RESEARCH PAPER

Gas chromatography–tandem mass spectrometry analysis of 52 monohydroxylated metabolites of polycyclic aromatic hydrocarbons in hairs of rats after controlled exposure

**Nathalie Grova · Guillaume Salquèbre ·
Brice M. R. Appenzeller**

Abstract A method based on gas chromatography–tandem mass spectrometry after derivatization with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide was developed for the analysis of monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in hair. The method focused on 52 target compounds corresponding to two- to six-ring monohydroxylated metabolites of polycyclic aromatic hydrocarbons (PAHs). The limits of quantification ranged from 0.2 to 50 pg mg^{-1}. The method was then applied to the analysis of hair samples collected from rats exposed to 12 PAHs at 0.01, 0.1, and 1 mg kg^{-1}, by intraperitoneal injection, for 28 days. The results of this study confirm that these metabolites can be incorporated in hair after intraperitoneal administration of the corresponding parent compound. Only 20 of the 52 metabolites were actually detected in hair samples and corresponded to nine parent PAHs. The mean concentrations of OH-PAHs in rat hair samples exposed to PAHs at 1 mg kg^{-1} ranged from 0.6±0.2 pg mg^{-1} for 8-hydroxybenzo[*b*]fluoranthene to 6.7±1.0 pg mg^{-1} for 1-hydroxypyrene. The results also demonstrated that hair pigmentation has no influence on the concentration of most OH-PAHs. This animal experiment confirmed the incorporation of PAH metabolites in hair and demonstrated that the method was sufficiently sensitive to detect low levels of exposure to PAHs. These results confirmed the usefulness of hair analysis in the biomonitoring of human exposure to PAHs.

N. Grova (✉) · G. Salquèbre · B. M. R. Appenzeller
Laboratory of Analytical Human Biomonitoring - CRP-Santé,
Université du Luxembourg, Campus Limpertsberg, 162A Avenue de
la Faïencerie, 1511 Luxembourg, Luxembourg
e-mail: nathalie.grova@crp-sante.lu

Introduction

Since exposure to polycyclic aromatic hydrocarbons (PAHs) is suspected to be responsible for numerous health issues, significant efforts have been made to develop efficient strategies for the assessment of human exposure to these ubiquitous compounds. Over the last few decades, PAH metabolites have been analyzed in urine [1–4], with a special focus on 1-hydroxypyrene and 3-hydroxybenzo[*a*]pyrene, which were considered the most representative biomarkers of global exposure to PAHs [5–14]. Measurements of these biomarkers in urine provided useful evaluation of recent exposure to pyrene and benzo[*a*]pyrene in occupationally exposed workers [9, 10, 15–19]. Since individuals are generally exposed to mixtures of PAHs, more extensive methods covering a number of PAH metabolites have been developed for a more comprehensive exposure assessment [3, 12, 20–22]. The urinary metabolites of PAHs have been analyzed by different methods, such as high-performance liquid chromatography with fluorescence detection [1, 2], gas chromatography (GC)–mass spectrometry (MS) after derivatization with an alkylating agent or a silylating agent [23–25] or with a fluorinating agent with use of negative chemical ionization (NCI) mode [4, 26], or liquid chromatography–tandem MS (MS/MS), directly [27–29] or after derivatization [30, 31].

Along with urine, which is the matrix classically used for the assessment of recent exposure to PAHs, the possibility to detect PAHs and their metabolites in hair has also been

demonstrated [32, 33]. Hair has been recognized to be a relevant matrix for analytical toxicology studies. Many studies performed on humans have demonstrated that this matrix is suitable for the analysis of drugs of abuse, medical drugs, metals, and organic pollutants [34–38]. The use of hair for the biomonitoring of long-term exposure to environmental pollutants offers several advantages: (1) this matrix allows the assessment of the average level of exposure (integrating all exposure routes) over several months depending on the hair sample length; (2) in contrast to biological fluids, the accumulation of pollutants in hair in the course of time and their presence even after exposure has stopped increases the likelihood of positive detection; (3) it relies on an easy sampling and simple storage conditions (no refrigeration). Some precautions have, however, to be taken with regard to hair analysis in order to avoid misinterpretation of the results. A main limitation concerns the possible effect of hair pigmentation, which was demonstrated to influence the incorporation of basic drugs such as codeine and cocaine [37]. Nevertheless, as the influence of pigmentation seems to be compound-dependent, with no or a limited effect for some chemicals [39, 40], this parameter has to be evaluated for each method development focused on the analysis in hair of new compounds.

Although hair analysis was first limited to the detection of illicit and medical drugs, technical advances in analytical methods resulting in increased sensitivity have allowed the analysis of pollutants such as dioxins [41–44] and pesticides [45–48]. PAHs were first detected in hair by Toriba et al. [33], who described the use of this matrix for the biomonitoring of human exposure to PAHs. More recently, human exposure to 3,6-dinitrobenzo[e]pyrene, a strong mutagen essentially produced by diesel exhaust, was also investigated in hair [49]. The presence of the latter compounds in air and dust can lead to contamination of hair surface. This external contamination corresponds to chemicals recently deposited on hair and is thus not representative of people's internal dose. In contrast, the chemicals incorporated from blood during hair growth following an individual's exposure (e.g., via ingestion or inhalation) are located inside the hair shaft [50]. For the proper determination of the level of exposure, external contamination has, therefore, to be removed prior to analysis. For this purpose, different solvents can be used. However, the efficiency of washing remains uncertain in that contamination can be only partially removed and also on the grounds that washing with organic solvents can alter hair structure and reach chemicals located inside the hair [50]. The origin of the removed chemicals is therefore unknown, and the interpretation of the results obtained from the analysis of washed hair or washing solvents remains contentious [32]. A possible way to avoid interferences due to contamination lies in the determination of metabolites which are produced during biological processes and are unlikely to be deposited via the air [32]. For

this purpose, monohydroxylated metabolites of PAHs appear to be suitable candidates since their atmospheric concentration is generally below the detection levels, except in areas where coal combustion is still an important source of energy production in facilities unable to efficiently remove this waste from released fumes [51–54]. For this purpose, recent studies performed in our laboratory have allowed the detection of hydroxylated metabolites of PAHs in hair [32, 55]. This method was based on NCI after derivatization of monohydroxylated PAHs (OH-PAHs) with (2S,4R)-N-heptafluorobutyryl-4-heptafluorobutoyloxyprolyl chloride [(S,R)-HFBOPCl] [32, 56]. Unlike with electron ionization (EI), whereby molecules are directly ionized through interaction with high-energy electrons, NCI generates interactions between analytes and a lower-energy reagent gas, such as methane. OH-PAH derivatives with (S,R)-HFBOPCl form negative ions through electron capture. NCI is particularly useful for providing molecular mass information as little or no fragmentation occurs, unlike with EI. For instance, the characteristic ions observed for (S,R)-HFBOPCl OH-PAH derivatives are $[M]^{\cdot-}$ and $[M - 20]^{\cdot-}$. Moreover, this derivatization produced higher molecular mass compounds, which allowed higher specificity (from m/z 629 for naphthols to m/z 799 for 3-hydroxydibenzo[a,h]anthracene). However, recently obtained results demonstrated that although this approach is suitable for two- to four-ring OH-PAHs, it is unsuitable for compounds with higher molecular mass [57]. In fact, (S,R)-HFBOPCl derivatives generated peak broadening, which did not allow appropriate resolution and separation of OH-PAH isomers, particularly those with more than four aromatic rings. A clearer separation of isomer analytes as well as a higher analytical response have been obtained by means of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) derivatization and EI–triple quadrupole detection [57].

