile–water (90:10) with 0.09% TFA.

Chemicals

Adrenocorticotropic hormone fragment 1–24 (ACTH 1–24) and BSA were purchased from Sigma (St. Louis, MO, USA). SCD expressed in *E. coli* was provided by Dr. Qi-Zhuang Ye of Parke-Davis (Ann Arbor, MI, USA). Details of the protein expression and purification is found elsewhere [36]. Trypsin-TPCK was from Pierce Chemical (Rockford, IL, USA) and the proteolytic digests (50:1 $w_{substrate}/w_{enzyme}$) were carried out in 0.1 *M* ammonium bicarbonate, pH 8.0, at 37°C for 16 h.

RESULTS AND DISCUSSION

A typical feature of ES-MS of peptides and proteins is the formation of a series of multiply protonated molecules which are represented as peaks in the mass spectrum. The measured m/z values for the protonated molecules are used to compute the molecular mass of the compound. In mixtures of peptides, hydrophobicity differences can result in pronounced differential ionization when analyzed by FAB. ES does not suffer as severely from this limitation [24], thus

permitting direct mixture analysis with minimal discrimination, although the spectra may be very complex. A separation method preceding ES-MS simplifies data interpretation in mixture analysis and may offer gains in sensitivity due to reduction of both charge competition among mixture components and buffer suppression of ionization [37]. However, Fig. 2 illustrates that a separation method preceding ES-MS analysis of mixtures may be necessary to assure correct interpretation of the mass spectral data. ES mass spectra are simulated for three hypothetical peptides in Fig. 2A–C. Although the peptides have different molecular masses, the simulated ES mass spectral peaks indicate that there are ions with identical m/z values found in the mass spectrum of more than one of the hypothetical peptides. It is a simple task to determine the M_r of the 'pure' peptides from the spectra in Fig. 2A–C. However, the same is not true for the spectrum in Fig. 2D which shows the composite spectrum of

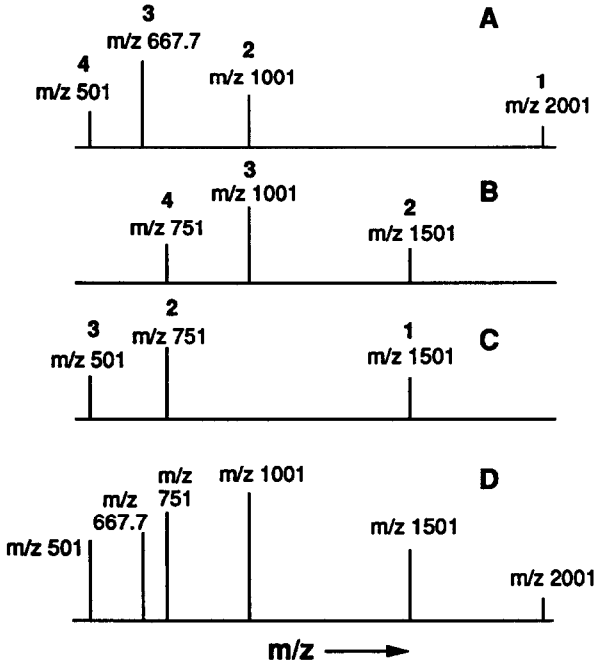

Fig. 2. Simulated electrospray mass spectra for hypothetical peptides with molecular masses of (A) 2000, (B) 3000 and (C) 1500 dalton. Fig. D is the hypothetical composite spectrum of the mixture of the three components in Fig. A–C. The numbers above the m/z labeled peaks in A–C represent the number of protons attached to the molecule represented by the mass spectral peak.

