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Journal of Chromatography A, 1067 (2005) 197–205
JOURNAL OF CHROMATOGRAPHY A
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Approaches for coupling solid-phase microextraction to nanospray

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Available online 8 December 2004

Abstract

Biocompatible solid-phase microextraction (SPME) devices were prepared using two restricted access materials (RAM) as the SPME coating. The restricted access materials were immobilized on steel and platinum wires. The selective coating eliminated most of the matrix interference, which allowed the coupling to mass spectrometry without further purification. The SPME devices were interfaced to mass spectrometry by electronanospray. Several experimental set-ups are described and discussed herein. For the in situ extraction of peptides from the tryptic digests, trypsin was immobilized both on steel wires and on the inside wall of a vial. The devices were incubated together with the RAM–SPME devices and a protein (casein) solution. After the protein digestion, the resulting peptides were analyzed by SPME/nanospray. The vial approach provided the best results; up to eight peptides could be identified which corresponds to a sequence coverage of 58%. The limit of detection of SPME/nanospray for the extraction of peptides from an aqueous solution was about 50 fmol/mL. The results demonstrate that the direct coupling of SPME to nanospray can reduce analysis time and is an attractive alternative to conventional approaches like Zip–Tip purification.

Keywords: Nanospray; Restricted access materials; Solid-phase microextraction

1. Introduction

Solid-phase microextraction (SPME) is a convenient and solventless extraction approach that offers many advantages over conventional analytical methods as it provides sampling, sample preparation and preconcentration in one step [1]. SPME reduces the time necessary for sample preparation, decreases the purchase and disposal cost of solvents and can improve detection limits. Therefore, the SPME technique is ideally suited for MS applications, combining a simple and efficient sample preparation method with good precision/accuracy and a very sensitive detection method [2]. It was previously demonstrated that by desorbing a SPME fiber directly inside an ion trap mass spectrometer, the sensitivity for the detection of toluene could be increased by two orders of magnitudes [3]. The direct coupling of SPME to mass spectrometry by fiber introduction mass spectrometry (FIMS) has also been reported for the analysis of volatile and semi-volatile compounds by Meurer et al. [4]. Using commercial fibers based on PDMS, this technique was suitable for the effective extraction of volatile and semi-volatile organic compounds (VOC, SVOC) and allowed simple introduction and thermal desorption directly into the ionisation region of a mass spectrometer [4].

Riter et al. demonstrated that SPME is suitable for direct coupling to a miniaturized portable mass spectrometer [5]. But with the current instrumental set-up, analysis is only suitable for on site measurement of VOCs in various matrices. To make the coupling of SPME to mass spectrometry suitable as a diagnostic tool in biomedicine, new approaches have to be developed for

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doi:10.1016/j.chroma.2004.11.047

the analysis of drugs and peptides from body fluids, for example.

The combination of SPME and MALDI for the analysis of biomolecules was recently introduced [6,7]. The elimination of matrix interferences, especially for the bioanalysis of body fluids, is crucial for mass spectrometry as they can lead to analyte suppression [8]. Newly developed biocompatible, tailor-made SPME extraction coatings like restricted access materials (RAM) eliminate most of the matrix interferences from body fluids and allow for the direct coupling to mass spectrometry without the time-consuming chromatography [9,10] step.

Similar to MALDI, mass spectrometry with electrospray ionization (ESI) allows for the fast analysis of drugs and polar biological molecules [11]. Nanoelectrospray (nanospray) has emerged as a promising ionization technique for the analysis of drugs and biological molecules because of its high ionization efficiency, low flow rates and high tolerance of salts compared to conventional electrospray (ESI) [12]. In this study, the direct coupling of SPME to electrospray/nanospray for the analysis of drugs and biomolecules is demonstrated.

For the direct coupling of SPME to electrospray and nanospray, SPME devices were coated with biocompatible restricted access materials containing C18 extraction centres (ADS) or strong cation exchange properties (XDS). The properties of these materials have been described previously [13,14]. The biocompatible device was used to extract drugs and peptides from biofluids and tryptic digests. For the latter, immobilized trypsin particles were coated on a wire and on the inside wall of a plastic vial. The devices are used to digest the protein α-casein. The resulting peptides are extracted by the RAM SPME devices and desorbed inside the nanospray tip.

2. Materials and methods

2.1. Materials

Stainless steel wires (147 μm diameter) were purchased from Small Parts Inc. (Miami Lakes, FL, USA); high temperature epoxy glue was obtained from Isolation Technologies Inc. (Hopedale, MA, USA); UV Loctite glue was purchased from Henkel (Duesseldorf, Germany); LiChrospher ADS-RP18 (alkyl diol silica) and XDS (strong cation exchange) particles (25 μm) were supplied by Merck KGaA (Darmstadt, Germany) as research samples.

Platinum wires (150 μm diameter were purchased from Sigma–Aldrich (St. Louis, MO, USA) Eppendorf-tips from Eppendorf (Hamburg, Germany).