The objective of this study consisted in the development of an analytical method based on GC–MS/MS after derivatization with MTBSTFA for the analysis of OH-PAHs in hair. The method focused on 52 target compounds corresponding to two- to six-ring monohydroxylated metabolites of PAHs. The method was then applied to the analysis of hairs of rats submitted to controlled exposure to PAHs in order to assess the incorporation of PAH metabolites in this matrix and to confirm that the method was sufficiently sensitive to highlight low levels of exposure.

Materials and methods

Reagents and standards

1-Hydroxyphenanthrene and 4-hydroxyphenanthrene standard solutions were supplied at 10 mg L^{-1} by Dr. Ehrenstorfer (Augsburg, Germany). Stable-isotope-labeled analogues 1-

hydroxypyrene-d_9 and naphthol-d_7 were obtained from Chiron (Trondheim, Norway) and Medical Isotopes (Pelham, NH, USA), respectively. 3-Hydroxyfluoranthene-$^{13}C_6$, 1-hydroxybenz[*a*]anthracene-$^{13}C_6$, and the 52 OH-PAHs investigated in this study were purchased in powder form from MRIGlobal (Kansas City, MO, USA). The purity of all the standards investigated was above 98 %, except for 2-hydroxychrysene, 9-hydroxybenzo[*a*]anthracene, 11-hydroxybenzo[*a*]anthracene, and 8-hydroxyindeno[1,2,3-*c*,*d*]pyrene, which had purity ranging from 91 to 96 %. The level of purity was taken into account for the preparation of the standard solutions. ENVI-Chrom P solid-phase extraction (SPE) columns and the derivatization reagent MTBSTFA (purity 97 % or greater) containing 1 % *tert*-butyldimethylchlorosilane were purchased from Chromoptic (Courtaboeuf, France) and Sigma-Aldrich (Bornem, Belgium), respectively. A stock solution of each compound at 1 g L^{-1} and mixed-standard solutions of OH-PAHs and internal standards were prepared in volumetric flasks at 10 mg L^{-1} in acetonitrile. Working solutions were prepared by successive tenfold dilutions at concentrations ranging from 10 to 1,000 μg L^{-1} and were stored at −20 °C. Ultrapure water was produced by means of an AFS-8 system from Millipore (Brussels, Belgium).

Instrumentation

Analyses were conducted with an Agilent 7890A gas chromatograph equipped with an HP-5MS capillary column (30 m, 0.25-mm inner diameter, 0.25-μm film thickness), coupled to an Agilent 7000A triple-quadrupole mass spectrometer operating in EI mode and an Agilent CTC PAL autosampler.

Gas chromatography

The inlet was at a temperature of 260 °C. Two microliters of extract was injected in pulsed splitless mode with a pressure of 47 psi for 1.5 min. Chromatographic separation was performed using the column described earlier. Helium was used as a carrier gas at 1.8 mL min^{-1}. The oven temperature was kept at 100 °C for 2 min. It was later increased to 235 °C at a rate of 40 °C min^{-1} and was then raised to 280 °C at 10 °C min^{-1}, and was maintained for 3 min. Finally, after it had been increased at a rate of 10 °C min^{-1}, the temperature was maintained at 300 °C for 14 min. After each run, the temperature was set to 300 °C for 4 min in backflush mode in order to remove high-boiling compounds through the split vent.

Mass spectrometry

The source temperature was set to 230 °C. The spectrometer was operated in EI mode with multiple reaction monitoring. Detailed parameters are presented in Table S1. The collision-induced-dissociation gas and the quench gas in the collision cell were nitrogen (1.5 mL min^{-1}) and helium (2.25 mL min^{-1}), respectively, and were used following the supplier's recommendations for flow and quality.

Administration of PAHs and sampling

Twenty healthy bicolor (white and black hair) Lister hooded rats (males of 180–200 g) obtained from Harlan (Horst, Netherlands) were kept in plastic cages and acclimatized to the conditions in the animal facility for 1 week under a timed 12 h/12 h light/dark cycle (light on at 7 pm) at 22±2 °C and 40±5 % relative humidity. Food and water were available ad libitum. The water, food, and oil were tested according to NF ISO 15302 to confirm that all these matrices were PAH-free down to a limit of detection (LOD) of 1 ng g^{-1} fat and 10 ng L^{-1} water. To avoid external contamination of OH-PAHs in hair by urine excretion, special bedding with a high absorption capacity (4.8 L kg^{-1}, Limogen® 8-15), was used and replaced every 2 days. All procedures complied with the rules set out in European Union Council Directive 2010/63/EU. The PAH stock solution (made of naphthalene, fluorene, phenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*c*,*d*]pyrene, and dibenzo[*a*,*h*]anthracene) was prepared in oil weekly. Five rats were randomly assigned to each of the experimental groups receiving the PAH mixture at a dose of 0.01, 0.1, or 1 mg kg^{-1}, the levels corresponding to the levels of exposure detected in smokers, heavy consumers of smoked or grilled meat and fish, or individuals with heavy occupational exposure [58, 59]. Each rat received intraperitoneal injections of the PAH mixture solubilized in vegetable oil (1 mL kg^{-1} body weight, ISIO4, Lesieur, Neuilly-sur-Seine, France) three times per week over a 28-day period. Control rats received blank vegetable oil only. The back of the rats was shaved with an electric shaver before and after the PAH exposure period to ensure that the hairs analyzed represented the period of exposure. White and black hairs were collected separately and stored in aluminum foil at room temperature until analysis.

Extraction and purification

Hair specimens were decontaminated by washing a 50-mg sample with 10 mL water (for 2 min) and were dried at room temperature. Hydrolysis was performed with 1 mL 1 N NaOH and samples were incubated overnight at 40 °C. Neutralization was achieved with 500 μL 2 N HCL and 1 mL ammonium acetate buffer (pH 1, 0.2 M). A first liquid–liquid extraction (LLE) was conducted twice with 2 mL dichloromethane (300 agitations per minute for 10 min and centrifugation for 3 min at 1,800 g at room temperature). The organic layer containing OH-PAHs was collected and dried under a nitrogen flow at 37 °C. Then, the residue was dissolved in 2 mL cyclohexane

and 2 mL methanol–water (80:20; v/v), shaken at 300 agitations per minute for 10 min, and centrifuged at 1,800 g for 3 min. The upper layer was collected with a glass Pasteur pipette and dried under a nitrogen flow at 37 °C. The residue was dissolved in 1 mL cyclohexane and applied onto an Envi-Chrom P SPE column that had previously been conditioned with water, methanol, and cyclohexane successively. After they had been cleaned with 1 mL cyclohexane, OH-PAHs were eluted with 2 mL cyclohexane–ethyl acetate (50:50; v/v). The extract that contained hydroxylated metabolites was evaporated to dryness (at 37 °C using nitrogen), and 50 μL MTBSTFA was added. Derivatization of target analytes was completed after 30 min at 60 °C, and 2 μL of sample was injected into the GC–MS/MS system.

Data analysis

Peaks were identified by absolute retention time and quantifier and qualifier ion peak area ratios. The transition yielding the highest signal-to-noise ratio for each analyte was used for quantification; the second and third transitions were used as qualifiers (Table S1). Data were processed as peak area ratios of target analytes and their internal standards.

Statistical analysis

The concentrations of OH-PAHs were analyzed using one-way analysis of variance with different doses of the PAH mixture as an independent factor. Given that the data did not follow a Gaussian distribution, nonparametric statistical procedures were used. The data from controls were compared with those from PAH-exposed rats using a Kruskal–Wallis test followed by a modified Dunn's procedure for post hoc comparisons. The statistical analysis was performed using SigmaPlot (Systat Software, Erkrath, Germany). Differences were considered to be significant at the level of $p < 0.05$.

Safety considerations

General guidelines for working with organic solvents, acids, and alkalis were respected. All standard compounds have been designated as "chemical carcinogens." This does not necessarily imply that the sample is a known carcinogen, but merely implies that it is intended for use in research involving chemical carcinogens and that it should be treated as a carcinogen.