the three hypothetical peptides. For example, the peaks at m/z 501, 751 and 1501 could be assigned as $(M+3H)^{3+}$, $(M+2H)^{2+}$ and $(M+H)^{+}$, respectively, indicating a species of M_r 1500 while the peaks at m/z 667.7, 1001 and 2001 could be assigned to $(M+3H)^{3+}$, $(M+2H)^{2+}$ and $(M+H)^{+}$ for a compound with M_r 2000. Thus the peaks in Fig. 2D may be inadvertently accounted for by components with M_r values of 1500 and 2000. However, because the component with M_r 3000 does not yield a singly protonated molecule (or the m/z range of the mass spectrometer is <3000 so it is not detected), it could easily be overlooked in the composite spectrum obtained by direct analysis. With chromatographic separation, the reconstructed ion chromatograms of individual m/z values would indicate three components. Coupling chromatographic separation with ES-MS simplifies data analysis of mixtures and minimizes the potential of misassignment of molecular masses.

Chromatographic profile at each detector

A wide range of capillary columns, from 0.01 mm to 0.5 mm in diameter, have been constructed for HPLC analyses [38]. Commercially available capillary HPLC columns of 0.3 mm I.D. and 0.5 mm I.D. have been used in this laboratory. Although lower detection limits can be achieved with a 0.3 mm I.D. column as the analyte is more effectively concentrated due to lower flow-rates, it has been our experience that the 0.5 mm I.D. columns are more durable, and maintain greater baseline stability with gradient RP-HPLC chromatography. So, for the most part 0.3 mm I.D. columns are reserved for the analysis of very low quantity samples (<5 picomoles). For routine separations, a 0.5 mm I.D. column is the most practical choice in the analysis of 10–500-pmol samples [38] and was selected as the column in measuring chromatographic profiles at each detector.

As a general rule, to ensure minimal extracolumn broadening by the detector, the detector cell volume should be less than one-tenth the volume of the peak of interest [39,40]. In the system described here, the UV and fluorescent detectors each satisfy this rule but there is 5 μl

dead volume between these two detectors. An important part of the evaluation of the system with three on-line detectors was to evaluate the chromatographic integrity by measuring chromatographic peak width at half heights ($w_{1/2}$) at each detector.

The $w_{1/2}$ values were measured from the chromatographic peak obtained from 25-pmol injections of ACTH (1–24) (M_r 2933.4) into the chromatograph. ACTH (1–24) was selected because the peptide contains a tryptophan residue and therefore is detected by fluorescence under the conditions described in the experimental section. For ES-MS detection, Q3 of the mass spectrometer was repetitively scanned from m/z 730–738 in order to detect only one of the prominent ions in the ES-MS of ACTH (1–24): $(M + 4H)^{4+}$. A scan rate of 80 $(m/z)s^{-1}$, yielding an acquisition rate of 10 scan $\cdot s^{-1}$ was used. Therefore, for this experiment the sampling interval for all three detectors was 0.1 s. A fast acquisition rate ensures that the chromatographic peak widths as observed by ES-MS are not artificially degraded by long sampling intervals while a slow mass spectrometer scan rate improves the signal-to-noise ratio of the peak. The measured $w_{1/2}$ for the chromatographic peak as observed at each detector is shown in Table I. The mixing which occurs in the flow cells along with laminar dispersion in the connecting tubing causes the 26% increase in the fluorescence $w_{1/2}$ *versus* UV, as shown in Table I. As evident by the small increase in $w_{1/2}$ for the TIC trace compared to the fluorescence trace, the data indicates that there is some minimal broadening of the chromatographic zone after fluorescence detection. This broadening occurs in the flow splitter, the connecting tubing prior to the electrospray interface, and in the electrospray interface itself.

TABLE I

MEASURED PEAK WIDTHS ($w_{1/2}$) WITH UV, FLUORESCENCE AND ES-MS DETECTION FOR ANALYSIS OF 25 pmol OF ACTH (1–24)

Detector	$w_{1/2}$ (s)	% Increase[c]
UV[a]	12.5 ± 0.2	–
Fluorescence[a]	15.7 ± 0.6	26
ES-MS[b]	17.9 ± 0.2	14

[a] Average of 8 analyses.
[b] Average of 2 analyses.
[c] Represents % increase in $w_{1/2}$ from that measured at the immediately preceding detector.