Controlled pore glass (CPG)-aminopropyl-glutaraldehyde-trypsin was supplied by Karen Waldron (University of Montreal, Montreal, Que.). Diazepam was purchased from Cerilliant (Austin, Texas, USA), leucine enkephalin and protein casein (bovine milk) were purchased from Sigma (St. Louis, MO, USA), Zip–Tips C18 were purchased from Millipore (Billerica, MA, USA), ammonium carbonate from Fisher Scientific (Fair Lawn, NJ, USA), formic acid from Merck KgaA (Darmstadt, Germany), and trifluoro acetic acid from Caledon Laboratories (Georgetown, Ont., Canada).

Nanopure deionised water from a Barnstedt/Thermodyne NANOpure Ultrapure water system was used (Dubuque, IA) to prepare all solutions.

An Intel Play QX3 (Santa Clara, CA) digital microscope was used to monitor the coating.

2.2. Preparation of SPME devices for bioanalysis

New SPME fibers were prepared by applying a uniform layer of RAM slurry on the surface end of the stainless steel and platinum wires. The wires were previously processed as follows: they were cut in 8.5 cm segments, etched for 2 min in concentrated hydrochloric acid, immediately washed with water, thoroughly cleaned by sonication (first in methanol and then in water) and finally dried at 120 °C. RAM (0.2 g) (ADS or XDS) particles were transformed into slurry by means of 0.8 mol of a 75:25 mixture of epoxy glue and chloroform. To create the extraction phase the wires were dipped 1.5 cm deep into the slurry, followed by 1 h of baking at 180 °C, to ensure complete polymerization of the epoxy glue.

Blood and urine were obtained with consent from healthy volunteers that did not receive any medical treatment prior to giving blood; samples were collected into blood collection tubes (BD Vacutainer) that contained of sodium heparin and the blood samples were stored at −20 °C until analysis.

For the extraction, the sample was placed on an orbital shaking platform and the extracting phase on the surface end of the wires was immersed in the sample for exactly 5 min. Afterwards, the SPME device was rinsed with water and inserted into the interfaces described in Section 2.5.

2.3. Preparation of SPME devices for extraction of peptides from tryptic digests

Two devices were prepared for the tryptic digestion experiments. (1) Trypsin particle fiber: CPG-aminopropyl-glutaraldehyde-trypsin particles were immobilized on a stainless steel wire as follows: the immobilized trypsin particles were prepared and reported by Waldron and co-workers [15]. The stainless steel wires were dipped into Loctite glue then stripped off by passing it right through a conventional HPLC vial septum to obtain a very thin layer of glue. Afterwards, the wires were dipped 1.5 cm deep into a 0.25 mL micro vial filled with 1 mg of trypsin particles to obtain a thin layer of particles (coating length 1.5 cm) on them, followed by UV-irradiation for 15 min using a Loctite UV-lamp. Weighing confirmed that 0.9 g of particles are bound to the surface of the wire. (2) Trypsin particle vials: 2 mg of CPG particles were immobilized onto the inside wall of a 0.25 mL Eppendorf vial, using the Loctite glue followed by 15 min curing under UV.

The two devices were stored in 20 mM ammonium bicarbonate solution (pH 8) before use.

2.3.1. Trypsin particle fiber digestion

For digestion, a solution of casein, 0.2 mg/mL, was prepared in 20 mM ammonium bicarbonate buffer (pH 8). For the fibre device, the trypsin particle fiber and XDS fiber were dipped into a 0.25 mL vial containing the buffered protein solution. The SPME devices were placed close to the trypsin particle fiber to avoid a large diffusion barrier between the CPG-trypsin wire and XDS wire, and incubated at 37 °C for 24 h. The digestion was stopped after 24 h by adding 25 μL of 0.1% TFA. An ADS SPME fiber was then placed in the vial to extract the remaining digested peptides at room temperature for 1 h.

For the vial device, the trypsin-coated vial was filled with 0.2 mg/mL casein solution, followed by incubation at 37 °C for 24 h. An XDS SPME fiber was then placed in the vial to extract the digested peptides at room temperature for 1 h. For ADS SPME extraction, the solution was transferred into another 0.25 mL Eppendorf vial after digestion, and then acidified to pH 4.0 by adding 25 μL of 5% formic acid. The ADS fiber was then placed in the vial. For ADS extraction, the fibers were exposed to the sample solution for 1 h.

2.4. Zip–Tip purification

For comparison studies, experiments were repeated with 1 mg of loose CPG-trypsin particles in 0.2 mg/mL casein solution. The peptide digest was analyzed afterwards by Zip–Tip purification.

Zip–Tips were conditioned by aspirating and dispensing 50% ACN in water for five times.

Next, an equilibration solution of 0.1% TFA in water was aspirated and dispensed five times.

To extract peptides, the sample was aspirated and dispensed 10 times. Then the tip was washed using a 0.1% TFA solution. Peptides were eluted by aspirating and dispensing 3 μL of 50% ACN/0.1% formic acid five times. The peptide containing solution was transferred into a nanospray tip.

