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Identification of various urinary metabolites of fluorene using derivatization solid-phase microextraction

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Abstract

A method for the qualitative analysis of various metabolites of fluorene is presented. The method uses solid-phase microextraction with an 85 μm polyacrylate fiber for extraction, headspace silylation with BSTFA and MTBSTFA without any catalyst for on-fiber derivatization and GC–MS in the selected ion monitoring mode for separation and detection. The suitability of the method for profile analysis of polycyclic aromatic hydrocarbon metabolites is shown by analyzing an urine of an occupationally exposed person after enzymatic cleavage of the excreted conjugates. Satisfactory separation of all investigated metabolites was achieved without interferences due to matrix peaks.

Keywords: Derivatization, GC; Fluorene

1. Introduction

As part of the every day background, polycyclic aromatic hydrocarbons (PAHs) steadily contribute to environmental pollution. PAHs were recognized as carcinogens by the International Agency for Research on Cancer in 1983 [1]. Although PAHs represent a very complex mixture of various compounds (900 of them are characterized), some substances predominate, depending on the source. Naphthalene, followed by fluorene, often largely contributes by mass to the total load of PAHs, e.g., in environmental tobacco smoke [2]. Fluorene is part of the occupational exposure in certain working places like coking plants or road construction. In the human body these substances are extensively metabolized leading predominantly to oxygenated compounds like phenols, dihydrodiols and higher oxidized products.

The determination of metabolites of hazardous substances in humans (''human biomonitoring'') is a feasible way to assess the total exposure to the parent compounds. Certainly the simultaneous detection of several metabolites provides a more comprehensive picture of the exposure and thus helps in uncovering possible sources of exposure [3,4].

Analysis of PAH metabolites mainly relies upon solid-phase extraction (SPE) – also providing some

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clean up effect – and quantification by high-performance liquid chromatography (HPLC). Most methods conform to the work published by Jongeneelen et al. in 1987 [5]. More sophisticated procedures like coupled column HPLC or liquid chromatography–mass spectrometric (LC–MS) techniques gained importance during the last years [6,7].

Fluorene, one of the most abundant PAHs originating from different sources, is not considered a carcinogen and mutagen on the basis of experimental data [1] – it is rated as non carcinogenic by Lee et al. [8]. Among the 17 PAHs determined in the tobacco smoke condensate of the reference cigarette 1R4F [2], fluorene ranks second after naphthalene with about 17% (w/w).

There are a few scientific publications dealing with the metabolism of fluorene. Most of them concentrate on environmental degradation of fluorene by bacteria in contaminated soils. Oxygenated metabolites, phenols and ketones, were found in airborne particulate matter, too [9]. But so far the identification and quantification of fluorene metabolites in urine was not reported in the accessible literature.

This paper deals with the determination of hydroxylated fluorenes in the urine of occupationally exposed persons: solid-phase microextraction (SPME) of the target compounds was followed by on-fiber derivatization and detection by gas chromatography–mass spectrometry (GC–MS) [10]. Silylation in combination with SPME was first reported by Snow et al. [11] and adapted to the analysis of PAH metabolites in our laboratory [10]. The method combines the speed of a screening method with the high sensitivity desirable for determining the minor concentrations to be expected.

Reference compounds not commercially available were specifically synthesized to provide an unambiguous identification of the target analytes in any case.

2. Experimental

2.1. Reference compounds and reagents

2- and 9-hydroxyfluorenes were obtained from Sigma–Aldrich (Vienna, Austria). 2-Hydroxy-5,6,7,8-tetrahydrophenanthrene was a gift from the Institute for Analytical Chemistry, University of Vienna, Austria, and was used as internal standard. 85 μm polyacrylate SPME fibers, fiber holders, *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and *N*- (*tert*.-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) were obtained from Supelco (Bellafonte, PA, USA). β-Glucuronidase (approx. 100 000 units/ml) and sulfatase (up to 5,000 units/ml) from *Helix pomatia* were obtained from Sigma (St. Louis, MO, USA), acetonitrile from Merck (Darmstadt, Germany).

2.2. Synthesis of reference compounds

1- and 4-hydroxyfluorenes were synthesized according to the following steps.

2.2.1. 1-Fluorenamine (analogous to Smith et al. [12])

A 2.5-g amount of 1-fluorenecarbonic acid was refluxed in 50 ml of thionylchloride for 2 h. Surplus thionylchloride was distilled off and the residue was diluted with acetone before a solution of 1.55 g of sodium azide in 5 ml of water was added. Further, 25 ml of water was added and the mixture was stored overnight in the refrigerator. The solid was filtered off, washed with water, dissolved in 30 ml of benzene and refluxed for 1.5 h. After addition of 12 ml of a 50% solution of KOH in water, the reaction mixture was refluxed for 22 h, then distributed between water and benzene and the aqueous layer was extracted twice with benzene. The combined organic phases were dried with sodium sulfate, filtered and the solvent was evaporated. The red crystals (1.62 g; 75%) were used for the following reaction without further purification.

