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*Article*

# High-Throughput Analysis of Selected Urinary Hydroxy Polycyclic Aromatic Hydrocarbons by an Innovative Automated Solid-Phase Microextraction

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**Abstract:** High-throughput screening of samples is the strategy of choice to detect occupational exposure biomarkers, yet it requires user-friendly apparatus that gives relatively prompt results while ensuring high degrees of selectivity, precision, accuracy and automation, particularly in preparation processes. In the last 10 years, miniaturization has attracted much attention in analytical chemistry and has driven solvent and sample savings and easier automation, the latter thanks to the introduction on the market of three axis autosampler. In light of the above, this contribution describes a novel user-friendly solid-phase microextraction (SPME) off- and on-line platform coupled with gas chromatography triple quadrupole-mass spectrometry to determine urinary 1- and 2-hydroxy-napthalene, 9-hydroxy-phenantrene, 1-hydroxy-pyrene, 3- and 9-hydroxy-benzoantracene and 3-hydroxy-benzo[a]pyrene, metabolites of the related polycyclic aromatic hydrocarbons. In this new procedure chromatography's sensitivity is combined with the user-friendliness of *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide on-fiber SPME derivatization previous direct immersion sampling, to which is added the quantitative accuracy afforded using specific isotope-labelled internal standards. The detection limits for the seven OH-PAHs were ranged from 0.28 to 1.87 ng/L. Intra-(from 2.5 to 3.0%) and inter-session (from 2.4 to 3.9%) repeatability was also evaluated. This method serves to identify suitable risk-control strategies for occupational hygiene conservation programs.

**Keywords:** SPME; OH-PAHs; gas-chromatography; MTBSTFA.

## 1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of complex organic chemicals of increasing concern for their occurrence in the environment. PAHs can be found in the atmosphere in both gaseous and particulate forms depending on their volatility which is governed by their chemical structure. Particle-bound PAHs are considered to be very hazardous to human health; many of the studies on the effects of air pollution and cancer identify solid aerosol and PAHs as the components most associated with cancer risk [1]. Benzo[a]pyrene (B[a]P) is often used as a marker for total exposure to carcinogenic PAHs, and Ohura et al. [2] reported that contribution of B[a]P to the total carcinogenic potential as being in the range 51–64%. Outdoor air pollution in both cities and rural areas was estimated to cause 4.2 million premature deaths worldwide in 2016 [3], mainly attributable

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to the airborne particulate matter (PM) [4,5]. Gherardi et al. indicates that 80% of the suspended PM1 is represented by PAHs [6]. The Institute of Occupational Medicine [7] estimated that in 2006 in the EU there were 234,000 workers who were potentially exposed to high levels of B[a]P and about 7 million to low levels. Recently, Stec et al. revealed that cancer incidence appears to be higher amongst firefighters compared to the general population [8].

Urinary hydroxylated-PAHs (OH-PAHs) have been used as biomarkers to assess total human exposure to PAHs, with 1-hydroxy-pyrene (1-OH-P) as the most commonly use indicator in biomonitoring studies [9]. The Center for Disease Control and Prevention (CDC) developed a OH-PAHs method used to analyze urinary samples from the National Health and Nutrition Examination Survey, a comprehensive survey that CDC performs annually to assess exposure of the U.S. general population to PAHs [10]. For many years the American Conference of Governmental Industrial Hygienist (ACGIH) had recommended the determination of urinary 1-OH-P as biomarker to occupational exposure to PAHs mixtures, without any indication of a limit value. In 2017, the ACGIH introduced a Biological Exposure Indices (BEI) value of 2.5 μg/L for 1-OH-P, and it proposed the urinary 3-OH-B[a]P, and the sum of 1- and 2-naphthols as non-quantitative markers [11].

Most analytical methods have been published to measure urinary OH-PAHs [12–25] which have two to three benzene rings, and only eleven study [26–36] have considered the determination of OH-PAHs with more than three benzene rings, particularly 3-OH-B[a]P. These existing assays have limitation, namely: their complexity, their use of solvents, and/or the need for clean-up steps to extract and eliminate interfering compounds from the urine, all of which involved lengthy manual operation, bigger costs, uncertainty in the determination analysis and the possible loss of analyte. For these reason, simultaneous and more sensitive assays methods than those available were needed.