Results and discussion

Extraction of OH-PAHs from hair

The efficiency of the extraction method was evaluated using blank rat hairs supplemented with OH-PAHs as well as hairs from animals after controlled exposure to PAHs. The hair samples were washed to remove external contamination such as residues of sebum and the dust typically present on hair and that generates an increase in the analytical noise. The hair washing with water was conducted as previously described by Schummer et al. [32]. This washing step allows impurities from hair to be removed without OH-PAHs being removed. In accordance with previous studies, hydrolysis with NaOH (1 N) was performed in order to increase the recovery of OH-PAHs from hair, and LLE with dichloromethane was used for the extractions of OH-PAHs [32]. Incubation of hair in NaOH, which leads to the complete hydrolysis of the matrix, is particularly suitable for chemically stable compounds such as drugs (Δ^9-tetrahydrocannabinol and methamphetamine) or organic micropollutants (PCBs and PAHs) [32, 33, 42, 60, 61].

Purification

The LLE using dichloromethane did not allow clear separation of the four- to six- ring OH-PAHs. Therefore, an additional SPE combining ethyl acetate–cyclohexane (50:50, v/v) as a mobile phase with Envi-Chrom P as a stationary phase was included in the method. Except for naphthols, the peak area increased by a factor ranging from 1.2 for 3-hydroxydibenzo[a,h]anthracene to 11.7 for 2-hydroxyfluorene. Although this SPE permitted clear separation and a higher response (peak area) of OH-PAHs, a greasy deposit remained in the extract, resulting in rapid clogging of the system and decreased performances. An additional purification step consisting in use of cyclohexane–methanol–water (50:40:10; v/v/v) LLE was thus added, as described in previous studies, with a view to removing impurities (e.g., lipids and proteins) [57, 62] (data not shown). The benefit of this additional LLE for the extraction of OH-PAHs from hair was tested through two different protocols: (1) SPE followed by LLE with cyclohexane–methanol–water (50:40:10; v/v/v); (2) SPE after LLE with cyclohexane–methanol–water (50:40:10; v/v/v). For most of the compounds tested here, the highest response (peak area and signal-to-noise ratio) was obtained when LLE was performed before SPE. For example, the signal-to-noise ratio increased by a factor ranging from 1.1 for 1-hydroxypyrene to 4.2 for 3-hydroxyfluoranthene. The purified extract was then dried before derivatization with MTBSTFA. Derivatization was completed after 30 min at 60 °C, and 2 μL of sample were injected into the GC–MS/MS system. Figure 1 displays the analysis of OH-PAHs in hair samples supplemented at 10 pg mg^{-1}.

Chromatographic aspects

Although most of the OH-PAHs were clearly separated, some of them which exhibited the same molecular mass and the same specific transitions ([M]$^{\bullet -}$ → [M-16]$^{\bullet -}$, [M]$^{\bullet -}$ → [M-
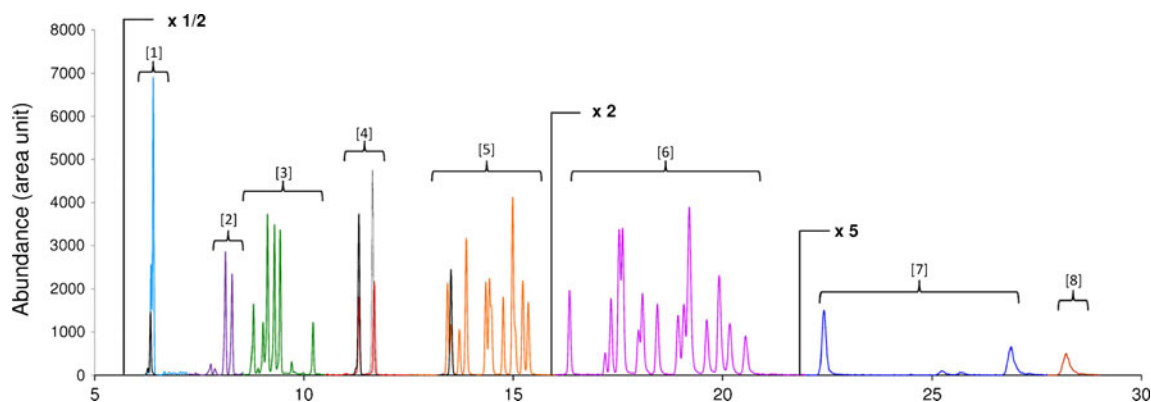
Fig. 1 Analysis of monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in hair samples supplemented at 10 pg mg^{-1}. OH-PAH standards were derivatized with *N-tert*-butyldimethylsilyl->*N*-methyltrifluoroacetamide. *[1]* hydroxynaphthalenes, *[2]* hydroxyfluorenes, *[3]* hydroxyphenanthrenes, *[4]* hydroxyfluoranthenes/hydroxypyrene, *[5]* hydroxybenzo[*c*]phenanthrene/hydroxybenzo[*a*]anthracene/hydroxychrysenes, *[6]* hydroxybenzo[*a*]pyrene/hydroxybenzo[*b*]fluoranthene/hydroxybenzo[*k*]fluoranthene, *[7]* hydroxyindeno[1,2,3-*c*,*d*]pyrene, *[8]* hydroxydibenzo[*a*,*h*]anthracene

57]$^-$, [M-57]$^- \rightarrow$ [M-57-16]$^-$, and [M-57]$^- \rightarrow$ [M-57-16-15]$^{\bullet-}$) remained coeluted. Despite many attempts to separate these isomers (alteration of stationary phase, column characteristics, and temperature program) ten OH-PAH pairs were still coeluted (Table S1). Therefore, for these specific OH-PAHs, the results were provided as the sum of the two compounds (Tables 1 and 2).

Analytical performances

The challenge mainly consisted in obtaining a sensitive and selective analytical method compatible with the low concentrations of the analytes, the complexity of the hair matrix (containing 1–9 % lipids, 65–95 % protein, and 0.1–5 % melanin), and the physicochemical properties of OH-PAHs (hydrophobic compounds). Table 1 details the LOD, limit of quantification (LOQ), calibration curve equations, recovery results, accuracy, and imprecision (relative standard deviation) for all the analytes. A calibration curve was constructed using hair specimens supplemented with the following concentrations of OH-PAHs: 0, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1,000 pg mg^{-1}. In accordance with several method validation guidelines, four replicates were analyzed for each concentration [63, 64]. Linearity was evaluated by linear regression and expressed by the coefficient of determination (R^2). The calibration curve was linear from the LOQ to 1,000 pg mg^{-1} for all the OH-PAHs tested (Table 1). The coefficient of determination (R^2) was above 0.997 for all the analytes, with the exception of 6-hydroxyindeno[1,2,3-*c*,*d*]pyrene (0.990) and 3-hydroxydibenz[*a*,*h*]anthracene (0.986).

Blank samples were analyzed to determine the initial concentration of OH-PAHs in the hairs of rats. Only naphthols were detected, and the recovery values were corrected by subtracting the concentration initially detected in blank samples. Recovery was evaluated by supplementing hair samples at three concentrations (1, 10, and 100 pg mg^{-1} hair) and by comparing the peak area with the peak area of samples treated identically but supplemented with the same concentrations of OH-PAHs just before the derivatization step. At the highest concentration, the recovery ranged from 4 to 69 % for all the OH-PAHs investigated in this work (Table 1). These results are in line with the recoveries for the OH-PAHs obtained from human hair samples that were mostly above 42 % with the GC–NCI-MS method [32]. Among the 52 OH-PAHs analyzed, only five compounds had recoveries below 25 %.

Regarding the specificity of the method, one or two confirmation transitions were used to ensure the presence of each target compound (Table S1). The variability in the ratio of the quantification transition to the confirmation transition was expected to be within 20 % to confirm the presence of the analytes.