2.2.2. 1-Hydroxyfluorene (analogous to Grantham et al. [13])

A solution of 1 g of sodium nitrite in 10 ml of water was added under cooling with ice to a suspension of 1.62 g of 1-fluorenamine, 30 ml of glacial acid, 30 ml of water and 15 ml of conc. sulfuric acid. After about half an hour 2.3 g of urea and after additional 15 min 350 ml of boiling 1.75 *M* sulfuric acid was added. The mixture was allowed to cool

down, the precipitate (0.27 g) was filtered off and recrystallized from water.

^{1}H-Nuclear magnetic resonance (NMR) ($CDCl_3$): δ (ppm)=7.83–7.79 (d, 1H, J=7.04 Hz, 5-H), 7.59–7.56 (d, 1H, J=6.78 Hz, 8-H), 7.47–7.27 (m, 4H, 3-, 4-, 6- and 7-H), 6.82–6.78 (d, 1H, J=7.72 Hz, 2-H), 5.35 (s, 1H, OH), 3.85 (s, 2H, 9-H).

2.2.3. 4-Fluorenamine (according to Smith et al. [12])

A 0.106-g amount of 4-fluorenecarbonic acid was refluxed in 4 ml of thionylchloride for 2 h. Surplus thionylchloride was distilled off and the residue was diluted with acetone before a solution of 0.07 g of sodium azide in 0.3 ml of water was added. Further 1.1 ml of water was added and the mixture was stored overnight in the refrigerator. The solid was filtered off, washed with water, dissolved in 1.3 ml of benzene and refluxed for 0.5 h. After addition of 0.55 ml of a 50% aqueous solution of KOH, the reaction mixture was refluxed for 18 h, then distributed between water and benzene and the aqueous layer was extracted twice with benzene. The combined organic phases were dried with sodium sulfate, filtered and the solvent was evaporated. The red crystals (0.09 g; quant.) were used for the following reaction without further purification.

2.2.4. 4-Hydroxyfluorene (according to Grantham et al. [13])

A solution of 0.054 g of sodium nitrite in 0.54 ml of water was added under cooling with ice to a suspension of 0.09 g of 4-fluorenamine, 1.8 ml of glacial acid, 1.8 ml of water and 0.9 ml of conc. sulfuric acid. After about half an hour 0.126 g of urea and after additional 15 min 20 ml of boiling 1.75 *M* sulfuric acid was added. The mixture was allowed to cool down and was extracted with benzene. The combined organic phases were extracted with a 2 *M* sodium hydroxide solution, the aqueous phase was acidified and was reextracted with benzene. The organic layer was dried with sodium sulfate, filtered and the solvent was evaporated. The red residue was boiled in light petroleum and the solution was filtered and evaporated to yield 53 mg of the product.

^{1}H-NMR ($CDCl_3$): δ (ppm)=8.15–8.11 (d, 1H, J=7.58 Hz, 8-H), 7.55–7.52 (d, 1H, J=7.06 Hz, 5-H), 7.44–7.14 (m, 4H, 1-, 2-, 6- and 7-H), 6.76–6.72 (d, 1H, 3-H), 5.25 (s, 1H, –OH), 3.93 (s, 2H, 9-H).

2.3. Hydrolysis and derivatization of urine samples

Urine samples were first adjusted to pH 5 with 0.1 *M* acetate buffer (pH 5). After adding 1 μl enzyme solution per ml urine, urine samples were hydrolyzed enzymatically with a mixture of β-glucuronidase and sulfatase at 37°C overnight and stored at −20°C until analysis.

SPME was performed by immersing the fiber into 3 ml sample liquid after addition of 5 μl internal standard solution (1 μg/ml) overnight at 35°C under magnetic stirring. Following extraction, the fiber was washed for about 1 min in deionized water and dried with a paper towel. Derivatization took place by exposing the fiber to the headspace in a 2 ml autosampler vial for 30 min at 60°C. The vial had been previously prepared by injecting 1 μl derivatizing reagent. Finally the fiber was transferred to the hot injection port of the GC system and desorbed for 3 min.