In the last 10 years, miniaturization has attracted much attention in analytical chemistry and has driven solvent and sample savings, sample enrichment, rapid sample preparation, and easier automation. Sample preparation remains one of the more time-consuming and error-prone aspects of analytical chemistry. To overcome drawbacks of conventional extraction techniques, innovative miniaturized methods have been mainly proposed as microextraction coupled to solid phase, specifically Solid Phase MicroExtraction (SPME) proposed by Supelco (Bellefonte, Pennsylvania, PA, U.S.), CTC (Zwingen, Switzerlandand), and Restek (Bellefonte, Pennsylvania, PA, U.S.) [37–41], SPME Arrows [42], MicroExctraction by Packed Sorbent (MEPS) [43], Stir Bar Sorptive Extraction (Twister, SBSE) [44], Solid Phase Dynamic Extraction (Magic Needle, SPDE) [45], In-Tube Extraction (ITEX) [46], HiSorb Sorptive Extraction [47], and Monolithic Material Sorptive Extraction (MonoTrap) [48].

Within analytical chemistry, the SPME analysis is considered one of major breakthroughs that shaped 20th-century analytical chemistry. SPME is the first powerful miniaturized sampling technique developed for GC. The SPME, which was invented by Pawliszyn in 1989 [49], integrates sampling, extraction, concentration and sample introduction into a single step and the extraction requires no polluting organic solvent. Through this, the principles of green chemistry are applied to not only chemical engineering and synthesis, but also increasingly analytical chemistry [10,21,50,51]. From 2009, a significant progress was achieved by the market introduction of the Fast Fit Fiber Assemblies (FFA) [52]. This new generation of SPME fiber was developed by Chromline (Prato, Italy), in cooperation with Supelco, expanding the applicability of SPME; the product line is centered around the SPME FFA barcoded that can be automatically exchanged on a three axis autosampler equipped with the Multi Fiber EXchanger (MFX) system[53].

Therefore, we sought to simplify sample treatment by using the SPME technique in off- and on-line mode for seven OH-PAHs, namely: 1-hydroxy-naphtalene (1-OH-Nap), 2-hydroxy-napthalene (2-OH-Nap), 9-hydroxy-phenantrene (9-OH-Phen), 1-OH-P, 3-hydroxy-benzoanthracene (3-OH-B[a]A), 9-hydroxybenzo-anthracene (9-OH-B[a]A) and 3-OH-B[a]P. The efficiency of their tert-

butyldimethylsilyl (TBDMS) derivatives has been demonstrated as has the success of the quantitative determination by gas chromatography (GC) coupled with triple quadrupole-mass spectrometry (QpQ-MS). By combining these procedures, we propose a new user-friendly SPME platform which provides relatively prompt results with a high degree of selectivity, precision and accuracy.

## 2. Results and Discussion

For many years, the 1-OH-P has been accepted as urinary biomarker to estimate PAHs exposure in the occupational and general population due to its relatively high concentration, even if pyrene is not carcinogenic. Conversely, the profile of PAHs is dependent on the emission sources, and therefore such extrapolation would introduce uncertainty, and determination of hydroxy metabolites of B[a]P and B[a]A – which contain "bay region" that favor production of reactive and potentially carcinogenic metabolites - should more accurately assess to internal exposure to carcinogenic OH-PAHs. Gundel et al. [54] proposed 3-OH-B[a]A as an indicator for internal exposure to PAHs, also due to the fact that it is excreted in relatively high concentration in the urine. Smoking is a significant source of exposure to PAHs representing a confounding factor and so a suitable smoking status on PAH biomarker levels is necessary. The largest different in PAH metabolite concentrations between smokers and non-smokers were observed with 2-OH-Nap, 1-OH-P and OH-phenanthrenes [55-57]. Several authors show that urinary OH-fluorene levels are positively correlated with smoking status, particularly 1-OH-fluorene [22,57].