Effect of mass spectrometer acquisition rate on chromatographic trace

The chromatographic peak width observed with mass spectrometric detection is a function of the mass spectrometer's acquisition rate. Increasing the acquisition rate (by shortening the sampling interval) results in a narrower and more accurate characterization of the chromatographic peak because there are more data points to define its shape [41,42]. It has been estimated that using 10–25 cm long columns with efficiencies between $1 \cdot 10^4$ and $2.5 \cdot 10^4$ plates, the sampling interval should be 1 s, preferably between 0.1–0.5 s. Otherwise, there is an apparent loss in chromatographic resolution [41]. If the m/z range scanned is kept constant, increasing the frequency at which mass spectra are collected requires an increase in the scan rate [measured as $(m/z)s^{-1}$]. However, increasing the acquisition rate by scanning faster results in a poorer S/N ratio because the sampling times (sampling time $\equiv$ scan rate^{-1}) for each m/z value are shortened. Fig. 3 displays 8-min windows of TIC traces from repetitive injections of 25 pmol of ACTH (1–24). Because the HPLC conditions for these repetitive analyses were identical, the variation in the chromatographic peak observed in the TIC trace is due to the different acquisition and scan rates of the mass spectrometer. The data obtained by scanning the mass spectrometer from m/z 500–2000 at a rate of 150 $(m/z)s^{-1}$, 300 $(m/z)s^{-1}$, 600 $(m/z)s^{-1}$ and 1250 $(m/z)s^{-1}$ is shown in Fig. 3A–D, respectively. Fig. 3A–D compares the measured $w_{1/2}$ for these traces. Of the four traces displayed in Fig. 3A–D, the $w_{1/2}$ in Fig. 3D is the narrowest as is expected because the acquisition rate was highest. However, the increased scan rate results in a decrease in the S/N ratio due to the decrease in sampling times. An alternative way to increase

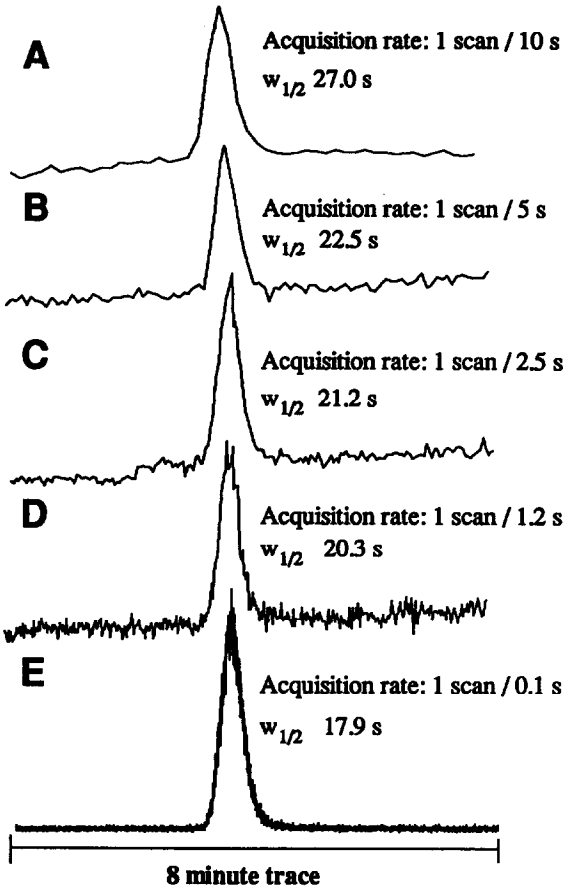

Fig. 3. The TIC chromatogram for analysis of 25 pmol of ACTH (1–24) with the mass spectrometer scanning parameters as follows: (A) m/z 500–2000 at 150 $(m/z)s^{-1}$, sampling time 6.67 ms$(m/z)^{-1}$, (B) m/z 500–2000 at 300 $(m/z)s^{-1}$, sampling time 3.33 ms$(m/z)^{-1}$, (C) m/z 500–2000 at 600 $(m/z)s^{-1}$, sampling time 1.67 ms$(m/z)^{-1}$, (D) m/z 500–2000 at 1250 $(m/z)s^{-1}$, sampling time 0.8 ms$(m/z)^{-1}$ and (E) m/z 730–738 at 80 $(m/z)s^{-1}$, sampling time 12.5 ms$(m/z)^{-1}$. The HPLC conditions were 90% A to 50% A in 25 min followed by re-equilibration at initial conditions.