2.4. GC–MS analysis

The GC–MS system comprised a Trace 2000 gas chromatograph and a Voyager mass spectrometer (Thermo Quest, Austin, TX, USA). Separation was performed on a DB1 fused-silica capillary column, 15 m×0.2 mm I.D., 0.11 μm film thickness (J&W Scientific, Folsom, CA, USA). Carrier gas was helium with an inlet pressure set to 100 kPa. The GC temperature program was: 100°C held for 1 min, 15°C/min to 160°C, 10°C/min to 315°C, held for 4 min. The split/splitless injector, equipped with a 3 mm I.D. glass liner, was set to 270°C. The desorption of the fiber took place during the splitless period of 3 min.

The mass spectrometer was operated in the electron impact (EI) mode at an ionization energy of 70 eV. The ion source was set to 250°C and the GC–MS interface to 280°C. In addition to analyses in the scan mode, for higher sensitivity selected ion monitoring (SIM) was applied. In this mode, two ion masses for each compound (as summarized in Table 1) were

Table 1
Composition of the spiking solution and essential key figures for spiked buffer

Compound	CON (μg/ml)	Masses (Dalton)	LOD (μg/l)	CC	SDP (%)	LWR (μg/l)	UWR (μg/l)
1-Hydroxyfluorene	1.00	254/239	0.03	0.995	8.2	1.7	16.7
2-Hydroxyfluorene	0.90	254/239	0.03	0.997	9.3	1.5	15.0
4-Hydroxyfluorene	1.50	254/239	0.04	0.995	10.4	2.5	25.0
9-Hydroxyfluorene	1.68	254/239	0.06	0.998	6.5	2.8	28.0
2-Hydroxy-5,6,7,8-tetrahydrophenanthrene (I.S.)	0.66	270/255	0.05	–		–	–

recorded, resulting in a much better signal/noise ratio compared to scanning acquisition.

3. Results and discussion

3.1. Analysis of reference compounds

Table 1 shows composition and concentrations (CON) of the individual compounds in the spiking solution (solvent: acetonitrile) used for identification of hydroxyfluorenes in urine samples. In addition are given: the ion masses for SIM detection of the silylated target hydroxyfluorenes, the limits of detection (LOD), the correlation coefficient (CC), the standard deviation of the entire procedure (SDP) and the lower and upper working range limits (LWR, UWR, respectively). These figures are determined according to a German calibration standard [14]. Calibration solutions were prepared by spiking 3 ml buffer with the appropriate volume of the spiking solution within the working range. Buffer was chosen for calibration purposes, because we could not find a urine without traces of 2-hydroxyfluorene within the scope of this work.

3.2. Identification of monohydroxylated metabolites

Consistent with the chemical structure of fluorene, there are five different monohydroxylated metabolites possible without additional hydration or oxidation of the rest of the molecule. As shown in Fig. 1, hydroxylation might occur in positions 1, 2, 3, 4 or 9.

Monohydroxylated fluorene metabolites were found in the urine of a person occupationally exposed to PAHs. The analysis was performed by SPME and on-fiber derivatization as outlined in Section 2.3. Identification was based on retention times and mass spectra, which were compared to data obtained from the following silylated reference substances: 2- and 9-hydroxyfluorenes, as already mentioned, were of commercial origin, 1- and 4-hydroxyfluorenes were synthesized via a two-step procedure from the corresponding carboxylic acids (Section 2.2).

3.3. Derivatization with BSTFA

All mass chromatograms in Fig. 2 were obtained by GC–MS analysis in the scanning mode. The sample preparation involved extraction by SPME and on-fiber silylation with BSTFA (as described in Sections 2.3 and 2.4). The ion 254 is the molecular ion (M^+) of silyl-hydroxyfluorenes. Traces (A) and (C) in Fig. 2 show the total ion chromatogram (TIC) and the mass trace 254 from a buffer solution, spiked with the reference compounds for four of the five

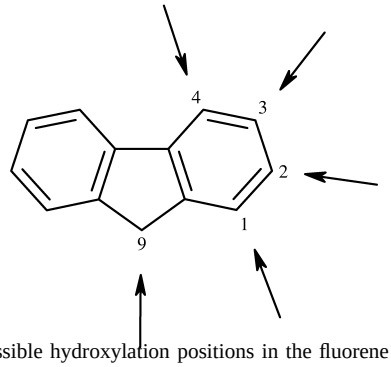

Fig. 1. Possible hydroxylation positions in the fluorene molecule.