The development of analytical methods to identify suitable risk-control strategies for occupational hygiene conservation programs have aroused interest of the scientific community. The use of MS techniques, particularly GC and liquid chromatography (LC), are indispensable tools in metabolomic science owing to their high sensitivity and specificity. Relatively to assessment of B[a]P exposure - the only PAH classified as category 1 by International Agency for Research on Cancer - urinary 3-OH-B[a]P determination plays a fundamental role; the hyphenated chromatographic MS procedures proposed for its analysis are based on age-old methodologies resulting in many manual operations with related uncertainty of the determination and higher overall costs of the method [26,27,29,33-36]. The use of liquid/liquid extraction (LLE) or SPE with evaporation to dryness of the collect analyte solution followed by reconstitution in a suitable solvent for injection into the chromatographic system - with or o not derivatization - are the typical sequences for monohydroxy PAHs in urine. Currently four GC methods using N-methyl-N-(trimethylsilyl) trifluoroacetamide (BSTFA) as derivatizing agent previous extraction with hexane or pentane and related analysis by single [27], QpQ [34], or high-resolution [26,35] MS were proposed. Regarding the LC-triple quadrupole analyses, Raponi et al. [29] and Zangh et al. [36] reported use of SPE, while Luo et al. [33] included also the reaction by dansyl chloride (DNS). From the analytical evaluation of the seven above indicated methods we revealed that i) phenolic compounds are susceptible to oxidation with related losses of OH-PAHs and addition of gallic acid (50 μg/mL urine) prior to evaporation and derivatization steps was effective for inhibiting losses (lower than 5%), in according by Jacob et al. [9]. Woudneh et al. [35] indicated that oxidation was controlled by a combination of employing 2-mercaptoethanol and utilizing a nitrogen atmosphere, ii) the photodegradation can be a key factor in recovering the OH-PAHs [58] and the amber glassware is not available for all sizes or types of glass, iii) the relevant amounts of BSTFA injected with conventional sample preparation methods quickly wear injector, column and GC detector, iv) we evaluated a more rapid and less solvent consuming derivatization step by 1.2-dimethylimidazole-4-sulfonyl (DMISC) instead than DNS. Therefore DMISC-derivatives show a retention time (RT) three times lower respect the DNS and the daughters spectrum that we have obtained were of good quality, as shown in our previous work [59] v) these five methods do not allow the possibility of fully automation.

Accordingly, we developed a method where on-fiber SPME technique was applied after direct immersion (DI), and then coupled with quantitative determination via GC/QpQ-MS. Three fundamental aspects motivated this choice.

*2.1 SPME extraction*

The absortive liquid 85 μm polyacrylate (PA) coating was choice for sampling of a very complex matrix as to urine, because there is no competition between analytes. Because of the properties liquid coating, which is applied in DI-SPME analysis, the extraction obeys the rules of liquid-liquid equilibrium

$$n = C_0V_1V_2K / KV_1+V_2$$

where K is the partition constant SPME fiber liquid polymeric coating/sample, $C_0$ is the initial concentration of the analyte in the aqueous solution, $V_1$ and $V_2$ are the volumes of the coating and the aqueous solution, in the equilibrium concentration of the analyte in the aqueous matrix. However, SPME is an equilibrium extraction but not an exhaustive extraction. The DI is effective for $K_H$ less than 0.17 atm $cm^3$/ mol. The $K_{ow}$ is a good estimated of K, however, the correlation has to be confirmed for the group of substances from a number of investigators. K values of the analytes are often very close to the gas phase partition coefficient/aqueous matrix partition coefficient ($K_2 = K_H/RT$) and to the SPME coating/gas phase partition coefficient ($K_1$); $K = K_2 \cdot K_1$, where it is more practical to say that both $K_1$ and $K_2$ values allowed to know in advance whether or not the SPME method offers the advantages. The equilibrium and kinetics of the OH-PAHs versus SPME fiber with liquid coating were investigated theoretically. Table 1 illustrated the physicochemical constants of the seven OH-PAHs obtained by Performs Automated Reasoning in Chemistry (ARChem, Danielsville, Georgia, USA) - a physicochemical calculator that uses computational algorithms based on the fundamental chemical structures to foresee a wide variety of reactivity parameters - to anticipate trends in sampling extraction.

**Table 1.** Physical properties and partition coefficients of OH-PAHs evaluated using SPARC. (M.W.= molecular weight; $T_{eb}$= boiling point; $D_{water}$= diffusion coefficient of the analyte in water; $K_H$= Henry's constant; $K_{ow}$= octanol-water partition coefficient; $P_{vap}$= vapour pressure).

| SMILES strings | M.W. (g/mol) | $T_{eb}$ (°C) | $D_{water}$ ($cm^2$/sec) | $K_H$ (atm/(mol/$m^3$)) | $K_{OW}$ (Log) | $P_{vap}$ (log(atm)) |
|---|---|---|---|---|---|---|
| OC1=CC=CC2=CC=CC=C21 | 144 | 269.7 | $8.08*10^{-6}$ | $8.39*10^{-8}$ | 3.04 | -6.0 |
| OC1=CC2=CC=CC=C2C=C1 | 144 | 269.8 | $8.08*10^{-6}$ | $9.10*10^{-8}$ | 3.11 | -6.14 |
| OC1=CC2=C(C3=C1C=CC=C3)C=CC=C2 | 194 | 378.9 | $6.92*10^{-6}$ | $6.93*10^{-9}$ | 4.49 | -8.67 |
| OC1=CC=C(C=C2)C3=C1C=CC4=CC=CC2=C34 | 218 | 454.6 | $6.41*10^{-6}$ | $6.37*10^{-9}$ | 5.01 | -8.9 |
| OC(C=C1)=CC2=C1C3=CC4=CC=CC=C4C=C3C=C2 | 244 | 537.2 | $6.15*10^{-6}$ | $3.24*10^{-10}$ | 5.71 | -11.86 |
| OC1=CC=C2C(C=C(C=CC3=C4C=CC=C3)C4=C2)=C1 | 244 | 537.2 | $6.15*10^{-6}$ | $3.21*10^{-10}$ | 5.71 | -11.86 |
| OC1=CC=C(C=C2)C3=C1C=CC4=CC5=CC=CC=C5C2=C43 | 268 | 564.5 | $5.74*10^{-6}$ | $4.56*10^{-10}$ | 6.28 | -11.81 |

