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Evaluation of Adsorption Preconcentration/Capillary Zone Electrophoresis/Nanoelectrospray Mass Spectrometry for Peptide and Glycoprotein Analyses

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The use of an on-line adsorption preconcentrator coupled with capillary zone electrophoresis/nanoelectrospray mass spectrometry (PC/CZE/nESMS) is described for the analysis of peptides and protein digests. The investigation was focused on the production of disposable preconcentrators made of large particle size (40 μm irregular packing), thereby eliminating the use of a retaining frit without loss of performance. These preconcentration devices were made of commercially available components which can be easily interfaced to current CZE/nESMS systems. Practical issues such as the composition of the stationary phase, the elution volume and sample breakthrough and carry-over were evaluated in order to optimize the analytical performance of this technique. Under optimized elution conditions, the PC/CZE/nESMS technique provided separation efficiencies in excess of 100 000 theoretical plates for a sample loading of 8 μl. Sample carry-over was minimized by proper reconditioning of the preconcentrator prior to the CZE separation. Alternatively, the sample carry-over resulting from small elution volumes could be used advantageously to provide multiple analyses from a single injection of sample. The application of this technique is demonstrated for the analysis of proteolytic peptides from a *Bauhinia purpurea* lectin at a concentration level of 30 nM. Further structural information was obtained using on-line tandem mass spectrometry to elucidate the structure of N-linked glycans and the amino acid sequences of the glycopeptides. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: nanoelectrospray; mass spectrometry; tandem mass spectrometry; peptides; glycoproteins

INTRODUCTION

Capillary zone electrophoresis (CZE) has several desirable characteristics as an analytical tool, including high resolving power and separation efficiency, low solvent consumption, relatively low cost and minimal sample consumption (nanoliters). The small injection volume associated with CZE is also a major limitation of this technique as relatively high concentrations are required for adequate detection of analytes. Concentration detection limits using UV absorption are typically in the low μM range, depending on the nature of the chromophore and wavelength for detection. Efforts to lower the concentration detection limits of CZE have focused on improving both detection and sample loading methods.

Enhancement of signal detectability using UV detection has been achieved through the use of a detector cell with increased irradiation pathlength.[1–3] Improvement of detection limits and analyte selectivity has also been accomplished using detectors such as laser-induced fluorescence (LIF),[4–8] thermo-optical absorbance[9] and mass spectrometry.[10,11] Amongst the different detectors currently available, LIF provides the highest sensitivity with detection limits approaching single molecule detection.[12,13] However, LIF detection is inherently limited to reactive functionalities on the native molecule and the availability of fluorescent reagents with spectral characteristics compatible with existing laser wavelengths. Thermo-optical detection of phenylthiohydantoin derivatives of amino acids gave detection limits two orders of magnitude better than conventional UV detection.[14] However, ultrapure solvents and reagents are required to minimize the background absorbance

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associated with this technique. Electrospray mass spectrometry (ESMS) is potentially the most versatile detector for CZE as it provides sensitivity, selectivity and structural information without the requirement of analyte derivatization. Improvements in interface design have further enhanced the sensitivity of CZE/ESMS, giving concentration detection limits approaching those of LC/MS[15–18]

On-line sample concentration techniques have been developed to improve the concentration detection limits of CZE by increasing the amount of sample injected without compromising separation efficiency. Sample stacking, isotachophoresis and adsorption methods have provided concentration detection limits similar to those achievable by high-performance liquid chromatography.

Sample stacking provides for increased sample loading (typically a 10-fold increase over that of conventional CZE) by making use of ionic strength differences between the sample and separation electrolyte. The low ionic strength sample acts as a voltage divider, so that most of the CZE potential is applied across the low-conductivity sample. As a result, the ions in the sample are concentrated in the capillary until the ionic strength of the sample equals that of the separation buffer. Chien and Burgi[19–21] have described reversed-polarity stacking conditions where the entire capillary is filled. In this case, the electroosmotic flow (EOF) removes the sample electrolyte while the analyte is focused inside the capillary. The polarity is switched before the analyte exits the capillary and the separation is allowed to proceed.

Improvements in sample loading have also been achieved using a discontinuous buffer system to perform isotachophoretic stacking prior to zone electrophoresis.[22] In this method, the capillary is first filled with a high-mobility background electrolyte, followed by the sample with lower mobility and finally a terminating electrolyte with mobility lower than that of the sample. As a result of the discontinuous buffer system, the field strength varies along the capillary. As the components separate, the field strength within individual zones changes until a steady state has been reached and all zones migrate with the same velocity. At this point the terminating buffer is replaced with the background electrolyte and regular zone electrophoresis of the analyte occurs. As with the sample stacking method described above, isotachophoresis is limited to sample sizes dictated by the dimensions of the capillary. Alternative separation formats such as capillary isoelectric focusing (CIEF) allow the loading of the entire column with the sample dissolved in an ampholite buffer (typically 0.5–1 μl sample loading). The application of CIEF/ESMS has been demonstrated for the analysis of proteins[23,24] and glycoproteins[25] and provided detection limits approaching 10 nM for different hemoglobin variants.[26]

The analysis of large volumes in excess of several μl of dilute solutions has been carried out using CZE with adsorption preconcentration. In this approach, a small bed of reversed-phase LC material was packed in a column and coupled directly to the separation capillary. A review of different chromatographic phases as adsorption preconcentrators has been presented recently.[27] Despite the large increase in sample loading possible with this technique, it has not gained wide acceptance owing to the degradation of CZE performance and the complexity of the instrumentation. Significant losses in separation efficiency, band broadening and column overloading often compromise the separation.[27,28] Different attempts, including the reduction of stationary phase volume and the use of discontinuous buffer systems, have been proposed to alleviate these difficulties.[28] A detailed study of the mechanism of on-line solid-phase extraction capillary electrophoresis with UV detection was published recently.[29] The technique has also been coupled with ESMS using both co-axial[30] and sheathless interfaces.[31]

In the present study, a simple on-line preconcentration device based on a small bed of large particle adsorbent was developed for the determination of submicromolar peptide concentrations using CZE/ESMS. The analytical performance of this device was first evaluated using a co-axial interface to determine the enhancement of sample loading and improvement in detection limits compared with conventional CZE. Comparison of different stationary phases was undertaken to assess factors such as sample breakthrough and washoff, in addition to memory effects. The improved sensitivity required for the analysis of unknown samples using full-scan data acquisition was achieved by coupling the preconcentrator with a nanoelectrospray (nESMS) interface. Application of the optimized preconcentration/CZE/nanoelectrospray mass spectrometry (PC/CZE/nESMS) system to the analysis of glycoprotein digests is presented for a lectin isolated from seeds of *Bauhinia purpurea.*

EXPERIMENTAL

Reagents and materials

Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillary column butt connector (part no. 2-3796) and Teflon tubing were obtained from Supelco (Oakville, Ont., Canada). Formic acid (99%), ammonium persulfate (98%), *Bauhinia purpurea* (Camel's foot tree) lectin and peptide standards were purchased from Sigma Chemical (St Louis, MO, USA) and used without further purification. Sequencing grade TPCK-trypsin was purchased from Boehringer Mannheim (Montréal, Qué., Canada). 7-Oct-1-enyltrimethoxysilane was obtained from Hüls America (Bristol, PA, USA) and the [(acryloylamino) propyl]trimethylammonium chloride (refered to as BCQ by the manufacturer) was supplied by Chemische Fabrik Stockhaussen (Krefeld, Germany). *N,N,N′,N′*-tetramethylethylenediamine (TEMED) was purchased from International Biotechnologies (New Haven, CT, USA). The preconcentrators were constructed using the following stationary phase materials: 40 μm irregular aminopropyl (Varian Canada, Mississauga, Ont., Canada), 40 μm irregular C_{18} particles (Waters, Milford, MA, USA) and 5 μm POROS R1 C_{18} (Perseptive Biosystems, Framingham, MA, USA).

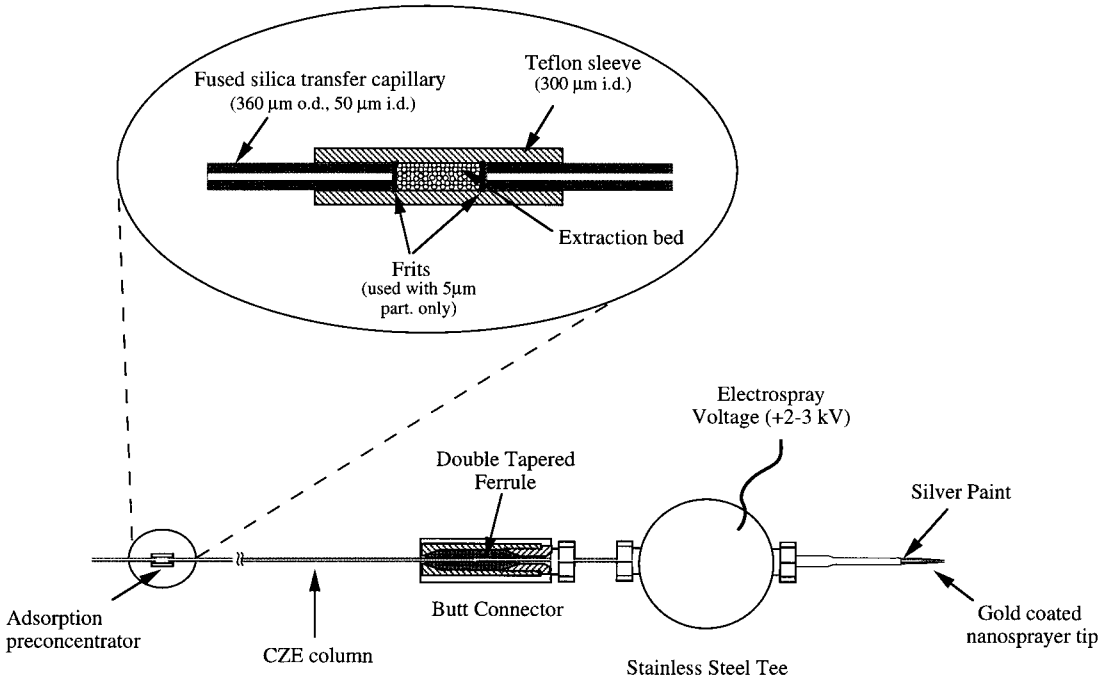

Figure 1. Schematic representation of the adsorption preconcentrator device and the nanoelectrospray interface.