the acquisition rate is to scan over a narrower mass range. The chromatographic trace displayed in Fig. 3E represents the data obtained at the highest acquisition rate, but the scan rate, 80 $(m/z)s^{-1}$, is the slowest because the mass spectrometer was only scanned from m/z 730–738. Of all the traces displayed in Fig. 3, the profile shown in Fig. 3E reflects the true chromatographic peak shape of the analyte most accurately. However, due to the small scan range, less information is available in the mass spectrum.

Limits of detection in the analysis of ACTH (1–24)

The detection limits for each of the three detectors were determined using dilutions of ACTH (1–24). The chromatographic profiles for UV, fluorescence and ES-MS detection following the injection of 1.2 pmol of ACTH (1–24) onto the 0.5-mm C_{18} column are shown in Fig. 4. With a 1:1 split of the eluent following fluorescence detection, approximately 600 fmol is introduced into the electrospray interface. The profiles shown in Fig. 4B and C were obtained when the mass spectrometer was scanned from m/z 500–800 at a rate of 120 $(m/z)s^{-1}$ (1 scan/2.5 s). Although background ion current obscures the

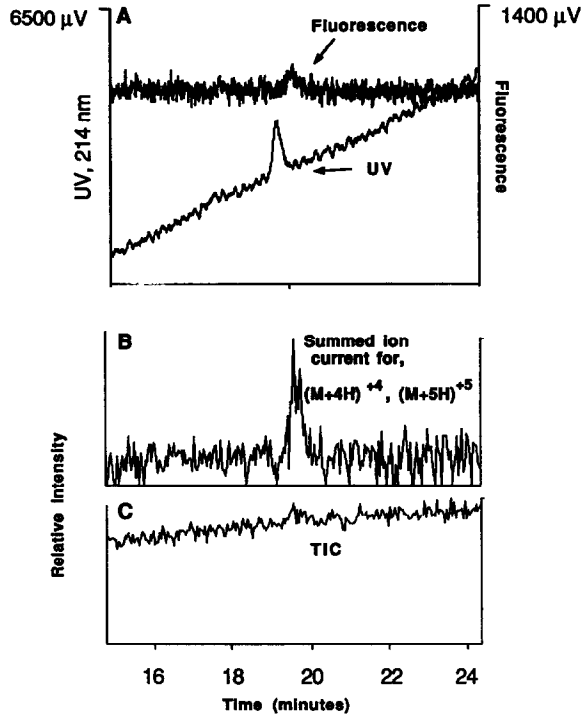

Fig. 4. Chromatographic profiles observed for (A) fluorescence and UV detection, (B) ion current detected at m/z 734 and m/z 588 and (C) TIC with ES-MS following injection of 1.2 pmol of ACTH (1–24) into the chromatograph. For mass spectrometric detection, Q3 was scanned from m/z 500–800 at a rate of 120 $(m/z)s^{-1}$ (acquisition rate, 1 scan/2.5 s).

analyte signal in the TIC trace as seen in Fig. 4C, the analyte signal is observed in the sum of the $(M+4H)^{4+}$ and $(M+5H)^{5+}$ ion current displayed as a function of time as shown in Fig. 4B. The detection limit for analysis of a targeted peptide or protein by HPLC–MS could be further lowered by using selected ion monitoring (SIM) rather than a scanning analysis as in this example. Furthermore, multiple charging with electrospray usually provides more than one m/z value per analyte so that specificity could be enhanced by using a multiple ion monitoring approach.