Sensitivity was evaluated by the LOD and LOQ. The LOD was determined for each analyte as the lowest concentration with a signal-to-noise ratio of at least 3:1 for the quantification transition (Table 1). The LOQ was defined as the lowest concentration for each analyte that can be measured using supplemented hair samples with variability within (±) 25 % ($n = 4$) based on the quantification transition (Table 1). Clearer separation of isomer analytes as well as a higher analytical response were obtained by means of MTBSTFA derivatization and EI–triple quadrupole detection compared with (*S,R*)-HFBOPCl derivatization and GC–NCI-MS detection [65]. The present method allowed us to achieve LOQs that were from 15 to 30 times lower than those previously obtained for the same OH-PAHs derivatized with (*S,R*)-HFBOPCl and detected by GC–NCI-MS. The LOD ranged from 0.05 to 10 pg mg^{-1} hair and the LOQ ranged from 0.2 to 50 pg mg^{-1} depending on the analyte investigated (Table 1). The LOD and LOQ determined for hydroxynaphthalenes were probably overestimated owing to the presence of these compounds in the blank sample (see Table 1). The sensitivity

Table 1 Monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in hair investigated by gas chromatography–tandem mass spectrometry: limits of detection (LOD), limits of quantification (LOQ), calibration curve equation, recovery results, accuracy, and imprecision (relative standard deviation, RSD)

Compound	R^2	Equation	Linear range (pg mg⁻¹)	LOD (pg mg⁻¹)	LOQ (pg mg⁻¹)	Recovery (%) (n=4)			Accuracy (percentage of the target)						RSD (%)					
									Intra-day (n=4)			Inter-day (n=30)			Intra-day (n=4)			Inter-day (n=30)		
						Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
1-Hydroxynaphthalene[a]	0.9996	$y=1.1x+0.04$	0.6–1,000	0.2	0.6	14	13	24	105	102	100	95	97	100	7	4	13	14	10	7
2-Hydroxynaphthalene[a]	0.9994	$y=2.1x+0.81$	1–1,000	0.3	1	17	26	36	82	78	96	98	95	99	4	8	4	16	11	10
9-Hydroxyfluorene	0.9965	$y=0.01x$	50–1,000	10	50	ND	ND	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3-Hydroxyfluorene	0.9988	$y=8.36x$	0.5–1,000	0.15	0.5	58	59	61	99	101	100	99	98	100	5	8	2	12	8	9
2-Hydroxyfluorene	0.9994	$y=2.23x$	0.5–1,000	0.15	0.5	63	62	66	99	100	103	102	100	102	4	5	4	14	11	10
4-Hydroxyphenanthrene	0.9979	$y=7.97x$	0.2–1,000	0.05	0.2	63	63	58	94	93	101	102	96	98	7	5	1	10	9	7
9-Hydroxyphenanthrene	0.9995	$y=2.77x$	0.5–1,000	0.15	0.5	20	16	17	100	98	101	94	89	96	18	8	2	14	10	12
3-Hydroxyphenanthrene	0.9994	$y=2.60x$	0.2–1000	0.05	0.2	67	68	66	101	101	102	99	95	98	10	8	6	12	12	14
1-Hydroxyphenanthrene	0.9985	$y=6.61x$	0.3–1,000	0.1	0.3	74	69	65	98	93	100	96	95	96	1	0	1	9	10	9
2-Hydroxyphenanthrene	0.9995	$y=2.94x$	0.2–1,000	0.05	0.2	70	67	68	100	96	102	99	99	97	6	6	7	10	12	12
3-Hydroxyfluoranthene	0.9995	$y=1.18x$	0.5–1,000	0.15	0.5	46	42	43	91	92	102	98	89	93	3	2	2	11	10	10
1-Hydroxypyrene	0.9995	$y=1.14x$	0.2–1,000	0.05	0.2	56	59	55	99	99	105	96	93	92	2	5	2	12	11	10
1-Hydroxybenzo[a]anthracene	0.9998	$y=3.55x$	0.3–1,000	0.1	0.3	58	56	58	97	97	100	97	95	94	6	1	1	5	6	6
4-Hydroxychrysene	0.9995	$y=3.59x$	0.2–1,000	0.05	0.2	43	43	43	94	94	100	96	93	93	3	1	3	8	6	6
6-Hydroxychrysene + 11-hydroxybenzo[a]anthracene	0.9997	$y=3.12x$	0.2–1,000	0.05	0.2	36	38	35	95	84	101	100	95	100	6	3	3	10	7	5
2-Hydroxybenzo[a]anthracene + 5-hydroxybenzo[a]anthracene	0.9996	$y=2.09x$	0.2–1,000	0.05	0.2	29	31	26	100	96	100	97	95	95	2	0	1	7	4	6
8-Hydroxybenzo[a]anthracene	0.9996	$y=1.50x$	0.3–1,000	0.1	0.3	29	31	28	94	91	100	96	91	100	6	1	0	11	11	8
3-Hydroxychrysene	0.9999	$y=1.64x$	0.2–1,000	0.05	0.2	54	57	51	100	96	99	96	92	95	2	1	2	7	7	5
1-Hydroxychrysene + 4-hydroxybenzo[a]anthracene	0.9998	$y=5.41x$	0.2–1,000	0.05	0.2	51	58	50	96	94	99	95	93	95	8	2	1	10	6	8
10-Hydroxybenzo[a]anthracene	0.9995	$y=0.98x$	1–1,000	0.25	1	28	30	27	100	97	101	99	95	101	3	14	3	11	10	9
3-Hydroxybenzo[a]anthracene + 9-hydroxybenzo[a]anthracene	0.9993	$y=2.18x$	0.2–1,000	0.05	0.2	40	45	37	98	89	100	97	93	96	14	2	2	5	4	4
2-Hydroxychrysene	0.9989	$y=1.64x$	0.2–1,000	0.05	0.2	47	59	47	100	99	101	103	96	94	3	5	3	11	6	5
8-Hydroxybenzo[b]fluoranthene	0.9998	$y=2.19x$	0.5–1,000	0.15	0.5	42	49	38	101	100	95	96	98	97	3	3	2	11	10	9
11-Hydroxybenzo[a]pyrene	0.9992	$y=0.56x$	3–1,000	1	3	ND	9	9	ND	100	102	98	99	106	ND	4	6	14	9	9
2-Hydroxybenzo[b]fluoranthene	0.9999	$y=1.82x$	2–1,000	0.5	2	53	54	47	102	102	101	93	96	93	8	4	1	11	9	12
1-Hydroxybenzo[b]fluoranthene + 7-hydroxybenzo[b]fluoranthene	0.9997	$y=7.90x$	0.3–1000	0.1	0.3	46	47	45	103	98	100	95	91	92	5	2	3	11	7	8
12-Hydroxybenzo[b]fluoranthene + 8-hydroxybenzo[k]fluoranthene	0.9995	$y=8.56x$	0.3–1000	0.1	0.3	46	48	45	98	94	100	94	94	98	6	4	2	10	8	8
10-Hydroxybenzo[a]pyrene	0.9997	$y=1.19x$	3–1000	1	3	ND	25	26	ND	95	101	93	97	94	ND	4	4	15	11	10
12-Hydroxybenzo[a]pyrene + 6-hydroxybenzo[a]pyrene	0.9996	$y=1.92x$	2–1000	0.5	2	36	34	35	99	96	101	92	95	99	6	2	2	13	13	12
5-Hydroxybenzo[a]pyrene	0.9984	$y=0.15x$	30–1000	10	30	ND	4	4	ND	94	98	ND	114	105	ND	9	3	ND	23	13

Table 1 (continued)