Proteolytic digestion

The lectin *Bauhinia purpurea* was incubated with endoproteinase Glu-C (Boehringer Mannheim), at an enzyme-to-substrate ratio of 1:60 (w/w), in ammonium bicarbonate buffer at pH 8.1 at 25 °C for 5 h. The digest was boiled briefly and then evaporated to dryness using a Speedvac concentrator. The residue was dissolved in 0.1 M ammonium bicarbonate and incubated with trypsin (30:1 substrate:trypsin ratio) for 19 h at 37 °C. The solution was evaporated to dryness and the residue was dissolved in deionized water (250 μl) and further diluted as required.

Capillary coating

The capillary columns were typically coated in 5 m lengths and cut as needed for use in CZE/ESMS experiments. The method used for preparing the BCQ coating was described previously.[15] The capillary was rinsed sequentially with 1 M NaOH, deionized water and methanol, each for 1 h at 20 psi. A solution of 7-oct-1-enyltrimethoxysilane (20 μl) and glacial acetic acid (20 μl) in methanol (4 ml) was passed through the column overnight (8–12 h) at 20 psi. The capillary was subsequently rinsed with methanol and deionized water (1 h each, 20 psi). TEMED (8 μl) and aqueous ammonium persulfate [15% (w/v), 56 μl] were added to a solution of BCQ in deionized water [2% (v/v), 4 ml] and this solution was immediately rinsed through the column for 8 h (or overnight) at 20 psi. The capillary was flushed with deionized water for 1 h and both ends of the columns were subsequently closed with septa for storage. Prior to use, the column was flushed with CZE buffer for 5–10 min.

CZE/ESMS interface

CZE/ESMS analyses were carried out using a Thermo CZE system (Thermo Capillary Electrophoresis, Franklin, MA, USA) with 0.1 M formic acid as the background electrolyte. Separations were achieved by applying −20 kV to the injection end of the column (1 m × 50 μm i.d.) and 2.5 kV to the nanoelectrospray tip or 5 kV to the electrospray needle when using the co-axial interface. Samples were introduced by pressure (100 mbar, 6 s), resulting in 10 nl injection volumes.

Data were acquired using an API III$^+$ triple-quadrupole mass spectrometer (Perkin-Elmer SCIEX, Concord, Ont., Canada). A separate power supply (Glassman EH Series, Glassman, Whitehouse Station, NJ, USA) was used to provide the electrospray voltage. Selected ion monitoring (SIM) experiments were carried out using eight ions with a dwell time of 100 ms per channel monitored. Full-scan acquisition used a 1 Da step size with a 3 ms dwell time. A Macintosh Quadra 950 computer was used for instrument control, data acquisition and data processing.

Detailed descriptions of the co-axial CZE/ESMS interface[32] and the nanoelectrospray interface[15] have been presented previously. The nanoelectrospray tip was connected to the separation capillary using a column connector (Supelco). Optimization of the interface was achieved by electro-infusion of a peptide standard (Leu-enkephalin, 10 μg ml^{-1}) dissolved in the CZE buffer.

PC/CZE capillary construction

The preconcentrator (Fig. 1) was constructed by drawing packing material suspended in methanol into a 2 cm piece of Teflon tubing (300 μm i.d.). The tubing was briefly dipped into the slurry while drawing on a syringe attached by fused-silica capillary to the free end of the tubing. A bed of 1 mm length, verified using a light microscope, was typically used. Frits were not necessary to prevent loss of packing material when the 40 μm irregular particles were used. However, the 5 μm material required the construction of frits, which was achieved by inserting a piece of 0.22 μm filter from a Millipore Ultrafree MC centrifuge filter unit into the Teflon tubing before and after addition of the packing material. Similar preconcentration devices using small particle adsorbents have been described previously.[33–35]

The preconcentrator was attached to 15 and 80 cm pieces of BCQ-coated capillary that served as the inlet and the outlet end, respectively. The outer diameter of the capillary (360 μm) was appropriate for a push-fit attachment to the Teflon tubing and did not require epoxy or other sealant to prevent leakage. The preconcentrator did not restrict the flow of solvent and could be replaced without changing the capillary. A further benefit of this system is that optimization of the mass spectrometric conditions can be achieved without the preconcentrator, an important advantage when using the nanoelectrospray interface.

Conditioning of preconcentrator

Reproducible conditions were maintained for the preconcentrator by adopting the following sequence. The capillary was rinsed with two column volumes of elution solvent (90:10 acetonitrile–1% HCl) followed by five column volumes of separation electrolyte. These rinses were carried out after each experiment (except where noted) in order to prevent memory effects as discussed below. In each experiment the sample was injected at 1000 mbar for 4–8 min, resulting in an injection volume of 4–8 μl. Larger volumes could be injected with a corresponding increase in sample loading time. Following injection, the capillary was rinsed with four column volumes of separation electrolyte. The sample was eluted from the stationary phase using a small plug of elution solvent, followed by a brief rinse with separation electrolyte to push the elution solvent through the stationary phase, thereby preventing re-adsorption of the analyte. Voltage was applied and the acquisition started after the sample was eluted from the preconcentrator.

RESULTS AND DISCUSSION

Co-axial CZE/ESMS and PC/CZE/ESMS

Initially the analytical performance of the preconcentrator was evaluated using the co-axial sheath flow CZE/ESMS interface. The use of BCQ-coated capillaries was previously demonstrated for the separation of peptide mixtures[15] and protein digests and this approach was used in the present experiments. SIM was used with a mixture of standard peptides in order to compare detection limits and to assess the effect of the preconcentrator on the CZE performance. The CZE/ESMS analysis of a mixture of seven peptides at a concentration level approaching the detection limits is shown in Fig. 2.

The corresponding CZE/ESMS analysis using an on-line adsorption preconcentrator is shown in Fig. 3 for a 4 μl injection of a 10 ng ml^{-1} solution of the same peptide mixture. The peak at 9 min is due to the organic solvent migrating with the EOF. This analysis was also obtained for a 100-fold dilution of the sample used in Fig. 2 and provided comparable signal-to-noise (S/N) ratios for the individual ion electropherograms. Detection limits based on a 4 μl injection volume are summarized in Table 1, together with those obtained using the conventional sample-loading method with the same CZE/ESMS co-axial interface. It is noteworthy that the two analyses gave comparable mass detection limits, in the low femtomole range, but the use of an on-line preconcentrator provided an inherent enhancement of the concentration detection limit by at least two orders of magnitude. The reproducibility of analyses obtainable with this preconcentration device was evaluated using the same peptide mixture at a concentration of 100 ng ml^{-1}. Replicate injections ($n = 3$) obtained with this preconcentrator provided relative standard deviation (RSD) values of 2.0–2.9% on migration times for the different peptides.

