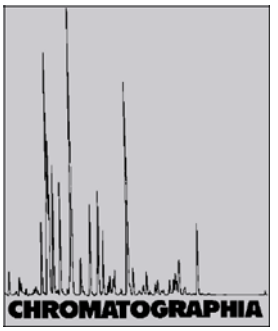
Analysis of 1-Hydroxypyrene in Urine as PAH Exposure Marker Using in-situ Derivatisation Stir Bar Sorptive Extraction-Thermal Desorption – Capillary Gas Chromatography – Mass Spectrometry



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Key Words

Capillary gas chromatography – mass spectrometry
Thermal desorption
Stir bar sorptive extraction
In-situ derivatisation
1-Hydroxypyrene

Summary

A fast and robust method for the determination of 1-hydroxypyrene (OH-pyrene) and other hydroxylated PAHs in urine by stir bar sorptive extraction (SBSE) combined with thermal desorption – capillary gas chromatography – mass spectrometry (TD-CGC-MS) is described. After enzymatic hydrolysis, in-situ derivatisation with acetic acid anhydride precedes SBSE sampling to improve the chromatographic behavior of the analytes. The performance of the method has been evaluated by the quantitative determination of OH-pyrene in some certified samples. Detection limits as low as 2 ng L^{-1} (ppt) can be obtained. The procedure is illustrated with the analysis of a urine sample of a firefighter.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are mainly formed as a result of pyrolytic processes, especially through incomplete combustion of organic materials during industrial or domestic activities [1]. Natural processes like carbonization, also contribute to their ubiquitous spread in the environment. PAHs are carcinogenic and mutagenic [2] and are readily taken up into the human body by ingestion, inhalation and skin absorption [3]. Possible health risks after environmental or occupational exposure to PAHs can successfully be assessed by the analysis of their major hydroxylated metabolites. Jongeneelen et al. analyzed OH-pyrene, which is the primary metabolite of pyrene, as a representative marker to measure the uptake of PAHs by man [4, 5].

The determination of hydroxy-PAHs in biological fluids encounters two major problems. First, the phenolic metabolites are partly Phase II metabolized [6] and occur as glucuronates or sulfates (Figure 1). The conjugates can be analyzed as such by high performance liquid chromatography coupled to fluorescence detection (HPLC-Fluo) but the technique lacks separation efficiency and selectivity, even in combination with a solid phase extraction (SPE) clean-up. An alternative is offered by liberation of the analytes prior to analysis by a β-glucuronidase enzyme complex from *Escherichia Coli K12* or from *Helix Pomatia*. The latter was used in this work since this enzyme complex shows sulfatase activity as well. Secondly, the analytes are present in biological fluids only at trace levels. The analytical methodology therefore should comprise an enrichment step. Recently, stir bar sorptive extraction (SBSE) was introduced as a sensitive and robust sample preparation technique for the analysis of aqueous samples [7]. Basically, a stir bar coated with a thick layer of polydimethylsiloxane (PDMS) is stirred in the sample and the solutes are enriched into the silicon gum. The stir bars are then desorbed in a thermal desorption (TD) system and the analytes are analyzed on-line by capillary gas chromatography – mass spectrometry (CGC-MS). The technique has already proven its versatility for the analysis of pharmaceutical drugs and drugs of abuse in a wide range of biological fluids [8, 9]. For more polar compounds in-situ derivatisation in the aqueous sample with ethyl chloroformate or acetic acid anhydride was developed [10]. The latter reaction is very suitable for conversion of phenolic groups into the respective acetates and this can be performed simultaneously with the SBSE sampling. The O-acetyl-PAHs show better chromatographic performance than their parent compounds. The performance of in-situ derivatisation-SBSE-TD-CGC-MS for the determination of OH-pyrene in urine was evaluated by the

