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## Determination of PAHs and OH-PAHs in Rat Brain by Gas Chromatography Tandem (Triple Quadrupole) Mass Spectrometry

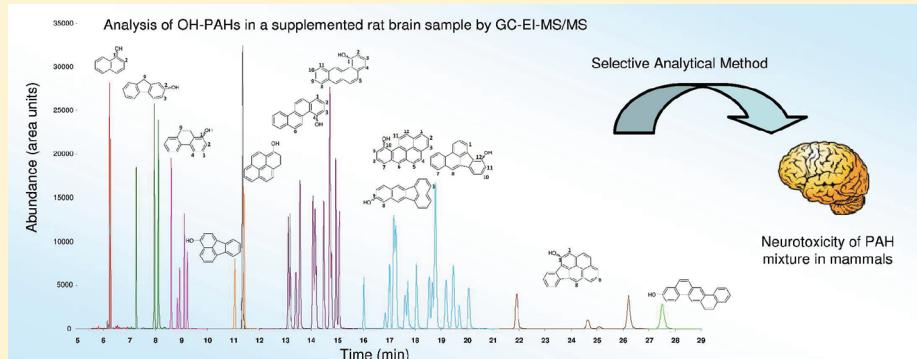
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Supporting Information

### ABSTRACT:



An efficient and selective method for the quantitative determination of polycyclic aromatic hydrocarbons (PAHs) and their monohydroxylated metabolites (OH-PAHs) in rat brain tissue using gas chromatography tandem (triple quadrupole) mass spectrometry (GC-MS/MS) was developed and validated. The list of molecules investigated comprised the 16 PAHs from the US-EPA list and 53 of their OH-PAHs. Brain extract was submitted to enzymatic hydrolysis, followed by liquid–liquid extraction, and then purified by solid-phase extraction. Limits of quantification ranged from 0.6 to 29 pg/mg and from 0.5 to 30 pg/mg for PAHs and OH-PAHs respectively. The analysis of rat brain samples exposed to PAH mixture (0.01–1 mg/kg, 28 days, ip) demonstrated that this method allowed the detection of 16 PAHs and 28 OH-PAHs out of the 69 analytes investigated. Mean concentrations of PAHs in animal brain samples exposed to 1 mg/kg of PAH mixture ranged from  $3.0 \pm 2$  pg/mg for benzo[*b*]fluoranthene to  $146 \pm 29$  pg/mg for phenanthrene. Concomitantly, mean concentrations of OH-PAHs ranged from  $0.49 \pm 0.4$  to  $26.5 \pm 23$  pg/mg for 2-OH-chrysene and 1-OH-pyrene respectively. This study proves, for the first time, the bioavailability of most of the PAHs and OH-PAHs in mammalian brain tissue and should provide an important new tool for future neurotoxicological studies.

### INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), formed as byproducts of incomplete combustion of organic material, are released into the atmosphere mainly from human activities such as residential heating, industrial activities and transportation.<sup>1–3</sup> Human exposure mostly occurs through ingestion of contaminated food and water and inhalation of contaminated air and tobacco smoke. Even if high exposure is limited to specific categories (smokers, occupationally exposed workers), exposure to these ubiquitous chemicals at environmental levels concerns the entire population of highly populated industrialized countries. While the carcinogenicity of PAHs is well established,<sup>4</sup> the other toxicological properties of these compounds, especially their neurotoxicity, have received much less attention. Neurotoxic symptoms are a part of common health problems that result from exposure to contaminated atmosphere. It is plausible that long-term exposure to ambient air pollutants affects neurocognitive

functions in humans.<sup>5–7</sup> For instance, short-term memory disorders were reported in workers of a coke processing plant in Poland.<sup>7</sup> There is growing evidence that early exposure to air pollutants from combustion of coal and other fossil fuels induces growth impairment and decrement in early child neurodevelopment.<sup>8–11</sup> Such observations have led to speculations about a possible relationship between neurodegenerative diseases and exposure to environmental PAHs.<sup>11–17</sup> To explore this hypothesis, several *in vivo* experiments have been carried out to investigate how PAHs can induce adverse neurobehavioral effects in rodents.<sup>6,13,18–25</sup> Recent findings suggest that there is a relationship between the level of benzo[*a*]pyrene (B[*a*]P) exposure, the modulation of anxiety-related behavior, changes in gene expression of glutamate NMDA receptor and its relative concentration in the brain.<sup>18,26</sup> Although the behavioral effects of a single compound

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(such as B[a]P or fluoranthene) were already established<sup>18,19,21–23</sup> in rodents, the impact of a PAH mixture on the mammalian brain has not been investigated yet.

Analytical methods currently used for B[a]P analysis in the brain are not selective enough to allow studying environmental exposure to mixture of PAHs. <sup>3</sup>H- and/or <sup>14</sup>C-radiolabeled PAHs were traditionally used for studies that focused on PAH distribution in the brain.<sup>27–29</sup> Their quantification by liquid scintillation counting is quite simple and can be combined with thin layer chromatography or a Flo-One Beta radioactive detector in order to separate native and metabolite forms.<sup>30</sup> Nevertheless, the limitations of this method are (i) the lack of radio-labeled compounds available from suppliers, (ii) the inability to measure more than two labeled compounds together and (iii) the cost of recycling radioactive waste material. An alternative approach is the use of HPLC coupled with fluorescence for the detection and quantification of PAHs and their respective metabolites in rodent brain.<sup>18,21</sup> This method is sensitive (e.g., 0.5–5 pg/mg of B[a]P and its metabolites in brain tissue<sup>18</sup>), but the lack of selectivity and cross reactions appear as major disadvantages. In previous literature, gas chromatography or liquid chromatography coupled with tandem (triple quadrupole) mass spectrometry (LC– or GC–MS/MS) has never been tested for the detection of PAHs and OH-PAHs in the mammalian brain. The first advantage in using MS/MS is the ability to identify and quantify low levels of concentration of PAHs and OH-PAHs present in brain. The second one is that the use of tandem MS (MS/MS) allows increasing the signal-to-noise ratio (S/N).<sup>31</sup>

PAHs are lipophilic compounds which are rapidly absorbed and distributed to a variety of tissues with a distinct tendency for localization in body fat.<sup>32</sup> For this reason, their extraction from fatty matrices such as brain is more difficult than from aqueous fluids (e.g., blood and urine). Therefore, the challenge of this analytical development is inherent to brain composition and to the relatively high lipid solubility of PAHs and OH-PAHs.

The aim of the present study was to develop a highly sensitive method for the analysis of PAHs and OH-PAHs in the brain by GC–MS/MS. The development of the full methodology includes the setting up of an adapted extraction method of analytes from brain, the comparison of various derivative reagents and the validation of the method. The list of molecules investigated comprised the 16 PAHs from the US-EPA list and the 53 OH-PAHs commercially available. Furthermore, an animal study was conducted to test the suitability of the analytical method developed. The validated method was thereafter applied to rat brain samples exposed to environmental levels of PAHs. The PAHs were administered at doses 0.01–1 mg/kg that correspond to the levels of exposure of smokers, heavy consumers of smoked or grilled meat and fish, or individuals with heavy occupational exposure.<sup>33,34</sup> This study proves, for the first time, the bioavailability of most of the PAHs and OH-PAHs in mammalian brain tissue and should provide an important new tool for neurotoxicological studies in the future.

## MATERIALS AND METHODS

**Reagents and Standards.** Methanol of HPLC grade, ethyl acetate of analytical grade and piston pellets were purchased from Fischer Bioblock (Tournai, Belgium). Except for 1-OH- and 4-OH-phenanthrene standard solutions, which were supplied at 10 mg/L by Dr. Ehrenstorfer (Augsburg, Germany), all OH-PAH standards were purchased in powder form from MRI/NCI (Kansas City, MO, USA).

Fluorene-*d*<sub>10</sub> was supplied by Dr. Ehrenstorfer (Augsburg, Germany), and benzo[*a*]pyrene-*d*<sub>12</sub> was purchased from Sigma Aldrich (Bornem, Belgium). 1-OH-pyrene-*d*<sub>9</sub> and naphthal-*d*<sub>7</sub> were obtained from Chiron AS (Trondheim, Norway) and Medical Isotopes Inc. (Pelham, NH, USA) respectively. Standard calibration mix of US-EPA PAHs (1 mL), Triton X-100, sulfatase and glucuronidase, sodium acetate, potassium hydroxide, cyclohexane of analytical grade, Supelclean ENVI-Chrom P (styrene-divinylbenzene copolymer resin, 100 mg) SPE columns and derivatization reagent *N*-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide, 1% TBDMCS (MtBSTFA, ≥ 97% purity) were purchased from Sigma-Aldrich (Bornem, Belgium). (2*S,4R*)-*N*-Heptafluorobutyryl-4-heptafluorobutoxy-prolyl chloride [(*S,R*)-HFBOPCl] tested for derivatization was produced in our laboratory as previously described.<sup>35</sup> A stock solution of each compound at 1 g/L, mixed-standard solutions of OH-PAHs at 10 mg/L and internal standards at 10 mg/L were prepared in acetonitrile. Working solutions were prepared by 10-fold successive dilutions at concentrations ranging between 1000 µg/L and 10 µg/L and were stored at –20 °C. Ultrapure water was produced by means of an AFS-8 system from Millipore (Brussels, Belgium).

