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Automated on-line ionic detergent removal from minute protein/peptide samples prior to liquid chromatography–electrospray mass spectrometry

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Abstract

An automated on-line ionic detergent removal pre-column system coupled to capillary liquid chromatography–electrospray mass spectrometry is described. The system involves two micro precolumns, composed of a specific ionic detergent trapping column and a preconcentration column, respectively, and a packed 300 μm I.D. analytical column. Sample loading to the micro precolumns and regeneration of the detergent trapping column were performed at a flow-rate of 50 μl/min, while the flow-rate through the analytical column was set at 5.0 μl/min. Ionic detergent-containing tryptic-digested protein samples were directly applied to the micro precolumns without sample pretreatment and were analysed by UV absorption detection and electrospray mass spectrometry. The presented system allows for the fully automated removal of SDS with virtually no loss in protein/peptides. Maximum SDS load and breakthrough have been determined. Excellent protein recovery and complete removal of SDS is found. The chromatographic separation after SDS removal was completely restored and equalled the reference chromatogram. Mass spectral data confirm these findings. Finally, this technique allows for SDS removal from minute protein samples without the need for any sample handling.

Keywords: Detergents; Ionic detergent removal; Proteins; Peptides

1. Introduction

Reversed-phase liquid chromatography–electrospray mass spectrometry (RPLC–ESI-MS) is currently the method of choice for the separation and identification of complex protein and peptide mixtures. However, protein/peptide samples are often at low concentrations and contain buffers and/or detergents, which hamper LC–ESI-MS analysis [1,2]. Commonly used purification and concentration techniques like liquid–liquid extraction [2,3], gel filtration [4–6] and precipitation [7–9] often fail to quantitatively recover the compounds of interest and are not accessible for minute sample volumes. Furthermore, these techniques are very time consuming and cannot be coupled easily with identification methods such as MS.

Different types of solid-phase extraction cartridges

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have been introduced recently for conventional and microbore LC to trap ionic and non-ionic surfactants. However, these cartridges are not compatible with capillary LC. The system employed in this study uses miniaturised precolumns to which the samples are applied. By using subsequent wash steps, using different wash solvents, interfering compounds can be selectively removed.

With the presented method, it is possible to fully automatically preconcentrate the sample on cartridge-type microcolumns with virtually no dead-volume. Typical loading and clean-up flow-rates are up to 50 μl/min, which results in very fast sample clean-up of less than only 1 min. Both steps are performed on-line. The use of micro-precolunms in combination with low dispersion injections valves allows for optimal clean-up of small and large sample volumes. The developed technique minimises the risk of sample loss, since no additional sample preparation steps are required. Results will be shown of the removal of the ionic detergent sodium dodecylsulphate (SDS) from minute proteinaceous samples using microcolumn switching in combination with capillary LC. Also demonstrated is the enhancement in selectivity and sensitivity, as is shown by capillary liquid chromatography and mass spectral analysis.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and water, both of HPLC grade, were purchased from LabScan (Dublin, Ireland). Trifluoroacetic acid (TFA), cytochrome *c* (from bovine heart), α-lactalbumin B (from bovine milk) and a HPLC peptide standard mixture were obtained from Sigma (St. Louis, MO, USA); trypsin (from bovine pancreas), tris(hydroxymethyl)aminomethane, sodium acetate and calcium chloride were from Fluka (Buchs, Switzerland).

2.2. Digest preparation

Typical amounts of 1–2 mg/ml of protein were dissolved in digest buffer [100 m*M* sodium acetate, 100 m*M* tris(hydroxymethyl)aminomethane and 1 m*M* calcium chloride, pH 8.3]. Protein samples were digested by addition of trypsin dissolved in 1 m*M* HCl (trysin–protein, 1:50, w/w) and incubated at 37°C for 4 h, followed by a second enzyme addition to give a final trypsin–protein concentration ratio of 1:25 (w/w). After 16 h of incubation, the samples were diluted five times in the mobile phase and stored at −20°C.

2.3. Capillary LC instrumentation

A FAMOS microsampling workstation (LC Packings, San Francisco, CA, USA) with two additional built-in six-port valves was used for sample injection, sample clean-up and preconcentration. A schematic overview of the instrumental set-up is given in Fig. 1. Sample preconcentration and sample clean-up were performed with an LC-10S pump (Shimadzu, Tokyo, Japan) that was operated at 50 μl/min. Cartridge-type precolumns (LC Packings) with a length of 5 mm and different inner diameters were used to trap SDS or to preconcentrate the peptides originating from the tryptic digest. The packing material in the SDS trapping column consists of an anion-exchange type of stationary phase with a nominal particle diameter of 5 μm. The preconcentration column was filled with a 5-μm standard reversed-phase stationary phase packing material. Both micro precolumns were typically replaced after fifty injections. The loading solvents for sample preconcentration and sample clean-up were CH_3CN–H_2O mixtures containing 0.1% TFA. Different loading solvent compositions have been studied. For details see Section 3. Regeneration of the SDS trapping column was done with 0.1% TFA in CH_3CN.