Compound	R^2	Equation	Linear range (pg mg⁻¹)	LOD (pg mg⁻¹)	LOQ (pg mg⁻¹)	Recovery (%) (n=4)			Accuracy (percentage of the target)						RSD (%)					
									Intra-day (n=4)			Inter-day (n=30)			Intra-day (n=4)			Inter-day (n=30)		
						Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
11-Hydroxybenzo[b]fluoranthene	0.9995	y=1.97x	2–1000	0.5	2	51	60	48	98	97	96	88	92	91	15	3	1	19	8	14
10-Hydroxybenzo[b]fluoranthene	0.9999	y=1.64x	2–1000	0.5	2	55	64	45	99	100	99	95	91	92	6	5	3	19	12	11
4-Hydroxybenzo[a]pyrene + 3-hydroxybenzo[k]fluoranthene	0.9996	y=1.93x	2–1000	0.5	2	36	33	28	100	101	100	92	93	99	6	3	2	15	13	13
9-Hydroxybenzo[k]fluoranthene + 7-hydroxybenzo[a]pyrene	0.9995	y=5.34x	3–1000	1	3	44	52	42	95	91	97	89	91	95	5	1	2	13	14	13
9-Hydroxybenzo[a]pyrene	0.9994	y=1.37x	3–1,000	1	3	52	52	48	100	103	99	91	98	97	7	3	3	18	11	10
2-Hydroxybenzo[a]pyrene + 1-hydroxybenzo[a]pyrene	0.9989	y=2.80x	2–1000	0.5	2	42	40	39	99	92	100	92	93	95	7	6	1	17	16	14
3-Hydroxybenzo[a]pyrene	0.9993	y=1.31x	3–1,000	1	3	35	35	34	102	93	99	97	101	100	3	3	2	21	14	13
8-Hydroxybenzo[a]pyrene	0.9997	y=1.23x	3–1,000	1	3	ND	49	47	ND	97	101	108	93	96	ND	3	1	21	16	15
6-Hydroxyindeno[1,2,3-c,d]pyrene	0.9982	y=0.55x	1–1,000	0.2	1	ND	43	38	ND	95	95	97	97	94	ND	6	1	24	15	11
1-Hydroxyindeno[1,2,3-c,d]pyrene	0.9895	y=0.11x	10–1,000	5	15	ND	ND	10	ND	ND	157	ND	90	104	ND	ND	19	ND	46	17
2-Hydroxyindeno[1,2,3-c,d]pyrene	0.9974	y=0.08x	20–1,000	5	20	ND	ND	6	ND	ND	98	ND	97	106	ND	ND	22	ND	25	16
8-Hydroxyindeno[1,2,3-c,d]pyrene	0.9971	y=0.26x	10–1,000	2	10	69	56	62	226	164	162	ND	107	103	6	16	29	ND	17	16
3-Hydroxydibenzo[a,h]anthracene	0.9859	y=0.21x	10–1,000	2	10	ND	56	54	ND	97	99	ND	103	102	ND	5	4	ND	20	15

Recovery was evaluated by supplementing hair samples with three concentrations (low, medium, and high levels) of each analyte (1, 10, and 100 pg mg⁻¹, respectively). Intraday and interday accuracy and imprecision were determined on hair samples supplemented with OH-PAHs at 1, 10, and 100 pg mg⁻¹ (low, medium, and high, respectively)

ND not determined

[a] Validation was done on a pulverized and homogenized hair sample that contained 1-hydroxynaphthalene and 2-hydroxynaphthalene at doses of 1.9 and 4.8 pg mg⁻¹, respectively

determined for 9-hydroxyfluorene in hair was clearly below that evaluated for all the other OH-PAHs. This might be explained by poor derivatization due to steric hindrance which might hamper the MTBSTFA derivatization and/or by the fact that the proton from the hydroxyl group is less labile than in other OH-PAHs, for which the negative charge is stabilized by the aromaticity. In contrast, the negative charge of oxygen resulting from the loss of hydrogen is delocalized all around the ring for all the other OH-PAHs, thereby increasing alkoxide stability and improving its reactivity regarding the derivatization agent. As previously described by Schummer et al. [65], MTBSTFA derivatization on a standard solution facilitates the separation of OH-PAH isomer analytes, whereas N,O-bis(trimethylsilyl)trifluoroacetamide seems more suitable for sterically hindered compounds such as 9-hydroxyfluorene. Nevertheless, in previous work we observed that the sensitivity obtained using MTBSTFA derivatization for the analysis of 9-hydroxyfluorene in brain was acceptable (LOQ of 0.6 pg mg^{-1} brain tissue and LOD of 0.2 pg mg^{-1} brain tissue) [57], leading us to suppose that the lack of sensitivity observed here for the analysis of this compound in hair might be due to the presence of impurities specific to this matrix.

Precision and accuracy were evaluated with hair samples spiked at three different concentrations (1, 10, and 100 pg mg^{-1}) for each compound (Table 1). The results are expressed as a percentage of the target value and the relative standard deviation. Except for hydroxyindeno[1,2,3-c,d]pyrenes, intraday precision (n=4) was always below 15 % (and often below 10 %) for all the compounds. Interday precision (n=30) was in most cases below 20 % and was always below 25 %. Except for 1-hydroxyindeno[1,2,3-c,d]pyrene and 8-hydroxyindeno[1,2,3-c,d]pyrene, intraday accuracy (n=4) and interday accuracy (n=30) evaluated at an OH-PAH dose of 100 pg mg^{-1} in hair were 95–105 % and 91–106 % (Table 1).

Although the present method exhibits satisfactory separation of most of the metabolites investigated here, 23 of the 75 possible monohydroxylated metabolites of PAHs were not analyzed because standards were not available from suppliers. As a consequence, it cannot be ruled out that the latter analytes were coeluted with the ones analyzed with the present method and possibly led to overestimation of the amount determined for some of the target compounds.

Application to hair samples from treated rats

The main disadvantages concerning hair analysis in the biomonitoring of human exposure to pollutants lie in (1) the possibility of external deposition of chemicals on the hair surface and (2) the possible influence of hair pigmentation (i.e., the melanin content of hair) [50]. To avoid misinterpretation due to external contamination, the method developed here focuses on the analysis of

OH-PAHs. Being produced during the metabolic processes, these compounds are not present in air and are unlikely to be deposited via air. Naphthols are an exception, since these compounds were detected in air in areas where coal combustion is an important source of energy production [51–54]. Our in vivo study reports on the quantitative analysis of 52 OH-PAHs in hair samples obtained from Lister hooded rats exposed to a PAH mixture at doses ranging from 0.01 to 1 mg kg^{-1} for 28 days. The results of this study confirm that these metabolites can be incorporated in hair after intraperitoneal administration of the corresponding parent compounds (Figs. 2 and 3). The 52 OH-PAHs investigated here were metabolites of the 12 PAHs administered to the rats. Only 20 of the 52 metabolites were actually detected in hair samples. These 20 metabolites corresponded to nine parent PAHs (Table 2, Fig. 2). No metabolites of fluorene, benzo[k]fluoranthene, and dibenzo[a,h]anthracene were detected.

Although frequently encountered in human hairs, 9-hydroxyfluorene, 2-hydroxyfluorene, and 3-hydroxyfluorene, were not detected in rat hairs regardless of the dose analyzed. These results suggest that 2-hydroxyfluorene and 3-hydroxyfluorene were probably minor metabolites, and that other monohydoxylated or dihydroxylated metabolites could be produced in higher quantities. This is certainly the case for 9-hydroxyfluorene, which is the most abundant metabolite of fluorene detected in human hair [55]. The high LOQ determined for this compound did not allow the detection of concentrations below 50 pg mg^{-1} hair. The absence of positive detection in hair samples could also be explained by the fact that the level of exposure of humans to fluorene was higher than the controlled exposure of rats.