2.5. Methodology

Initial experiments for coupling SPME to electrospray were done on an Agilent MSD instrument (Delaware, USA). A scheme of a conventional ESI source is shown in Fig. 1.1A. The conventional ESI sprayer was removed and replaced by the modified one shown in Fig. 1.1B (Method I). Compared to the conventional ESI sprayer, the metal sheath was removed and the ESI capillary was replaced by a coated stainless steel wire (length 8.5 cm, diameter 147 μm) to which high voltage (3.5 kV) was applied. The dry gas flow was set to 8 L/min. The ion with *m*/*z* 287.5 $(M+H)^+$ of diazepam was monitored in SIM mode with an acquisition time of 200 ms.

As the outer metal sheath was removed, no nebulizer pressure could be applied to support ionization. A RAM coated steel wire was exposed to a diazepam solution for 5 min. After washing the wire in water for 1 min, it was inserted into the described holder. The solvent from the HPLC-pump (methanol/water 0.1% formic acid 70/30, v/v) formed droplets that run down the steel wire. The flow rate was ramped from 0.1 to 0.7 mL/min. At the bottom of the steel wire, the analytes were desorbed from the ADS-coating by solvent, and due to the applied electric field (3.5 kV), ions formed. After evaporation of the solvent, the ions were transmitted into the MS system.

For the second experiment (Method II), a conventional Agilent ESI sprayer was used and the ESI capillary was replaced by the coated SPME device (Fig. 1.1C). The solvent (methanol/water 0.1% formic acid 70/30, v/v) from the HPLC-pump (Agilent 1100) formed droplets that run down the steel wire. The flow rate was also ramped from 0.1 to 0.7 mL/min. Nebulizer pressure (30 psi) created a turbulent spray inside the nebulizer, which theoretically should help to desorb and ionize the analyte from the coating. Dry gas was maintained at a flow of 8 L/min.

For the next experiment (Method III) a Micromass Q-TOF Ultraflex (Manchester, UK) instrument with a dedicated nanospray source was used. A schematic of the extraction and desorption process, using Waters nanospray tips (Milford, MA, USA), is shown in Fig. 1.2. Platinum wires were coated with the RAM material. Diazepam (1, 5, 10, 200 ng/mL) was extracted with the RAM devices. A 1 mL pipette tip (Eppendorf, Hamburg, Germany) was fixed on the *x*, *y*, *z* stage to hold the nanospray tip. The nanospray tip filled with 3 μL of desorption solvent (ACN/water 0.1% formic acid 50/50, v/v), that was pushed through the hole of the Eppendorf tip to enable alignment in front of the MS inlet. Then, the platinum wire was carefully inserted into the nanospray tip for the analyte desorption. High voltage was applied to the coated platinum wire via an alligator clip. Nanoelectrospray was performed at 1.5 kV spray voltage, while the nanospray emitter was positioned ∼1 mm from inlet of the mass spectrometer using the *x*, *y*, *z* stage (see Fig. 1.3). Diazepam ions were scanned in the range of *m*/*z* 200–1000 with an acquisition time of 1 s and an interscan delay of 0.1 s in positive TOF MS Mode to detect possible cluster ions and matrix interferences. The optimized cone voltage for Diazepam was 25 V, respectively. The source temperature was set to 80 °C. Data collection and processing were performed using the Masslynx software.

For the peptide experiments, the cone voltage was set to 35 V. Data were scanned in the range of *m*/*z* 100–3000, with a scan time of 2 s and an interscan time of 0.1 s. The source temperature was maintained at 80 °C. Fifty scans were integrated for each experiment. Data were processed using the Masslynx software without prior smoothing of the data.

In the last experiment (Method IV), fibers were desorbed inside commercial nanospray tips. After a 30 s period of de-