An excellent SPME extraction sensitivity for the urinary OH-PAHs was generally achieved by immerging the PA fiber in the diluted urine (1:5 v/v). Dilution of the urine with distilled water reduces the sensitivity of the method but increases the precision and the fiber lifetime. The better results were obtained with DI times up to 30 minutes with temperature-controlled agitation (60 °C and 500 rpm). To remove any liquid sample remaining on the SPME PA fiber after DI extraction, the fiber was placed for 45 seconds into an SPME fiber conditioning station set at 100 °C.

The results confirmed that DI-SPME is efficient under such conditions, considering that the extraction time in the unagitated case is limited by the transport of analyte in the aqueous phase and a decrease in the diffusion coefficient of the analyte in water ($D_{water}$) by an order of magnitude produce about an order of magnitude increase in equilibration time as discussed by Louch et al. [60]. Moreover, since the reduction in vial diameter by a factor of 3 resulted in an order of magnitude decrease in extraction time, where t, the average time of the diffusion through the aqueous layer is

expected to be proportional to the square of the migration distance, x, and inversely proportional to the $D_{water}$ [60],

$$t = x^2/2D_{water}$$

for high-concentration samples, 2-mL can be used instead of 10-mL amber vials.

*2.2 SPME-Derivatization*

*N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) as a TBDMS derivatizing agents was used in GC analysis of amino acids and in GC-MS analysis of hydroxylated fluorenes, and it was shown that TBDMS derivatives were thermally stable and had favorable fragmentations upon electron impact (EI) ionization [22,61] (Figure 1).

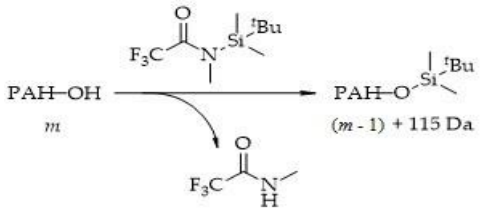


**Figure 1.** Derivatization of OH-PAHs with MTBSTFA.

These spectra were simple and gave intense fragment ion peaks corresponding to the [M-57] + ion due to the loss of a tert-butyl fragment from the molecular ion. We found that the intensity due the base peaks of OH-PAHs-TBDMS was about five times higher than that of OH-PAHs-TMS even if the TBDMS derivatives had later eluting times than the corresponding TMS derivatives. So, the effects of time, temperature and volume of urine and derivatization reagent for automated analysis were evaluated. For on-fiber derivatization low and high values for three variables (15 and 100 µL MTBSTFA, 25 and 60 °C, and 10 and 60 minutes) were selected on the basis of previously reported results [62]. The volume of MTBSTFA, the derivatization time and temperature were fixed to 15 µL, 30 minutes, and 60 °C, respectively. In order to avoid contamination problems between consecutive samples, on-fiber derivatization was performed in argon atmosphere in 2-mL silanized amber vials, placed in a 98-position vial tray set to +4 °C.

*2.3 Automation of SPME procedure*

New fully automation of the procedure was achieved using a *xyz* robotic autosampler coupled by FFA-SPME fibers. In off-line SPME sampling mode by Multi Off-Line Sampler, the fibers - previous extraction and derivatization steps manually performed - are placed into *xyx* autosampler and transported from the MFX 45-position tray to the injector by SPME holder equipped with a plunger/magnetic system; at the end of the analysis each desorbed fiber is moved back to the tray and the cycle is repeated with a new loaded SPME fiber. Instead, in on-line SPME fully automated mode, the FFA fiber is transported from the vials - containing urine or derivatization agents -to the injector. In Figure 2 an example of the advantages of using an SPME-FFA Multi Off-Line Sampler calculating a urine extraction time of 30 minutes, followed by 30 minutes time of MTBSTFA derivatizating reaction plus an analysis time of 20 minutes; the results are excellent, with a reduction in total analysis time of 2,200 minutes for 60 samples, compared to SPME on-line analysis. The initial economic commitment for the purchase of SPME fibers, as well as for the manual transport steps for the extraction and derivatization, is superseded by the possibility that the off-line method offers regarding a high-throughput approach.