**Instrumentation.** Analyses were carried out with an Agilent 7890A gas chromatograph equipped with HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness), coupled with an Agilent 7000A triple quadrupole mass spectrometer operating in electron impact ionization mode and an Agilent CTC PAL autosampler. Other equipment used: Sigma Laborzentrifugen 4-15 sold by Meditec (Contern, Luxembourg), WTW InoLab pH-meter with a glass-calomel electrode, IKA Labortechnik HS 501 Digital shaker, Reacti-Therm III and a Reacti-Vap system used for heating, evaporating and concentrating under nitrogen from Perbio Science (Erembodegem, Belgium), and Supelco Visiprep 24 DL from Sigma-Aldrich (Bornem, Belgium).

**Administration and Sampling of PAHs.** Twenty male Lister hooded rats (180–200 g) from Harlan (Horst, Netherlands) were kept in plastic cages and acclimatized to the animal facility for 1 week under timed 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, feed and oil were tested according to NF ISO 15302 to confirm that all these matrices were free of PAHs down to a detection limit of 1 ng/g of fat matter and 10 ng/L of water. All procedures were in compliance with the rules provided by the European Communities Council Directive of 24 November 1986 (86/609/EEC). The PAH stock solution was prepared in oil weekly. Five rats were randomly assigned to each of the experimental groups receiving 0 to 1 mg/kg of PAH mixture. Each animal received intraperitoneal injections of PAH mixture solubilized in vegetable oil (1 mL/kg body weight, ISIO4, Lesieurs, Neuilly sur Seine, France), 3 times per week over a 28-day period. Control animals solely received vegetable oil. Animals were then sacrificed 60 min after the last injection. The brains were dissected and stored at –20 °C until further use.

**Extraction–Purification.** Each brain sample (100 mg) was homogenized in a small Eppendorf grinder with 200 µL of ultrapure H<sub>2</sub>O, 0.1% of Triton X-100 and 20 µL of mixed internal standard solution (0.1 mg/mL). Then, 200 µL of sodium acetate buffer (pH 5.6, 1 M) was added. Hydrolysis was performed using 5 µL of purified sulfatase (10 units/µL) and 5 µL of glucuronidase (127 units/µL) from *Helix pomatia* juice, and samples were incubated overnight at 37 °C. The brain samples were transferred to 10 mL tubes, and volume was adjusted to 2 mL with water. The first extraction was carried out with 2 mL of ethyl acetate–cyclohexane (50:50, v/v). The mixture was agitated for 15 min and centrifuged for 5 min at 1800g at room temperature. Supernatants were collected, 50 µL of *n*-heptane was added in order to prevent evaporation until dryness and solvents were evaporated under N<sub>2</sub> at 37 °C. The residue was dissolved in 1 mL of cyclohexane and applied onto an Envi-Chrom P SPE column previously conditioned with cyclohexane. After purification with 2 mL of cyclohexane, PAHs and OH-PAHs

**Table 1.** GC–MS/MS Method Parameters for PAHs and OH-PAHs in Rat Brain

compound <sup>a</sup>	peak label	mol wt	<i>t</i> <sub>R</sub> <sup>b</sup> (min)	analytical parameters				
				quantification transition	CE <sup>c</sup> (V)	confirmation transition	CE (V)	confirmation transition
naphthalene		128	10.7	128.0→102.0	25	128.0→128.0	5	126.0→100.0
acenaphthylene		152	14.6	152.0→126.0	32	150.0→97.9	34	152.0→152.0
acenaphthene		154	15.0	152.0→126.0	32	152.0→152.0	3	150.0→98.0
fluorene- <i>d</i> <sub>10</sub>		176	16.2	175.0→175.2	45			
fluorene		166	16.2	166.0→115.0	54	166.0→139.1	47	165.0→165.0
phenanthrene		178	18.5	176.0→149.8	22	151.0→125.2	17	152.0→126.0
anthracene		178	18.6	176.0→149.8	22	152.0→126.0	22	151.0→125.0
fluoranthene		202	22.0	202.0→174.1	55	174.0→174.0	13	200.0→174.1
pyrene		202	22.8	202.0→174.1	55	200.0→174.1	22	174.0→174.0
B[a]A		228	27.5	228.0→201.8	33	226.0→226.2	8	228.0→228.0
chrysene		228	27.7	228.0→201.8	33	228.0→228.0	4	226.0→226.2
B[b]F		252	32.1	126.0→113.0	13	252.0→226.2	39	252.0→252.1
B[k]F		252	32.2	126.0→113.0	13	252.0→252.1	4	252.0→226.0
benzo[a]pyrene- <i>d</i> <sub>12</sub>		264	33.3	264.0→260.0	39			
B[a]P		252	33A	252.0→252.1	4	252.0→226.0	39	126.0→113.0
I[1,2,3-c,d]P		276	37.9	276.0→276.2	23	276.0→274.2	47	
Dib[a,h]A		278	38.0	278.0→278.2	23	278.0→276.2	50	
B[g,h,i]P		276	38.6	276.0→276.2	23	276.0→274.2	23	
1-OH-naphthalene- <i>d</i> <sub>7</sub>	1	151	6.2	265.5→208.2	13			
1-OH-naphthalene	2	144	6.2	201.4→185.0	14	258.5→201.2	13	
2-OH-naphthalene	3	144	6.3	201.4→185.0	14	258.5→201.2	13	
9-OH fluorene	4	182	13	239.5→165.0	27	296.5→165.0	43	239.5→139.0
3-OH fluorene	5	182	8.0	239.5→165.0	27	296.5→165.0	43	239.5→139.0
2-OH-fluorene	6	182	8.1	239.5→165.0	27	296.5→239.0	11	296.5→165.0
4-OH-phenanthrene	7	194	8.6	251.4→235.0	18	308.5→235.0	36	251.4→220.0
9-OH-phenanthrene	8	194	8.8	251.4→235.0	18	308.5→251.2	14	308.5→235.0
3-OH-phenanthrene	9	194	8.9	308.5→251.2	14	251.4→176.0	49	251.4→151.0
1-OH-phenanthrene	10	194	9.1	251.4→235.0	18	308.5→251.2	14	251.4→220.0
2-OH-phenanthrene	11	194	9.2	251.4→176.0	49	308.5→251.0	14	251.4→151.0
3-OH-fluoranthene- <sup>13</sup> C <sub>6</sub>	12	224	11.1	338.5→281.0	16			
3-OH-fluoranthene	13	218	11.1	332.5→275.0	16	275.4→259.0	22	
1-OH-pyrene- <i>d</i> <sub>9</sub>	14	227	11.4	341.5→284.0	17			
1-OH-pyrene	15	218	11.4	332.5→275.0	17	275.4→259.0	29	
1-OH-B[a]A- <sup>13</sup> C <sub>6</sub>	16	250	13.2	364.5→307.0	17			
1-OH-B[a]A	17	244	13.2	301.5→285.0	23	358.5→301.0	17	301.5→270.0
4-OH-chrysene	18	244	13.4	301.5→285.0	23	358.5→301.0	17	301.5→270.0
6-OH-chrysene + 11-OH-B[a]A	19	244	13.6	358.5→301.0	17	301.5→285.0	23	301.5→270.0
2+5+8-OH-B[a]A	20	244	14.1	358.5→301.0	17	301.5→285.0	23	
3-OH-chrysene	21	244	14.5	358.5→301.0	17	301.5→285.0	23	301.5→226.0
1-OH-chrysene + 4-OH-B[a]A	22	244	14.7	301.5→285.0	23	358.5→301.0	17	
10-OH-B[a]A	23	244	14.8	358.5→301.0	17	301.5→285.0	23	
3+9-OH-B[a]A	24	244	14.9	358.5→301.0	17	301.5→285.0	23	301.5→226.0
2-OH-chrysene	25	244	15.1	358.5→301.0	17	301.5→285.0	23	301.5→226.0
8-OH-B[b]F	26	268	16.0	325.4→309.0	30	382.6→325.0	18	325.4→294.0
11-OH-B[a]P	27	268	16.8	325.4→309.0	19	382.6→325.0	20	325.4→294.0
2-OH-B[b]F	28	268	17.0	382.6→325.0	20	325.4→309.0	19	325.4→294.0
1-OH + 7-OH-B[b]F	29	268	17.2	325.4→309.0	30	382.6→325.0	20	325.4→294.0
12-OH-B[b]F + 8-OH-B[k]F	30	268	17.3	325.4→309.0	19	382.6→325.0	20	325.4→294.0
10-OH-B[a]P	31	268	17.6	325.4→309.0	19	382.6→325.0	20	325.4→294.0
12-OH + 6-OH-B[a]P	32	268	17.7	382.6→325.0	20	325.4→309.0	19	325.4→294.0
5-OH-B[a]P	33	268	17.8	382.6→325.2	18	325.4→309.0	19	
11-OH-B[b]F	34	268	18.1	382.6→325.2	20	325.4→309.0	19	