LC experiments were performed with an LC-10-AD high-pressure gradient system (Shimadzu). Micro flows were generated by an AC-400-VAR variable microflow processor (LC Packings) that was connected to the gradient system [10,11]. The micro outlet flow was transferred directly to the workstation. Peptide samples were separated on a 15 cm× 300 μm I.D. capillary LC column (LC Packings) packed with 3 μm C_{18} base deactivated stationary phase at a flow-rate of 5 μl/min. The gradient was

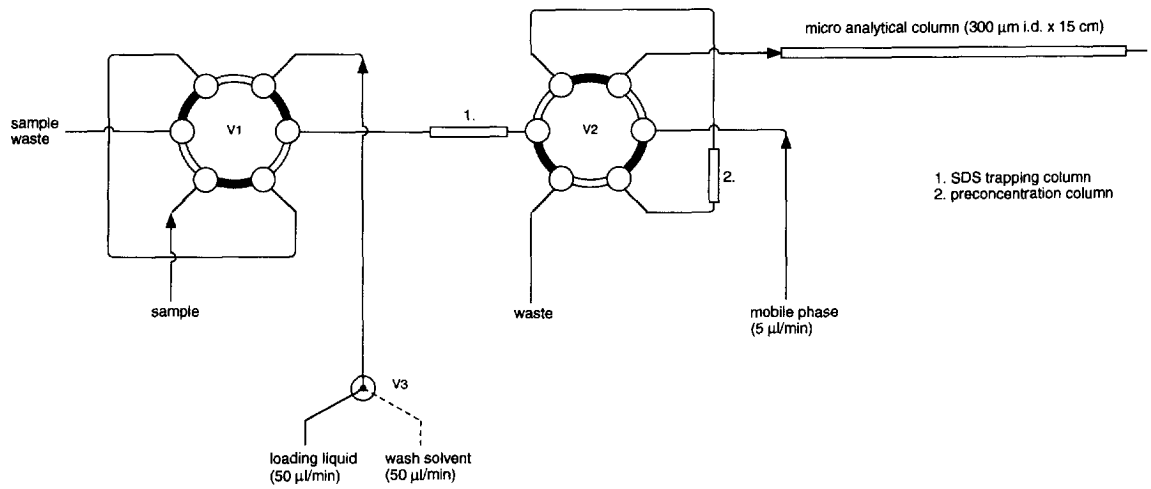

Fig. 1. Scheme of the instrumental set-up for on-line SDS removal consisting of three six-port valves; injection valve (V1), switching valve (V2) and a third six-port valve that was used as a solvent selection valve (V3). For details see Section 2.

developed over 20 min from 5 to 80% B for the HPLC peptide mixture, from 5 to 35% B for the cytochrome *c* tryptic digest and from 5 to 40% B for the α-lactalbumin B tryptic digest. Mobile phase A consisted of 0.1% TFA in CH_3CN–H_2O (5:95, v/v) and mobile phase B of 0.08% TFA in CH_3CN–H_2O (80:20, v/v).

Detection was performed at 214 nm using an SPD-10A UV–Vis absorbance detector (Shimadzu) equipped with a 35-nl, 8 mm longitudinal U-shaped capillary flow cell (LC Packings) [12,13]. Data acquisition was performed with MT-2 chromatography software (Kontron Instruments, Milan, Italy).

2.4. Mass spectrometry

Pneumatically assisted electrospray (ion spray) ionisation mass spectrometry was conducted with an API 300 triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada) in the positive ion, single scan mode. Scans were taken from *m/z* 300 to 1500 with a scan duration of 2 s, using a step size of 1 a.m.u. and a 2.5-ms dwell time per step. The mass spectrometer was set to the following parameters; ion spray voltage, 5.5 kV; orifice voltage, 35 V. The nebulizer gas (air) and curtain gas (nitrogen) were adjusted to 1.5 l/min and 0.8 l/min, respectively.