Utility of on-line UV, fluorescence and ES-MS detection

a) Analysis of BSA tryptic digest. The utility of this combination of detectors is illustrated in the analysis of the tryptic digest of bovine serum albumin (BSA, M_r 66 500). BSA was digested with trypsin, and a 50 pmol aliquot was injected for analysis. Fig. 5 shows the chromatographic profiles obtained with UV and fluorescence detection. Based on the recently corrected primary sequence of BSA [14], the protein consists of 583 amino acid residues, two of which are tryptophan. The fluorescence detector trace indicates immediately that the peptides eluting at 13.1 min and 14.4 min contain tryptophan. The third highly fluorescent peak (*ca.* 58 min) is undigested BSA. The TIC profile and the m/z values for ion current detected at times corresponding to the fluorescent peptides is displayed in Fig. 6. The reconstructed ion chromatograms shown in Fig. 6B and D indicates the peak at 13.1 min consists of two peptides. The measured M_r values determined for these two peptides are consistent with two anticipated tryptophan-containing tryptic fragments AWSVAR (M_r 688.4) and FWGK (M_r 536.3). The fluorescent peptide eluting at

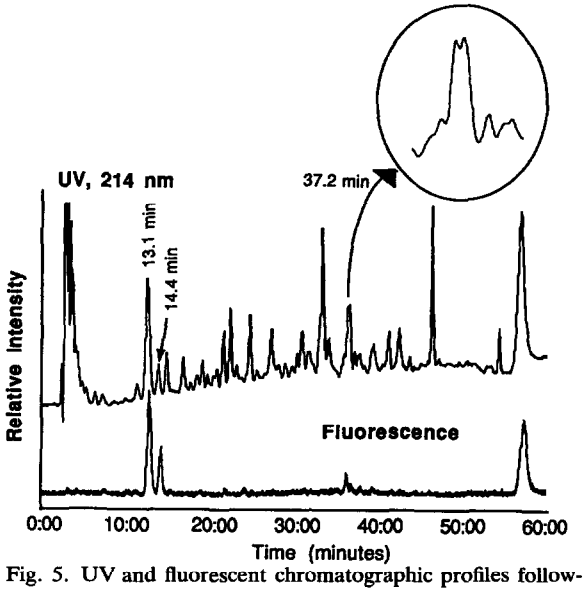

Fig. 5. UV and fluorescent chromatographic profiles following injection of 50 pmol aliquot of BSA tryptic digest. HPLC conditions were 90% A for 4 min, ramped to 40% A at 49 min and to 25% A at 66 min. Inset is a 3-min window centered around the peaks detected with UV at 37.2 min.