Table 1. Continued

compound <sup>a</sup>	peak label	mol wt	<i>t</i> <sub>R</sub> <sup>b</sup> (min)	analytical parameters					
				quantification transition	CE <sup>c</sup> (V)	confirmation transition	CE (V)	confirmation transition	CE (V)
10-OH-B[b]F	35	268	18.5	382.6 → 325.2	20	325.4 → 309.0	19	325.4 → 294.0	48
4-OH-B[a]P + 3-OH-B[k]F	36	268	18.7	382.6 → 325.2	20	325.4 → 309.0	19	325.4 → 294.0	48
9-OH-B[k]F + 7-OH-B[a]P	37	268	19.0	382.6 → 325.2	20	325.4 → 309.0	19		
9-OH-B[a]P	38	268	19.2	382.6 → 325.2	18	325.4 → 309.0	19		
2-OH + 1-OH-B[a]P	39	268	19.5	382.6 → 325.2	18	325.4 → 294.0	41	325.4 → 309.0	19
3-OH-B[a]P	40	268	19.7	382.6 → 325.2	20	325.4 → 294.0	48	382.6 → 309.0	40
8-OH-B[a]P	41	268	20.1	382.6 → 325.2	20	325.4 → 309.0	19		
6-OH-I[1,2,3-c,d]P	42	292	21.9	406.1 → 349.0	21	349.0 → 333.0	29		
1-OH-I[1,2,3-c,d]P	43	292	24.6	406.1 → 349.0	21	349.0 → 293.0	23	349.0 → 333.0	29
2-OH-I[1,2,3-c,d]P	44	292	25.1	406.1 → 349.0	21	349.0 → 293.0	23	349.0 → 333.0	12
8-OH-I[1,2,3-c,d]P	45	292	26.2	406.1 → 349.0	21	349.0 → 293.0	23	349.0 → 321.0	12
3-OH-Dib[a,h]A	46	294	27.5	408.1 → 351.0	19	351.0 → 295.0	21	351.0 → 335.0	21

<sup>a</sup>B[a]A, benz[a]anthracene; B[b]F, benzo[b]fluoranthene; B[k]F, benzo[k]fluoranthene; B[a]P, benzo[a]pyrene; I[1,2,3-c,d]P, indeno[1,2,3-c,d]perylene; Dib[a,h]A, dibenzo[a,h]anthracene; B[g,h,i]P, benzo[g,h,i]perylene; OH-B[a]A, OH-benz[a]anthracene; OH-B[b]F, OH-benzo[b]fluoranthene; OH-B[k]F, OH-benzo[k]fluoranthene; OH-B[a]P, OH-benzo[a]pyrene; OH-I[1,2,3-c,d]P, OH-indeno[1,2,3-c,d]perylene; OH-Dib[a,h]A, OH-dibenzo[a,h]anthracene; OH-B[g,h,i]P, OH-benzo[g,h,i]perylene. <sup>b</sup>*t*<sub>R</sub>: retention time. <sup>c</sup>CE: collision energy.

were eluted with 2 mL of ethyl acetate–cyclohexane (50:50; v/v). 50  $\mu$ L of water were also added to avoid evaporation until dryness and the brain extracts were evaporated. Then, the residues were dissolved in 2 mL of cyclohexane and 2 mL of methanol–water (80:20; v/v), shaken at 300 agitations per minute for 15 min, and then centrifuged at 1800g for 5 min; the two layers were separated with a glass Pasteur pipet. This step allowed the separation of PAHs (cyclohexane fraction) from hydroxylated metabolites (methanol–water fraction). 50  $\mu$ L of water was added to the cyclohexane fraction, which was dried under N<sub>2</sub> and submitted to saponification for 1 h at 60 °C (1 mL of alcoholic KOH 7% w/v). Then, 2 mL of ultrapure water and 2 mL of ethyl acetate–cyclohexane mixture (50:50; v/v) were added. The mixture was shaken again, agitated (300 agitations per min) for 15 min and centrifuged at 1800g for 5 min at room temperature. The upper phase containing PAHs was collected and dried under N<sub>2</sub> until 50  $\mu$ L; 1  $\mu$ L was injected into the GC–MS/MS. The lower phase that contained hydroxylated metabolites was evaporated to dryness (at 37 °C using N<sub>2</sub>), 40  $\mu$ L of MTBSTFA was added and derivatization of target analytes was completed after 30 min at 60 °C; 2  $\mu$ L was injected into the GC–MS/MS.

**Gas Chromatography.** Two different methods were developed for sample analysis depending on target analytes studied: PAHs or OH-PAHs.

**PAH Analysis.** The inlet was at a temperature of 260 °C. One microliter of brain extract was injected in pulsed splitless mode with a pressure of 35 psi for 1.5 min. Chromatographic separation was carried out using the column described above. Helium at 1.8 mL/min was used as carrier gas. Oven temperature was kept at 70 °C for 5 min. It was later increased to 200 °C at a rate of +10 °C/min with a 0.5 min plateau, and then raised to 244 °C at +5 °C/min, maintained for 0.5 min. Next, the oven was heated up to 266 °C at +5 °C/min and maintained at this temperature for 0.5 min. Finally, the temperature was taken to 300 °C at a rate of +5 °C/min and maintained for 5.5 min. After each run, the temperature was set at 300 °C for 4 min more in a backflush mode in order to remove hard-boiling compounds through the split vent.

**OH-PAH Analysis.** Several parameters remained unchanged between the two methods, so only differences are exposed hereafter. This time, 2  $\mu$ L of the brain extract was also injected in pulsed splitless mode but with higher pressure at 47 psi since initial oven temperature was higher. An initial temperature was set and kept at 100 °C for 2 min, then raised by +40 °C/min to 235 °C, then to 280 at 10 °C/min and maintained for

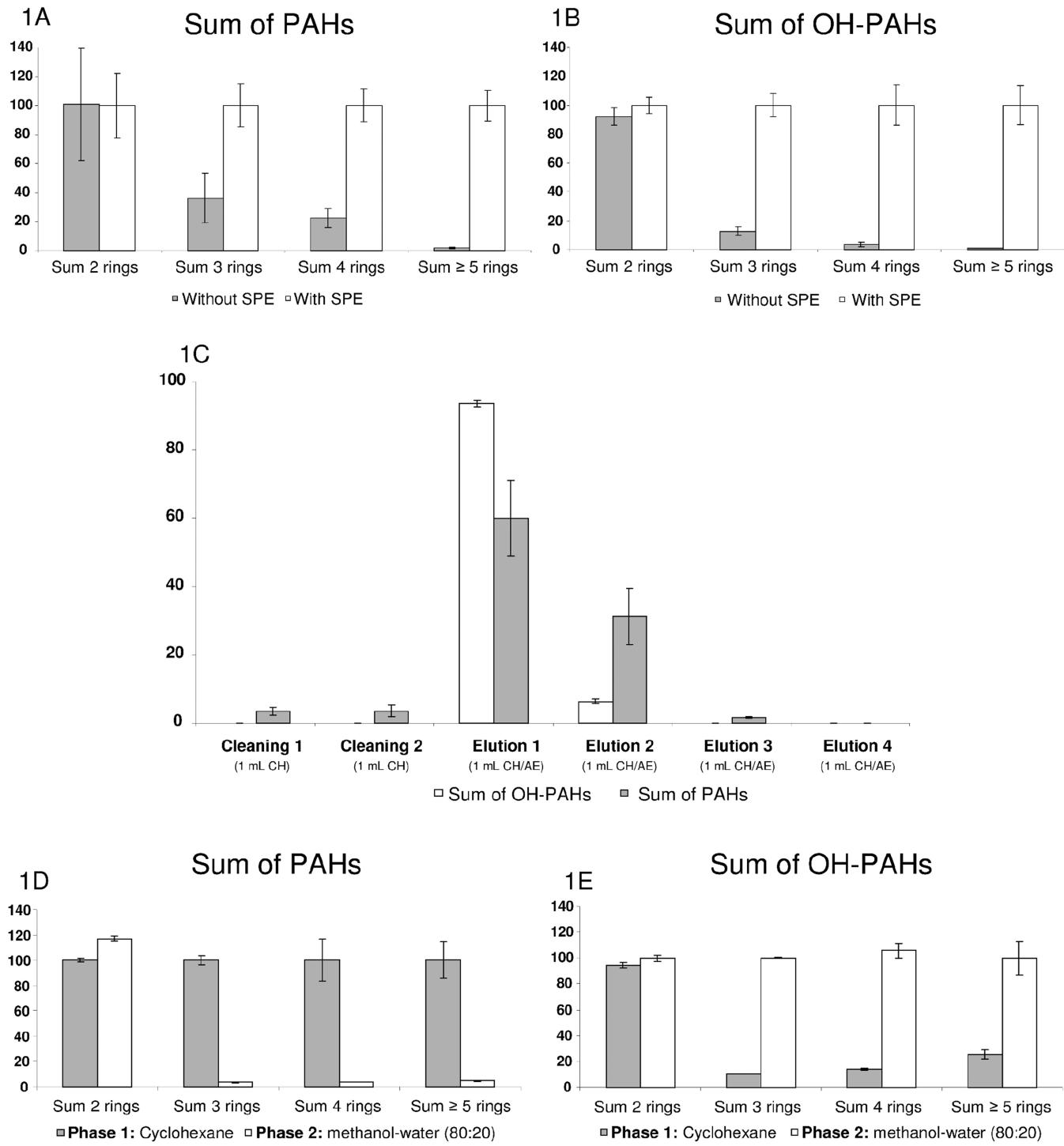
3 min. Finally, after being heated at 10 °C/min, the temperature stayed at 300 °C for 14 min. Backflush was also carried out after each run.