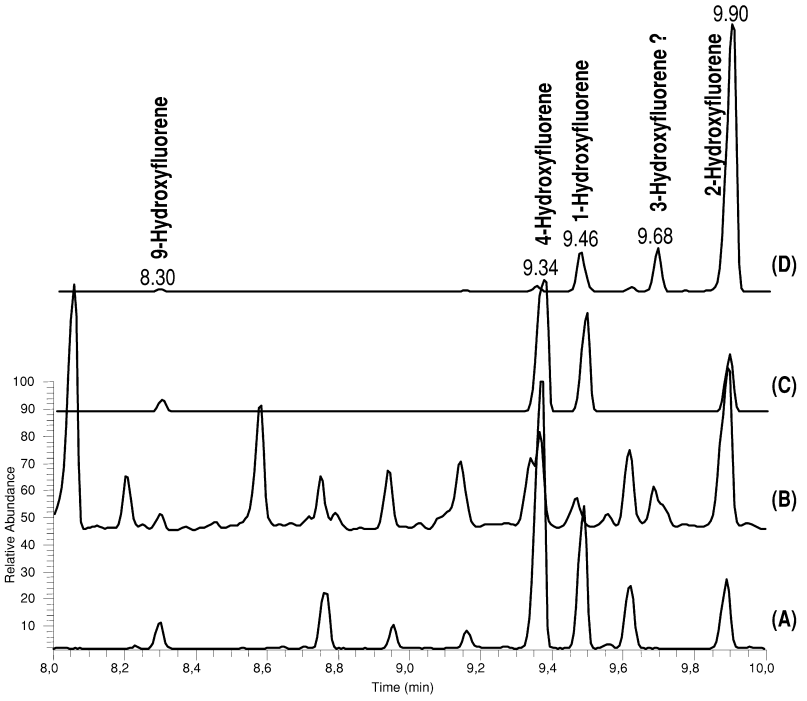

Fig. 2. GC–MS analysis after SPME sampling and derivatization with BSTFA: scanned acquisition as described in Section 2.4. (A) TIC and (C) mass trace 254 of a buffer solution, spiked with 1-, 2-, 4-, and 9-hydroxyfluorene at the upper working range. (B) TIC and (D) mass trace 239 of a urine sample, obtained from a worker occupationally exposed to PAHs.

possible metabolites: 1-, 2-, 4-, and 9-hydroxyfluorene.

(B) and (D) in Fig. 2 show the corresponding traces of an urine obtained from a worker occupationally exposed to PAHs. In the traces of the urine sample signals appear at retention times coinciding with the reference peaks in trace (C), thus allowing the tentative identification of the two most abundant unknowns as 2-hydroxyfluorene (retention time, t_R= 9.90 min) and 1-hydroxyfluorene (t_R=9.46 min). This identification is confirmed by the perfect match of the corresponding mass spectra in Figs. 3 and 4: (A) shows the reference solutions' mass spectrum, (B) the urine samples'.

Furthermore, the small but clearly observable signals in the urine traces in Fig. 2 appearing at t_R=9.34 min coincide with 4-hydroxyfluorene. Yet in the respective mass spectrum (Fig. 5B), the most abundant masses of the reference spectrum (223, 239, and 254 in Fig. 5A) are still present, but not as dominant as in the standard and accompanied by a lot of other peaks. This finding points to a co-eluting background substance.

To nevertheless achieve an unambiguous identification of the presumed metabolite, a complementary derivatization was tried using MTBSTFA as outlined in Section 3.4.

In trace (D) of Fig. 2 at t_R=9.68 min another weak signal shows up in the urine traces. The respective mass spectrum, reproduced in Fig. 6, exhibits a notable similarity to the other TMS-hydroxyfluorene spectra. This explicitly points to the only missing monophenolic metabolite 3-hydroxyfluorene.

The SPME method described so far using BSTFA as a derivatization reagent, is not very sensitive for 9-hydroxyfluorene: the slope of its calibration curve is only about 0.24 compared to 0.85 for 1-hydroxyfluorene. Fig. 7 presents mass spectra of 9-hydroxyfluorene (t_R=8.30 min), showing comparable frag-