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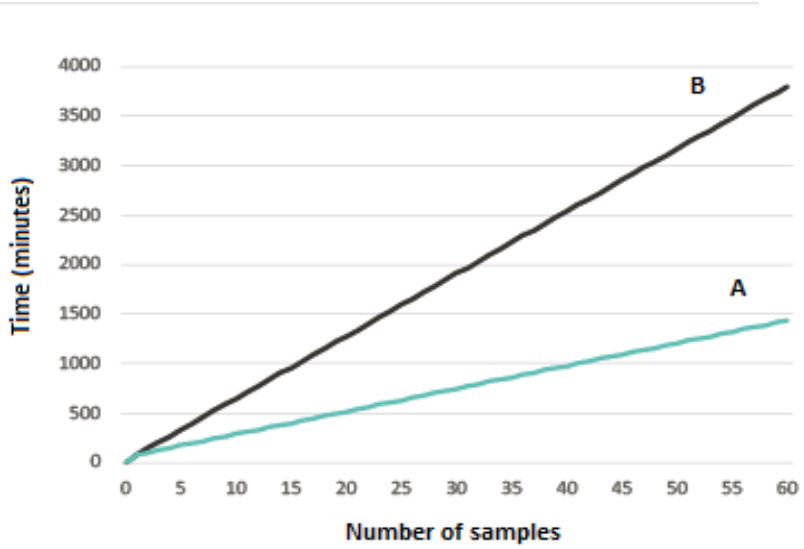


**Figure 2.** Comparison between SPME-FFA Multi Off-Line Sampler (A) and SPME on-line (B) for the analysis of 60 urinary OH-PAHs samples.

In light of what indicated above the authors present the final results in Table 2.

**Table 2.** LOD, LOQ, accuracy and precision for each OH-PAHs measured in urine samples.

| **Response factor Plot and Limit of Detection and Quantification** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **1-OH-Nap** | **2-OH-Nap** | **9-OH-Phen** | **1-OH-P** | **3-OH-B[a]A** | **9-OH-B[a]A** | **3-OH-B[a]P** |
| Least-squares linear regression parameters | m | 1.0924 | 1.0922 | 1.1125 | 1.1126 | 1.1244 | 1.1240 | 1.1245 |
| | b | 0.1893 | 0.2204 | 0.0769 | 0.0828 | 0.0455 | 0.0453 | 0.0368 |
| Coefficient of Correlation | | 0.99 | 0.99 | 1.00 | 0.99 | 0.99 | 1.00 | 1.00 |
| LOD (ng $L^{-1}$) | | 1.87 | 1.52 | 0.94 | 1.63 | 0.34 | 0.38 | 0.28 |
| LOQ (ng $L^{-1}$) | | 6.3 | 5.1 | 3.2 | 5.5 | 1.2 | 1.4 | 0.91 |
| **Accuracy and precision (%)** | | | | | | | | |
| Within-session accuracy | | 10.0 | 9.3 | 10.3 | 9.8 | 10.5 | 10.2 | 10.7 |
| Within-session repeatability | | 2.7 | 2.7 | 3.0 | 2.7 | 2.5 | 2.6 | 2.5 |
| Inter-session repeatability | | 3.0 | 3.0 | 3.6 | 3.9 | 3.4 | 2.4 | 3.4 |

The resulting calibration curves were linear, in the investigated range for all the considered OH-PAHs, with correlation coefficients >0.99. The precision of the assay (reported as a coefficient of variation, C.V.%), estimated both as within-session and as inter-session repeatability resulted in the range 2.5-3.0 and 2.4-3.9%, respectively. Accuracy was within 15% of the theoretical concentration, in line with the requirement of US Food and Drug Administration for the bioanalytical methods validation. To demonstrate the applicability of the method to urinary samples, the content of these compounds in human urines of no-exposed, smoking (n= 19) and no-smoking (n= 21) subject was analyzed and indicated in Table 3.

| | **No-smoker** Average (ng/L) ± S.D. (min-max value) | **Smoker** Average (ng/L) ± S.D. (min-max value) |
|---|---|---|
| 1- OH-Nap | 1040.6 ± 340.7 (150.3-1500.2) | 2966.6 ± 904.3 (240.1-3500.6) |
| 2-OH-Nap | 1879.2 ± 402.4 (201.6-2001.3) | 4297.5 ± 1151.2 (2898.3-8214.4) |
| 9-OH-Phen | <LOD ± 0.54 (<LOD-3.2) | <LOD ± 0.66 (<LOD-3.6) |
| 1-OH-P | 59.3 ± 27.4 (25.1-166.7) | 291.4 ± 89.3 (178.0-647.2) |
| 3-OH-B[a]A | 0.43 ± 0.21 (<LOD-1.2) | 0.60 ± 0.23 (<LOD-1.6) |
| 9-OH-B[a]A | <LOD ± 0.25 (<LOD-1.41) | 1.44 ± 0.59 (<LOD-2.3) |
| 3-OH-B[a]P | <LOD ± 0.17 (<LOD-0.91) | 0.98 ± 0.14 (<LOD-1.32) |