**Mass Spectrometry.** The source temperature was set at 230 °C. The spectrometer was operating in EI mode and monitored multiple reactions (MRM), the parameters of which are presented in Table 1. The collision induced dissociation (CID) gas and the quench gas in the collision cell were respectively nitrogen and helium and were used following the supplier's recommendations on flow and quality. Flow rates were 1.5 mL/min for collision gas nitrogen and 2.25 mL/min for quench gas helium.

**Data Analysis.** Peaks were identified by absolute retention time, quantifier and qualifier ion peak area ratios. The transition yielding the highest signal-to-noise ratio of each analyte was used for quantification; the second and the third transitions served as qualifiers (Table 1). Data were processed as peak area ratios of target analytes and respective internal standards. Calibration curves were established using linear regression with 1/*x* weighting.

**Validation.** Calibration was performed using brain specimens supplemented with the following concentrations of PAHs and OH-PAHs: 0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 pg/mg for PAHs; 0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500 pg/mg for OH-PAHs with fewer than 5 aromatic rings and 0, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, and 5000 pg/mg for the other OH-PAHs ( $\geq 5$  aromatic rings). According to several method validation guidelines, 3 replicates were analyzed at each concentration level.<sup>36,37</sup> Linearity was assessed using a least-squares regression line calculated from all standard concentrations and expressed by the squared correlation coefficient (*R*<sup>2</sup>). Individual calibrator concentrations were recalculated against the full calibration curve and were required to be within 20% of the target. In addition, the *R*<sup>2</sup> value was required to be greater than 0.98 for each analyte on all analyses. Sensitivity was evaluated by the limit of detection (LOD) and limit of quantification (LOQ). LOD was determined as the lowest concentration of each analyte with a signal-to-noise ratio of at least 3:1 for the quantifier transition. LOQ was defined as the lowest measured concentration (based on LOD) of each analyte that can be recovered from the supplemented brain sample within ( $\pm$ ) 20% of true value (*n* = 3) based on the quantifier transition. LOQ was most of the time equivalent to a signal-to-noise of 10:1.

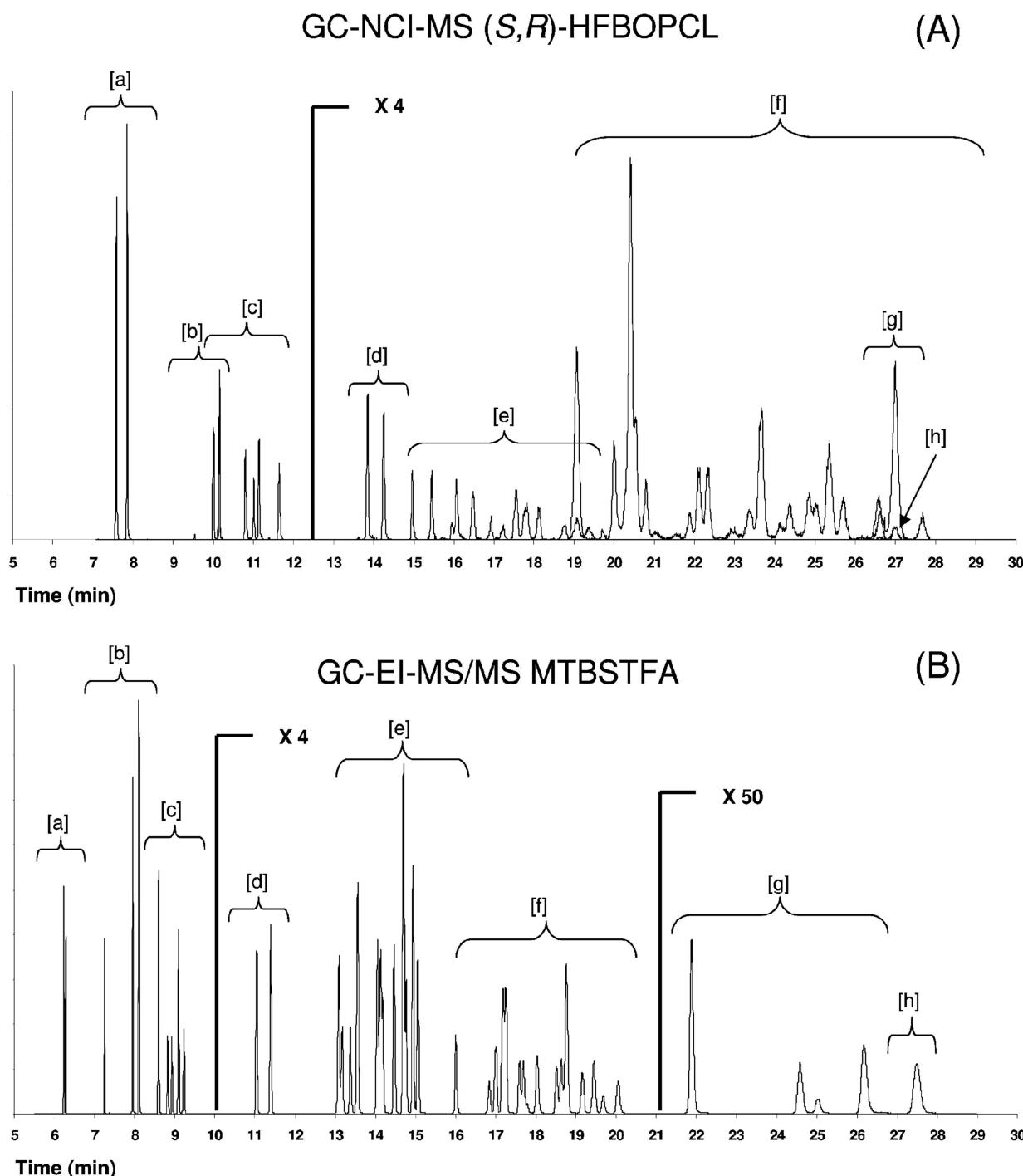
Intraday and interday variability and accuracy were determined on brain samples supplemented at 10 pg/mg of brain tissue for all PAHs,



**Figure 1.** Optimization of extraction conditions. Charts 1A,B display the recovery of PAHs and OH-PAHs from supplemented brain obtained with or without a solid phase extraction (SPE) procedure. Chart 1C concerns the optimization of the Envi-chrom P SPE column (CH, cyclohexane; CH/AE, cyclohexane/ethyl-acetate (50:50). Charts 1D,E show the separation of PAHs (cyclohexane fraction) from OH-PAHs (methanol/water fraction). For all charts, the best response was set to 100 and the response obtained with the second condition was normalized accordingly. Mean values ( $n = 3$ ) are represented as the sum of all PAHs and OH-PAHs in chart 1C and separated according to the number of aromatic rings in charts 1A–D.

10 pg/mg for OH-PAHs with less than 5 aromatic rings and 100 pg/mg for the other OH-PAHs ( $\geq 5$  aromatic rings). Variability was expressed as % of relative standard deviation (RSD) of the calculated concentrations, and the accuracy was calculated as % of relative error from target concentration. Intraday variability and accuracy were determined on 4 replicates of each concentration. Interday variability and accuracy were

evaluated on 8 separate days. Considering the complex composition of brain or of other biological matrices,<sup>38</sup> 25% of the target concentration was considered as acceptable accuracy. Analytical recovery was evaluated by supplementing brain samples with two concentrations of each analyte (10 and 200 pg/mg of brain tissue for PAHs, 1 and 10 pg/mg for OH-PAHs with fewer than 5 aromatic rings, 10 and 100 pg/mg for the other



**Figure 2.** Ion chromatograms corresponding to OH-PAH standards derivatized with (*S,R*)-HFBOPCL (A) or MTBSTFA (B): [a] OH-naphthalenes, [b] OH-fluorenes, [c] OH-phenanthrenes, [d] OH-fluoranthenes/OH-pyrene, [e] OH-B[a]A/OH-chrysenes, [f] OH-B[a]P/OH-B[b]F/OH-B[k]F, [g] OH-I[1,2,3-c,d]P, [h] OH-Dib[a,h]A.

OH-PAHs,  $\geq 5$  aromatic rings) and by comparing the results to those of samples treated identically, but supplemented with the same concentrations of PAHs or OH-PAHs during the last evaporation step. Matrix effect was determined by comparing these same samples supplemented just before the derivatization step to PAH and OH-PAH standards ( $n = 4$ ) with the same concentrations of PAH and OH-PAH standard solutions.