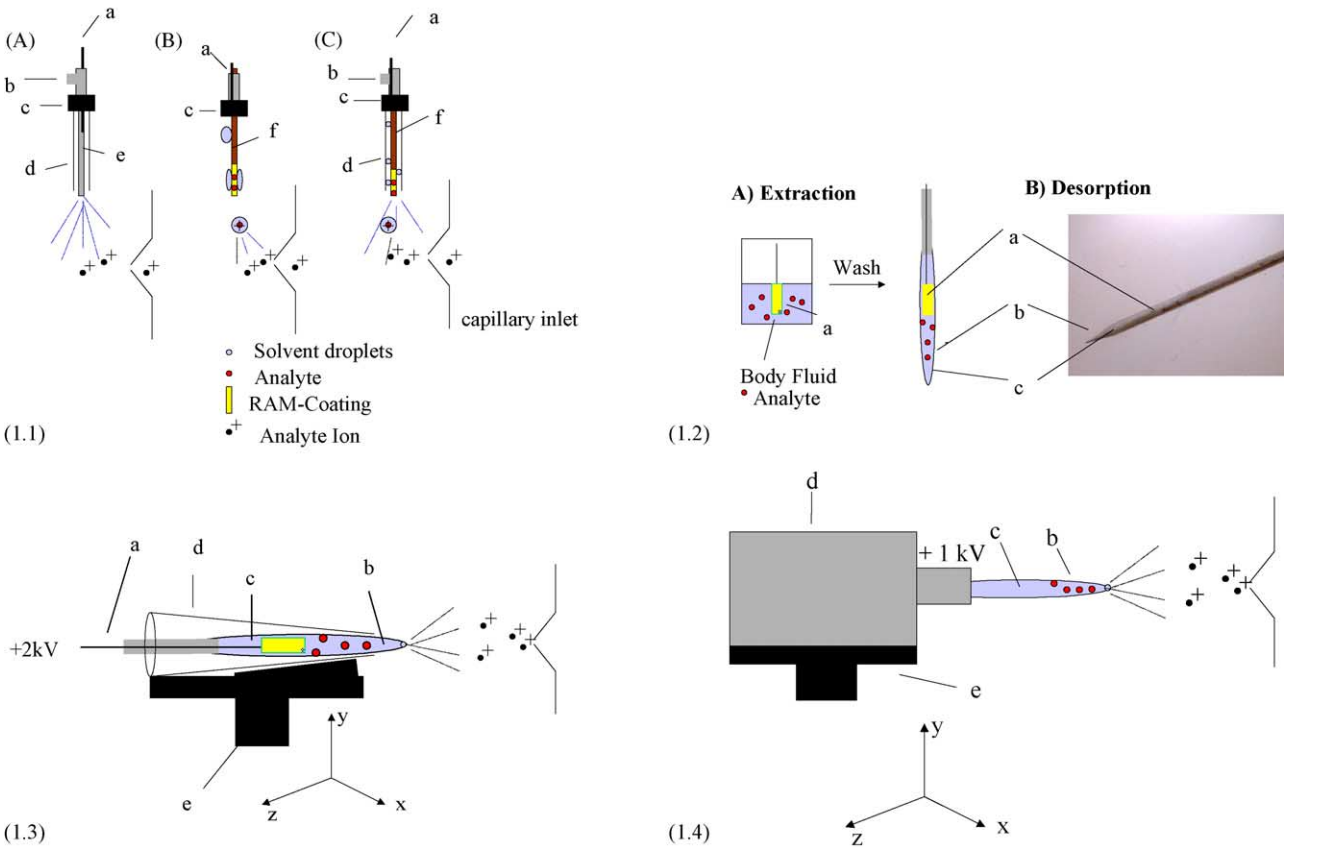

Fig. 1. (1.1) (A) Scheme of the commercial Agilent ESI source: (a) solvent inlet; (b) nebulizer pressure inlet; (c) septum; (d) stainless steel sheath; (e) electrospray capillary. (B) Scheme of modified sprayer with removed stainless steel sheath (Method I): (a) solvent inlet; (c) septum; (f) RAM-coated SPME device. (C) Scheme of modified sprayer (Method II): (a–d) see above; (f) RAM-coated SPME device. (1.2) (A) Scheme of SPME/nanospray extraction: (a) RAM-coated SPME device. (B) Scheme of SPME/nanospray desorption (digital image shows SPME/device inside nanospray tip with 20-fold magnification): (a) RAM-coated SPME device; (b) nanospray-tip; (c) desorption solvent. (1.3) Scheme showing desorption and ionization of analytes using of RAM coated platinum wires (Method III): (a) RAM coated platin wires; (b) nanospray-tip; (c) desorption solvent; (d) 1 mL Eppendorf tip; (e) *x*, *y*, *z* moveable stage. (1.4) Scheme showing ionization of analytes using the commercial nanospray source (Method IV): (b–c) see above; (d) commercial tip holder; (e) *x*, *y*, *z* moveable stage.

sorption, the devices were removed from the nanospray tips, and the tips were then inserted in the dedicated nanospray source of the instrument (see Fig. 1.4). For analysis, 1 kV was applied between the capillary and the nanospray tip. The other experimental conditions were maintained as previously described.

In addition to testing the diazepam solutions, aqueous solutions and body fluids (blood, urine) were spiked with leucine enkephalin in the range of 50–300 fmol/mL. The solutions were then extracted for 5 min. After washing the SPME-devices in water for 1 min, they were inserted into the nanospray tip for desorption.

For the peptide experiments cone voltage was set to 120 V. Data were scanned in the range of *m*/*z* 100–3000 with a scan time of 2 s and an interscan time of 0.1 s. The source temperature was kept at 80 °C. Fifty scans were integrated for each experiment. Data were processed using the Masslynx software without prior smoothing of the data. The peptide charges were determined by isotopic spacing.

3. Results and discussion

3.1. Fiber characterization

The extracting phase of the new SPME fibers consisted of RAM particles, which have been described previously [9,16]. These particles were retained on the stainless steel fiber by means of high temperature epoxy glue. Several binding procedures and adhesives were assessed for their chemical stability and mechanical strength. The epoxy glue proved to be chemically inert in contact with the mobile phase, providing at the same time enough physical endurance; a fiber could be used more than 150 times before a noticeable decrease in extraction capacity was observed. To immobilize the CPG-glutaraldehyde-trypsin-particles, Loctite glue was used, since the trypsin would have been destroyed by heating. In order to demonstrate the uniform distribution of RAM particles on the surface of the fiber, optical microscope images were recorded.

3.2. Experimental set-up

As outlined before, the direct insertion of SPME-fibers into a mass spectrometer could improve the sensitivity for the analysis of VOCs dramatically. As this approach is not suitable for polar, non volatile compounds, we considered the direct insertion of fibers into the electrospray ion source of LC–MS instruments appropriate for fast and sensitive determination of drugs and peptides. Several experimental set-ups were developed and are described in Section 2.5.