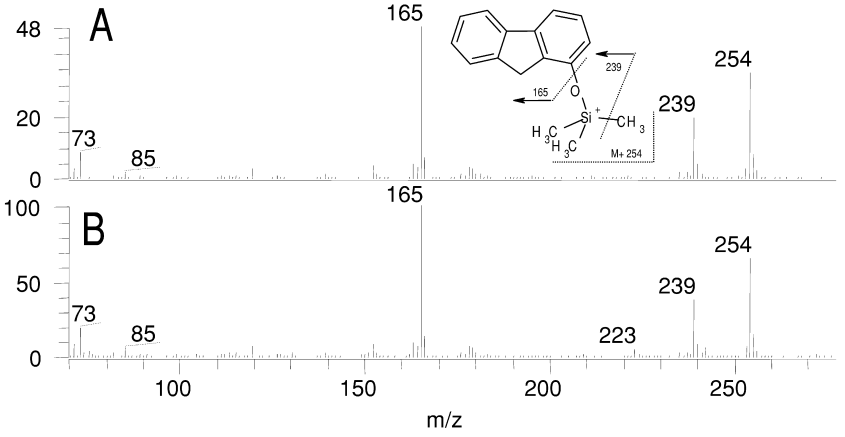

Fig. 3. Mass spectra of 1-hydroxyfluorene TMS ether after SPME and derivatization with BSTFA: (A) standard and (B) urine sample.

mentation, but differences in the relative abundances of the fragment ions.

3.4. Silylation with MTBSTFA

To confirm the presence of monohydroxylated fluorene metabolites, a supplementing silylation was performed using MTBSTFA as derivatization reagent. The conditions were the same as for BSTFA, viz. 1 μl reagent and a 30-min derivatization time at 60°C. By the way, the fibers proved more stable by using MTBSTFA instead of BSTFA. One reason for that finding might be the lower vapor pressure of MTBSTFA, resulting in a smaller amount of reagent on the fiber.

In the resulting mass chromatograms shown in Fig. 8 three peaks could easily be identified, viz. 4-hydroxyfluorene (t_R=11.72 min), 1-hydroxyfluorene (t_R=11.96 min), and 2-hydroxyfluorene (t_R=12.30 min). No signals in any of the traces could be assigned to 9-hydroxyfluorene. As in silylation using BSTFA, 1- and 2-hydroxyfluorenes are the

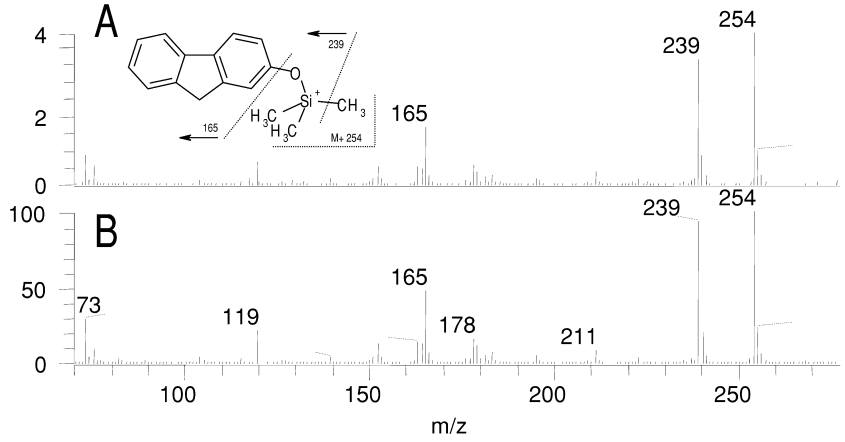

Fig. 4. Mass spectra of 2-hydroxyfluorene TMS ether after SPME and derivatization with BSTFA: (A) standard and (B) urine sample.

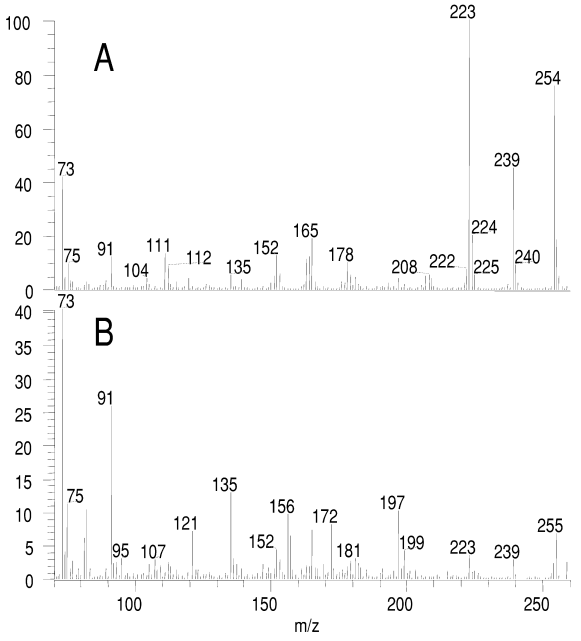

Fig. 5. Mass spectra of 4-hydroxyfluorene after SPME and on-fibre derivatization with BSTFA: (A) standard and (B) urine sample.

most abundant metabolites found in urine and their mass spectra show a good correlation to the standards.