**Statistical Analysis.** The concentrations of PAHs and OH-PAHs were analyzed using one way ANOVA with different doses of PAH mixture as an independent factor. Whenever significant ( $p < 0.05$ ), post

hoc comparisons between pairs of treatments were performed using the Student–Newman–Keuls procedure. Given that most concentration data did not follow a Gaussian distribution, nonparametric statistical procedures were used. Data from controls were compared to those of PAH-exposed animals using a Kruskal–Wallis test followed by a modified Dunn's procedure for post hoc comparisons. The statistical analysis was performed using the Sigma Plot software for windows (Erkrath, Germany). Differences were considered to be significant at the level of  $p < 0.05$ .

**Table 2.** PAHs and Monohydroxylated PAHs in Brain Tissue by GC–MS/MS: Validation Parameters of the Analytical Method

compound	peak label	$R^2$	accuracy <sup>a</sup> (% of target)		RSD <sup>a</sup> (%)		LOQ (pg/mg) (n = 3)	LOD (pg/mg) (n = 3)	recovery <sup>b</sup> (%)			matrix effect <sup>c</sup> (%)
			intraday (n = 4)	interday (n = 8)	intraday (n = 4)	interday (n = 8)			LL <sup>d</sup>	HL <sup>d</sup>	means	
naphthalene <sup>f</sup>		0.977	NA	NA	NA	NA	29	9	NA	NA	NA	NA
acenaphthylene		0.995	92	105	14	20	1.0	0.3	25	22	24	+32
acenaphthene		0.988	92	89	14	12	5.6	1.8	40	24	32	+30
fluorene		0.991	96	111	3	15	6.3	1.9	31	26	29	+31
phenanthrene <sup>f</sup>		0.996	NA	NA	NA	NA	12.1	3.6	NA	NA	NA	NA
anthracene		0.990	101	99	9	16	0.9	0.3	45	44	45	+20
fluoranthene		0.995	94	119	6	13	7.4	2.2	49	59	54	+7
pyrene		0.998	98	135	3	42	4.4	1.0	77	62	70	-3
B[a]A		0.992	96	103	7	11	1.1	0.3	66	68	67	+14
chrysene		0.995	98	108	5	11	0.6	0.2	69	70	70	+7
B[b]F		1.000	89	101	8	13	1.0	0.5	73	71	72	+16
B[k]F		0.992	109	110	4	7	3.0	1.0	74	69	72	-19
B[a]P		0.987	96	98	8	11	0.7	0.2	69	71	70	+13
I[1,2,3-c,d]P		0.996	91	85	4	11	3.6	1.2	71	72	72	+49
Dib[a,h]A		0.998	112	110	6	16	7.5	2.5	69	75	72	+15
B[g,h,i]P		0.995	100	81	4	15	3.0	1.0	72	72	72	+16
1-OH-naphthalene	2	0.999	97	94	4	8	0.6	0.2	9	12	11	+4
2-OH-naphthalene	3	0.997	109	120	3	19	1.5	0.5	33	28	31	-3
9-OH-fluorene	4	0.981	98	122	5	14	0.6	0.2	28	38	33	N/A
3-OH-fluorene	5	0.998	105	107	3	6	0.8	0.3	64	66	65	-6
2-OH-fluorene	6	0.996	117	86	12	16	0.3	0.1	69	74	72	-8
4-OH-phenanthrene	7	0.998	99	96	5	7	0.3	0.1	38	45	42	-7
9-OH-phenanthrene	8	0.995	76	38	14	60	0.5	0.15	7	12	10	-12
3-OH-phenanthrene	9	1.000	104	107	8	7	0.4	0.12	61	66	64	-13
1-OH-phenanthrene	10	0.999	93	91	6	12	0.5	0.15	49	54	52	-4
2-OH-phenanthrene	11	1.000	99	107	12	11	0.4	0.12	67	72	70	-12
3-OH-fluoranthene	13	1.000	99	100	3	3	0.2	0.08	22	26	24	-10
1-OH-pyrene	15	1.000	101	99	5	7	0.2	0.06	31	42	37	-11
1-OH-B[a]A	17	0.999	118	116	8	6	0.1	0.04	50	58	54	-21
4-OH-chrysene	18	0.999	90	87	1	7	0.1	0.04	34	45	40	-15
6-OH-chrysene + 11-OH-B[a]A	19	1.000	80	75	12	14	0.1	0.02	21	28	25	-17
2+5+8-OH-B[a]A	20	0.999	98	93	4	6	0.1	0.04	34	40	37	-18
3-OH-chrysene	21	1.000	112	113	7	6	0.2	0.05	62	69	66	-18
1-OH-chrysene + 4-OH-B[a]A	22	1.000	103	103	6	5	0.1	0.02	53	60	57	-15
10-OH-B[a]A	23	0.998	84	91	2	24	0.2	0.08	34	36	35	0
3+9-OH-B[a]A	24	0.999	101	91	5	14	0.2	0.05	51	57	54	-18
2-OH-chrysene	25	0.999	118	116	8	6	0.2	0.05	70	70	70	-17
8-OH-B[b]F	26	0.994	77	83	7	23	3.0	1.00	21	30	26	+16
11-OH-B[a]P	27	0.989	97	100	16	15	7.5	2.50	5	10	8	-19
2-OH-B[b]F	28	0.999	127	125	8	11	6.0	2.00	53	62	58	-22
1-OH + 7-OH-B[b]F	29	0.987	102	108	17	13	0.9	0.30	54	60	57	-25
12-OH-B[b]F + 8-OH-B[k]F	30	0.990	124	122	9	7	0.9	0.30	41	53	47	-18
10-OH-B[a]P	31	0.998	77	80	19	17	6.0	2.00	12	19	16	-19
12-OH + 6-OH-B[a]P	32	0.995	81	93	9	17	12.0	4.00	15	22	19	-12
5-OH-B[a]P	33	0.987	41	87	22	37	30.0	10.00	5	5	+2	
11-OH-B[b]F	34	0.995	100	107	25	18	2.4	0.80	61	65	63	-10
10-OH-B[b]F	35	0.996	104	105	9	10	3.0	1.00	69	71	70	-26
4-OH-B[a]P + 3-OH-B[k]F	36	0.990	100	81	15	34	3.0	1.00	13	18	16	-12
9-OH-B[k]F + 7-OH-B[a]P	37	0.993	98	92	7	15	1.2	0.40	39	46	43	-19
9-OH-B[a]P	38	0.992	95	97	11	12	3.0	1.00	42	50	46	-16
2-OH + 1-OH-B[a]P	39	0.985	98	103	11	11	2.4	0.80	31	33	32	+5

**Table 2.** Continued

compound	peak label	$R^2$	accuracy <sup>a</sup> (% of target)		RSD <sup>a</sup> (%)				recovery <sup>b</sup> (%)				matrix effect <sup>e</sup> (%)
			intraday (n = 4)	interday (n = 8)	intraday (n = 4)	interday (n = 8)	LOQ (pg/mg) (n = 3)	LOD (pg/mg) (n = 3)	LL <sup>c</sup>	HL <sup>d</sup>	means		
3-OH-B[a]P	40	0.995	92	77	14	20	12.0	4.00	-	11	11	+7	
8-OH-B[a]P	41	0.994	98	101	10	16	6.0	2.00	51	55	53	-19	
6-OH-I[1,2,3-c,d]P	42	0.983	94	107	24	23	0.9	0.30	21	29	25	+31	
1-OH-I[1,2,3-c,d]P	43	0.986	135	138	15	13	6.0	2.00	7	10	9	-34	
2-OH-I[1,2,3-c,d]P	44	0.996	94	105	17	27	15.0	5.00	2	4	3	-22	
8-OH-I[1,2,3-c,d]P	45	0.997	86	81	20	23	1.8	0.60	61	67	64	-41	
3-OH-Dib[a,h]A	46	0.989	91	102	20	8	1.2	0.40	64	70	67	-43	

<sup>a</sup> Intraday and interday accuracy and variability (RSD %) were determined on brain samples supplemented at 10 pg/mg of brain tissue for all PAHs, 10 pg/mg for OH-PAHs with fewer than 5 aromatic rings and 100 pg/mg for the other OH-PAHs,  $\geq 5$  aromatic rings). <sup>b</sup> Recovery was evaluated by supplemented brain samples with two concentrations (low and high levels) of each analyte (10 and 200 pg/mg of brain tissue for PAHs, 1 and 10 pg/mg for OH-PAHs with fewer than 5 aromatic rings; 10 and 100 pg/mg for the other OH-PAHs ( $\geq 5$  aromatic rings)). <sup>c</sup> Low level. <sup>d</sup> High level. <sup>e</sup> Matrix effect was determined by comparing the same samples as in footnote b supplemented just before the derivatization step to PAH and OH-PAH standards (n = 4) with the same concentrations of PAH or OH-PAH standard solutions. <sup>f</sup> Naphthalene and phenanthrene linear range: 200–500 pg/mg of brain.

**Safety Considerations.** General guidelines for work with organic solvents, acids and alkalines were respected. All standard compounds have been designated as “chemical carcinogen”. This is not necessarily meant to imply that the sample is a known carcinogen, only that it is intended for use in research involving chemical carcinogens and it should be treated as a carcinogen.