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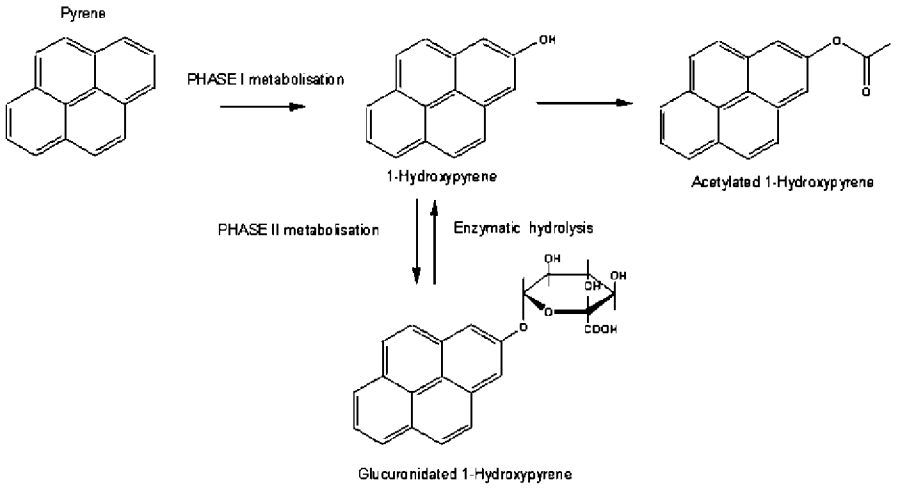

Figure 1. Pyrene metabolism and acetylation reaction of OH-pyrene.

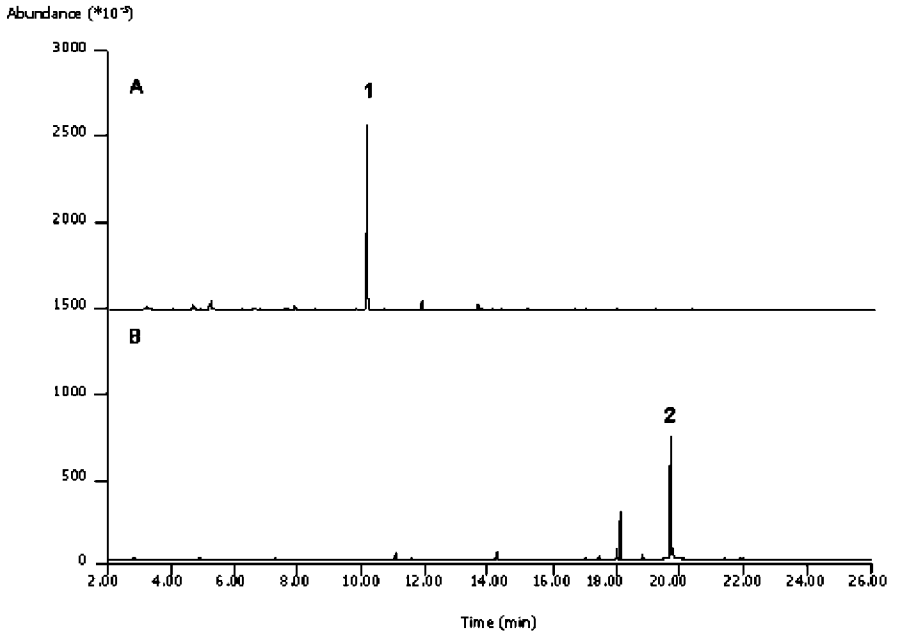

Figure 2. Selected ion chromatogram at *m/z* 218 (**A**) and 252 (**B**) of the in-situ derivatisation-SBSE-TD-CGC-MS analysis of a water sample spiked with 2,4-dibromophenol and OH-pyrene to concentrations of 1 μg L^{-1}. Peaks **1** = 2,4-dibromophenoxy acetate, **2** = 1-O-acetoxypyrene. Sampling and chromatographic conditions see text.

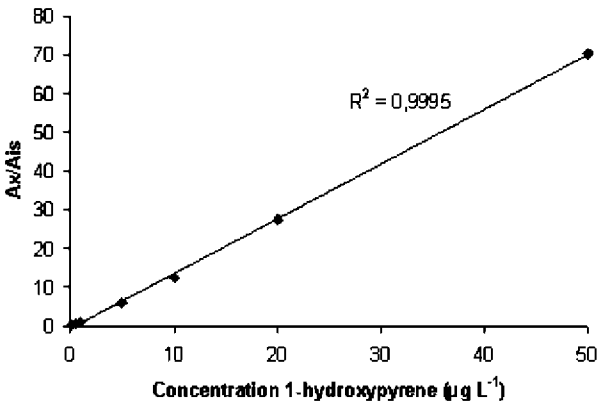

Figure 3. Internal standard calibration curve of OH-pyrene in blank urine.

analysis of some certified urine samples. The analysis of urine of a firefighter taken the morning after a fire call illustrates the versatility of SBSE.