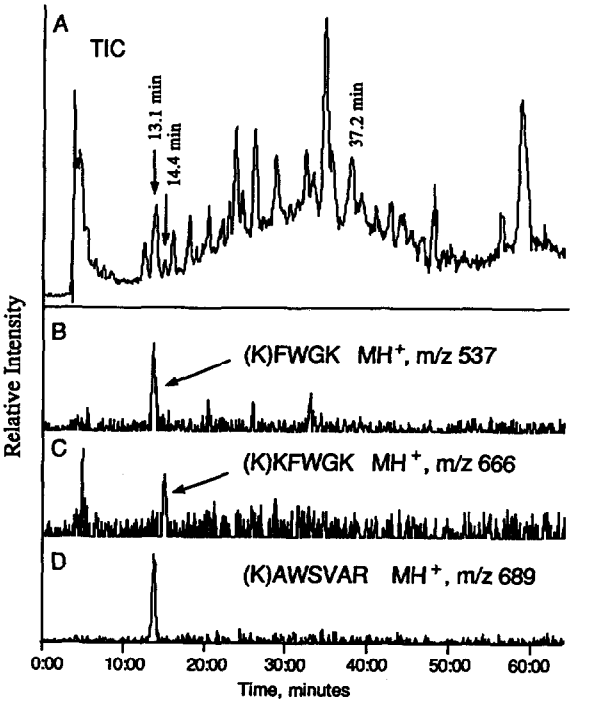

Fig. 6. Chromatographic profile observed for (A) TIC with ES-MS detection, (B) ion current at m/z 537 representing the tryptic peptide FWGK (M_r 536.3), (C) ion current at m/z 666 representing tryptic peptide KFWGK (M_r 664.8) and (D) ion current at m/z 689 representing tryptic peptide AWSVAR (M_r 688.4) for analysis of 50 pmol of BSA tryptic digest. This data was obtained from the same run as the data shown in Fig. 5.

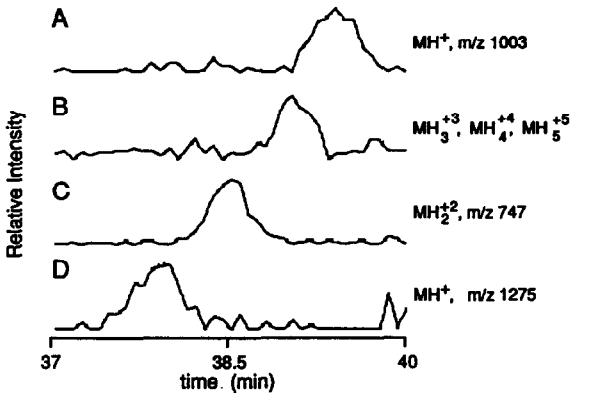

Fig. 7. Reconstructed mass chromatograms for time corresponding to the inset shown in Fig. 5 (37.2 min) for ions detected representing (A) tryptic peptide T 209–217, M_r 1001.6, (B) tryptic peptide with M_r 5262 (sequence uncertain), (C) tryptic peptide T 205–217, M_r 1490.8 and (D) tryptic peptide T 185–196, M_r 1274.4 in the analysis of a tryptic digest of BSA.

14.4 min (Fig. 6C) has a measured M_r of 665 which is consistent with the peptide KFWGK (M_r 664.8) resulting from incomplete digestion.

This analysis also demonstrates how well the resolution provided by the capillary separation is maintained by the three detectors. As evident from the magnification shown in Fig. 5, the peak at 37.2 min in the UV trace consists of multiple components which are not completely resolved. Reconstructed mass chromatograms corresponding to this time frame are shown in Fig. 7 A–D. The computer programs ProComp [43] and MacProMass [44] were used to aid sequence assignments for the peptides detected. Three of the four tryptic peptides detected within this 3-min window were tentatively identified as indicated in Fig. 7. These identifications suggest incomplete digestion. Two of the tryptic fragments represented in Fig. 7, T 205–

TABLE II

SEQUENCE AND MOLECULAR MASSES OF PEPTIDES OBSERVED IN THE HPLC–ES-MS ANALYSIS OF THE TRYPTIC DIGEST OF EXPRESSED SCD

Peak	t_R (min)	Residues	Sequence	$M_{r(theor)}$	$M_{r(exp)}$
1	7.1	10–11	(K)WR(K)	360.2	360.5
2	7.1	10–12	(K)WRK(T)	488.3	488.4
3	15.6	12–18	(R)KTHLTYR(I)	917.5	918.0
4	23.1	3–9	(R)TFPGIPK(W)	758.4	759.0
5	23.1	19–28	(R)IVNYTPDLPK(D)	1158.6	1159.2
6	26.1	10–28	(K)WRKTHLTYRI VNYTPDLPK(D)	2401.8	2401.2
7	26.1	19–37	(R)IVNYTPDLPKD AVDSAVEK(A)	2073.0	2073.4
8	27.7	152–174	(R)LSQDDINGIQSL YGPPPDSPETP	2439.1	2439.8
9	31.0	41–52	(K)VWEEVTPLTFS R(L)	1462.7	1463.0
10	31.0	150–174	(R)FRLSQDDINGIQSL YGPPPDSPETP	2742.3	2743.4
11	34.3	38–52	(K)ALKVWEEVTPL TFSR(L)	1775.0	1775.8
12	35.2	68–106	(R)EHGDFYPFDGP GNVLAHAYAPGP GINGDAHFDDDEQ WTK(D)	4255.8	4255.3
13	36.5	53–67	(R)LYEGEADIMISF AVR(E)	1712.8	1713.4
14	47.5	107–149	(K)DTTGTNLFLVA AHEIGHSLGLFHS ANTEALMYPLYHS LTDLTR(F)	4726.4	4726.9