In parallel, three hypotheses may account for the absence of hydroxybenzo[k]fluoranthene and hydroxydibenzo[a,h]anthracene in hairs. Firstly, the metabolites of hydroxybenzo[k]fluoranthene and hydroxydibenzo[a,h]anthracene investigated here might not be produced or incorporated in hair. This assumption is supported by the low bioavailability of high molecular mass PAHs [66] and by recent findings of the absence of these metabolites in urine of control and asphalt workers and poor recovery in urine of coke oven workers [67]. Secondly, the high LOQ obtained for hydroxybenzo[k]fluoranthene and hydroxydibenzo[a,h]anthracene as compared with the other metabolites could also explain why these five-ring metabolites were not detected in rat hairs. Finally, rat metabolism may produce monohydroxylated metabolites different from those (3-hydroxybenzo[k]fluoranthene, 8-hydroxybenzo[k]fluoranthene, 9-hydroxybenzo[k]fluoranthene, and 3-hydroxydibenzo[a,h]anthracene) investigated in this study (standards unavailable from suppliers). Chromatographic

Table 2 OH-PAHs (pg mg^{-1} hair) measured in hair samples collected from rats treated with a mixture of polycyclic aromatic hydrocarbons (PAHs; 0.01, 0.1, or 1 mg kg^{-1}, 28 days, intraperitoneal administration)

	Hair color	PAH doses (mg kg^{-1})			
		0	0.01	0.1	1
1-Hydroxynaphthalene	W	2.77 (1.16–4.52)	1.75 (1.42–2.09)	1.91 (1.52–2.35)	2.73 (2.37–2.96)
	B	2.46 (1.40–3.42)	2.16 (1.81–2.64)	1.96 (1.40–2.59)	2.88 (2.48–3.14)
2–Hydroxynaphthalene	W	2.65 (1.29–4.29)	2.03 (1.76–2.42)	2.04 (1.92–2.19)	2.6 (2.26–3.06)
	B	2.48 (1.60–3.32)	2.66 (1.84–3.21)	1.91 (1.61–2.34)	2.25 (2.02–2.36)
4-Hydroxyphenanthrene	W	ND	ND	<LOQ	0.58 (0.30–0.63)
	B	ND	ND	<LOQ	0.78* (0.63–0.90)
9-Hydroxyphenanthrene	W	ND	ND	ND	<LOQ
	B	ND	ND	ND	ND
3-Hydroxyphenanthrene	W	ND	ND	0.23 (0.18–0.27)	0.95 (0.50–1.14)
	B	ND	ND	0.24 (0.22–0.27)	1.2 (1.12–1.29)
1-Hydroxyphenanthrene	W	ND	ND	ND	0.61 (0.46–0.72)
	B	ND	ND	ND	0.56 (0.38–0.69)
2-Hydroxyphenanthrene	W	ND	ND	ND	0.45 (0.20–0.60)
	B	ND	ND	ND	0.48 (0.33–0.59)
3-Hydroxyfluoranthene	W	ND	ND	ND	0.63 (0.72–0.46)
	B	ND	ND	ND	0.58 (0.69–0.38)
1-Hydroxypyrene	W	ND	ND	0.42 (0.32–0.51)	3.88 (2.03–5.51)
	B	ND	ND	0.52[a] (0.38–0.64)	6.70* (5.30–7.68)
4-Hydroxychrysene	W	ND	ND	<LOQ	0.25 (0.20–0.29)
	B	ND	ND	<LOQ	0.33 (0.25–0.40)
6-Hydroxychrysene + 11-hydroxybenzo[a]anthracene	W	ND	ND	ND	0.35 (0.35–0.35)
	B	ND	ND	ND	0.28 (0.28–0.28)
3-Hydroxychrysene	W	ND	ND	<LOQ	0.62 (0.46–0.88)
	B	ND	ND	<LOQ	0.84 (0.62–0.94)
1-Hydroxychrysene + 4-hydroxybenzo[a]anthracene	W	ND	ND	<LOQ	0.31 (0.24–0.45)
	B	ND	ND	<LOQ	0.46 (0.34–0.54)
8-Hydroxybenzo[b]fluoranthene	W	ND	ND	ND	1.01 (0.5–1.47)
	B	ND	ND	ND	0.6 (<LOQ to 0.81)
1-Hydroxbenzo[b]fluoranthene +7-hydroxybenzo[b]fluoranthene	W	ND	ND	ND	<LOQ
	B	ND	ND	ND	<LOQ
3-Hydroxbenzo[a]pyrene	W	ND	ND	ND	ND
	B	ND	ND	ND	<LOQ
6-Hydroxyindeno[1,2,3-c,d]pyrene	W	ND	ND	ND	1.78 (1.28–2.39)
	B	ND	ND	ND	1.3 (1.04–1.55)

The results are expressed as the mean and quartiles (in *parentheses*) of five rats per group

B black, *W* white

*$P < 0.05$ statistically significant differences from control rats (Dunn's procedure for post hoc comparisons)

[a] Tendency ($p < 0.1$), statistically significant difference from control rats (Dunn's procedure for post hoc comparisons)

analysis seems to confirm the latter hypothesis. On comparison of chromatograms of blank ($n = 4$) and supplemented (1, 10, and 100 pg mg^{-1}) hair samples with hair samples from rats exposed to 1 mg kg^{-1} ($n = 4$) (data not shown), two peaks were detected at retention times of 18.15 and 18.31 min. Both peaks corresponded to the transitions common to hydroxybenzo[a]pyrene, hydroxybenzo[k]fluoranthene, or hydroxybenzo[b]fluoranthene with a precursor ion at $m/z = 382.6$ and a product ion at $m/z = 325.2$. Given that the retention time was identified for all the hydroxybenzo[a]pyrene isomers, these two peaks likely to correspond to isomers of hydroxybenzo[k]fluoranthene and/or hydroxybenzo>[b]fluoranthene.
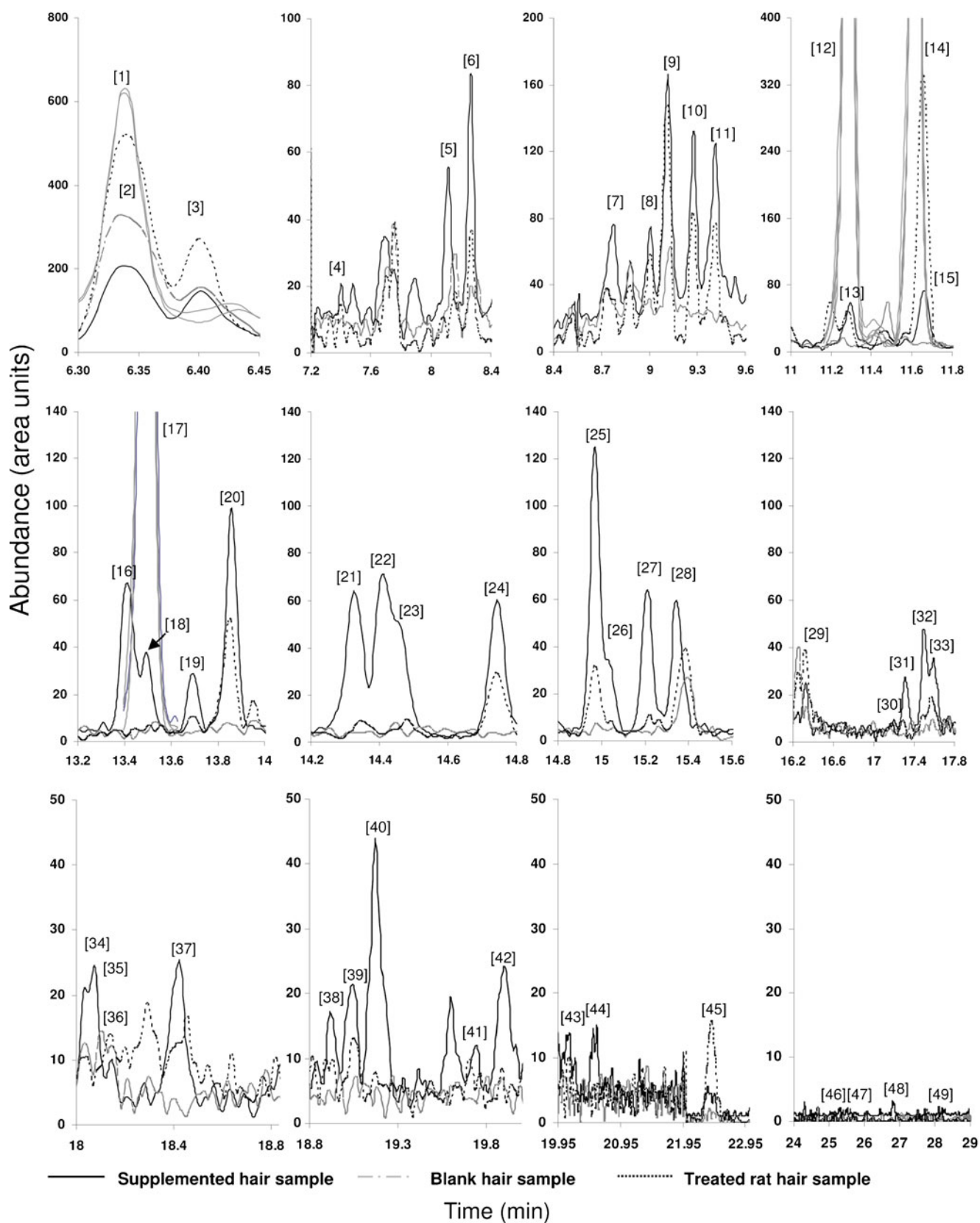
The concentrations of the OH-PAHs in white hair samples of rats exposed to PAHs are presented in Table 2 and Fig. 3.