Experimental

Chemicals

OH-pyrene, 2,4-dibromophenol, potassium hydrogen carbonate, sodium acetate and β-glucuronidase from *Helix pomatia* were purchased from Sigma-Aldrich (Bornem, Belgium). Methanol and water of HPLC grade and acetic acid anhydride were obtained from Riedel-de-Haën (Seelze, Germany). Certified urine samples (ClinCheck®) containing three levels of OH-pyrene were obtained from Recipe (Munich, Germany). The stir bars (Twister™) containing 24 μL PDMS were purchased from Gerstel GmbH (Mülheim/Ruhr, Germany).

Sample Preparation

Water or urine samples (10 mL) are poured in 40 mL vials and buffered at a pH of 5 using sodium acetate buffer (0.1 M) resulting in a total volume of ca. 13 mL. Ten μL of a 1 μg L^{-1} solution of 2,4-dibromophenol in methanol is added as internal standard (IS) to each sample. Twenty μL β-glucuronidase extract from *Helix Pomatia* is added and the mixture is incubated overnight at 37 °C (Figure 1). This is in principle not needed for spiked water and urine samples but was performed to uniform the sample conditions and SBSE enrichment. Acetylation is performed by adding 500 μL acetic acid anhydride and 0.5 g of potassium hydrogen carbonate (pH 11). The stir bar is added immediately after and stirred at 100 rpm. After completion of the reaction (10 min), which is indicated by the termination of carbon dioxide gas production, the solution is stirred at 1000 rpm for another 50 minutes. After sampling, the Twister is taken out with tweezers and 2 mL of water is poured with a pipette over the stir bar to remove traces of acetic acid anhydride. The Twister is dipped shortly on a clean tissue to remove residual water droplets and placed in an empty glass tube (187 mm long, 6 mm O.D., 4 mm I.D.) for thermal desorption. When not analyzed immediately, the stir bars are kept in a closed autosampler vial and stored in the