3. Results and discussion

3.1. SDS presence

The results in Fig. 2 demonstrate the effect of the presence of SDS in a sample on the chromatographic separation of a tryptic digest of α-lactalbumin B. The upper trace represents the chromatogram of the digested α-lactalbumin B sample in the presence of 0.1% SDS and the lower trace represents the same sample without SDS. Peak broadening, increased retention and loss of resolution are usually observed in the presence of SDS. SDS is known to bind to proteins and peptides resulting in more hydrophobic species and overall similar polarity which causes the majority of the individual compounds of the tryptic digest to co-elute. In addition to proteins and peptides, SDS also binds to the surface of the applied stationary phase packing material resulting in insufficient protein/peptide separations on reversed-phase surfaces. No useful mass spectra could be obtained (as shown later) in the presence of SDS.

3.2. Sample preconcentration

The maximum tolerable amount of CH_3CN present in the carrier liquid was determined in order to

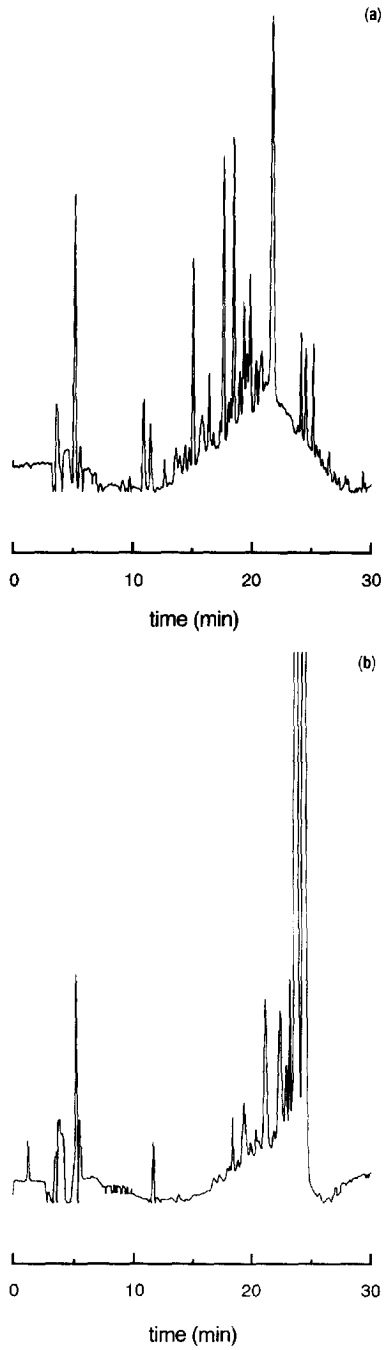

Fig. 2. Comparison of peptide separations from tryptic digested α-lactalbumin B on a 15 cm×300 μm I.D. packed capillary LC column. The upper trace shows the chromatographic separation of an SDS-free sample. The lower trace shows the separation of the same sample in the presence of 0.1% SDS. For chromatographic conditions see Section 2.

investigate whether partial SDS removal could be achieved during sample preconcentration [14]. Digest and peptide samples were directly loaded onto the preconcentration column. The SDS trapping column (as shown in Fig. 1) was not used in these experiments.

Sample breakthrough was observed for the more hydrophilic tryptic peptide with as little as 5% CH_3CN in acidified water (0.1% TFA) as the loading solvent. This is in contrast to work published earlier [14], where up to 20:80 (v/v) CH_3CN–H_2O could be used for sample loading and complete SDS removal. In the present study, sample loading with CH_3CN–H_2O (20:80, v/v) resulted in almost complete sample breakthrough and only partial SDS removal. No sample breakthrough was observed with 0.1% TFA in H_2O as the carrier liquid. Therefore, all further experiments were performed with 0.1% TFA in H_2O as the loading solvent for sample preconcentration.

3.3. Sample recovery

The recovery of the sample preconcentration was determined by comparing peak heights and peak areas of direct 1-μl and 5-μl sample injections to those using preconcentration. The I.D. of the 5 mm long micro preconcentration columns were 0.3 or 0.5 mm, respectively.

Complete sample recovery was obtained for the tryptic digest of α-lactalbumin and cytochrome *c* mixtures for the 0.3 and 0.5 mm I.D. micro preconcentration columns and was independent of the injected sample volumes (between 1 and 5 μl). For the HPLC peptide standard mixture, consisting of glycine–tyrosine, valine–tyrosine–valine, methionine enkaphalin, leucine enkaphalin and angiotensin II, the sample recovery was equal to 100% for the tripeptide and the three pentapeptides. Partial sample breakthrough was observed for the dipeptide and was equal to about 50%. However, since the tryptic digest mixtures do have much higher retention factors than the dipeptide it was experimentally determined that the tryptic digest samples could be applied to the micro precolumns using 0.1% TFA in H_2O at flow-rates of up to 50 μl/min without sample loss.