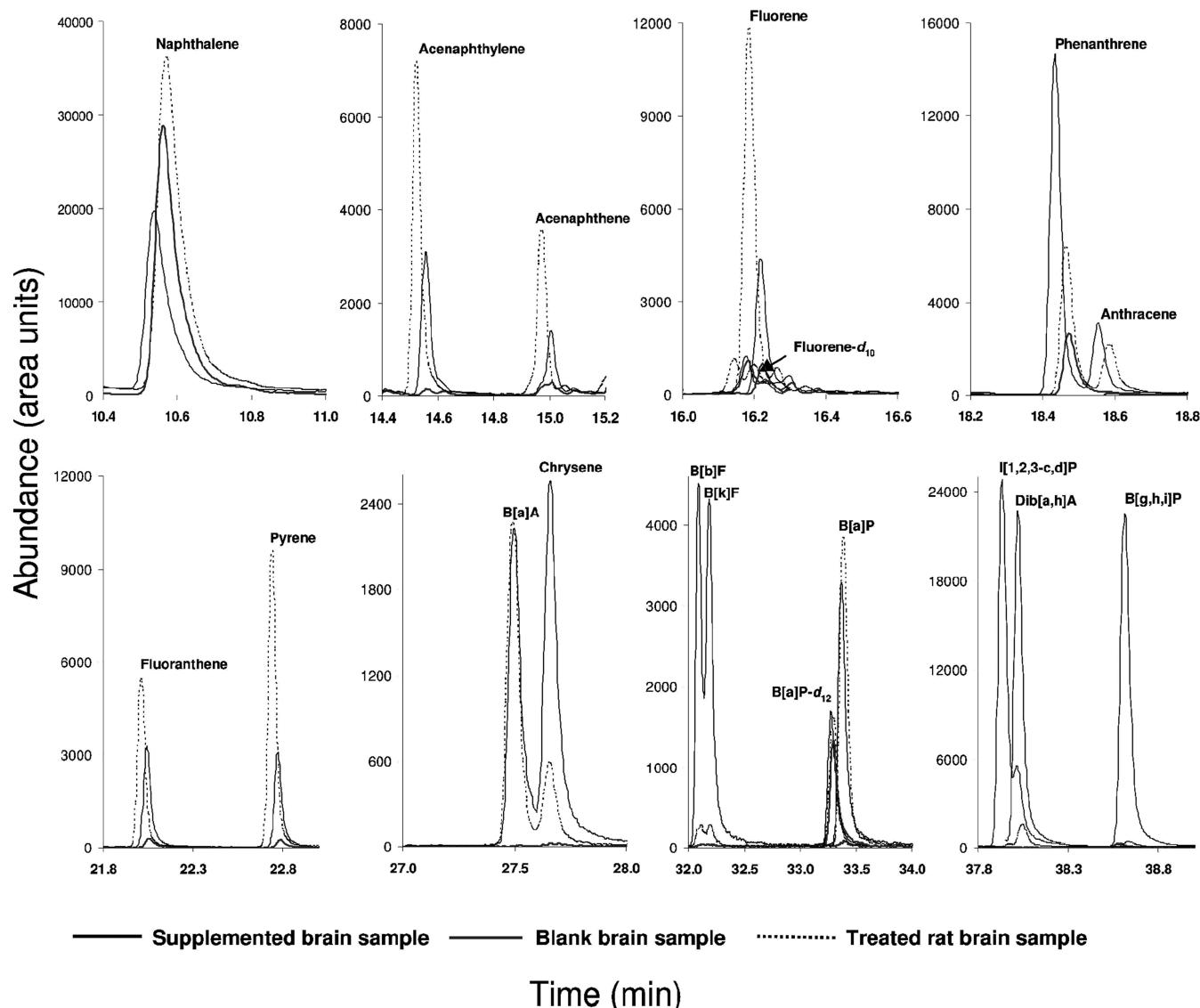
## RESULTS AND DISCUSSION

**Extraction of PAHs and OH-PAHs from Brain: Analytical Development.** The efficiency of the extraction method was evaluated using rat brain supplemented with PAHs and OH-PAHs as well as brain from animals after controlled exposure to PAHs. The sample preparation method presented in previous papers<sup>18,39,40</sup> was used as starting point in the development of our method. A preanalytical treatment with Triton X-100 and sodium acetate buffer (pH 5.6, 1 M) was applied to the brain samples before destructure of the matrix by enzymatic digestion. Enzymatic hydrolysis using sulfatase and glucuronidase from *Helix pomatia* (16 h at 37 °C) was compared to alkaline hydrolysis (1 M NaOH at 60 °C) usually used for the analysis of OH-PAHs in hair.<sup>40</sup> The latter process, less specific than the enzymatic one, led to the formation of many “byproducts” such as amino acids, fatty acids and esters from sphingolipids, cerebrosides, hydrolysates, and so on, that can interfere with PAH and OH-PAH signals.<sup>41</sup> Therefore, enzymatic hydrolysis was preferred to alkaline hydrolysis for the following experiment. PAHs and OH-PAHs were then extracted with an equivalent volume of cyclohexane as previously described with other biological fluids and tissues.<sup>18,39</sup> A chromatographic (SPE) system combining ethyl acetate–cyclohexane (50:50, v/v) as mobile phase with Envi-Chrom P as stationary phase was selected for PAH and OH-PAH purification. Envi-Chrom P SPE columns were conditioned with cyclohexane as described in Materials and Methods. The residues were preliminary dissolved in 1 mL of cyclohexane and applied on the column. Figures 1A and 1B show the recovery of PAHs and OH-PAHs from supplemented brain obtained with or without SPE procedure. Although no difference was observed for naphthalene and naphthols, recovery was increased from 64% to 98% for PAHs

and from 87% to 99% for OH-PAHs according to their number of aromatic rings (Figures 1A and 1B). On the basis of these results, the Envi-Chrom P SPE column was used for the following experiments. The volume of solvent that was required for the complete elution of PAHs and OH-PAHs was also evaluated. Each fraction of 1 mL eluted was separately collected and analyzed by GC–MS/MS after evaporation and reconstitution in cyclohexane for PAHs and a derivatization step for OH-PAHs. Thus, 2 fractions of 1 mL of cyclohexane (corresponding to the cleaning step) and 4 fractions of 1 mL of ethyl acetate–cyclohexane (50:50, v/v) (corresponding to the elution step) were tested. This assay demonstrated that 2 mL of ethyl acetate–cyclohexane (50:50; v/v) were sufficient to elute 100% of OH-PAHs and 91.2% of PAHs (Figure 1C).

The separation of PAHs and remaining fatty acids from the OH-PAH fractions was carried out with LLE (2 mL of cyclohexane/2 mL of methanol–water (80:20; v/v)) as described by Lapole et al., 2007.<sup>42</sup> This treatment initially developed for the analysis of 3 PAHs (phenanthrene, pyrene and B[a]P) and their major metabolites in milk was applied to our list (16 PAHs and 53 OH-PAHs). PAHs and OH-PAHs were measured in both layers in order to verify their total separation. The added value of this step was clearly demonstrated in charts 1D and 1E (Figure 1). This LLE allows removing fatty acid from the OH-PAHs phase with an acceptable decrease in their recovery (11 to 25% according to the number of aromatic rings). The purified OH-PAH extract was then dried before the derivatization procedure. Fatty acids were definitively removed from the PAH fraction by saponification (alcoholic KOH 7% w/v, see experimental design).

Given that evaporation of organic extract was proven to induce the loss of most volatile PAHs (20–70% loss of 3-ring PAHs) in various biological matrices (e.g., mussels),<sup>43,44</sup> our analytical method was tested with dry and wet residues at each of the steps described above. Recovery was increased by a 9-fold factor in wet residues for naphthalene and a 2-fold factor for acenaphthene, acenaphthylene and fluorene respectively, leading us to take it into account in the full method (data not shown). Finally, two derivatization procedures were investigated here. TMS (trimethylsilyl) derivatives were obtained by adding 50  $\mu$ L of