The mass spectrum of the spurious peak appearing at the t_R of 4-hydroxyfluorene matches very well with the corresponding reference compound (Fig. 9) and thus proves the presence of this metabolite in the urine of the person under study.

The mass spectrum of 4-hydroxyfluorene highlights an interesting detail, as its base peak, mass 223, does not appear at all in the spectra of the other hydroxyfluorenes. As described by Jacob et al. [15], this fragment arises by cyclization involving the silyl group as shown in Fig. 10. It appears after silylation using BSTFA as well as MTBSTFA and – in the case of the parent compound fluorene – only can form in the 4-hydroxy position.

Finally, considering the signals at t_R=12.04 min in Fig. 8 and the respective mass spectrum shown in Fig. 11, this component can be presumed to be 3-hydroxyfluorene without ambiguity: the spectrum exhibits all characteristics typical for the TBDMS derivatives of the other hydroxyfluorenes.

3.5. Quantification

Calibration solutions were prepared by dilution of the mixture described in Table 1. These solutions as well as the samples contained 2-hydroxy-5,6,7,8-tetrahydrophenanthrene as internal standard. The detection limits for each detected metabolite were calculated using a 10-point calibration curve over a range of two orders of a magnitude. For sample quantification, a three-point calibration (forced though the origin) was used. Prior to analysis, the urine samples were diluted with buffer solution to meet the dynamic range of the calibration (the

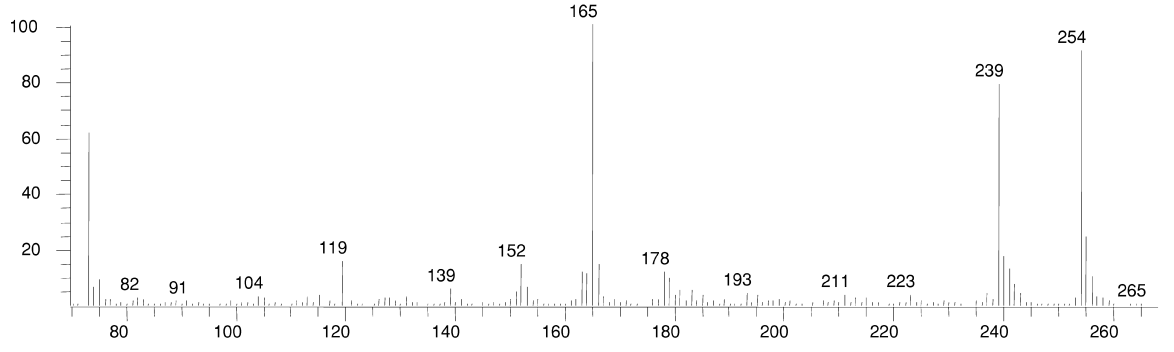

Fig. 6. Mass spectra of the substance, eluting at t_R=9.68 min after SPME and derivatization with BSTFA.

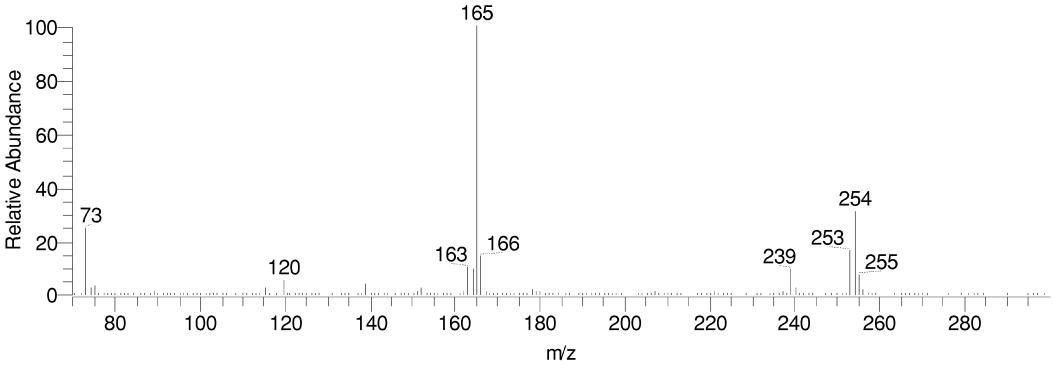

Fig. 7. Mass spectra of 9-hydroxyfluorene after SPME and on-fibre derivatization with BSTFA.

outcomes of course were recalculated to the original amount).

Table 2 shows the results for the fluorene metabolites using SPME and two different derivatization reagents. For each metabolite and for both types of silylation reagent the mass trace exhibiting the lowest background noise was selected for quantification.