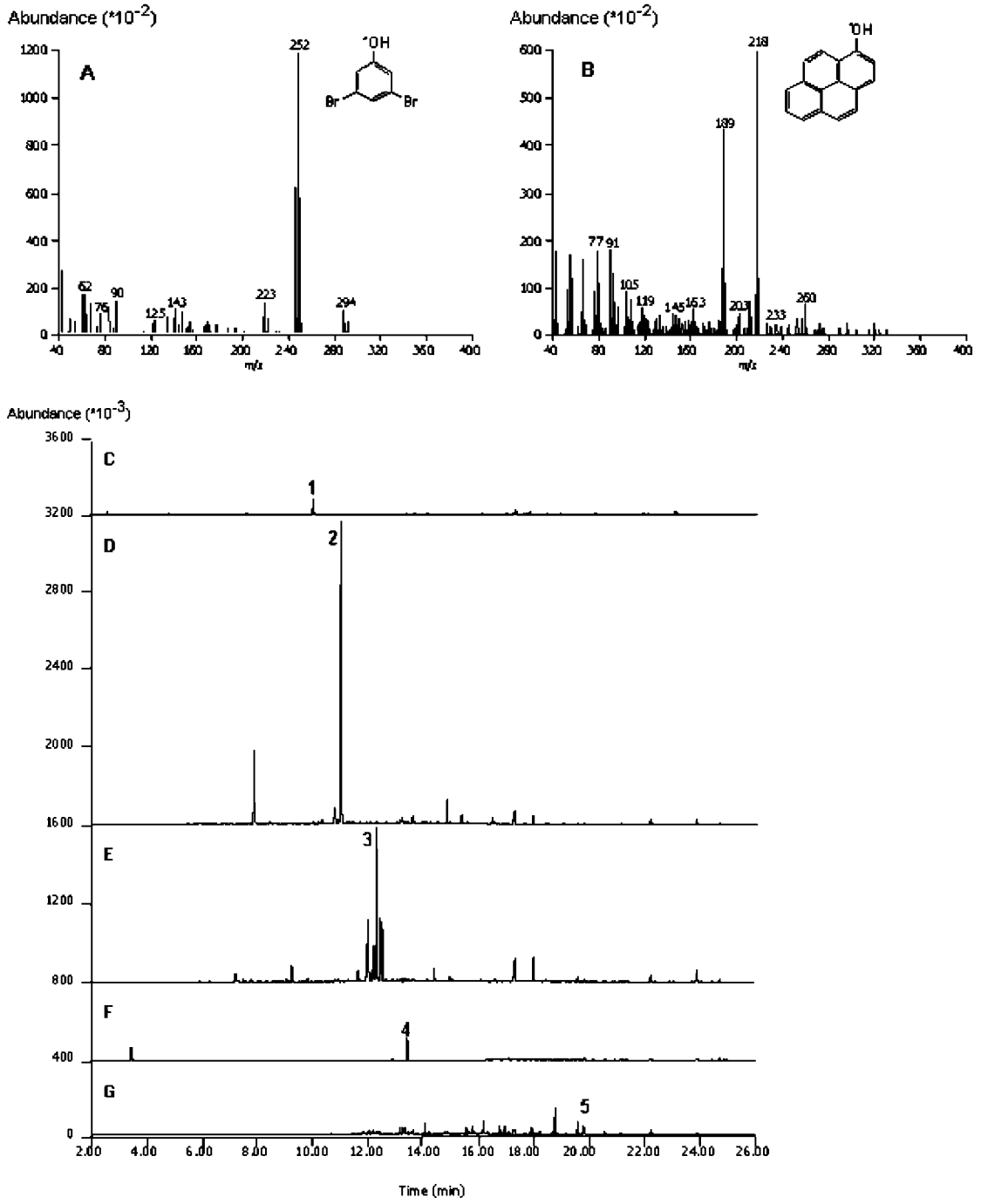

Figure 4. Mass spectra of the acetates of 2,4-dibromophenol **(A)** and OH-pyrene **(B)** and extracted ion chromatogram at *m/z* 252 **(C)**, 144 **(D)**, 158 **(E)**, 266 **(F)**, 218 **(G)** for the acetates of **1** = 2,4-dibromophenol, **2** = 1-naphthol, **3** = 3-methyl-1-naphthol, **4** = pentachlorophenol and **5** = OH-pyrene, respectively, in the urine of a fireman. Sampling and chromatographic conditions see text.

refrigerator (4 °C). This does not affect the recovery of the target analytes.

Instrumental Conditions

An Agilent 6890 GC coupled to an Agilent 5973 mass spectrometric detector (MSD, Agilent Technologies, Little Falls, USA) was equipped with a TDS-2 thermal desorption system (Gerstel). The stir bars are thermally desorbed programming the TDS from 50 °C (1 min) to 280 °C (10 min) at 60 °C min $^{-1}$. The analytes are transferred on-line into a programmed temperature vaporization (PTV) injector (CIS-4, Gerstel) using a total flow of 100 mL min $^{-1}$ and trapped at a temperature of −150 °C in an empty baffled liner. After desorption, splitless injection is performed by heating the PTV from −150 °C to 300 °C (2 min) at 12 °C s $^{-1}$. The splitless time is 2.5 min. Separation is done on a 20 m L × 0.25 mm I.D., 0.25 µm d_f HP5-MS column (Agilent Technologies). Helium is used as carrier gas at a constant flow of 0.9 mL min $^{-1}$. The oven is programmed from 50 °C (2.5 min) to 300 °C (5 min) at 10 °C min $^{-1}$. For screening, the MSD is used in the electron impact mode (70 eV), generating full scan spectra between *m/z* 40 and 350. For quantitative analysis, the MSD is programmed in the

Table I. Repeatability (RSD %) and accuracy of in-situ derivatisation-SBSE-TD-CGC-MS analysis of OH-pyrene in urine at three concentration levels. Reference urine samples are from Recipe, Munich (Germany). Sampling and chromatographic conditions see text.

Sample	Reference concentration ($\mu g\,L^{-1}$)	Control range ($\mu g\,L^{-1}$)	RSD (%)	Measured concentration ($\mu g\,L^{-1}$)
Level I	0.80	0.6 1.0	6.3	0.83
Level II	3.18	2.5 3.8	5.0	2.76
Level III	11.7	9.4 14.0	5.8	12.5

ion-monitoring mode at *m/z* 218 (OH-pyrene) and 252 (2,4-dibromophenol, IS) using a dwell time of 100 ms.