**Table 3.** OH-PAHs in human urines of smoking and no-smoking subject. (S.D.= standard deviation)

## 3. Materials and Methods

### *3.1 Hydrolysis of conjugated OH-PAHs*

Sample processing was conducted in a dark room with limited yellow light. Three-mL of urine were spiked with 5 µL di β-Glucuronidase from Helix pomatia (Sigma-Aldrich, Saint Louis, MO, U.S. cat. no. G7017-5ML) in 10-mL amber vial (Sigma-Aldrich, Saint Louis, MO, U.S., cat. no. 27389). The headspace (HS) over each sample was purged with argon, sealed with screwed caps (Agilent Technologies, St. Clara, CA, U.S. cat.no. 8010-1039) and incubated in the dark at 37 °C. After 17 hours the samples were diluted with 7-mL of water and doped by deuterated internal standards (ISs) for on- or off-line analysis.

### *3.2 On-line DI-SPME and xyz robotic apparatus*

Automated DI-SPME and on-fiber derivatization experiments were carried out by Flex Autosampler (EST Analytical, Fairfield, CT, U.S.). The *xyz* robotic system was assembled with 32-position tray for 10-mL vials, 98-position tray for 2-mL vials, tray cooler-Peltier (set to 4 °C), MFX 6-positions SPME system, SPME fiber conditioning station, and agitator. The 10-mL amber vial containing standards/sample was taken automatically from the 32-position tray and was inserted into the agitator, heated (60 °C), and agitated (pulsed agitation, 2 seconds at 500 rpm and off 4 sec). During that period, the FFA-SPME 85 µm PA fiber (Supelco, Bellefonte, PA, U.S., cat. no. FFA 57294-U) was immersed directly into sample solution. After SPME extraction, the fiber was placed for 45 seconds into an SPME fiber conditioning station set at 100 °C. Subsequently, the SPME on-fiber HS derivatization was performed into the agitator for 30 minutes at 60 °C, exposing the SPME fiber in 2-mL amber silanized vials (Thermo Fisher Scientific, Waltham, MA, US, cat. no. MSCERT 5000-S41W) assembled with screw thread caps for magnetic transport (Thermo Fisher Scientific, Waltham, MA, US, cat. no. 9-MSC(BG)-ST101) and containing 15 µL MTBSTFA (Sigma-Aldrich Saint Louis, MO, U.S., cat. no. 394882-10X1ML). Finally, the fiber was inserted into the GC injector equipped with Merlin Microseals (Sigma-Aldrich, Saint Louis, MO, U.S., cat. no. 24817-U) for the thermal desorption of analytes.

### *3.3 Off-line DI-SPME and xyz robotic apparatus*

The SPME Multi Off-Line Sampler (Chromline, Prato, Italy) is a holder designed (Figure 3) to be used with FFA SPME fibers; in our case PA 85 µm SPME FFA were used. The holder acts as a support when exposing the SPME fibers in the 10-mL amber vials (60 °C for 30 minutes), after which they are placed on 15-position magnetic stirrer plates (Chromline, Prato, Italy). After extraction, the FFAs are

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removed from the Multi Off-Line Sampler and placed for 45 seconds into an SPME fiber conditioning station set at 100 °C. Subsequently, the SPME on-fiber HS derivatization (30 minutes for 60 °C) was performed into 2-mL amber silanized vials, placed into SPME Multi Off-Line Sampler. For desorption the fiber was put into MFX 45-position SPME system installed on the Flex autosampler coupled with GC instrumentation.