For Method I (see Fig. 1.1B), the SPME device was inserted directly into the ionization chamber of the mass spectrometer. A droplet running down the wire should desorb the analyte from the coating and assist the ionization of the analyte. In the first step, the flow rate of the mobile phase was optimized between 0.1 and 0.7 mL/min. A low flow rate should be suitable for this approach, as small single droplets are formed that desorb and preconcentrate the analyte in a small volume. In fact, one single droplet is not sufficient for a complete desorption of the analyte.

At low flow rates between 0.1 and 0.3 mL/min, multiple droplets are necessary to desorb the analyte, which leads to several peaks in the time resolved-acquisition (Fig. 2A). As not all analytes can be desorbed in one droplet, the sensitivity is reduced.

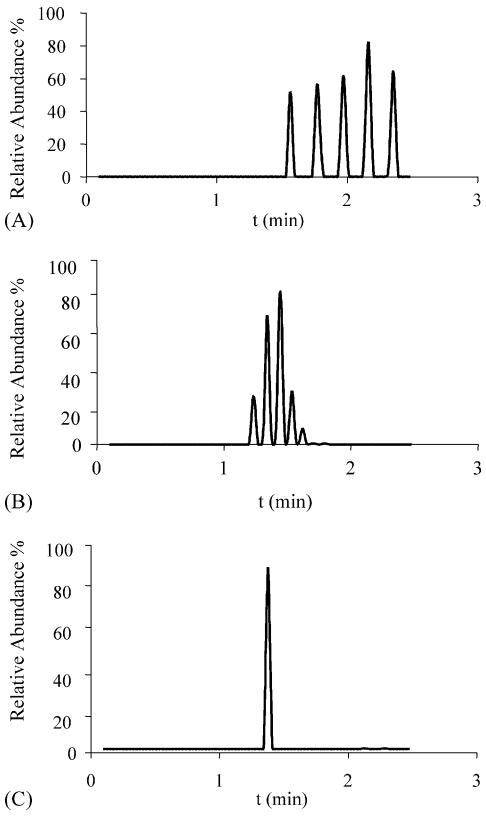

Fig. 2. Intensity–time-profile for desorption of diazepam (50 ng/mL) in dependence of flow rate: flow rate (A) 0.2 mL/min; (B) 0.3 mL/min; (C) 0.6 mL/min.

Droplet formation increases with increasing flow rates, up to 0.5 mL/min, so that the distance between the peaks in the intensity–time plot narrows (Fig. 2B). At the same time, the analyte is diluted in a higher solvent volume. At 0.6 mL/min, only one peak is observed in the intensity–time plot (Fig. 2C). Although analyte elution seems to be optimal at 0.6 mL/min since only one peak is observed, the MS ion abundance decreased with a corresponding increase in the flow rate. Diminished ion evaporation at higher flow rates might lead to a signal decrease in the MS.

To evaluate the reproducibility of Method I, an aqueous diazepam (50 ng/mL) solution was extracted for 5 min ($n = 5$) and diazepam was desorbed at a flow rate of 0.6 mL/min. The R.S.D. was measured at 14.5%. The method's linearity was evaluated for a concentration range from 500 to 2500 ng/mL. In this range, a good linear coefficient ($R^2 = 0.998$) could be obtained, but it has to be improved for lower concentration ranges. The alignment of the holder and the positioning of the wire were crucial for the method's performance, as the coating has to be wetted equally.

For Method II, the conventional Agilent ESI sprayer was used and the ESI capillary was replaced with the coated SPME device (see Fig. 1.1C). In contrast to Method I, nebulizer pressure was applied here to generate a small mist of droplets. It is known that the droplet size affects the ionization. But the addition of nebulizer gas did not improve the method's performance. In contrast to Method I, a sharp peak in the intensity–time plot could not be obtained (data not shown). The obtained peak always exhibited strong tailing, therefore the sensitivity and reproducibility were not evaluated. Even the modified desorption solvent (by increasing the methanol content from 70 to 90 vol.%) did not lead to an improved peak shape.

To overcome the low sensitivity observed, the SPME devices were desorbed inside a nanospray tip (Method III, Fig. 1.2). By desorbing the extracted analytes in a small amount of solvent, a high concentration factor and low detection limit should be obtained. At the same time, problems with the droplet size and the wetting of the coating are eliminated, as the whole coating is immersed in the desorption solvent.

Initial experiments were conducted with coated platinum wires, which were desorbed inside of nanospray tips (Fig. 1.3). For ionization, a high voltage (2 kV) was directly applied to the platinum wire. Direct ionization from platinum wires in nanospray capillaries has been reported previously by Van Berkel et al. [17]. The method is sufficient for the ionization of diazepam, but the disadvantage of this approach is that the applied electrical field affects the stability of the SPME coating. To overcome this problem, RAM-based SPME devices were desorbed inside the commercial nanospray tips, which were filled with 2 μL of desorption solvent (Method IV, Fig. 1.4). A desorption time of 30 s was sufficient to quantitatively desorb the analytes from the tip. The SPME devices were then removed, and nanospray tips

were inserted in the dedicated nanospray source of the instrument, followed by mass spectra acquisition.