However, a significant limitation of the adsorption preconcentrator is the concurrent loss in separation efficiency resulting from the relatively large elution volume of organic solvent. Separation efficiencies obtained

Table 1. Limits of detection (LOD) for peptides analyzed by co-axial CZE/MS and PC/CZE/ESMS

			LOD (nM)		LOD (fmol)	
Peptide	m/z	M_r	CZE	PC/CZE	CZE	PC/CZE
Cholecystokinin$_{10-20}$	418	1251	300	6.0	8.0	6.0
Angiotensin I	433	1296.5	300	4.0	7.0	4.0
Val$_4$,Ile$_7$-Angiotensin III	442	883	100	4.0	4.0	3.4
Substance P$_{3-11}$	548	1094	260	2.0	7.0	2.3
Substance P$_{1-9}$	553	1104	150	1.0	4.0	0.9
Leu-enkephalin	556	555	50	1.0	1.5	1.0
Glu-fibrinopeptide B	786	1570.6	100	1.0	3.0	1.3

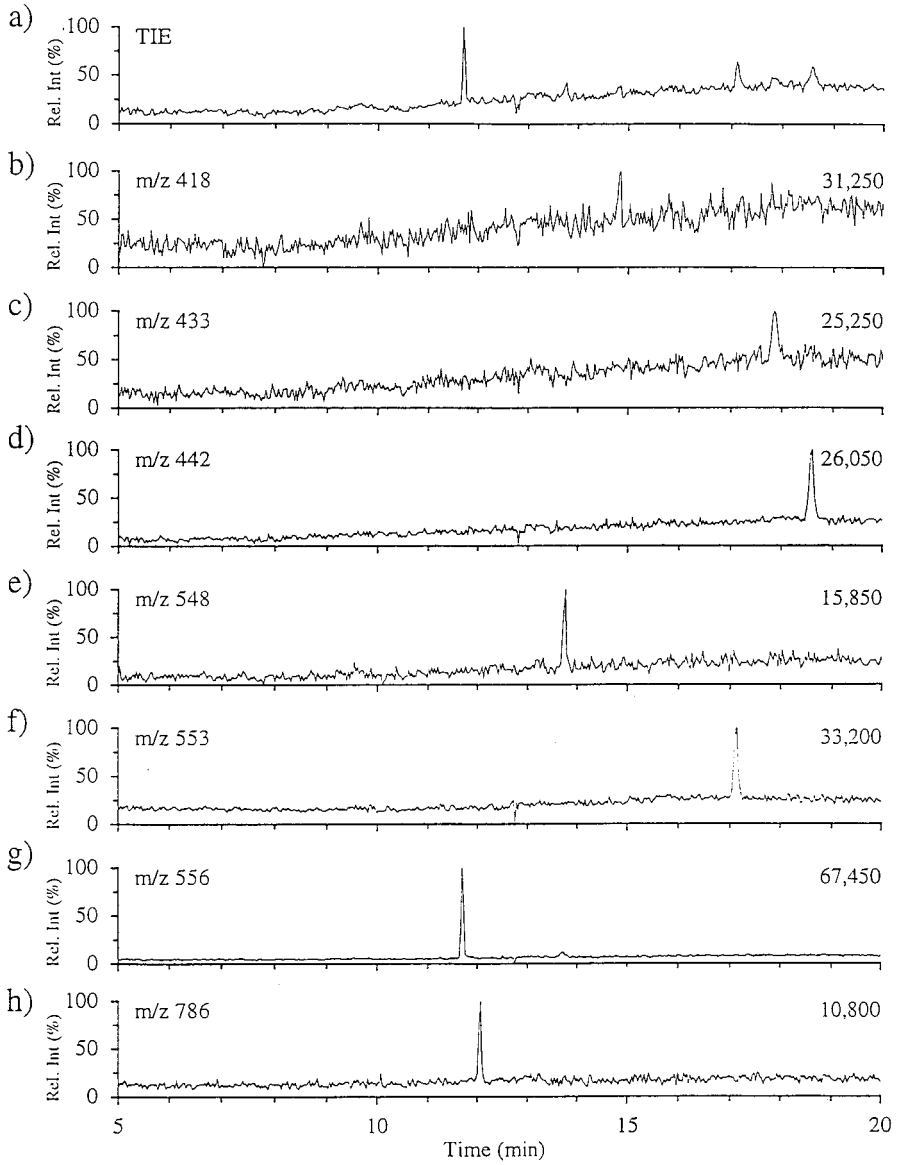

Figure 2. Co-axial CZE/ESMS analysis of a mixture of seven peptides using selected ion monitoring. (a) Total ion electropherogram; (b) cholecystokinin$_{10-20}$; (c) angiotensin I; (d) Val$_4$,Ile$_7$-angiotensin III; (e) substance P$_{3-11}$; (f) substance P$_{1-9}$; (g) Leu-enkephalin; (h) Glu-fibrinogen B. Separation conditions: BCQ-coated capillary (80 cm × 50 μm i.d.), 0.1 M formic acid, −30 kV effective, 10 nl injection of 1 μg ml^{-1} of each peptide. Numbers indicated at the top right of each electropherogram correspond to ion counts for the selected *m/z* value.

using the preconcentrator are significantly lower than those achievable with conventional CZE format (Table 2). The incorporation of an on-line adsorption preconcentrator gave rise to significant peak broadening which resulted in a decrease of 10-fold in separation efficiencies. Clearly, improvement in separation performance would be required to enhance concentration detection limits and S/N ratios further. Signal detectability can also be improved using an nESMS interface provided that the conditions necessary for on-column

Table 2. Theoretical plate values and signal-to-noise ratios for the analysis of peptides by co-axial CZE/ESMS and coaxial PC-CZE/ESMS

		Theoretical plates (*N*)		S/N	
Peptide	*m/z*	CZE	cPC/CZE	CZE	cPC/CZE
Cholecystokinin$_{10-20}$	418	216000	13600	1.5:1	4:1
Angiotensin I	433	122000	10300	2:1	6:1
Val$_4$,Ile$_7$-angiotensin III	442	234000	9900	9:1	9:1
Substance P$_{3-11}$	548	288000	18900	4:1	12:1
Substance P$_{1-9}$	553	197000	11850	7:1	30:1
Leu-enkephalin	556	207000	15600	40:1	46:1
Glu-fibrinopeptide B	786	221000	16570	7:1	16:1

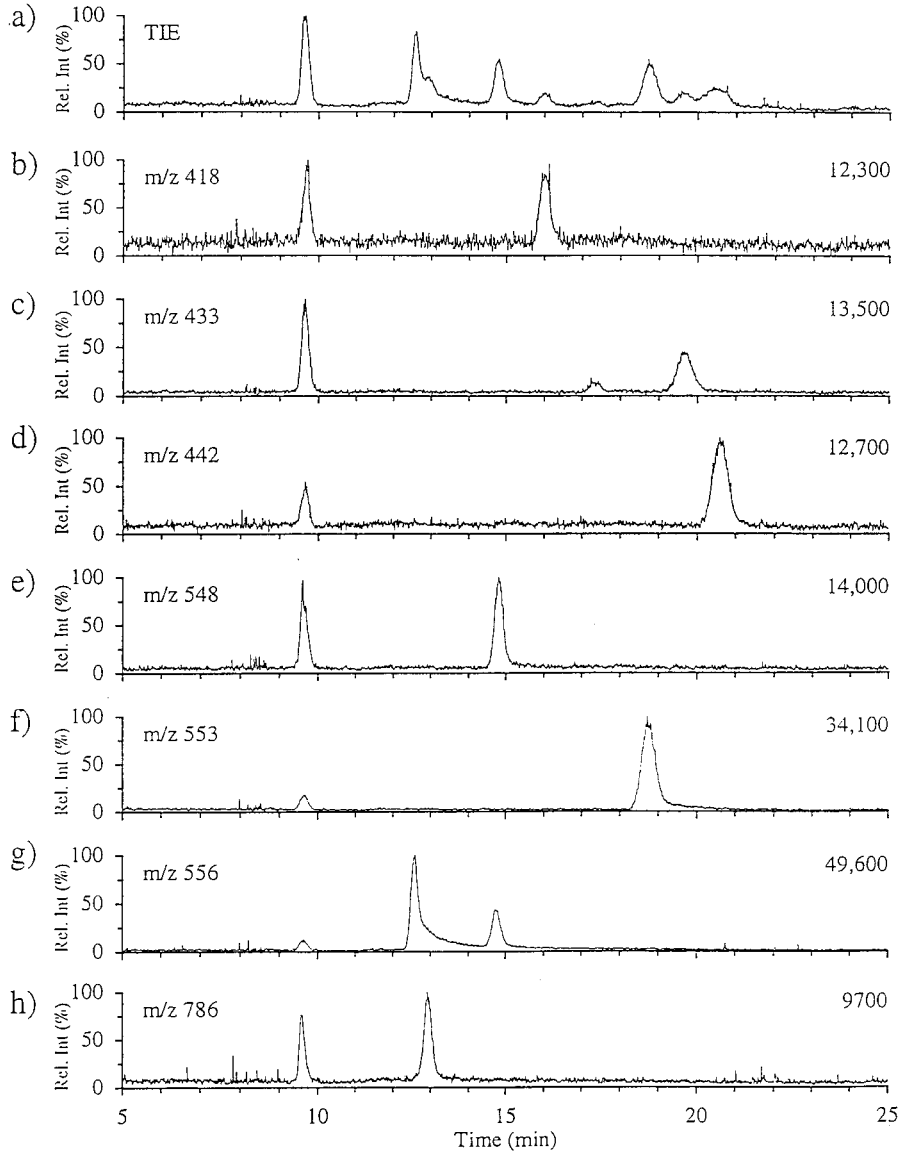

Figure 3. Co-axial PC/CZE/ESMS analysis of a mixture of seven peptides using selected ion monitoring. (a) Total ion electropherogram; (b) cholecystokinin$_{10-20}$; (c) angiotensin I; (d) Val$_4$,Ile$_7$-angiotensin III; (e) substance P$_{3-11}$; (f) substance P$_{1-9}$; (g) Leu-enkephalin; (h) Glu-fibrinopeptide B. Conditions as in Fig. 2 except that a 4 μl injection of a peptide mixture of 10 ng ml^{-1} was loaded on the C_{18} preconcentrator (40 μm irregular packing).

adsorption preconcentration are compatible with those required for proper electrophoretic separation and analyte ionization.