Results and Discussion

In-situ Derivatisation-SBSE Optimization

2,4-Dibromophenol is used as internal standard because its physical-chemical characteristics are similar to those of OH-pyrene. Both phenolic compounds are readily acetylated in water in the presence of acetic acid anhydride and both acetates show high affinity for the apolar PDMS stir bar, which is expressed by their relative high $K_{o/w}$ -values of 4.5 (OH-pyrene) and 3.4 (2,4-dibromophenol) [7]. Initially, a water sample (10 mL) was spiked with OH-pyrene and 2,4-dibromophenol to individual concentrations of 1 $\mu g\,L^{-1}$ (ppb) and analyzed as described above using several sampling times (10, 30, 60, 90 and 120 min). The MSD was used in the SIM mode. Figure 2 shows the resulting chromatogram at a sampling time of 60 min. TD-CGC-MS analysis resulted in maximum abundances for both compounds from 60 min extraction time on.

Figures of Merit of in-situ Derivatisation-SBSE-TD-CGC-MS

The linearity of the full procedure was verified by making a calibration graph for OH-pyrene between 0.1 and 50 $\mu g\,L^{-1}$ in urine (Figure 3). Solutions (10 µL) of OH-pyrene in methanol at concentrations of 0.1, 0.5, 1, 5, 10, 20 and 50 $mg\,L^{-1}$ were spiked in seven blank urine sub-samples. The internal standard (IS) was spiked each time at a constant concentration of 1 $\mu g\,L^{-1}$. Linear regression was performed on the internal standard curve and a correlation coefficient of $R^2 > 0.999$ was obtained. At the lowest level, the signal-to-noise was S/N = 151 for OH-pyrene giving a calculated limit of detection (LOD) for S/N = 3 of 2 $ng\,L^{-1}$. This is far lower than the limit of quantitation (LOQ) of 0.1 $\mu g\,L^{-1}$ requested in nominal bio-monitoring screenings [5].

Three certified lyophilized urine samples (ClinCheck®) were prepared by dissolution of the powder in HPLC grade water and contained average levels of 0.8, 3.2 and 11.7 $\mu g\,L^{-1}$ OH-pyrene, respectively. The concentration levels of each sample were measured 6 times using the described in-situ-derivatisation-SBSE-TD-CGC-MS procedure and the mean values are summarized in Table I. For the three concentration levels, the relative standard deviations (RSD%) approximated ca. 5%. The measured values were within the prescribed control concentration range (Table I) proving the accuracy of the method.

Analysis of a Urine of a Fireman

A morning urine sample of a firefighter taken the day after a fire call was analyzed using the described procedure. For OH-pyrene, an incubation time of two hours is considered sufficient to liberate this marker quantitatively from its glucuronic acid or sulfate conjugates [5] but hydrolysis was performed overnight to ensure splitting of other conjugated PAH metabolites as well. The urine sample was first monitored in the full scan mode to allow the identification of unknowns. Figure 4A and 4B show the recorded mass spectra for the acetates of the IS (2,4-dibromophenol) and OH-pyrene, respectively. Both quantitation ion *m/z* 218 (OH-pyrene acetate) and 252 (dibromophenol acetate) result from the respective deacetylated fragments shown in Figure 4A and 4B. Besides OH-pyrene, other hydroxylated polyaromatic substances could be identified. Figure 4C shows the extracted ion chromatogram at *m/z* 144, 158 and 218 for the acetates of 1-naphthol (peak 2), 3-methyl-1-naphthol (peak 3) and OH-pyrene (peak 5), respectively, in the urine of the fireman. The naphthalene metabolites are present in much higher concentrations compared to OH-pyrene (ca. 30–50 $\mu g\,L^{-1}$). The ion trace at *m/z* 266, overlaid in Figure 4C, is showing the presence of pentachlorophenol (peak 4), detected as its acetate, which is a commonly used biocide for wood protection. Quantitation of OH-pyrene was done in the selected-ion-monitoring (SIM) mode using 2,4-dibromophenol as IS (peak 1) as described above. The OH-pyrene output was normalized from the creatinine concentration and is expressed as $\mu mol\,mol^{-1}$ creatinine. The detected 0.4 $\mu mol\,mol^{-1}$ creatinine normalized concentration, corresponding with 1.7 $\mu g\,L^{-1}$ OH-pyrene, is relatively low and indicates that, although the firefighter was in contact with the visible smoke for half an hour, his protection mask worked effectively.

Conclusion

Stir bar sorptive extraction in combination with thermal desorption-capillary gas chromatography is an accurate innovative sample preparation method for the fast and sensitive analysis of hydroxylated PAH metabolites in urine. In-situ derivatisation with acetic acid anhydride provides good chromatographic behavior of the analytes. A concentration range of 0.1 to 50 $\mu g\,L^{-1}$ OH-pyrene can easily be covered.

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