The experimental set-up of Method IV is the most robust of the four tested, as it does not affect the stability of the SPME devices and can be used with commercial nanospray sources.

3.3. Applications in bioanalysis

The feasibility of SPME/nanospray (Method IV) is demonstrated in the field of peptide analysis. Bioanalysis of peptides is important, since some peptides, such as angiotensin I, II or leucine enkephalin, possess significant biological activity [18]. The properties of the RAM-based SPME devices to extract peptide like angiotensin I, II and III was demonstrated recently using an SPME–LC–MS interface [16]. The physiological concentrations of these peptides are in the lower pmol–fmol range (for angiotensin I 60–70 pM) and the sensitivity obtained with SPME coupled to LC/MS using single quadrupole instrument was not sufficient to determine the peptide concentration in whole blood samples.

Because the goal of the study was to demonstrate the feasibility of the SPME/nanospray approach for bioanalysis, whole blood and urine samples were spiked with the peptide leucine enkephalin, since this peptide is not naturally present in blood or urine and would not lead to a high background signal. Fig. 3 illustrates the spectra following extraction of blank and spiked urine samples. The spectra of urine samples contain some interference in the lower molecular range, which could not be eliminated by the usage of restricted access materials. The spectrum obtained from the 300 fmol/mL spiked urine sample shows presence of the molecular ion of leucine enkephalin ($[M+H]^+ = 556$) (Fig. 3). The LOD of the method is 50 fmol/mL for an aqueous solution, 250 fmol/mL for a spiked urine, and 300 fmol/mL for blood samples, which is in the physiological concentration range of peptides.

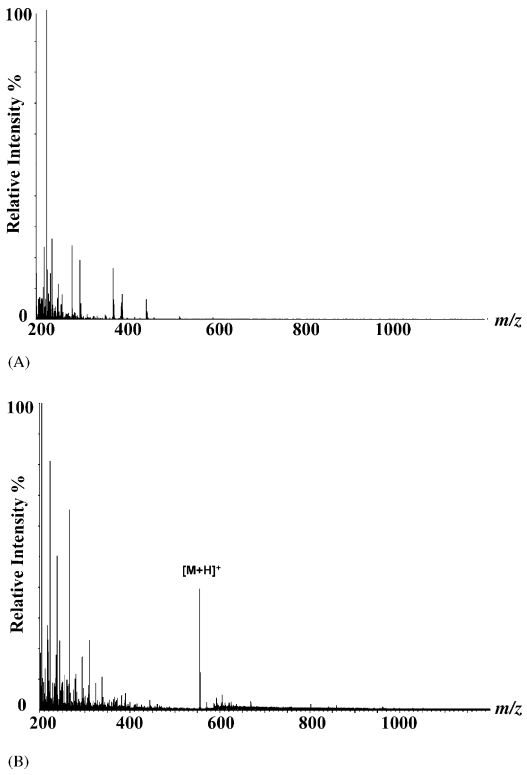

Fig. 3. SPME/nanospray: MS spectra after extraction of (A) blank urine; (B) spiked urine with 300 fmol/mL of leucine enkephalin $(M+H)^+ = 556$.

The SPME fibres can be miniaturized (by using smaller diameter particles), which facilitates an interesting tool for in-vein sampling [19]. Hence, SPME/nanospray with the coupling to mass spectrometers is an interesting approach for the monitoring of drugs in blood and other in vivo applications.

3.4. Application of SPME/nanospray for peptide mixtures from tryptic digestion

The feasibility of SPME/nanospray for the extraction of single peptides has been demonstrated, and the method's performance for the extraction of peptide mixtures was also evaluated.

The analysis of peptide mixtures (peptide mapping) from a tryptic digest is important for proteomics studies. For sequencing gel-separated-proteins, nanoelectrospray is a very sensitive, fast and reliable approach. Usually, proteins are analyzed after digestion by on-line capillary HPLC coupled to quadrupole TOF mass spectrometry [20]. However, the highest sensitivity is obtained by directly analyzing the peptide mixture in the nanospray emitter [21]. Zip–Tip purification is usually the method of choice for sample clean-up. In this study, we compared SPME/nanospray after tryptic digestion on fibers and in a coated vial with a conventional Zip–Tip method. For the initial studies, we used casein (MW 22974.87).

In this study, CPG-glutaraldehyde-trypsin particles were immobilized on steel wires with the help of a binding agent. The RAM-coated SPME device and the trypsin particle fiber were dipped close to each other in the casein solution for 24 h, since the protein digestion and peptide extraction should occur at the same time. As the digestion of protein casein was performed at pH 8 by dipping the trypsin device and the RAM (ADS/XDS)-based SPME device into protein solution for 24 h. Previous studies using this material in reactors show that digestion times of 24 h are suitable for a complete digestion [21]. As the peptide recovery of the ADS-based RAM was poor in neutral solution, the ADS coated wires were placed in an acidified solution (with 0.1 vol.% TFA), followed by extraction for 1 h. Desorption was then carried out on the RAM-based SPME fibre inside the nanospray tip.