Performance of CZE/nESMS and PC/CZE/nESMS

The use of nanoelectrospray with CZE/ESMS has been shown previously to provide detection limits in the low-femtomole range for full-scan analysis of peptides.[15] An example of a CZE/nESMS analysis of a mixture of nine peptides at the 10 μg ml^{-1} level is shown in Fig. 4. Representative full-scan spectra extracted from the crests of peaks are shown in Fig. 4(b)–(e). The corresponding analysis using an on-line preconcentrator is shown in Fig. 5 for the injection of 4 μl of peptide standards each present at a concentration of 100 ng ml^{-1}.

Comparison of the total ion electropherograms (TIE) observed in Figs 4(a) and 5(a) indicates significant changes in the separation profiles and the relative abundances of peptide ions. For example, peaks annotated 3, 6, 7 and 8 in Fig. 4(a) are of significantly lower relative intensities in the corresponding PC/CZE/nESMS analysis presented in Fig. 5(a). This change in retention characteristics was associated not only with the amino acid composition of the peptide but also with other factors such as non-retention of analyte during the sample loading or buffer rinsing steps. Further understanding of these effects is important for the reliable use of this technique and subsequent investigations were focused on the optimization of the stationary phase composition and elution volume to reduce sample loss and maximize separation efficiency.

Comparison of adsorptive media

Three different stationary phases were investigated for use as on-line preconcentrators in CZE/nESMS experi-

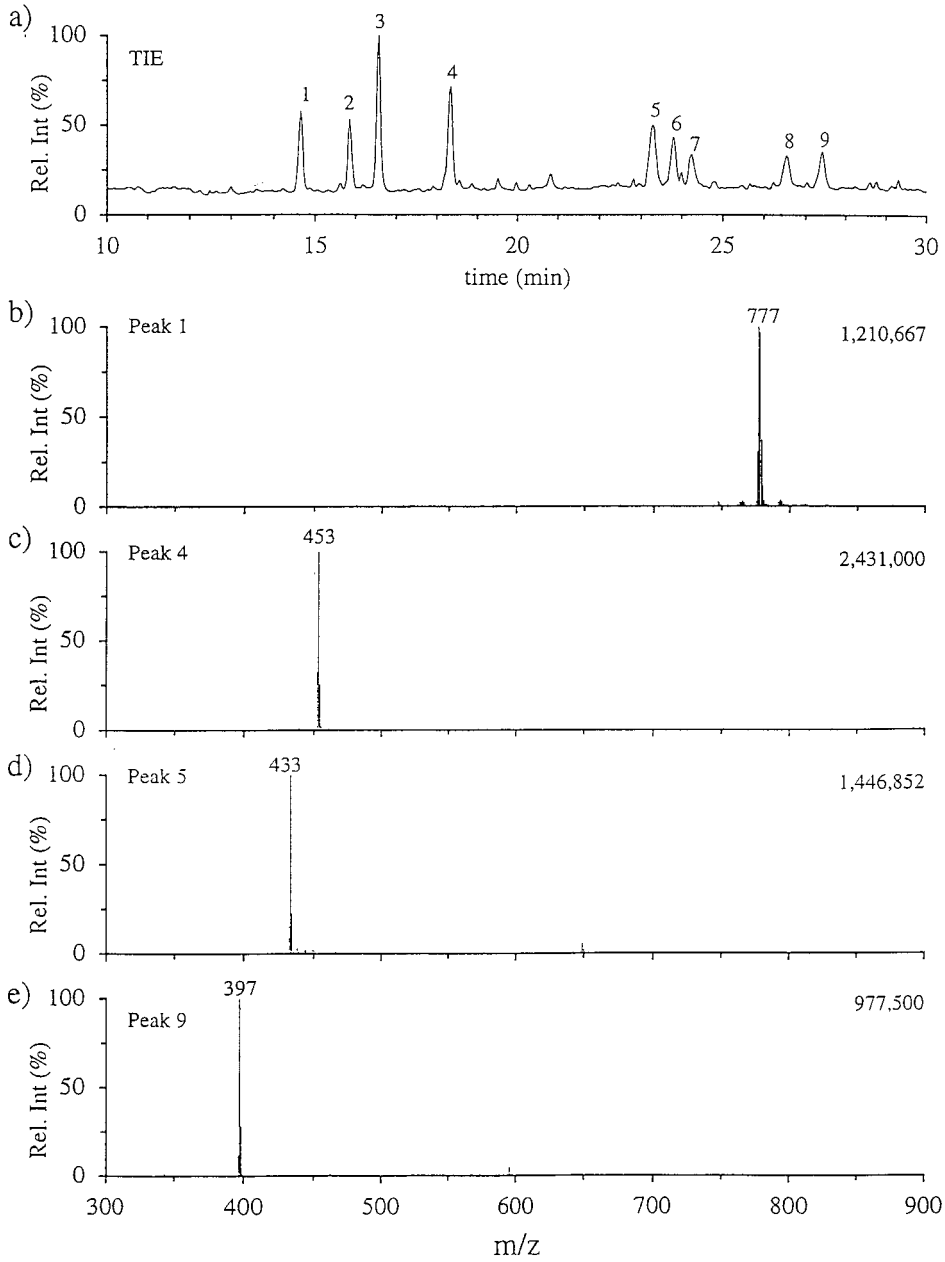

Figure 4. CZE/nESMS analysis of a mixture of nine peptides at the 10 μg ml^{-1} level using full-scan acquisition. Separation conditions: BCQ-coated capillary (50 μm i.d.), 0.1 M formic acid, −22 kV effective, 6 s injection at 100 mbar. (a) Total ion electropherogram (*m/z* 300–900); (b)–(e) Extracted mass spectra for peaks at 14.6, 18.2, 23.2 and 27.3 min, respectively. Peaks: 1 = neurotensin$_{1-6}$; 2 = Glu-fibrinopeptide B; 3 = Lys$_3$-bombesin; 4 = des-Arg-bradykinin; 5 = angiotensin I; 6 = des-pGlu-LHRH; 7 = melittin; 8 = thymopoietin$_{32-36}$; 9 = Lys-bradykinin. Separation conditions as in Fig. 2.

ments. As part of this comparative study we evaluated stationary phases such as 40 μm aminopropyl particles, 40 μm C_{18} particles and 5 μm C_{18} particles. The first two adsorptive media could be easily packed in a small Teflon tube without blockage of the transfer capillary or the CZE column but frits made of 0.22 μm cellulose membrane were used to retain the small 5 μm C_{18} particles inside the Teflon tube. Each phase was evaluated for its ability to retain analytes during the sample loading and buffer rinsing steps and to release the adsorbed peptides selectively with minimal memory effects. In each case the preconcentrators were initially conditioned by rinsing with the elution solvent and separation buffers as described in the Experimental section. The TIE profiles (sum of individual electropherograms for each protonated peptide ion) obtained during the sample application and replicate desorption of a peptide solution of 100 ng ml^{-1} are shown in Fig. 6 for each of the three stationary phases investigated.

This preliminary comparison indicated that the aminopropyl phase [Fig. 6(a)] had the least retentive properties of the three stationary phases evaluated. This result is not surprising considering the polar nature and short hydrophobic chain of this adsorptive medium This pelicular packing was originally selected because of its compatibility with the cationic coating of the capillary, thereby preventing change in the extent and direction of the EOF. Early experiments used a mixed C_{18}-