217 (FGERALKAWSVAR) and T 209–217 (ALKAWSVAR), both contain a tryptophan residue, so the identifications are supported by the fluorescence emission (Fig. 5). The chromatographic traces for m/z values representing singly or multiply protonated tryptic peptides of BSA show how these components may be resolved by mass even though they are not completely resolved by time. This data indicates that the chromatographic resolution, although not perfectly preserved throughout the ES interface, is sufficient to discern incompletely resolved chromatographic peaks.

A problem with the use of multiple detectors is correlation of the data from the different detector traces. Throughout these experiments, the time delay in transfer of the eluent from the UV to the fluorescence to the ES-MS detector was considered in determining which peaks from the different traces represented the same component. To assist in assigning peaks from different traces, a simple mixture containing components detectable by all three detectors but with various retention characteristics can be prepared. Analysis of such a mixture under gradient elution conditions benchmarks the delay times and therefore aids correlation of peaks observed in the analysis of unknown mixtures.

b) Characterization of expressed SCD. The metalloproteinase stromelysin is thought to be involved in cartilage degradation in arthritis [36]. SCD was expressed in *E. coli* [36] and introduced into the mass spectrometer by capillary RP-HPLC to remove the urea buffer and to assess sample purity. The M_r of the expressed protein measured by HPLC–ES-MS was $19494.1 \pm 0.05\%$, in good agreement with the theoretical M_r of 19492.7.

The protein was further characterized by digesting some of the material with trypsin. The tryptic map of the expressed material is shown in Fig. 8. As shown in Table II, the M_r values measured for the tryptic fragments are consistent with those anticipated for SCD, and all of the major fluorescent peaks are assigned to tryptic fragments containing a tryptophan residue. The italicized numbers above the chromatographic peaks in the fluorescent and UV traces shown in Fig. 8 correspond to the tryptic peptides listed in Table II. Determining ratios of UV absorbance

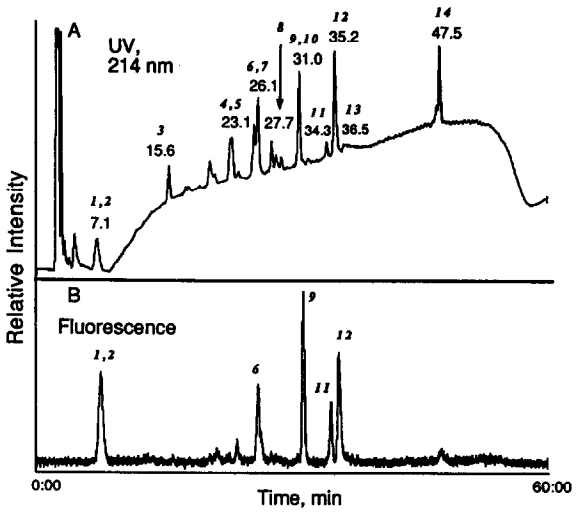

Fig. 8. The (A) UV and (B) fluorescent chromatographic profiles of analysis of tryptic digest of expressed SCD. The sequence for the labeled peaks (italicized numbers) is listed in Table II. The HPLC conditions were 95% A for 4 min followed by 95% A to 35% A from 4 to 40 min and from 35% A to 30% A from 40–45 min. The mobile phase was maintained at 30% A for 5 min before re-equilibration. The mass spectrometer was scanned from m/z 300–2000 at 340 $(m/z)s^{-1}$ which yields an acquisition rate of 1 scan/5 s.

and fluorescence signals may be useful in indicating the number of tryptophan residues, an approach used by other investigators [33], but not examined in this work.