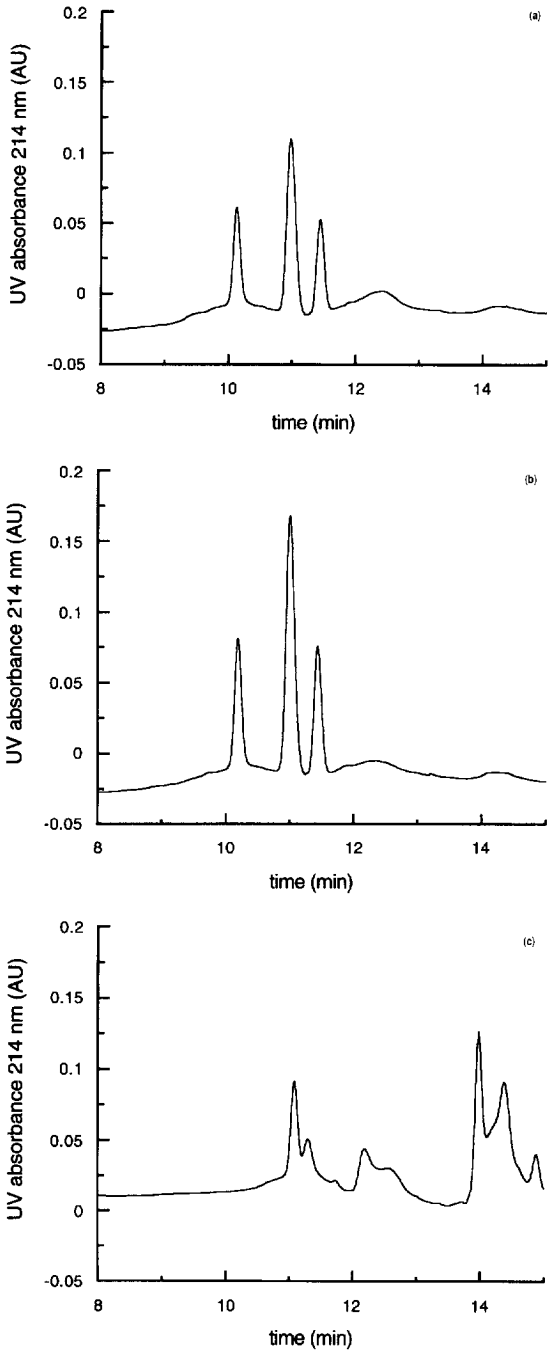

Fig. 3. SDS loadability and breakthrough. Chromatographic separation of a 5-μl sample injection containing three pentapeptides on a 15 cm×300 μm I.D. LC column in the absence of SDS (upper trace); with 0.5% SDS present in the sample (middle trace); with 1.0% SDS present in the sample (lower trace). For chromatographic conditions see Section 2.

3.4. SDS loadability and breakthrough

Besides sample breakthrough and sample recovery, it is also very important to determine how much SDS can be loaded onto the SDS-trapping micro precolumn without breakthrough of SDS. For these particular experiments the HPLC standard peptide mixture was used for evaluation. The three pentapeptides elute in a very narrow time space and are very sensitive to the presence of SDS. The amount of SDS that could be loaded onto the SDS trapping columns was determined for 0.3 and 0.5 mm I.D. micro precolumns. The injection volume was equal to 5 μl.

An example of SDS breakthrough is given in Fig. 3. The upper chromatogram shows the separation of the three pentapeptides when there is no SDS present in the sample. The middle chromatogram corresponds to an injection with 0.1% SDS present in the sample. As can been seen, chromatographic resolution is preserved. The bottom chromatogram shows the effect of partial breakthrough of SDS on the separation of the HPLC standard peptide mixture. Only the hydrophobic pentapeptides are separated well, meanwhile, the more hydrophilic pentapeptides start to co-elute, as can been seen from the bottom trace in Fig. 3. It was found that by injecting 5-μl samples containing different amounts of SDS, 0.025 mg of SDS could be loaded on the 0.3 mm I.D. micro precolumn and 0.050 mg of SDS on the 0.5 mm I.D. micro precolumn, both of 5 mm length.