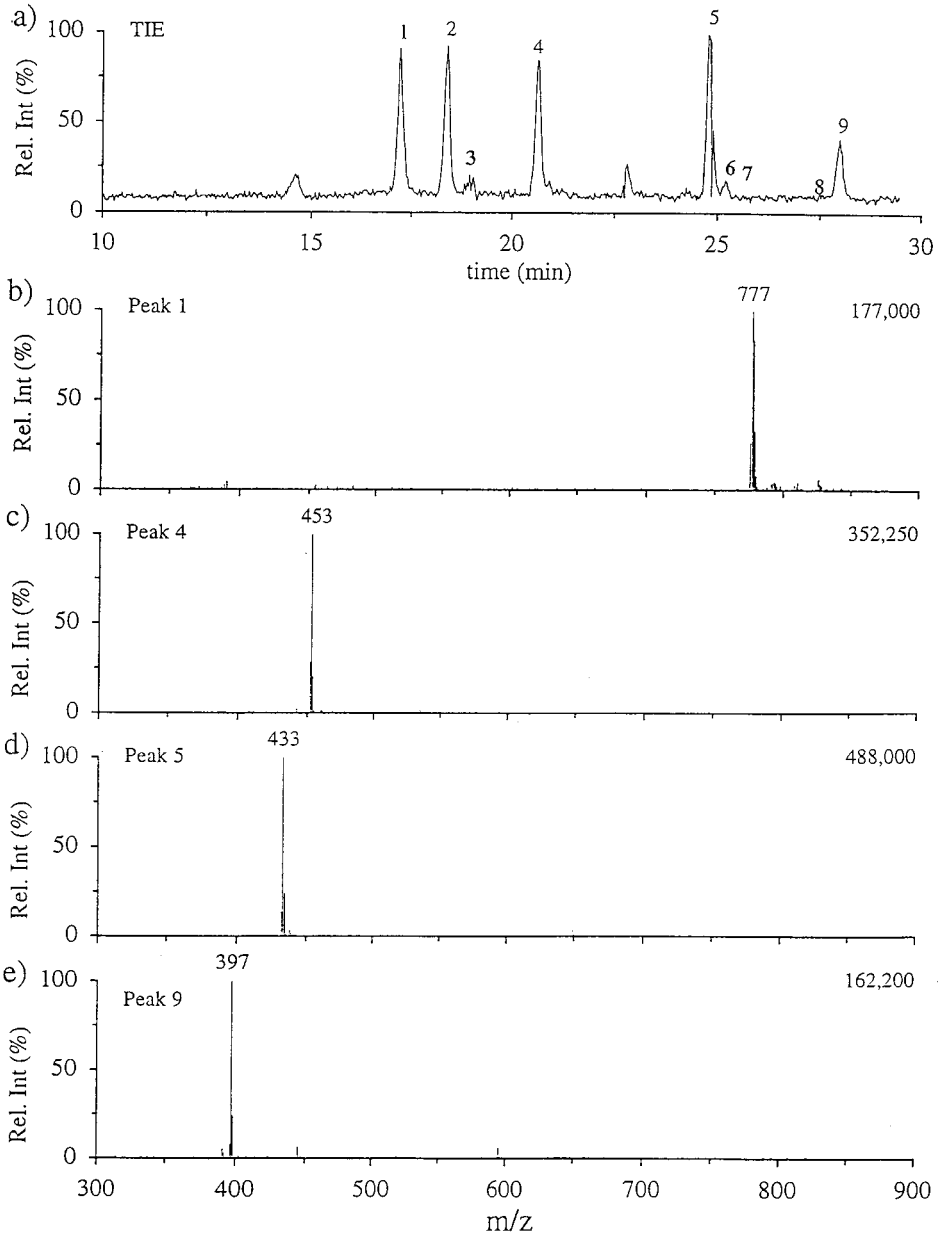

Figure 5. Co-axial PC/CZE/nESMS analysis of nine peptides each at the 100 ng ml^{-1} level using full-scan acquisition (*m/z* 300–900). Separation conditions as in Fig. 3. Peak numbers as in Fig. 4.

aminopropyl phase to prevent loss of current due to the low EOF generated in the neutral C_{18} phase (data not shown). In subsequent experiments, this problem was overcome by reducing the volume of adsorptive media to a smaller bed of material (typically 1 mm length).

The second stationary phase examined was a 5 μm C_{18} pelicular packing [Fig. 6(b)]. When compared with the aminopropyl adsorptive medium [Fig. 6(a)], this stationary phase yielded stronger analyte adsorption, as evidenced by the lower intensity of the sample breakthrough peak observed between 6 and 7 min in Fig. 6(b). This stationary phase also appeared to have better elution properties as the tailing of the elution peak was considerably less noticeable than for any of the other phases investigated. However, the construction of a preconcentrator made of this packing material required the incorporation of retaining frits, which was time consuming and thus a less attractive device for regular use.

The 40 μm C_{18} phase [Fig. 6(c)] provided the most desirable practical features in terms of sample capacity and ease of use. This phase exhibited the least sample breakthrough and wash-off, as revealed by the low abundance of peptide ions observed between 4 and 8 min. The elution of sample appeared to be efficient, although memory effects were apparent with multiple elutions. As indicated in Fig. 6(c), complete recovery of the adsorbed peptides could not be achieved using a single elution of 200 nl of organic solvent (90:10% acetonitrile, 1% HCl). Subsequent application of elution buffer provided variable amounts of the different peptides ranging from 20–40% of the first elution plug. This memory effect was found to be less pronounced than that observed using the other two stationary phases investigated. For these reasons, the 40 μm irregular C_{18} packing material was selected in all following experiments. It is noteworthy that the use of an ad-

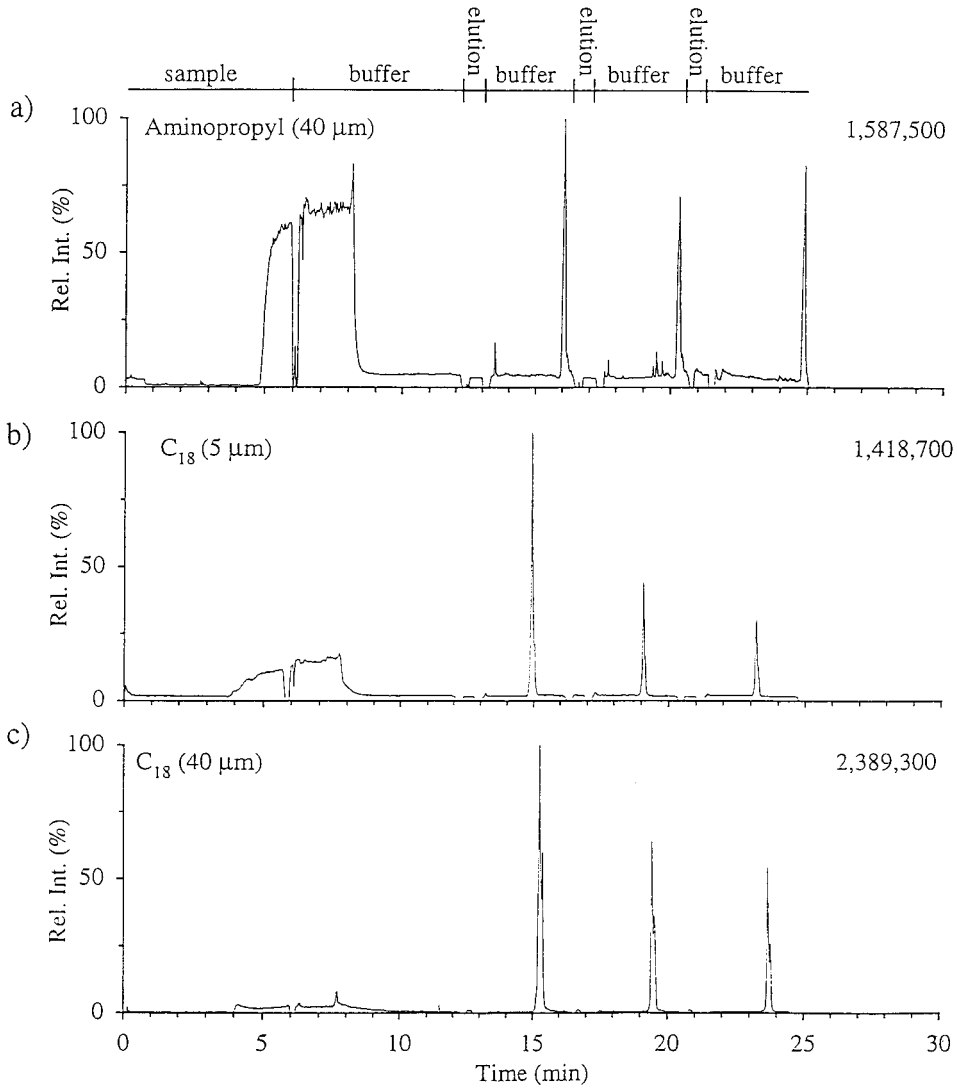

Figure 6. Comparison of stationary phases using flow injection nESMS. (a) 40 μm aminopropyl particles; (b) 5 μm C_{18} particles; (c) 40 μm C_{18} particles. Electrolyte, 0.1 M formic acid; elution solvent (400 nl), 90:10 acetonitrile–1% HCl. The TIE correspond to the sum of individual electropherograms for each protonated peptide ions shown in Fig. 2.

sorption preconcentrator coupled to BCQ-coated column proved to be very reliable and the lifetime of a single device often exceeded 5 days of continuous use.

Multiple elution and memory effects

The evaluation of the different phases also revealed that some peptides were retained more strongly than others, with some peptides being lost during the sample loading and rinsing stages while others remained bound to the preconcentrator even after multiple elutions. Figure 7 illustrates the selectivity of the technique with the analysis of a single injection of analyte followed by five separate elutions and separations. A relatively large elution volume (~500 nl) was used to maximize the sample recovery, which obviously had deleterious effects on separation efficiencies. Correspondingly, maximization of theoretical plates in CZE separation thus requires the use of significantly lower elution volumes.