Surprisingly, the levels of 1-hydroxynapthalene and 2-hydroxynaphthalene in the control group were

◀ **Fig. 2** Chromatograms obtained from the analysis of all the investigated OH-PAHs in a supplemented hair sample, a blank hair sample, and a treated rat hair sample (rat exposed to polycyclic aromatic hydrocarbons at a dose of 1 mg kg^{-1} by intraperitoneal administration for 28 days). *[1]* 1-hydroxynaphthalene-d_7, *[2]* 1-hydroxynaphthalene, *[3]* 2-hydroxynaphthalene, *[4]* 9-hydroxyfluorene, *[5]* 3-hydroxyfluorene, *[6]* 2-hydroxyfluorene, *[7]* 4-hydroxyphenanthrene, *[8]* 9-hydroxyphenanthrene, *[9]* 3-hydroxyphenanthrene, *[10]* 1-hydroxyphenanthrene, *[11]* 2-hydroxyphenanthrene, *[12]* 3-hydroxyfluoranthene-$^{13}C_6$, *[13]* 3-hydroxyfluoranthene, *[14]* 1-hydroxypyrene-d_9, *[15]* 1-hydroxypyrene, *[16]* 2-hydroxybenzo[*c*]phenanthrene, *[17]* hydroxybenzo[*a*]anthracene-$^{13}C_6$, *[18]* 1-hydroxybenzo[*a*]anthracene, *[19]* 4-hydroxychrysene, *[20]* 6-hydroxychrysene and 11-hydroxybenzo[*a*]anthracene, *[21]* 2-hydroxybenzo[*a*]anthracene and 5-hydroxybenzo[*a*]anthracene, *[22]* 3-hydroxybenzo[*c*]pyrene, *[23]* 8-hydroxybenzo[*a*]anthracene, *[24]* 3-hydroxychrysene, *[25]* 1-hydroxychrysene and 4-hydroxybenzo[*a*]anthracene, *[26]* 10-hydroxybenzo[*a*]anthracene, *[27]* 3-hydroxybenzo[*a*]anthracene and 9-hydroxybenzo[*a*]anthracene, *[28]* 2-hydroxychrysene, *[29]* 8-hydroxybenzo[*b*]fluoranthene, *[30]* 11-hydroxy[*a*]pyrene, *[31]* 2-hydroxybenzo[*b*]fluoranthene, *[32]* 1-hydroxybenzo[*b*]fluoranthene and 7-hydroxybenzo[*b*]fluoranthene, *[33]* 12-hydroxybenzo[*b*]fluoranthene and 8-hydroxybenzo[*k*]fluoranthene, *[34]* 10-hydroxybenzo[*a*]pyrene, *[35]* 12-hydroxybenzo[*a*]pyrene and 6-hydroxybenzo[*a*]pyrene, *[36]* 5-hydroxybenzo[*a*]pyrene, *[37]* 11-hydroxybenzo[*b*]fluoranthene, *[38]* 10-hydroxybenzo[*b*]fluoranthene, *[39]* 4-hydroxybenzo[*a*]pyrene and 3-hydroxybenzo[*k*]fluoranthene, *[40]* 9-hydroxybenzo[*k*]fluoranthene and 7-hydroxybenzo[*a*]pyrene, *[41]* 9-hydroxybenzo[*a*]pyrene, *[42]* 2-hydroxybenzo[*a*] and 1-hydroxybenzo[*a*]pyrene, *[43]* 3-hydroxybenzo[*a*]pyrene, *[44]* 8-hydroxybenzo[*a*]pyrene, *[45]* 6-hydroxyindeno[1,2,3-*c*,*d*]pyrene, *[46]* 1-hydroxyindeno[1,2,3-*c*,*d*]pyrene, *[47]* 2-hydroxyindeno[1,2,3-*c*,*d*]pyrene, *[48]* 8-hydroxyindeno[1,2,3-*c*,*d*]pyrene, *[49]* 3-hydroxydibenzo[*a*,*h*]anthracene

considerably higher than in the other treated groups. The concentrations of naphthols detected in the hairs of the control group probably result from environmental exposure to naphthalene. Moreover, no statistical difference was observed between the control group and the treated groups, suggesting that for the naphthols, the contribution of the controlled exposure (0.01–1 mg kg^{-1} for 28 days) was negligible in comparison with environmental exposure.

In contrast, 3-hydroxyphenanthrene and 1-hydroxypyrene, although not detected in blank samples, were detected above the LOQ in rats which received the 0.1 and 1 mg kg^{-1} doses for 28 days. The method developed here did not allow the detection of most of the OH-PAH metabolites in rats which received the two lowest doses of PAHs (0.01 and 0.1 mg kg^{-1} for 28 days). Two hypotheses may explain these results: (1) PAH doses of 0.01 and 0.1 mg kg^{-1} were below the thresholds at which the metabolism starts to be induced (Fig. 3) and/or (2) the method is not sensitive enough. These results are in accordance with previous data obtained from rat brain after exposure of rats to PAHs at concentrations ranging between 0.01 and 1 mg kg^{-1} [57]. Except for 6-hydroxyindeno[1,2,3-*c*,*d*]pyrene, which was detected
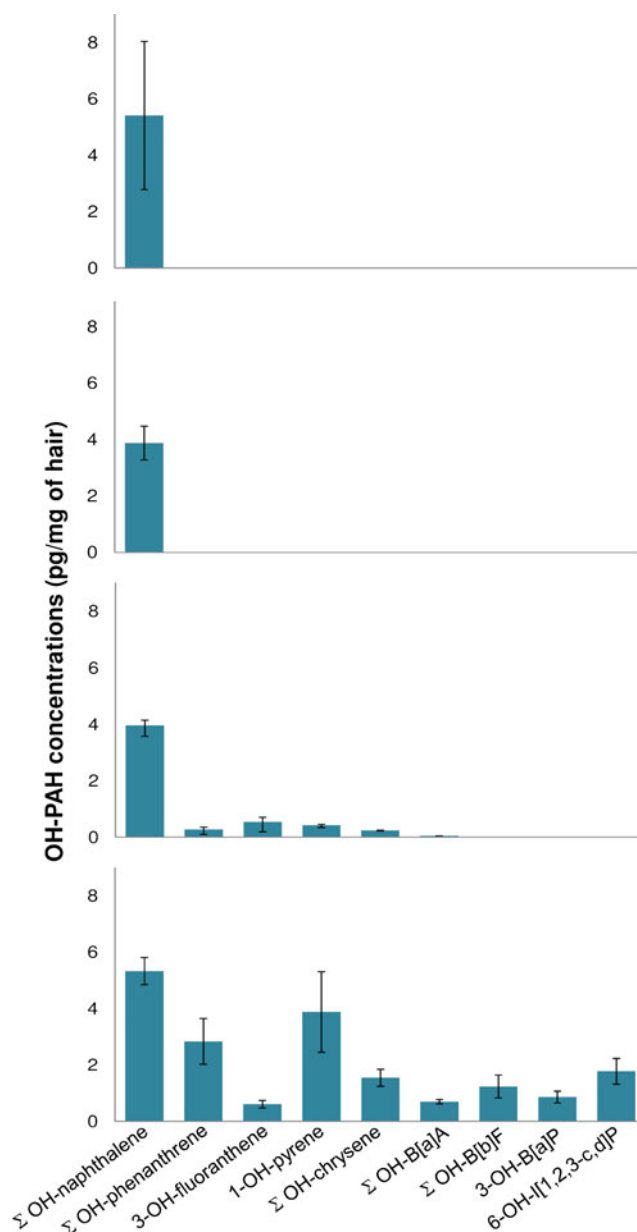