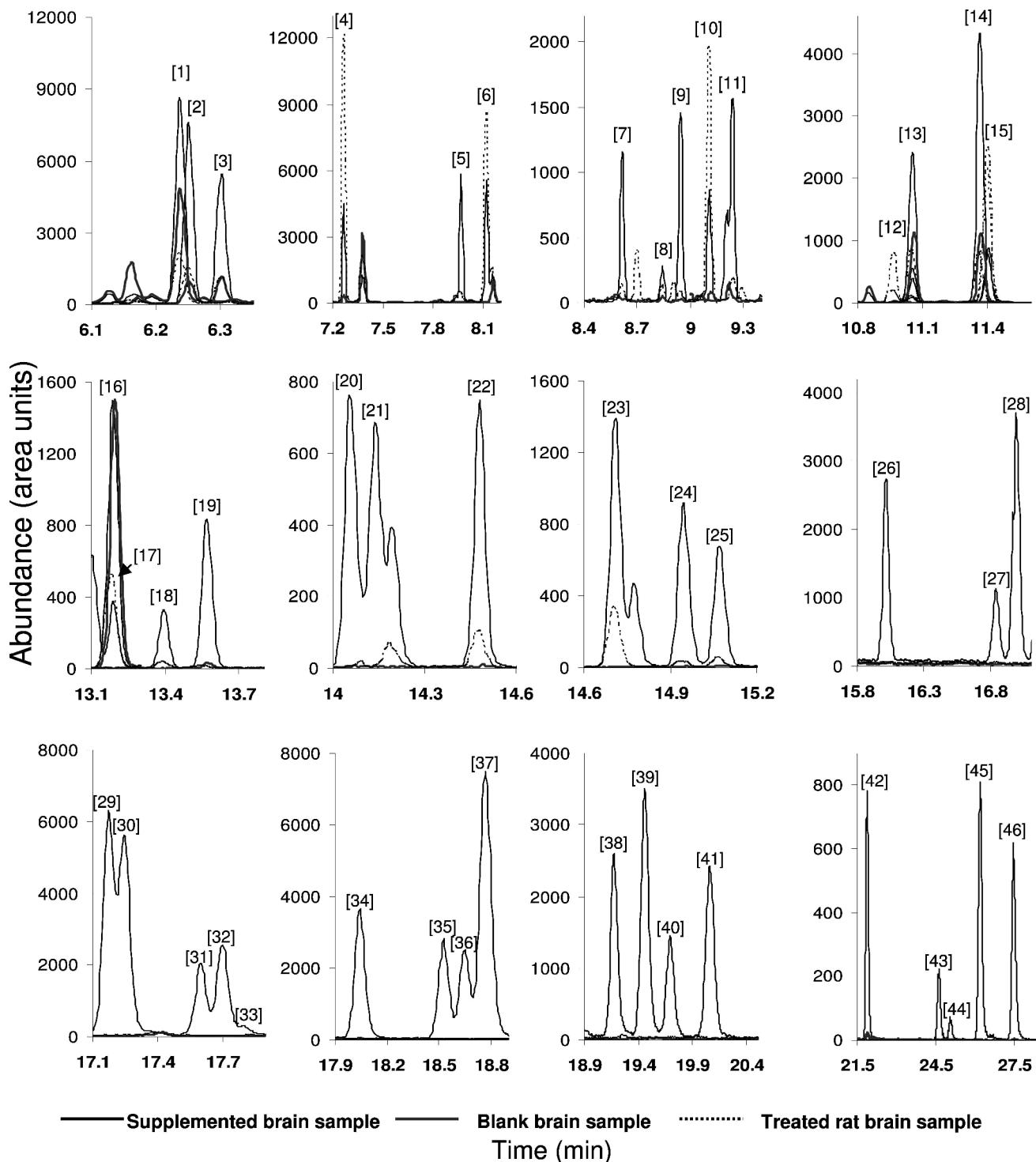


**Figure 3.** Chromatograms obtained from the analysis of all investigated PAHs in a supplemented brain sample, a blank brain sample and a treated rat brain sample (rat exposed to 1 mg/kg of PAHs by ip during 28 days).

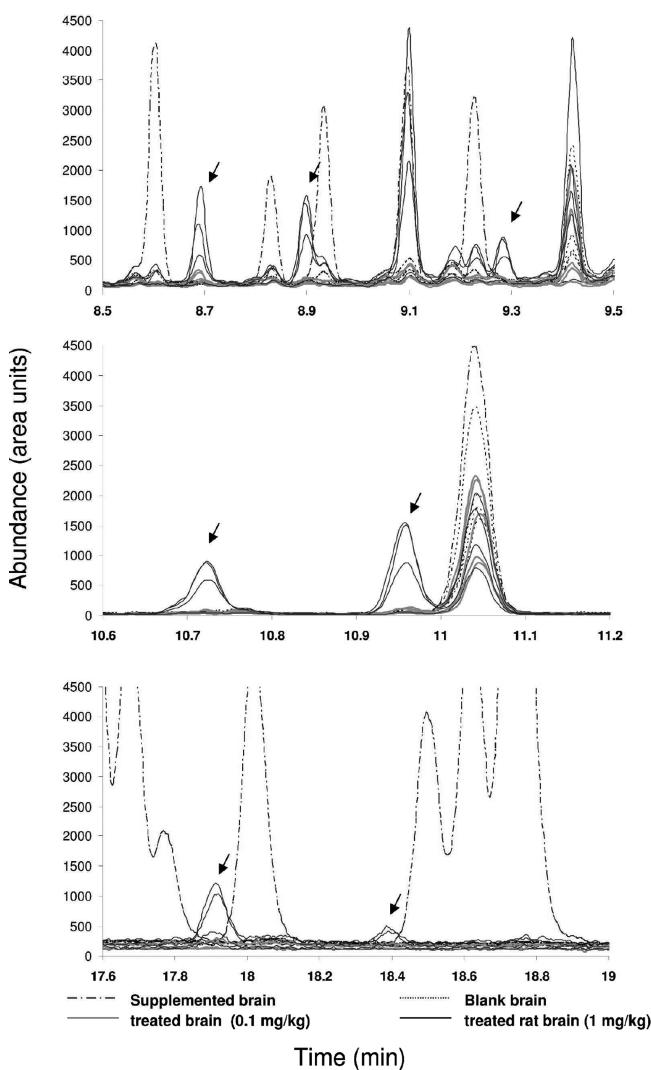
MTBSTFA to the extract, and total derivatization of target analytes was achieved after 30 min at 60 °C; 2 µL was injected into the GC–EI-MS/MS. In order to form derivatives compatible with negative chemical ionization (NCI), the (2S,4R)-N-heptafluorobutyryl-4-heptafluorobutoxy-prolyl chloride [(S,R)-HFBOPCl] was tested as fluorinated reagent for the derivatization of OH-PAHs. The derivatization procedure and GC–NCI-MS parameters were previously detailed.<sup>35,40</sup> Even if this derivatization produced higher molecular mass compounds from *m/z* 629 for naphthols up to *m/z* 799 for 3-OH-Dib[a,h]A; which allowed high specificity, and even if we noticed comparable sensitivities between NCI-single quadrupole and EI-triple quadrupole, allowing us to expect higher sensitivities in NCI-triple quadrupole, the results obtained were unsatisfying compared to those obtained with MTBSTFA (Figure 2). Thus, the chromatographic resolution and separation observed for MTBSTFA derivatives were improved compared to those obtained for (S,R)-HFBOPCl derivatives. In addition, the limited concentration of (S,R)-HFBOPCl derivative agent usable did not

allow complete derivatization of all OH-PAHs present in the sample.<sup>35</sup> On the contrary, the 50 µL of silylating reagent appeared to be enough for the derivation of all aromatic alcohols of OH-PAHs. These results are in accordance with previous works investigating OH-PAHs in complex biological matrices such as milk, tissue or urine (e.g., *m/z*: 216, 201 for naphthols; 266, 251 for OH-phenanthrenes; and 290, 275 for OH-pyrene).<sup>39,45,46</sup> Thus, MTBSTFA derivative agent seems suitable in the case of brain analysis.

**Analytical Performances.** The challenge mainly consisted of obtaining a sensitive and selective analytical method by taking into account the complexity of the brain matrix (containing fatty acids, amino acid, lipids) and the physicochemical properties of PAHs (highly hydrophobic compounds). Moreover, the determination of the specificity of the PAH method was difficult because control brain samples contained varying amounts of most of the PAHs investigated (Table 2). Naphthalene and phenanthrene showed mean concentrations of 111 ± 23 and 97 ± 19 pg/mg of brain tissue, respectively, corresponding to the



**Figure 4.** Chromatograms obtained from the analysis of all investigated OH-PAHs in a supplemented brain sample, a blank brain sample and a treated rat brain sample (rat exposed to 1 mg/kg of PAHs by ip during 28 days): [1] 1-OH-naphthalene- $d_7$ , [2] 1-OH-naphthalene, [3] 2-OH-naphthalene, [4] 9-OH-fluorene, [5] 3-OH-fluorene, [6] 2-OH-fluorene, [7] 4-OH-phenanthrene, [8] 9-OH-phenanthrene, [9] 3-OH-phenanthrene, [10] 1-OH-phenanthrene, [11] 2-OH-phenanthrene, [12] 3-OH-fluoranthene- $^{13}C_6$ , [13] 3-OH-fluoranthene, [14] 1-OH-pyrene- $d_9$ , [15] 1-OH-pyrene, [16] 1-OH-B[a]A- $^{13}C_6$ , [17] 1-OH-B[a]A, [18] 4-OH-chrysene, [19] 6-OH-chrysene + 11-OH-B[a]A, [20] 2+5+8-OH-B[a]A, [21] 3-OH-chrysene, [22] 1-OH-chrysene + 4-OH-B[a]A, [23] 10-OH-B[a]A, [24] 3+9-OH-B[a]A, [25] 2-OH-chrysene, [26] 8-OH-B[b]F, [27] 11-OH-B[a]P, [28] 2-OH-B[b]F, [29] 1-OH + 7-OH-B[b]F, [30] 12-OH-B[b]F + 8-OH-B[k]F, [31] 10-OH-B[a]P, [32] 12-OH + 6-OH-B[a]P, [33] 5-OH-B[a]P, [34] 11-OH-B[b]F, [35] 10-OH-B[b]F, [36] 4-OH-B[a]P + 3-OH-B[k]F, [37] 9-OH-B[k]F + 7-OH-B[a]P, [38] 9-OH-B[a]P, [39] 2-OH + 1-OH-B[a]P, [40] 3-OH-B[a]P, [41] 8-OH-B[a]P, [42] 6-OH-I[1,2,3-c,d]P, [43] 1-OH-I[1,2,3-c,d]P, [44] 2-OH-I[1,2,3-c,d]P, [45] 8-OH-I[1,2,3-c,d]P, [46] 3-OH-Dib[a,h]A.