3.5. On-line SDS removal

The analytical system utilised to remove SDS prior to LC–ESI-MS analysis has been described already in detail in the Section 2. The second additional six-port valve of the microsampling workstation was used as a solvent selection device. A 1-ml loop was mounted on the valve and filled with 0.1% TFA in CH_3CN which was used to regenerate the SDS trapping column. The loop was switched into the carrier liquid stream for 1 min. This means that theoretically the micro precolumn can be regenerated twenty times before the 1 ml loop has to be refilled with 0.1% TFA in CH_3CN. In practice however, this was done every tenth injection. By

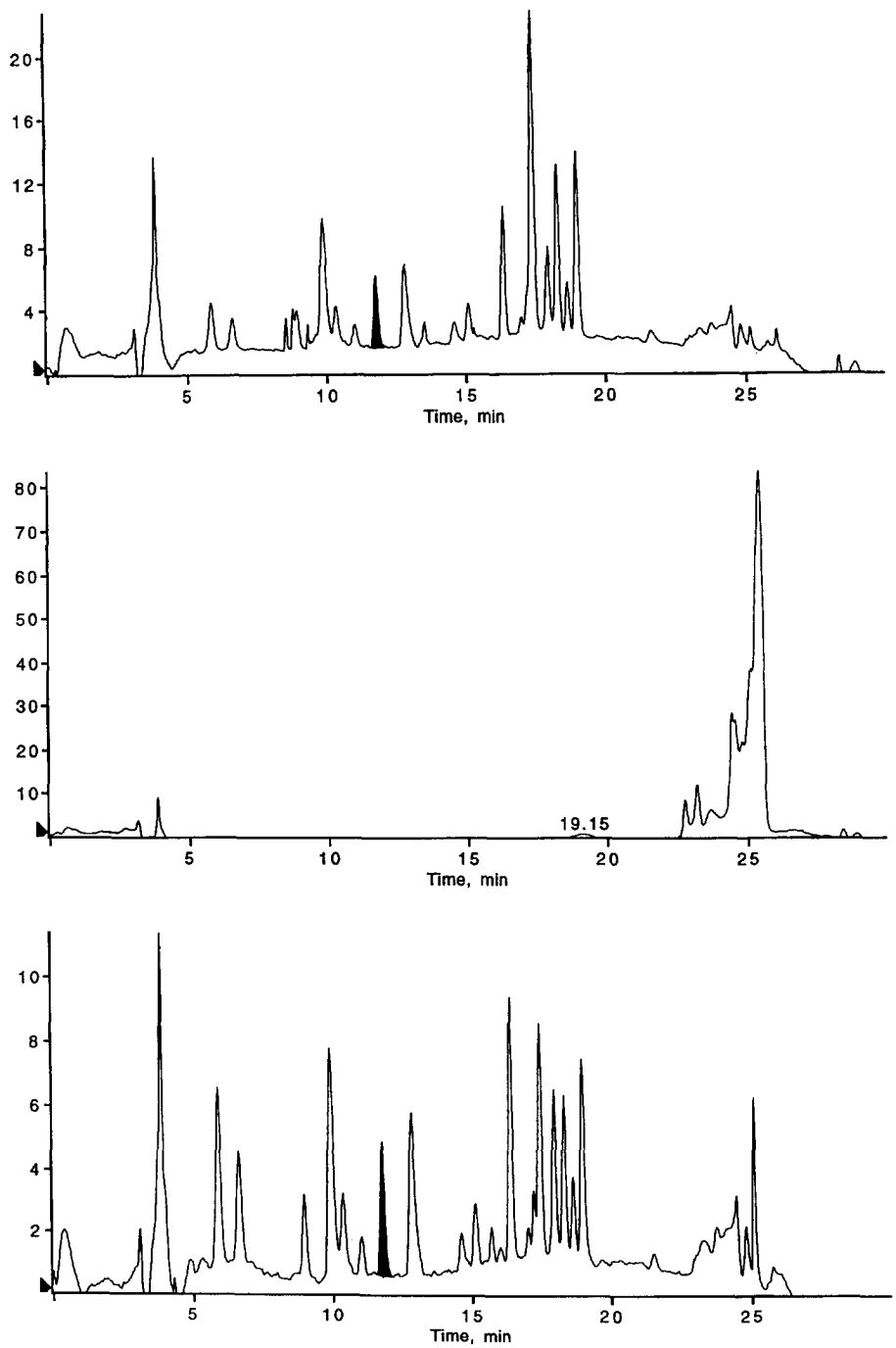

Fig. 4. Automated on-line SDS removal from a cytochrome *c* tryptic digest prior to capillary LC. The sample was run on a 15 cm×300 μm I.D. LC column. The upper UV absorption chromatogram shows the separation of a SDS-free cytochrome *c* digest (reference chromatogram). The middle trace corresponds to the separation of the same sample in the presence of 0.1% SDS. The bottom trace shows the separation of the cytochrome *c* digest after removal of SDS using the trapping column. For chromatographic conditions see Section 2.

simply increasing the loop volume size, larger numbers of unattended sample analyses are feasible.