The spectra are shown in Fig. 4. The first spectrum (Fig. 4A) shows the MS peaks obtained after digestion of casein with loose CPG-glutaraldehyde-trypsin particles in solution followed by Zip–Tip purification. Among these peptides, four matches could be identified that originated from the complete digestion of casein (see Table 1) by

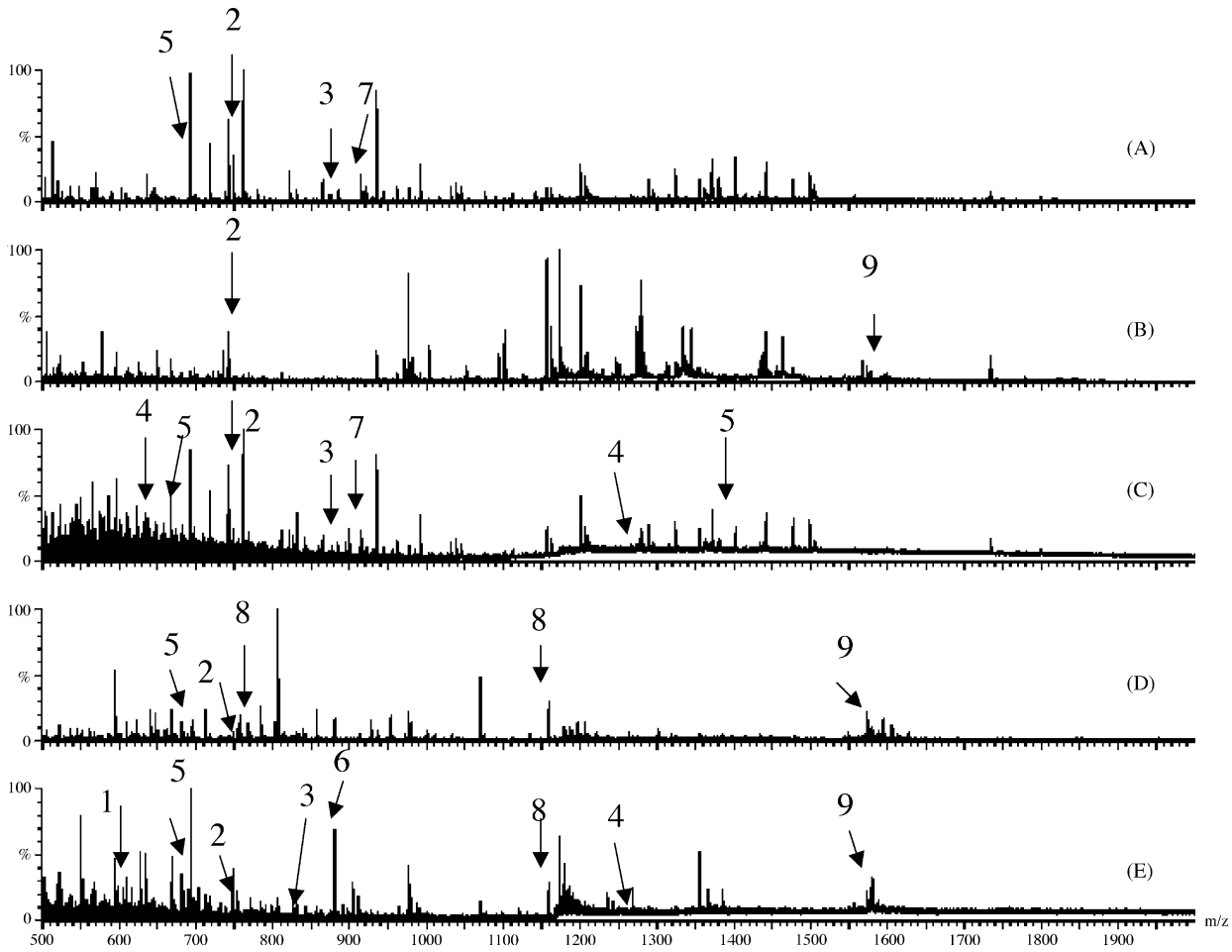

Fig. 4. MS spectra obtained from bovine α-casein tryptic digestion (A) Zip–Tip purification; (B) digestion using the first trypsin particles fibre device, extraction and purification using C18 ADS/SPME fiber; (C) digestion using the first trypsin particles fibre device, extraction and purification using XDS/SPME fibre; (D) digestion using the trypsin particles vial device, extraction and purification using C18 ADS/SPME fiber; (E) digestion using the trypsin particles vial device, extraction and purification using XDS/SPME fiber. For analysis, 200 scans were recorded and averaged. The number in the spectra refers to the identified peptide from Table 1.