After each sample elution, peak areas observed for the different peptide ions were calculated and the results obtained are summarized in Table 3. As indicated, the first elution generally yields between 25 and 90% recovery of the peptide ions compared with the total recoveries for all five elutions combined. Replicate elutions ($n = 5$) shown in Fig. 7 gave RSD values ranging from 1.7–2.3% for the migration times of the different peptides. The retention characteristics varied depending on the composition of the analyte. For example, the pentapeptide thymopoietin$_{32-36}$ appeared in low abundance only in the first elution, thus suggesting weak adsorption to the reversed-phase packing. The hydrophilic nature of this small peptide is attributed to the presence of two basic amino acids which could possibly explain its poor retention on the C_{18} phase. Neurotensin$_{1-6}$ behaves almost ideally, with an initially large signal which rapidly decreases in subsequent elutions. The recovery of this peptide was >75%, assuming no losses during loading and rinsing. In contrast, mellitin exhibited an unusual retention behavior which was partly attributed to its higher hydrophobicity. The multiply protonated ions for this peptide were observed only following the second and third

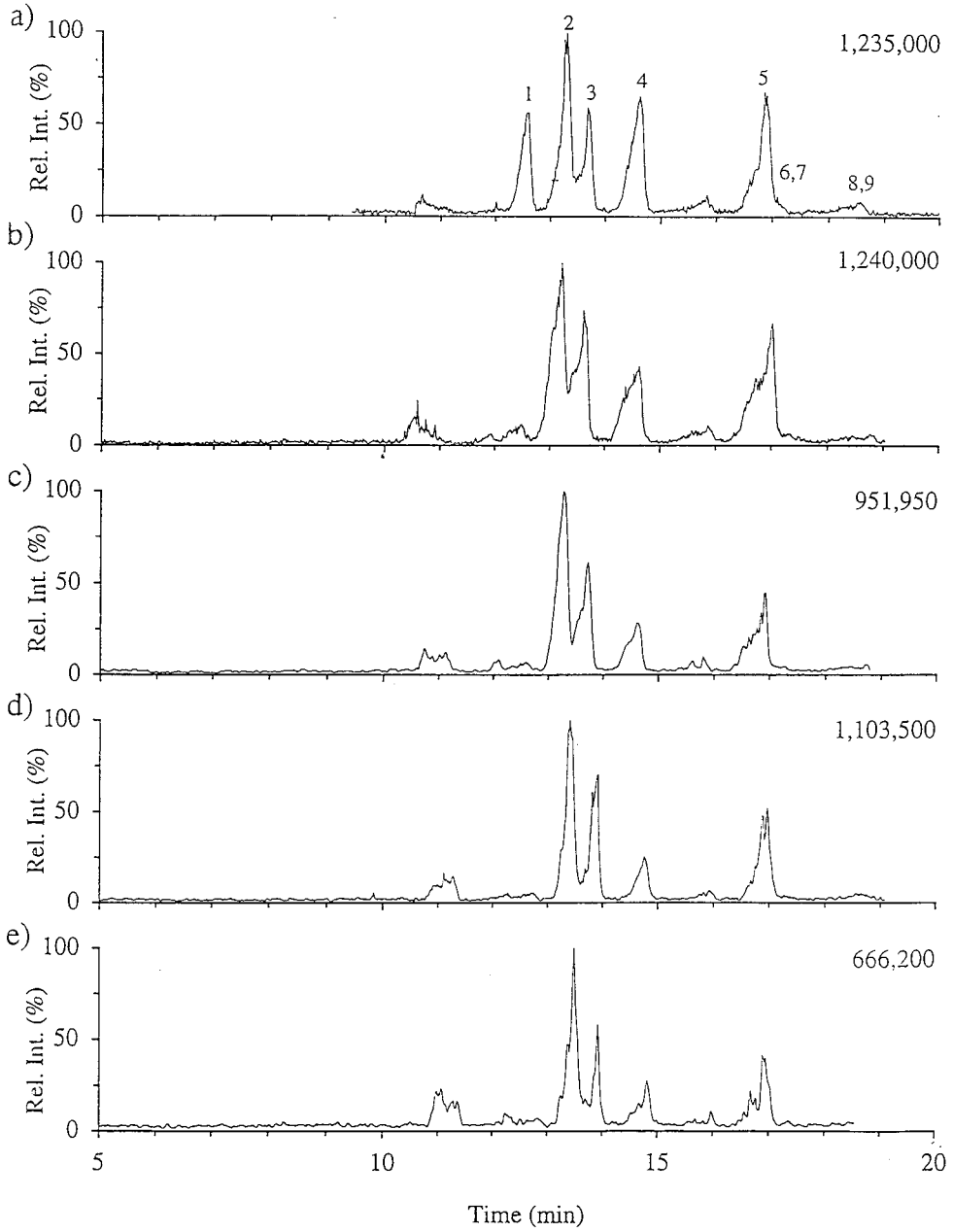

Figure 7. Memory effect observed in PC/CZE/nESMS analysis of a mixture of nine peptides each at 100 ng ml^{-1}. Conditions as in Fig. 5. (a) Injection of a 4 µl sample injection followed by 150 nl of elution buffer; (b)–(e) subsequent elutions without further sample injection.

sample elutions. This can be rationalized by assuming that the peptide was eluted and readsorbed by the stationary phase after moving progressively through the bed.

Previous reports describing the use of on-line preconcentration methods indicated that post-analysis rinsing with elution buffer was necessary to minimize sample carry-over (memory effects).[36] The injection of a larger plug of organic solvent to extract the analytes more efficiently prior to separation is not a feasible option, as it leads to poor separation efficiency (see below). An interesting possibility that takes advantage of incomplete elution of sample would be to carry out multiple analyses from a single sample injection, as illustrated in Fig. 7. The objective of the PC/CZE approach is to analyze dilute samples such as peptides present in biological fluids. By using a 'single injection–multiple elution' protocol, higher sample utilization could be obtained. This would be particularly useful when PC/CZE is combined with tandem mass spectrometry (MS/MS) in order to obtain peptide sequence information. Multiple elutions would also be beneficial for the acquisition of tandem mass spectra of co-eluting components.

Optimization of elution buffer volume

As described in the previous section, the use of a larger volume of elution buffer leads to significant degradation of separation efficiency. An example of this effect is demonstrated in Fig. 8 for elution volumes ranging

Table 3. Peak areas observed for multiple elutions of a single sample injection in PC/CZE/nESMS experiments

			Peak areas ($\times 10^6$) Elution number				
Peptide[a]	*m/z*	M_r	1	2	3	4	5
Thymopoietin$_{32-36}$ (8)	341	680	0.16	—	—	—	—
Lys-bradykinin (9)	397	1188	1.3	1.2	0.40	0.54	0.050
Angiotensin I (5)	433	1296.5	9.5	11.7	4.9	5.8	1.9
des-Arg-bradykinin (4)	452	904	9.9	8.1	3.4	2.8	1.6
des-pGlu-LHRH[b] (6)	536	1071	0.93	0.56	0.10	0.24	0.056
Melittin (7)	570	2847	—	0.39	0.0087	—	—
Neurotensin$_{1-6}$ (1)	777	776	2.8	0.39	0.10	0.11	0.097
Glu-fibrinopeptide B (2)	786	1570.6	7.3	9.4	6.8	6.0	3.4
Lys$_3$-Bombesin (3)	797	1592	5.7	9.1	4.9	4.8	1.6

[a] The numbers in parentheses refer to Fig. 7 and correspond to the migration order.
[b] Luteinizing hormone-releasing hormone.

from 150–300 nl. Analyte desorption in an elution volume of 150 nl [Fig. 8(a)] provided separation efficiencies of 116000–193000 theoretical plates, which were on average only 10% lower than those observed for the same peptides during the conventional CZE/nESMS experiment. As the volume of the elution buffer was increased the efficiency decreased rapidly, with the peaks becoming unresolved and much broader. When the volume of elution buffer was increased above 500 nl no peaks were detected unless a small inlet pressure (50 mbar) was applied concurrently with the separation voltage. This may reflect the low conductivity of the

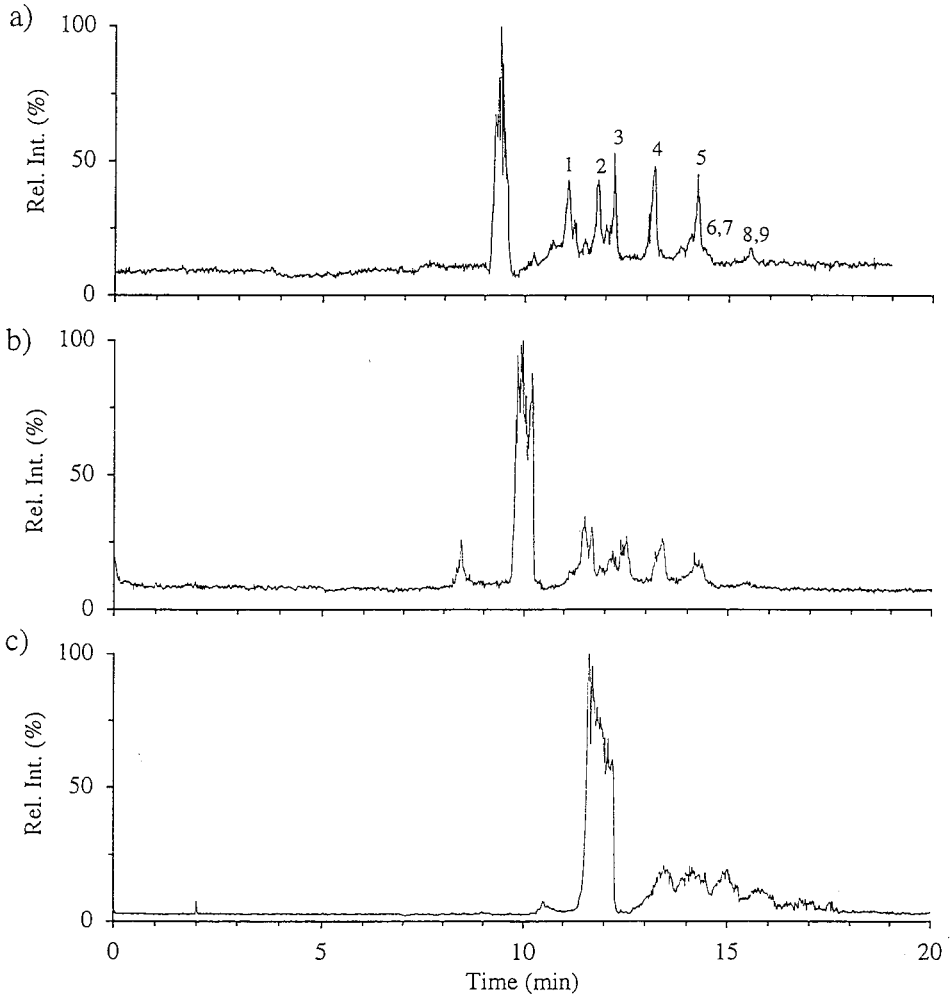

Figure 8. Effect of elution volume on separation efficiency observed in the PC/CZE/nESMS analysis of a peptide mixture. TIE (*m/z* 300–900) using (a) 150, (b) 225 and (c) 300 nl injections of elution buffer. Other conditions as in Fig. 3.