Fig. 3 Concentration of OH-PAHs in white hair of rats exposed to polycyclic aromatic hydrocarbons (0.01–1 mg kg^{-1}, intraperitoneal administration, 28 days). The results are expressed as the mean ± the standard deviation. *OH-naphthalene* hydroxynaphthalene, *OH-phenanthrene*, hydroxyphenanthrene, *3-OH-fluoranthene* 3-hydroxyfluoranthene, *1-OH-pyrene* 1-hydroxypyrene, *OH-chrysene* hydroxychrysene, *OH-B[a]A* hydroxybenzo[*a*]anthracene, *OH-B[b]F* hydroxybenzo[*b*]fluoranthene, *3-OH-B[a]P* 3-hydroxybenzo[*a*]pyrene, *6-OH-I[1,2,3-c,d]P* 6-hydroxyindeno[1,2,3-*c*,*d*]pyrene

only in hair samples, all the OH-PAHs found in hairs were also detected in brain [57]. For example, 1-hydroxypyrene is the most abundant metabolite in both matrices (26.5±22.7 pg mg^{-1} in brain tissue versus 6.7± 1.0 pg mg^{-1} in hairs of rats that received PAHs at a dose of 1 mg kg^{-1} for 28 days). These data are also in accordance with recent work that reported the presence of two- to four-ring OH-PAHs (1-hydroxynapthalene, 2-hydroxynaphthalene, 2-

Fig. 4 Concentration of OH-PAHs in white and black hair of rats exposed to polycyclic aromatic hydrocarbons (1 mg kg^{-1}, intraperitoneal administration, 28 days). The results are expressed as the mean ± the standard deviation. *Asterisk* $P < 0.05$ statistically significant differences from control rats (Dunn's procedure for post hoc comparisons)
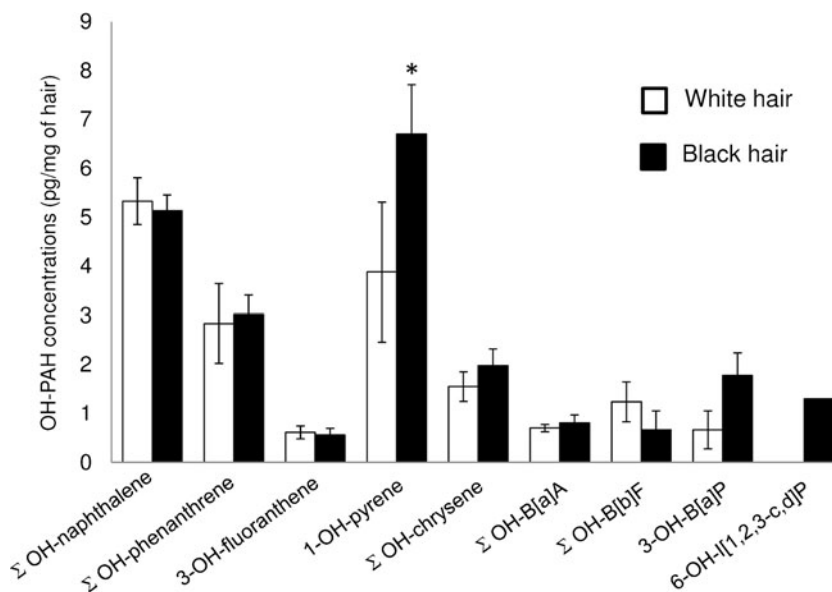
hydroxyfluorene, 9-hydroxyfluorene, 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene, 4-hydroxyphenanthrene, 9-hydroxyphenanthrene, 1-hydroxypyrene, 2-hydroxybenzo[c]phenanthrene, and 6-hydroxychrysene) in the hairs of humans exposed to environmental levels of PAHs [55].

The influence of hair pigmentation has also been mentioned among the possible limitations to the use of hair as a biomarker of exposure [50]. For instance, rats which were received methadone in their drinking water exhibited significantly higher concentrations in pigmented hair than in white hair in a ratio of 21:1 [68]. For this reason, the 52 OH-PAHs investigated in this work were analyzed in both black and white hairs that were separately collected from each rat. Although the concentration was significantly higher in black hair for 4-hydroxyphenanthrene and 1-hydroxypyrene ($p < 0.05$), the difference between black and white hair was not significant for the other metabolites (Table 2). For 1-hydroxypyrene, the concentrations were significantly higher in pigmented hair for exposure at both 0.1 mg kg^{-1} ($p = 0.105$) and 1 mg kg^{-1} ($p < 0.05$). Nevertheless, the black hair–white hair concentration ratios of 4-hydroxyphenanthrene and 1-hydroxypyrene remain weakly affected (1.3:1 and 1.7:1, respectively). In addition, taking into account the sum of the five hydroxyphenanthrenes, we found that the difference observed between black and white hairs for 4-hydroxyphenanthrene was not significant (Fig. 4). Similar differences were observed for dimethylphosphate between pigmented and white hairs of bicolor rabbits after long-term exposure to dimethoate (an organophosphate used as a pesticide) [46]. Although the concentration of this

metabolite was higher in pigmented hairs after 4 and 6 months' exposure and for the two levels tested, the pigmented hair–white hair concentration ratio did not exceed 1.8 [46]. Margariti et al. [46] expressed some reservations about their results (such as the metabolic difference between species and the limited number of rabbits) and concluded that hair pigmentation may affect the incorporation of dimethylphosphate into hair. Thus, our results demonstrate that, at least for rats, hair pigmentation has no influence on the concentration of most OH-PAHs and only a limited influence on the concentrations of 4-hydroxyphenanthrene and 1-hydroxypyrene. These results still have to be confirmed in human hair.

Conclusions

This study has described the development of a sensitive method for the simultaneous determination of OH-PAHs in hair by GC–EI-MS/MS. We have presented a validated, highly sensitive, and selective method for the determination of very low concentrations of 52 OH-PAHs in rat hairs ranging between 0.2 and 50 pg mg^{-1}. This rat experiment confirmed the incorporation of PAH metabolites in hairs and demonstrated that the method was sensitive enough to highlight low levels of exposure to PAHs. These results confirm the usefulness of hair analysis for the biomonitoring of human exposure to PAHs.

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