Although the results obtained with BSTFA are generally lower than with MTBSTFA, the mean

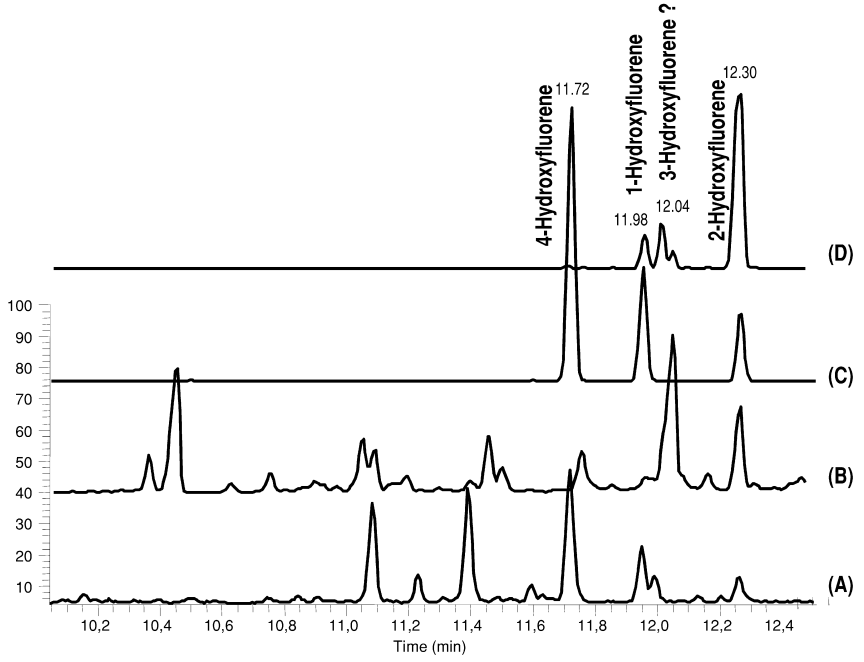

Fig. 8. GC–MS analysis after SPME sampling and derivatization with BSTFA: scanned acquisition as described in Section 2.4. (A) TIC and (C) mass trace 239 of a buffer solution, spiked with 1-, 2-, 4-, and 9-hydroxyfluorene at the upper working range. (B) TIC and (D) mass trace 239 of a urine sample, obtained from a worker occupationally exposed to PAHs.

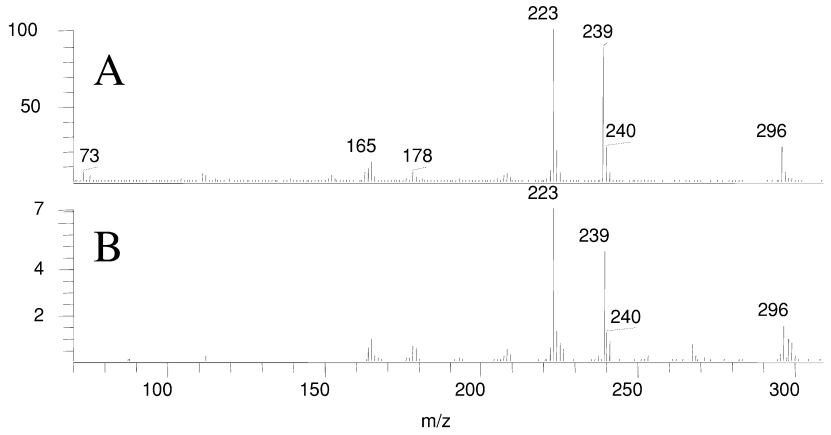

Fig. 9. Mass spectra of 4-hydroxyfluorene after SPME and on-fibre derivatization with MBSTFA: (A) standard and (B) urine sample.

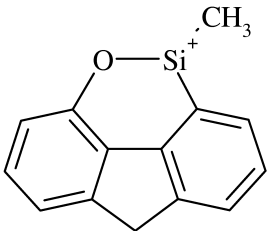

Fig. 10. Cyclic fragment (m/z=223) of silylated 4-hydroxyfluorene.

values of three determinations for each reagent lie in the same range. The relative standard deviations do not exceed 7% for the MTBSTFA method, and are even lower for BSTFA (max. 4%).