Table 1
Molecular weight (MW) of identified peptides[a] originating from bovine α-casein tryptic digestion

No.	MW	Charge state	Position	Sequence	Trypsin particle solution	Trypsin particle fiber		Trypsin particle vial	
					Zip–Tip	ADS	XDS	ADS	XDS
1	614.321	+1	135–139	LHSMK					x
2	747.363	+1	209–214	TTMPLW	x	x	x	x	x
3	830.377	+1	99–105	EDVPSER	x		x		x
4	1266.697	+1/+2	106–115	YLGYLEQLLR			x		x
5	1383.730	+2	38–49	FFVAPFPEVFGK	x	x	x	x	x
6	1758.938	+2	23–37	HQGLPQEVLNENLLR					x
7	1766.752	+2	58–73	DIGSESTEDQAMEDIK	x		x		
8	2315.1296	+2/+3	148–166	EPMIGVNQELAYFYPELFR				x	x
9	4715.166	+3	176–208	QFYQLDAYPSGAWYYVPLGTQ YTDAPSFSDIPNPIGSENS EK		x		x	x
	Sequence coverage (%)				28	21	36	28	57
					28		42		57

[a] Molecular weights of the peptides were obtained from http://www.expasy.org.

comparing the peptides to a sequence from the database (http://www.expasy.org).

Fig. 4B and C shows the spectra obtained after digestion/extraction using the ADS/XDS and immobilized trypsin fiber devices; two sequence matches could be identified after extraction with ADS fiber and five matches with the XDS fiber. Although a comparable number of peptides could be identified using the ADS/XDS SPME devices, the better signal to noise (s/n) ratio was obtained following Zip–Tip purification. One reason for the improved s/n ratio following Zip–Tip purification could be that a larger volume of CPG-trypsin particles were used for digestion. In addition, the glue curing procedure might affect the activity of the immobilized trypsin (e.g. less peptides are formed), which could also contribute to a reduction in the s/n ratio. The s/n ratio of the spectrum in Fig. 4B looks much better than in Fig. 4C, although with the XDS/SPME fiber much more peptides could be identified.

The XDS/SPME fiber seems to be more specific towards the digested peptides while the ADS/SPME result in interferences, which appear as highly abundant ions in the mass spectrum.

Fig. 4D and E shows the spectra obtained after ADS/XDS extraction from the immobilized CGP-glutaraldehyde-trypsin-containing vials. Up to eight matches could be identified using these vial devices. The sequence coverage (57%) and s/n ratio of these spectra is even better to the one obtained after Zip–Tip purification (28%, see Fig. 4A and Table 1).

Although the XDS fibers were exposed for 24 h to the protein solution for the trypsin fiber approach, compared to 1 h for the trypsin vial approach, the s/n ratio and sequence coverage is improved with the trypsin vial approach. Because more trypsin particles are used for the trypsin vial approach, more peptides might be formed during the digestion process, which would result in higher levels of signal abundancies for the peptides. As SPME is an equilibrium-based extraction technique, longer extraction times for the fiber approach should not affect the peptide recovery.

In this small trypsin microreactor, the peptides of the protein are released in a very small volume after digestion. Extraction from this volume leads to a high preconcentration on the SPME RAM device compared to Zip–Tip purification. Theoretically, an improved preconcentration factor could be obtained when both trypsin and RAM particles are immobilized on the same fiber and the digestion/extraction are performed in a very small volume. The diameter of the trypsin particles does not allow a direct insertion of the trypsin fiber into the nanospray tip. We currently work on smaller size of particles to enable digestion of proteins and extraction of peptides on the same fiber, without losing the coating during desorption inside the nanospray tip.

For the SPME/nanospray method, different extraction phases can be synthesized complementarily. Fig. 4 demonstrates that the XDS extraction phases extract different peptides than the ADS C18 extraction phase. Custom-made mixed mode SPME extraction phases (C4, C8, C18, cation exchange, anion exchange) will enable the identification of a wide variety of peptides.

In addition, the SPME/nanospray approach can be automated. Multiple fibers and 96-well plates could be coated with different extraction phase and immobilized trypsin, so that robot-controlled analysis is feasible similar to the commercial nanospray-based robotic procedures [22]. Automation and high sample throughput are very important for proteomic studies, as often several thousand proteins from 2D gels have to be characterized.

4. Conclusions

The coupling of SPME to electrospray and nanospray has been successfully demonstrated for several experimental set-ups. Among these experimental set-ups evaluated, the desorption of SPME-devices inside of commercial nanospray tips was the most successful.

The RAM-based SPME coating eliminates most matrix interferences like salts and high molecular weight proteins, allowing direct coupling to mass spectrometry. The coupling with nanospray allows fast determination of peptides in body fluids and tryptic digests with a good limit of detection down to 50 fmol/mL in aqueous solution. Lower detection limits can be expected when tandem MS is used. For the tryptic digestion of proteins, the immobilization of trypsin on the inside walls of a vial followed by extraction with a RAM–SPME device lead to the best results. Using this method, improved sequence coverages could be obtained than with the conventional Zip–Tip purification. The combination of different extraction phases (C18, cation exchange) and functionalization of fibers using immobilized enzymes like trypsin, as well as the in vivo extraction capability and—miniaturized—properties (smaller particle diameter) make SPME/nanospray an interesting tool for the bioanalysis of drugs, peptides and small proteins up to 15,000 Da. Currently, the automation capability of the method is being explored for proteomic applications.

Acknowledgement

The author's would like to thank NSERC for financial support and the German Academic Exchange Service (DAAD) for postdoctoral fellowship support for Markus Walles.

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