Cytochrome *c* tryptic-digested samples were applied to the SDS-removal system and analysed with a standard gradient. The results of these experiments are shown in Fig. 4. The upper trace of Fig. 4 shows the separation of the peptides without the presence of any SDS in the sample. The middle trace of Fig. 4 shows the same type of sample, however, now in the presence of SDS with a final concentration of 0.1%. The bottom trace of Fig. 4 corresponds to the sample as in the middle trace, however in this case after removal of SDS using the trapping column. The retention times of the peptides are almost identical to those obtained for the digest separation without any SDS. The recovery of the peptides is component dependent and was found to be between 85 and 95% for most peptides. Only in the case of one particular peptide fragment were recoveries of approximately 50% obtained. Apparently, a part of the peptide binds irreversibly to the SDS that is trapped on the SDS-trapping column. Identification of all the peptides is however only possible after removal of SDS. It must be noted that peptide mapping solely based on peak area patterns and the number of peaks (used, for example, to determine the consistency of protein production) can be strongly hampered by incomplete peptide recovery. However, with the use of the presented method the majority of the peptides can unambiguously be identified based on retention time and peak area.

The developed method can be extended to the automated on-line removal of non-ionic detergents and species, for sample concentration or for the de-salting of samples. By using micro precolumns with different chemistries (and appropriate sample loading and clean-up solvents), the removal of a wide range of interfering compounds can be addressed. The proposed system can be used for two-dimensional LC separations too. For these types of applications however a second gradient system would be required to fully accommodate such a

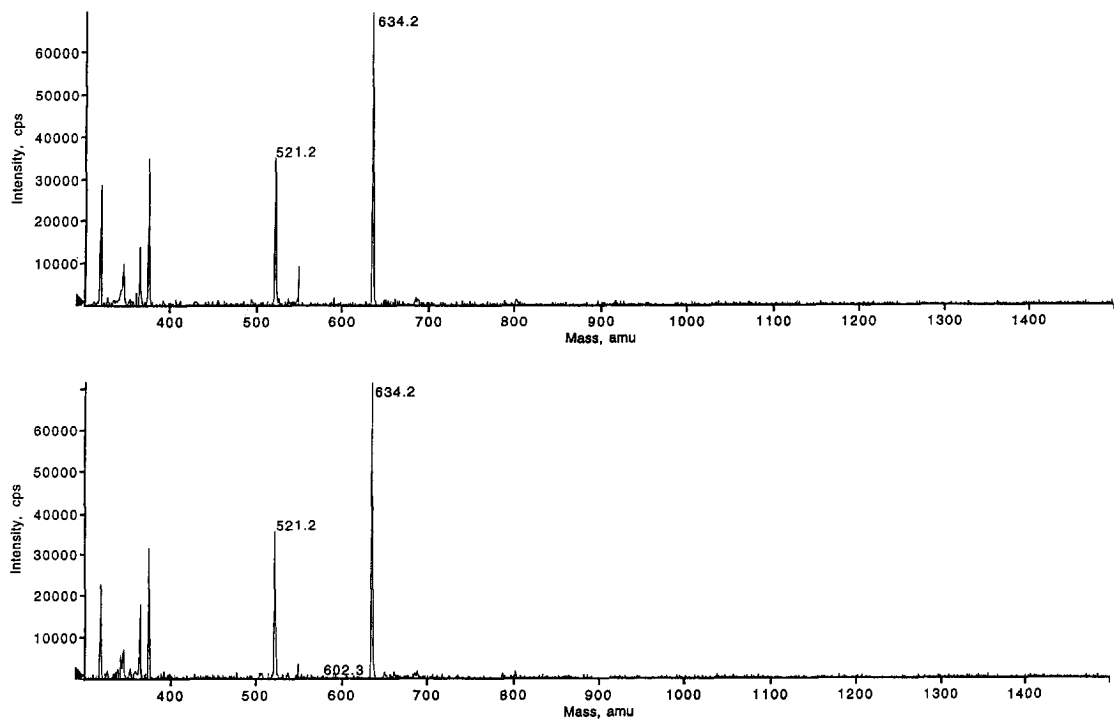

Fig. 5. Capillary LC–electrospray mass spectra of a typical digest peptide fragment in the absence of SDS (upper trace; reference electrospray mass spectrum) and after SDS removal (lower trace). The mass spectra correspond to the peaks labelled in black in Fig. 4.

system. Typical two-dimensional LC column set-ups are ion-exchange–reversed-phase and reversed-phase–reversed-phase.