This tryptic map also serves to demonstrate the compatibility of electrospray ionization with gradient elution HPLC. The mass spectra for two tryptic fragments which eluted at 78% H_2O and 36% H_2O are shown in Fig. 9A and B. Electrospray ionization mass spectrometry is able to tolerate a wide range of CH_3CN–H_2O compositions, providing good quality mass spectra.

CONCLUSIONS

On-line combinations of detectors following chromatographic separation by HPLC provides complementary information which is useful in the characterization of proteins/peptides. A novel aspect of this study is the use of fluorescence detection on-line with UV and ES-MS to monitor RP capillary HPLC separations. UV detection at 214 nm provides a complete map or fingerprint of the peptides present in a mixture.

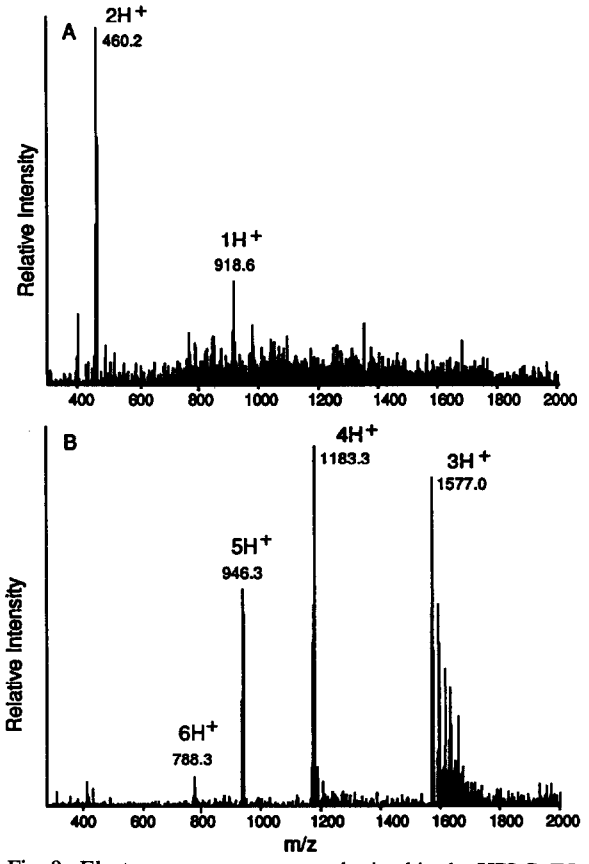

Fig. 9. Electrospray mass spectra obtained in the HPLC–ES-MS analysis of a tryptic digest of expressed SCD for (A) peak 3, which represents the tryptic peptide T 12–18 and has a M_r 917.5 eluting at 78% water and (B) peak 14, which represents the tryptic peptide T 107–149, M_r 4726.4 and elutes when the mobile phase composition is 36% water.

Fluorescence detection affords selective identification of tryptophan containing peptides, and ES-MS provides molecular mass measurements for each peptide. This combination of detectors may prove particularly useful in the analysis of proteins digested with enzymes which lack specificity, such as thermolysin. Knowledge of the presence or absence of tryptophan in a peptide along with the M_r is helpful in assigning a primary sequence. In addition, fluorescence monitoring at the appropriate conditions could provide enhanced detectability and selectivity in the analysis of peptides derivatized with a fluorescent tag [45,46]. As a result of band broadening, some components which were resolved as indicated by the UV trace, may have overlapping profiles in the mass spectrometric trace. However, the mass spectrometric detection allows these components to be resolved by mass. Other detectors in combination with ES-MS, such as electrochemical detection [47] which provides identification of disulfide containing peptides, may be useful in the future for characterization of peptide mixtures.

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