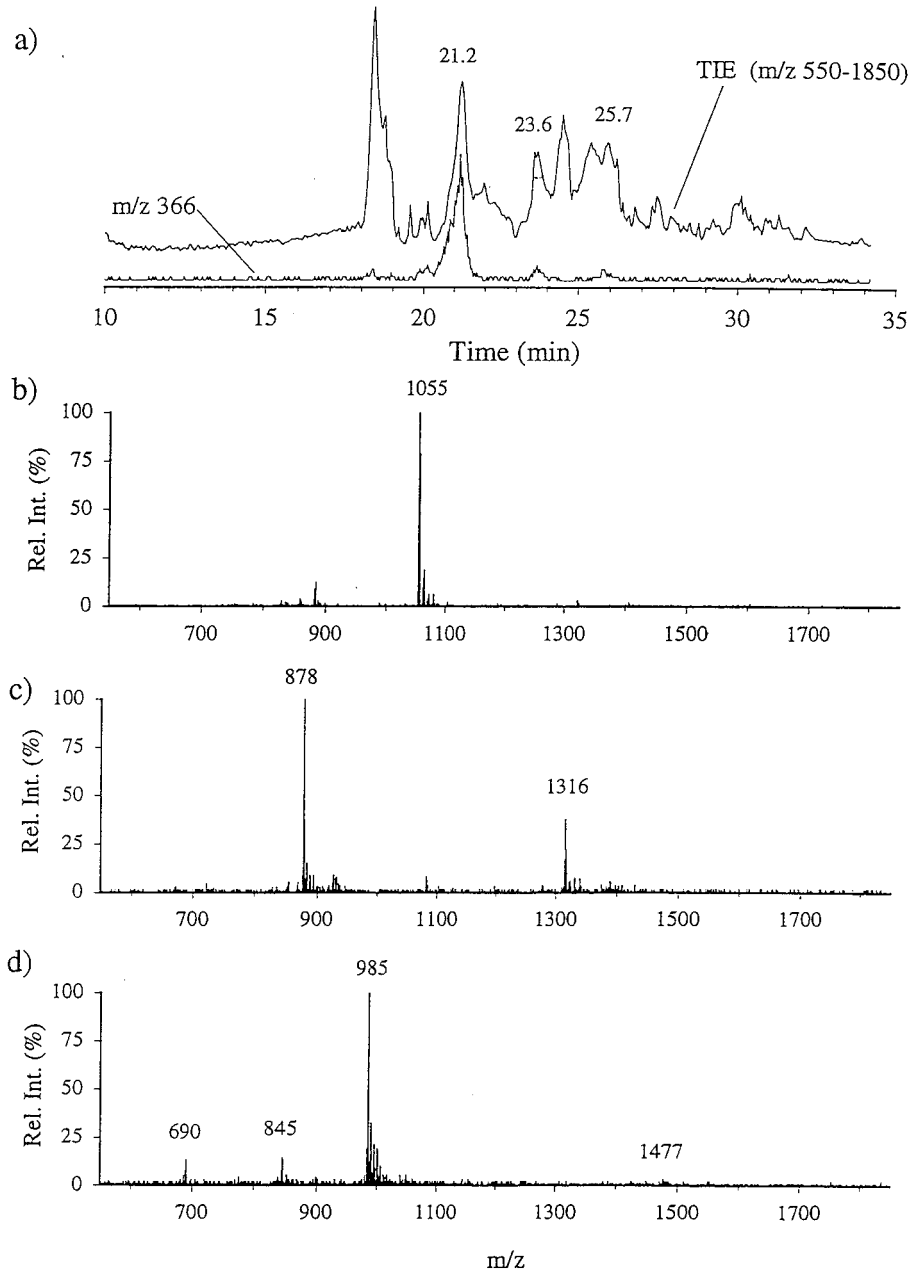

Figure 9. PC/CZE/ESMS analysis of a tryptic digest from the *B. purpurea* seed lectin. (a) TIE (*m/z* 550–1850, orifice voltage 50 V and *m/z* 366, orifice voltage 120 V), extracted mass spectra for peaks observed at (b) 21.2, (c) 23.6 and (d) 25.7 min. Conditions: 4 µl injection of 250 fmol of digest; other conditions as in Fig. 4.

elution buffer and resultant voltage drop across the elution buffer zone, which led to reduction of field strength in the remaining regions of the capillary.[29]

Analysis of glycoprotein digests using PC/CZE/nESMS

The optimized conditions for on-line preconcentration with nESMS detection were used for the analysis of a proteolytic digest of the *N*-acetyl-D-galactosamine specific lectin from *B. purpurea*. The *N*-terminal sequence of this lectin showed high homology with those of other leguminous lectins[37] and a complete sequence based on cDNA cloning and expression was presented previously.[38] An earlier investigation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) determined that the purified lectin consisted of a major band at approximately 34 kDa and a minor component at 36 kDa was presumed to reflect the heterogeneity of the carbohydrate chain of *B. purpurea*.[39] The open reading frame of the cDNA encodes a protein of 290 amino acids with a signal peptide of 28 amino acids, thereby giving a molecular mass of 28971 Da for the expressed lectin.[38] The mass difference observed between that predicted from the gene sequence and that

obtained from the SDS-PAGE measurement was assumed to originate from N-linked oligosaccharides attached to five potential sites. However, the nature and sites of glycosylation were not established unambiguously.[38]

The ESMS mass spectrum of the intact lectin obtained from infusion experiments (data not shown) gave rise to multiply protonated ion series extending from *m/z* 2300 to 3000. The reconstructed molecular mass profile showed a predominant glycoprotein peak at 32 036 ± 4 Da with a minor component at 33 110 ± 3 Da. In order to establish the nature of the oligosaccharides and the site of glycosylation, the proteolytic digest of the *B. purpurea* lectin was analyzed using PC/CZE/nESMS. The total ion electropherogram (TIE) corresponding to the injection of 8 µl of this lectin (250 fmol of the original protein), incubated sequentially with the endoproteinases Glu-C and trypsin, is presented in Fig. 9(a).

To facilitate the identification of glycopeptides, a mixed scan function[15,40] was used to promote the in-source formation of selected oxonium ions under high orifice voltage conditions (120 V), while enabling detection of multiply protonated ions using a low orifice voltage (50 V). The extracted ion electropherogram for the characteristic oxonium ion Hex-HexNAc at *m/z* 366 is shown in Fig. 9(a) together with the TIE (*m/z* 550–1850). As indicated, a prominent glycopeptide candidate is observed at 21.2 min with two minor components at 23.6 and 25.7 min. The extracted mass spectra for these three glycopeptides are presented in Fig. 9(b)–(d). The spectrum of the major glycopeptide [Fig. 9(b)] contains an abundant $[M + 2H]^{2+}$ ion at *m/z* 1055 corresponding to a molecular mass of 2108 Da. The other two glycopeptides displayed $[M + 3H]^{3+}$ and $[M + 2H]^{2+}$ ions at *m/z* 878 and 1316 [Fig. 9(c)] and *m/z* 985 and 1477 [Fig. 9(d)], corresponding to molecular masses of 2631 and 2952 Da, respectively.