4. Conclusion

The method presented relies on SPME in combi-

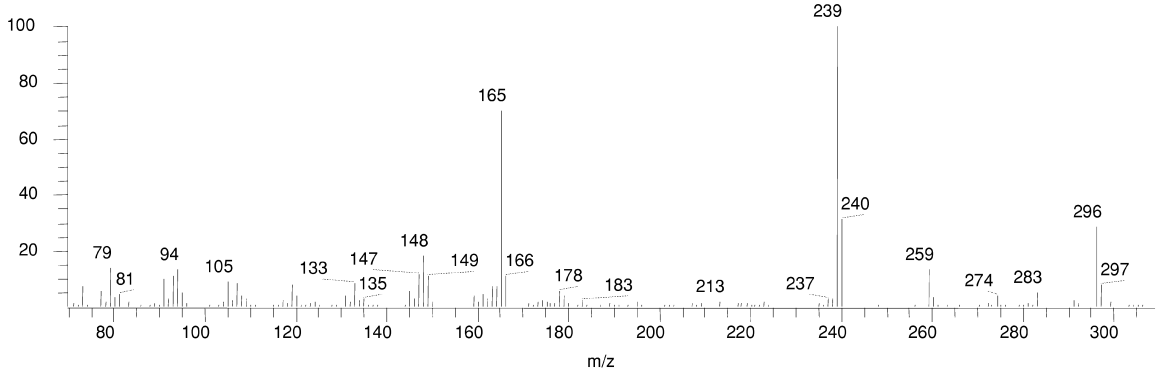

Fig. 11. Mass spectrum of the substance eluting at t_R=12.04 min (Fig. 8).

Table 2
Quantification results for a urine sample from a person occupationally exposed to PAHs

	BSTFA			MTBSTFA		
	Quantification mass m/z	Mean (μg/l)	SD, μg/l (RSD, %)	Quantification mass (m/z)	Mean (μg/l)	SD, μg/l (RSD, %)
1-Hydroxyfluorene	254	12.2	0.3 (2)	296	13.2	0.75 (6)
2-Hydroxyfluorene	254	154	6 (4)	296	181	12 (7)
4-Hydroxyfluorene	223	0.9	0.03 (3)	223	1.5	0.08 (6)
9-Hydroxyfluorene	165	17.8	0.3 (2)	–		

Quantification mass, mean values of three determinations, standard deviations (SDs) and relative standard deviations (RSDs) are given for two different derivatization reagents.

nation with silylation. It proved satisfactory for the identification of monohydroxylated metabolites in urine and quantification of four of them. Substantial means in achieving these targets was the utilization of reference compounds: two of these were not commercially available and hence synthesized in the laboratory.

Acknowledgements

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References

[1] IARC Monographs on the Evaluation Carcinogenic of the Risk of Chemicals to Humans, Polynuclear Aromatic Compounds, Part 1, Chemical, Environmental and Experimental Data, IARC, Lyon, 1983.
[2] G. Gmeiner, G. Stehlik, H. Tausch, J. Chromatogr. A 767 (1997) 163.
[3] G. Grimmer, G. Dettbarn, J. Jacob, Int. Arch. Occup. Environ. Health 65 (1993) 189.
[4] J. Angerer, C. Mannschreck, J. Gündel, Int. Arch. Occup. Environ. Health 69 (1997) 323.
[5] F.J. Jongeneelen, R.B.M. Anzion, P.T. Henderson, J. Chromatogr. 413 (1987) 227.
[6] R. Koeber, R. Niessner, J.M. Bayona, Fresenius J. Anal. Chem. 359 (1997) 267.
[7] Y. Yang, W.J. Griffiths, J. Sjovall, J.A. Gustafsson, J. Rafter, J. Am. Soc. Mass Spectrom. 8 (1997) 50.
[8] M.L. Lee, M.V. Novotny, K.D. Bartle, Analytical Chemistry of Polycyclic Aromatic Compounds, Academic Press, New York, 1991.
[9] R. Tomingas, W. Moench, U. Matthiesen, Chromatographia 22 (1986) 191.
[10] G. Gmeiner, C. Krassnig, E. Schmid, H. Tausch, J. Chromatogr. B 705 (1998) 132.
[11] N.H. Snow, P. Okeyo, S.M. Rentz, J. High Resolut. Chromatogr. 20 (1997) 171.
[12] P.A.S. Smith, J.M. Clegg, J.H. Hall, J. Org. Chem. 23 (1958) 529.
[13] P.H. Grantham, E.K. Weisburger, J.H. Weisburger, J. Org. Chem. 26 (1961) 1009.
[14] DIN 38 402, Teil 51, Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung, Allgemeine Angaben (Gruppe A), Kalibrierung von Analysenverfahren, Auswertung von Analysenergebnissen und Lineare Kalibrierfunktionen für die Bestimmung von Verfahrenskenngrössen (A 51), Beuth Verlag, Berlin, May 1986.
[15] J. Jacob, A. Schmold, G. Grimmer, Toxicology 25 (1982) 333.