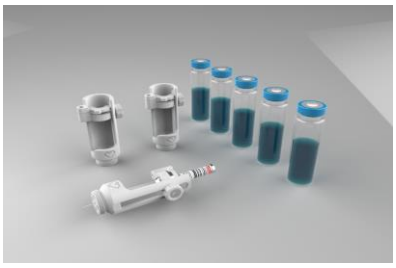

**Figure 3.** SPME Multi Off-Line Sampler.

*3.4 GC/QpQ-MS*

Analyses were performed with a Varian 3900 GC equipped with electronic flow control and a Varian 320-QpQ-MS (Agilent Technologies, St. Clara, CA, U.S.) detector (Table 4).

| **GC conditions** | | | |
|---|---|---|---|
| Injection | 300°C, 20:1 split mode. Liner 0.75mm i.d. | | |
| Oven | 40 °C (1 min) increased at 20 °C/min to 320 °C (5 min) | | |
| Column flow | Helium (99.999%) at a flow rate of 1.2 mL/min | | |
| Retention time | 1-OH-Nap (8.90 min); 2-OH-Nap (9.05 min); 9-OH-Phen (11.96 min); 1-OH-P (13.38 min); 3-OH-B[a]A (14.93 min); 9-OH-B[a]A (14.93 min); 3-OH-B[a]P (17.72 min) | | |
| GC interface | 280 °C | | |
| **MS parameters** | | | |
| Mode | EI | | |
| Filament | Electron energy, 70eV. Filament current 50μA | | |
| Source | Temperature, 200 °C. Pressure, 8 Torr. | | |
| Collision gas | CID gas, Argon. CID gas pressure, 2.00mTorr | | |
| Collision energy | 1-OH-Nap 15 eV; 2-OH-Nap 15 eV; 9-OH-Phen 15 eV; 1-OH-P 15 eV; 3-OH-B[a]A 15 eV; OH-9-B[a]A 15 eV; 3-OH-B[a]P 10eV. | | |
| **SRM transition** | | | |
| 1-OH-Nap | Fragment Q1>Q3 | Quantification *m/z* 258.5→201.2 | Confirmation *m/z* 201.4→185.0 |
| 2-OH-Nap | Q1>Q3 | 258.5→201.2 | 201.4→185.0 |
| 9-OH-Phen | Q1>Q3 | 308.5→251.2 | 251.4→235.0 |
| 1-OH-P | Q1>Q3 | 332.5→275.0 | 275.4→259.0 |
| 3-OH-B[a]A | Q1>Q3 | 358.5→301.1 | 301.5→285.0 |
| 9-OH-B[a]A | Q1>Q3 | 358.5→301.1 | 301.5→285.0 |
| 3-OH-B[a]P | Q1>Q3 | 382.6→325.2 | 382.6→309.6 |

**Table 4.** GC/QpQ-MS method parameters.

A VF-5ms +10m EZ-Guard fused silica capillary column (internal diameter 0.25 mm, length 30 m and film thickness 0.25 μm) (Agilent Technologies, St. Clara, CA, U.S., cat. no. CP9013) was used (Figure 4). For desorbing the analytes, the SPME fiber was introduced into the 1177 Varian GC injector port. A connection with the Laboratory Information Management System (Bika Lab System) provides a user-programmable suite of options.

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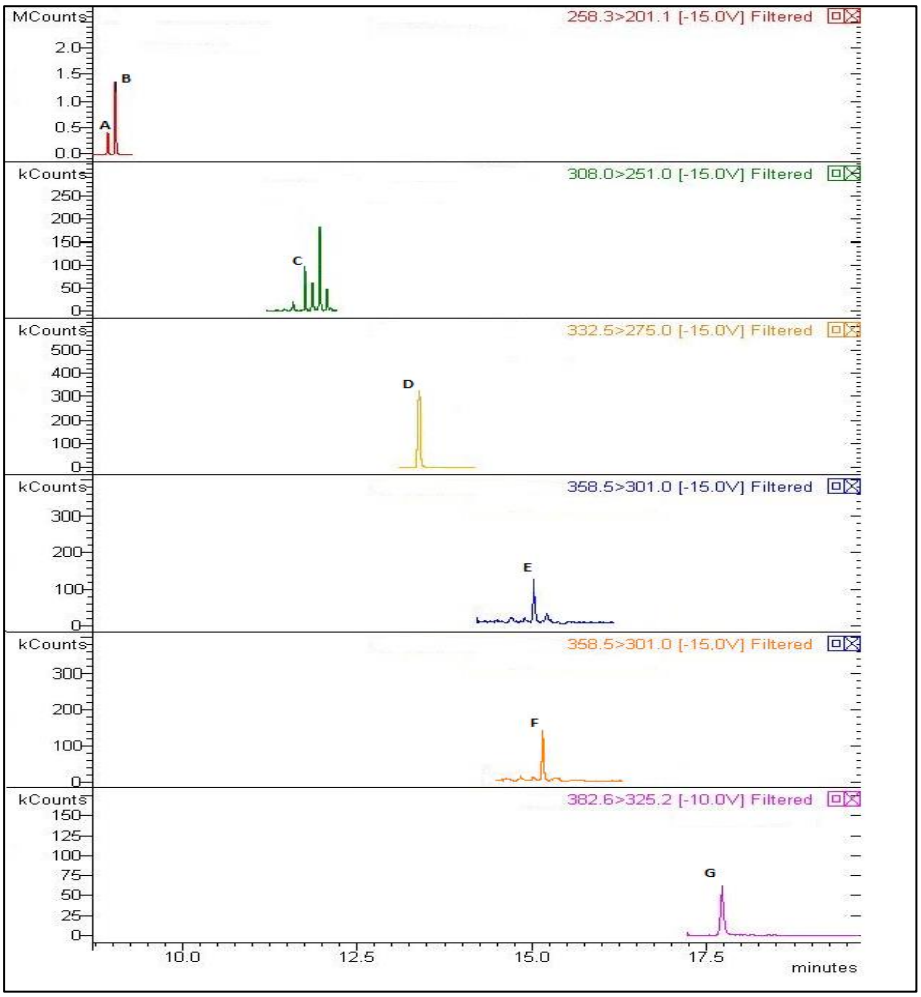