3.6. Mass spectrometry

To confirm that SDS was completely removed from the tryptic digest samples, capillary LC–ESI-MS was performed. The collected ESI-MS spectra correspond to the peaks marked in black in Fig. 4. The ESI-MS spectra in the presence and absence of SDS are given in Fig. 5. All peak spectra were averaged and background-subtracted. The upper trace shows the spectrum acquired in the absence of SDS and the lower trace the spectrum after SDS has been removed from the sample. Very pure mass spectra were obtained and spectral integrity was preserved. Furthermore, no complex or adduct formation was observed, nor was a reduction in the signal intensity of the mass spectrometer.

A complete overview of the efficiency of the developed SDS-removal technique is given by the two-dimensional plot in Fig. 6 where m/z is depicted as a function of the retention time t_R. The upper graph shows m/z vs. t_R for tryptic-digested cytochrome c that is free of SDS. The middle graph shows the same sample type, however, in this case, in the presence of 0.1% SDS. The bottom graph of Fig. 6 corresponds to the same sample as in the middle trace of Fig. 6, however in this case, after removal of SDS using the trapping column. The middle trace shows the pronounced effect of SDS on retention time and mass assignment of the individual peptide fragments of the cytochrome c digest. Due to the complexation of SDS to the peptides, increased retention times and excessive broadened peaks are obtained. Furthermore, the formation of adducts can be observed by MS. The retention times and assigned masses of the peptides after the removal of SDS are almost identical when compared to the SDS-free reference sample.

The gain in sensitivity is shown by the mass

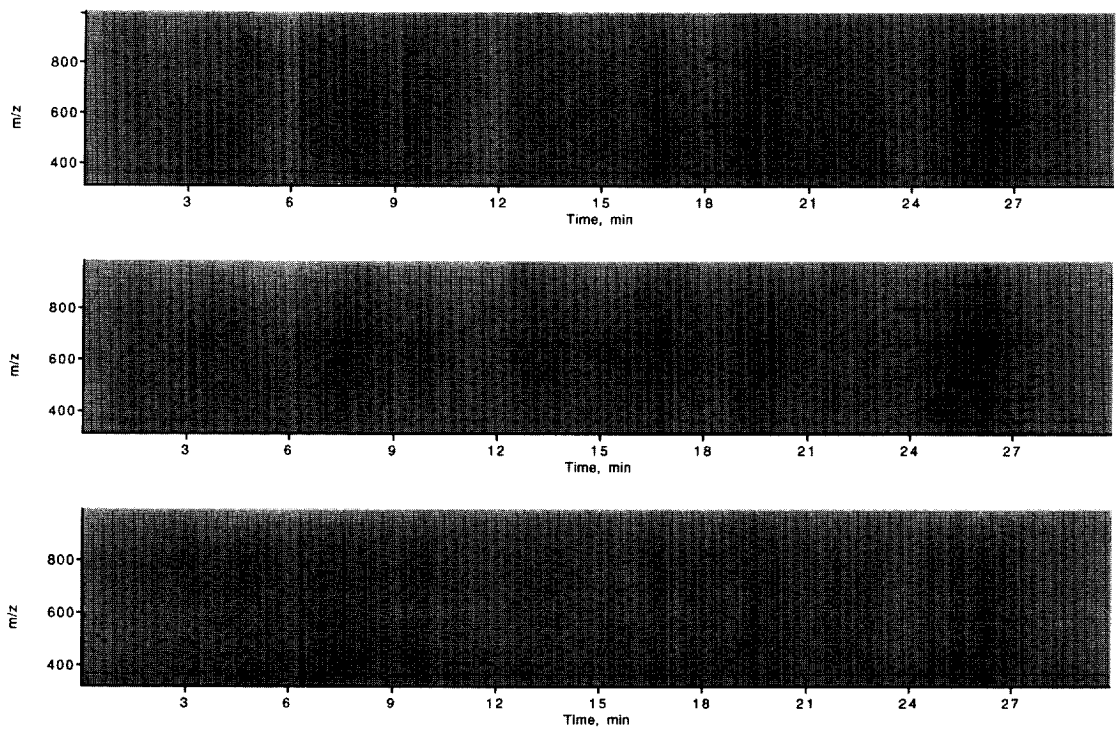

Fig. 6. Two-dimensional plot of m/z vs. retention time of tryptic digested cytochrome c. The upper graph shows the separation of a SDS-free, cytochrome c digest (reference graph). The middle graph corresponds to the separation of the same sample in the presence of 0.1% SDS. The bottom graph shows the separation of the cytochrome c digest after removal of SDS. Conditions as in Fig. 4.