Additional confirmation of the structural identity of these proteolytic glycopeptides was obtained using on-line MS/MS. Structural features of the glycopeptide were probed using low-energy collisional activation of the multiply protonated precursor ions. The tandem mass spectrum of the $[M + 2H]^{2+}$ ion of the glycopeptide at *m/z* 1055 [Fig. 9(b)] is shown in Fig. 10(a) and was produced from the injection of 5 pmol of digest of the original lectin. Prominent doubly charged fragment ions are observed for consecutive cleavages of the glycosidic bonds from the precursor ion, together with their singly charged oligosaccharide fragment ion counterparts indicated in the inset. Interestingly, an abundant

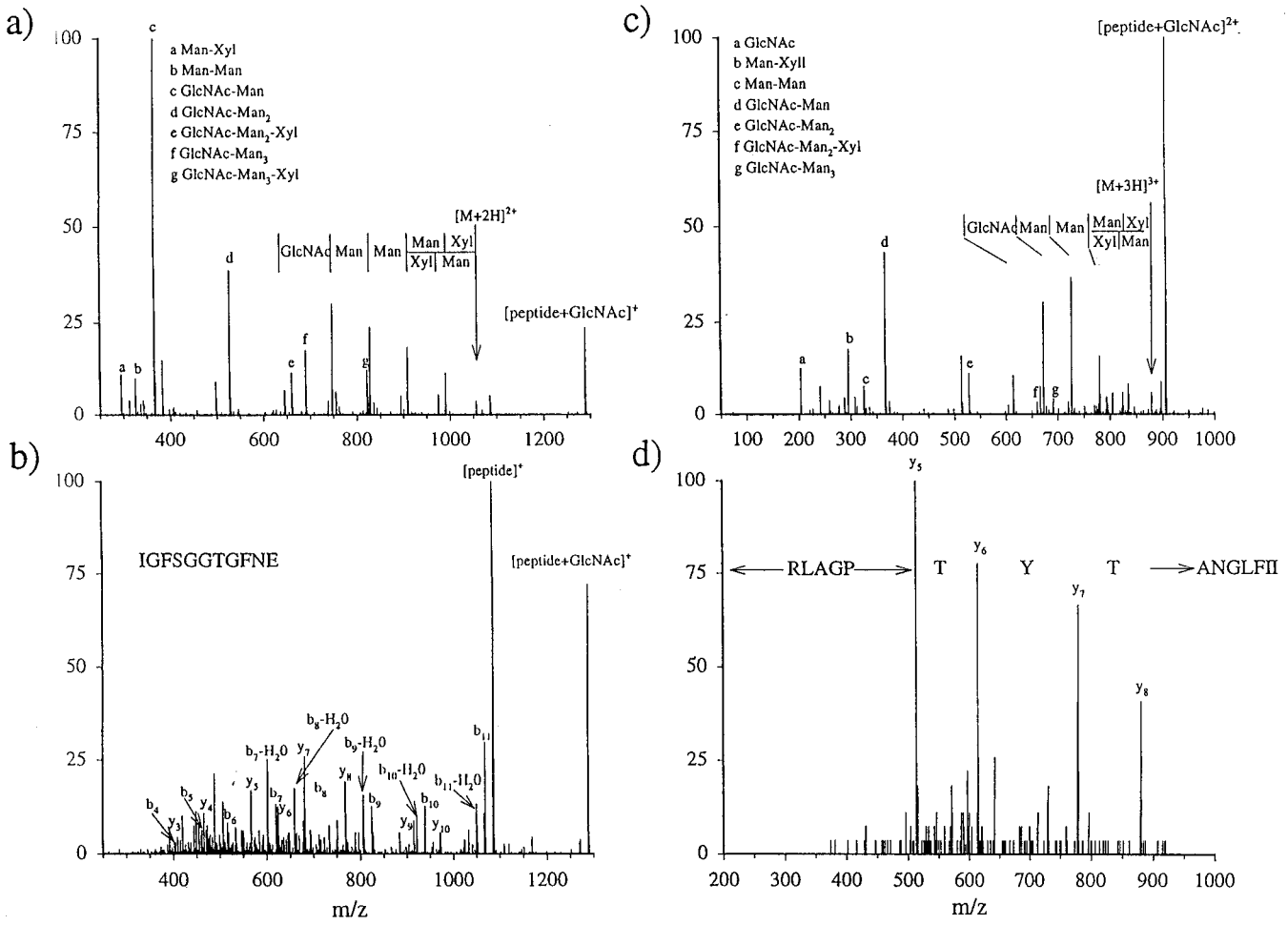

Figure 10. PC/CZE/MS/MS analysis of a tryptic digest from the *B. purpurea* seed lectin. Tandem mass spectra of (a) *m/z* 1055, (b) *m/z* 1288, (c) *m/z* 878 and (d) *m/z* 906. Separation conditions as in Fig. 9 except that the orifice voltage was set to 120 V in (b) and (d), collision energy 60 eV (a, c) and 50 eV (b, d), Ar target gas.

fragment ion corresponding to the peptide + GlcNAc is observed at m/z 1288, suggesting that the molecular mass of the deglycosylated peptide is 1085 Da. The observation of a peptide fragment ion containing a single GlcNAc residue is a characteristic feature of the tandem mass spectra of N-linked glycopeptides[15,41] and can be used to deduce the molecular mass of the tryptic peptide backbone. From the fragment ion series observed in this tandem mass spectrum, the structure of the oligosaccharide was assigned as $GlcNAc_2Man_3$ with a pentose sugar, presumably Xyl, attached to the central mannose residue. The proposed location of the pentose residue is based on the ion pairs associated with loss of either mannose or pentose and from the oligosaccharide fragments ions observed below m/z 900 [Fig. 10(a)].

The only peptide containing a consensus sequon for an N-linked glycosylation site that would have a molecular mass of 1085 Da is the proteolytic fragment of m/z 217–228: (R)IGFSGGTGFNE(T) (M_r 1085.14 Da). The residues in parentheses indicate adjacent terminal amino acids. Confirmation of this assignment was obtained by conducting MS/MS analysis on first-generation fragment ions corresponding to the peptide backbone plus GlcNAc formed in the orifice–skimmer region.[15] In this case we used the singly charged fragment ion at m/z 1288 formed at high orifice voltage in the ion source as precursor ion for the MS/MS experiment. The spectrum generated by this technique is shown in Fig. 10(b). The most abundant fragment ion observed here corresponds to the naked peptide ion at m/z 1085 arising from the loss of the GlcNac residue. Sequence information was derived from the observation of a set of consecutive fragment ions that were in good agreement with those expected from the b-type ion series of the corresponding peptide. The predominance of this ion series, as opposed to the y-type fragment ions typical of tryptic peptides, suggests that the ionizing proton is located at or near the *N*-terminus.

The same approach was used to determine the nature and site of glycosylation of the other two glycopeptide candidates found in this digest. The tandem mass spectrum of the minor glycopeptide observed at 23.6 min in Fig. 9 is presented in Fig. 10(c). Interestingly, this tandem mass spectrum yielded oligosaccharide fragment ions below m/z 800 identical with those observed previously for the most abundant glycopeptide [Fig. 10(a)], suggesting that the glycan $GlcNAc_2Man_3Xyl$ could be appended to the peptide backbone. This was further supported by the triply charged fragment ions corresponding to the consecutive losses of pentose and hexose residues from the $[M + 3H]^{3+}$ precursor ion. The molecular mass of this peptide was calculated to be 1607 Da based on the observation of an abundant doubly charged peptide + GlcNAc ion at m/z 906. This result is in excellent agreement with that of the calculated molecular mass for the peptide segment 24–38: (E)IIFLGNATYTPGALR(L) (M_r 1606.89). Confirmation of this sequence assignment was achieved in a manner similar to that described above, except that the doubly charged fragment ion of m/z 906 was selected as precursor. The corresponding tandem mass spectrum [Fig. 10(d)] gave several singly charged y-type fragment ions from which the amino acid string TYT could be deduced. It is noteworthy that sequence segment plus the molecular mass was sufficient to identify this lectin uniquely in a database search (Peptide scan, EMBL).

Mass spectral analyses on the third glycopeptide of M_r 2952 Da determined that the oligosaccharide chain $GlcNAc_2Man_3Xyl$ was attached at residue Asn_{156} of the peptide (R)YPHIGINVNSTVSVATTR(I) (data not shown). It is interesting that all three glycosylation sites found on *B. purpurea* lectin display the same oligosaccharide chain and that no sign of microheterogeneity in the glycan structure was found. Based on this information, the molecular mass calculated for the intact glycoprotein was found to be 32 039 Da, in good agreement with that measured (32 036 $\pm$ 4 Da) from the nanoelectrospray mass spectrum discussed above.

CONCLUSIONS

The use of a C_{18} preconcentration device with on-line CZE/nESMS provides an enhancement of concentration detection limits by up to two orders of magnitude compared with those obtained using the conventional CZE format and allowed reliable peptide identification for low nanomolar concentrations. The development of such preconcentration devices was found to be compatible with the operation of nanoelectrospray and separation conditions using covalent amine coated capillaries and acidic buffers. Careful consideration of the type of stationary phase used is important in order to maximize the effectiveness of this technique and to provide reliable analysis of dilute samples. Sample carry-over was found significant, especially when small elution volumes ($<$200 nl) had to be used to maintain adequate separation efficiencies ($N > 100\,000$). Awareness of the inherent memory effects of PC/CZE allows this characteristic to be used to the advantage of the operator by permitting multiple analyses from a single injection of sample.

Under optimized conditions, the PC/CZE/nESMS technique allowed the identification of tryptic peptides and glycopeptides at concentrations of 50 fmol μl^{-1}. These data are of sufficient quality for reliable database searching. In cases where an unknown protein is encountered, MS/MS could be used to obtain sequence data for more rigorous database searching. Stepped-orifice voltage scanning methods were used to identify glycopeptides in a tryptic digest of the *B. purpurea* lectin. MS/MS analyses of separated glycopeptides generated information on the types of glycans appended to the protein backbone. By increasing the orifice voltage, specific glycopeptide fragment ions were obtained which were subsequently used to confirm the peptide sequence of the target peak. These techniques should provide a powerful tool for the structural characterization of glycoproteins, particularly in situations where the amount of sample is limited.

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