**Figure 4.** Chromatogram of urinary OH-PAHs by GC/QpQ-MS. A=1-OH-Nap; B= 2-OH-Nap; C= 9-OH-Phen; D= 1-OH-P; E= 3-OH-B[a]A; F= 9-OH-B[a]A; G= 3-OH-B[p]A.

*3.5 Synthesis*

1-OH-Nap (cat. no. N1000), 2-OH-Nap (cat. no. 185507), 9-OH-Phen (cat. no. 211281), 1-OH-P (cat. no. 361518) were purchased by Sigma-Aldrich (Saint Louis, MO, U.S.). 3-OH-B[a]P was synthesized as described by Harvey [63], while 3-OH-B[a]A, 9-OH-B[a]A were prepared following Gelboin's procedure [64]. The deuterated compounds 1-hydroxy-naphtalene-D7, 2-hydroxy-napthalene-D7, 9-hydroxy-phenantrene-D9,1-hydroxypyrene-D9, 3-hydroxy-benzoanthracene-D11, 9-hydroxybenzo-anthracene-D11 and 3-hydroxy-benzo[a]pyrene-D11 were prepared by perdeuteration of the unlabeled starting material under the conditions described by Siegel [65]: in all cases two reaction cycles were enough to reach a deuteration of above 98%.

*3.6 Method Validation*

Six calibration standards were obtained (1, 2, 4, 8, 16, 32 ng/L) and five analyses for each of the calibration samples were performed. Least-square linear regression (LSLR) analysis was used to estimate slopes (m) and intercepts (b) of calibration lines y = mx + b, where y is the ratio between the chromatographic area of the analyte and the relative IS, and x the concentration of analytes (ng/L of urine). The limit of detection (LOD) of the assay was calculated according to the following equation 1: LOD = (3SEb + b)/m (1) where SEb is the internal standard error of the intercept. The precision of the assay (as a coefficient of variation, CV%) was based on both within-session and inter-session repeatability. Accuracy was evaluated by the recoveries (calculated from the percentage ratio

between the measured and the nominal concentration solutions) at all concentrations used for the calibration plot and from certified analytical standards for 1-OH-P (Chromsystems Instruments & Chemicals GmbH, Gräfelfing, German, cat. no. 53003). Values of accuracy were then compared with the requirements of the US Food and Drug Administration for analytical method validation. Low (2 ng/mL) and high (20 ng/mL) level quality control samples were prepared and processed in every analytical session from a fresh solution with the IPA with ISs to ensure the precision validity of reported results.

## 4. Conclusions

Occupational studies indicate that there is a correlation between PAHs exposure and cancer incidence for various human tissue such as lung, skin and bladder. As results a regular control of the concentrations in the workplace and in life environments by the measurement of their metabolites become mandatory. PAHs metabolites in human urine can be used as biomarkers of internal dose to assess recent exposure to PAHs. In previous studies, the oft-reported use of solvent and/or clean-up steps were necessary to extract and eliminate most of the interfering compounds from the urine. These laboratories use techniques based on age-old methodologies with low level of automation. A clear and optimized sample preparation strategy is necessary to minimize the number of steps because each step represent additional time and potential source of error.

Our data suggests that automated SPME extraction coupled with GC/QpQ-MS is a viable alternative for OH-PAHs analyses. Customized and automatized MS systems for high-throughput screening are not only user-friendly, but they reduce the costs of monitoring occupational health hazards. New sample preparation techniques are currently being increasingly explored because of the considerable need for information management, the automation of sample preparation, and the integration of data management into the analytical process.

**Conflicts of Interest:** The authors declare no conflict of interest.

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