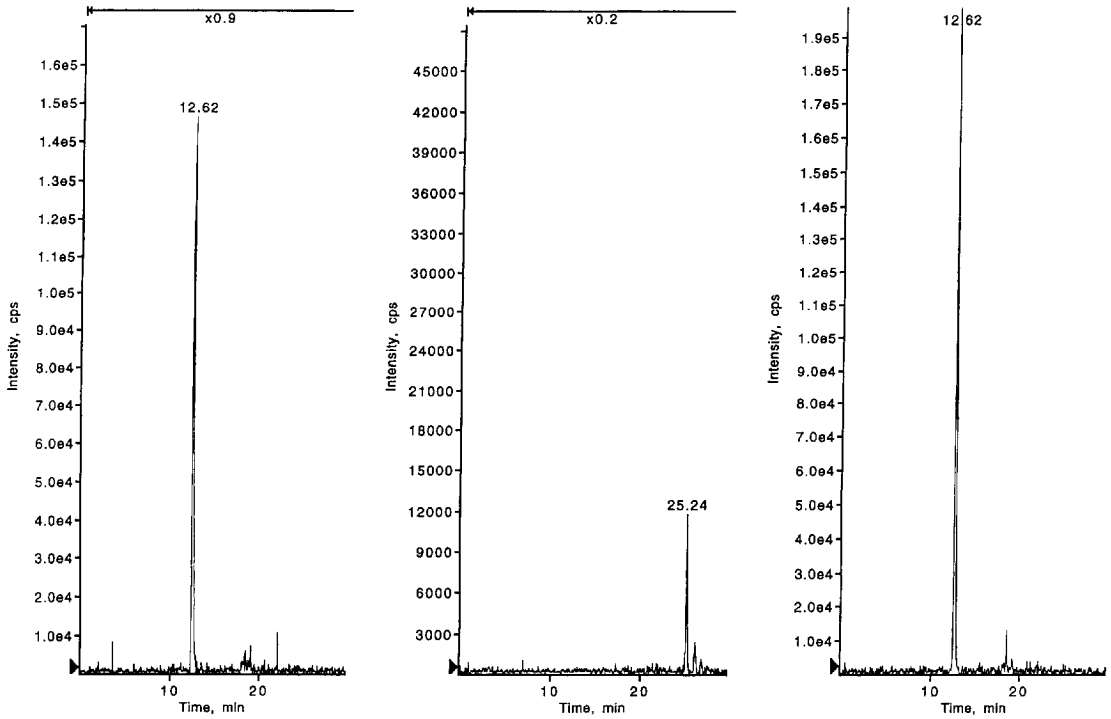

Fig. 7. Mass chromatograms of the selected m/z 634.2 ion for the cytochrome c tryptic digest that is free of SDS (left trace; reference mass chromatogram), in the presence of SDS (middle trace) and after removal of SDS (right trace).

chromatograms that are depicted in Fig. 7. The selected ion m/z 634.2 corresponds to the base peak of the mass spectra of Fig. 5. This ion is typical for the peaks labelled in black in Fig. 4. The left trace of Fig. 7 is the mass chromatogram of the cytochrome c tryptic digest in the absence of SDS and the middle trace is the mass chromatogram of the same sample in the presence of SDS. The right trace of Fig. 7 corresponds to the mass chromatogram after SDS has been removed from the sample. The calculated gain in sensitivity, using the SDS removal precolumn, is approximately three to four fold. The results given in Fig. 7 also show the enormous shift in retention as a result of the binding of SDS to the peptides and to the stationary phase.

4. Conclusions

The presented method allows for the automated and unattended on-line ionic detergent removal from protein digest samples in reversed-phase LC–ESI-MS. The data show that the separation capacity of the capillary analytical column can be completely preserved by using micro column switching techniques, i.e. a SDS trapping column, followed by a preconcentration column. Ionic detergents are selectively removed before the sample is applied to the capillary analytical system, thereby identification of the peptides is feasible solely based on retention. The removal of SDS is conducted on-line. Sample preparation, handling and transfer are therefore eliminated. The overall recovery was found to be component-dependent and ranged from 50 to 100%.

Mass spectral analysis confirmed the selective removal of SDS. Furthermore, no adduct formation was observed, indicating that the SDS was completely removed. Pure mass spectra were obtained allowing unambiguous identification of the peptide fragments. Finally, due to the selective removal of SDS, the gain in sensitivity (based on reconstructed selective-ion monitoring mass spectra) was by a factor 10 to 30.

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