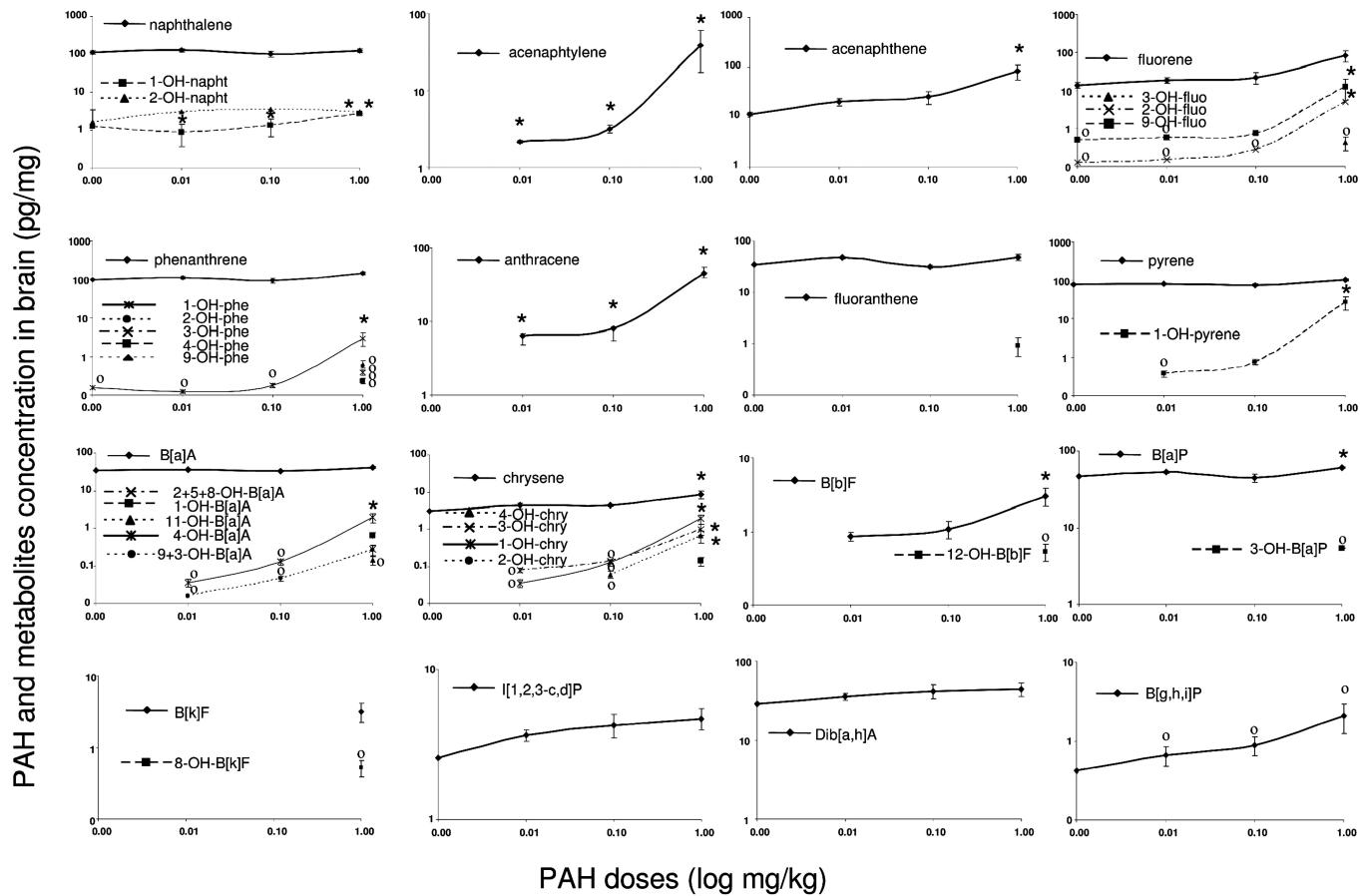
**Figure 5.** Analysis of unknown OH-PAHs by comparing chromatograms of blank brain samples ( $n=4$ ), supplemented brain samples at 2 or 20 pg/mg of tissue ( $n=1$ ) and treated rat brain samples at 0.1 ( $n=5$ ) and 1 mg/kg of PAH mixture ( $n=4$ ).

ante-penultimate concentration levels of the calibration curves. Even at the highest concentration (1 mg/kg of the PAH mixture), the levels of naphthalene and phenanthrene detected in the brain were included in the 25% of RSD and did not allow distinction between the treated and control brain samples. Except for naphthalene and phenanthrene, satisfactory linearity was obtained for all the analytes over the calibration ranging between 0.5 and 500 pg/mg for PAHs and OH-PAHs with fewer than 5 aromatic rings and 5 to 5000 pg/mg for the other OH-PAHs ( $\geq 5$  aromatic rings). Thus, the results presented in Table 2 were considered semiquantitative for phenanthrene and naphthalene.

The coefficient of determination ( $R^2$ ) was always over 0.981 for all the analytes of interest. The recovery of PAHs and OH-PAHs was evaluated on samples fortified at high and low concentrations as required by guidance documents.<sup>36,47</sup> First, blank samples were analyzed to determine the initial concentration of PAHs in rat brain. The recovery values were corrected by subtracting the concentration initially detected in blank samples. Therefore, recovery ranged between 24% and 72% for all the

PAHs investigated in this work (Table 2). These results are in line with PAH recoveries obtained in different food samples that were mostly over 70% with both HPLC and GC methods.<sup>48</sup> Among the 53 OH-PAHs analyzed, 41 compounds showed recovery between 25% and 72% and 12 showed very low recovery that varied from 3% to 19%. Nevertheless, the selectivity of the GC-MS/MS system used and the low matrix effect observed counterbalanced the low recovery observed for some compounds. Tandem mass spectrometry is among the most reliable tools currently available for discriminating between background noise generated by matrix interference and signal from molecules investigated. Two transitions were performed to confirm the presence of most of the analytes tested (Table 1). The variability in the ratio quantification transition/confirmation transition had to be within 20% to confirm the presence of the analyte. Table 2 displays ion suppression or enhancement caused by the matrix effect; values ranged from -3 to +49% for PAHs and from -43 to +31% for OH-PAHs. Moreover, the use of stable isotope labeled analogues ( $^{13}\text{C}$  or deuterated) as internal standards limited the effect of the low recoveries. Most authors agree that the recovery value appears not "significant", as long as the data for LOQ, LOD, variability and accuracy are acceptable.<sup>36,37,49</sup> LODs ranged between 0.2 and 8.7 pg/mg for PAHs and between 0.02 and 10 pg/mg for OH-PAHs and LOQs ranged between 0.6 and 29 pg/mg for PAHs and between 0.5 and 30 pg/mg for OH-PAHs. The LODs and LOQs determined for naphthalene and phenanthrene were probably overestimated due to the presence of these compounds in the blank sample (see Table 2 and Table S-1 in the Supporting Information). Since the detection of PAHs and OH-PAHs in brain was never performed by GC-MS or GC-MS/MS before the present work, results cannot be compared to any similar study. For instance, in food matrices, the LODs for PAHs typically range between 0.1 and 1  $\mu\text{g}/\text{kg}$  but higher amounts of material were systematically used.<sup>50</sup> Table 2 contains intra- and interday variability and accuracy data for each analyte. Intraday variability (% RSD) was less than 25% for all analytes ( $n=4$ ). Except for fluoranthene, 9-OH-phenanthrene, 4-OH, 5-OH-B[a]P and 3-OH-B[k]F interday variability was also less than 25% for all the analytes ( $n=8$ ). Intra- and interday accuracy was within 25% of the target, except for pyrene, 5-OH-B[a]P, 2-OH-B[b]F and 1-OH-I[1,2,3-c,d]P. Validation parameters obtained for this analytical method appear quite satisfactory for analyses performed upon a biological matrix as complex as brain.

**Application to Treated Rat Brain Samples.** Figures 3 and 4 display ion chromatograms of PAHs and OH-PAHs corresponding to blank sample, sample supplemented with standard solution, and sample from animal exposed to PAH mixture at 1 mg/kg. By comparison with previous works that identified only B[a]P and its metabolites in brain by HPLC coupled with fluorimetry,<sup>18,21</sup> the method developed here simultaneously quantified all the PAHs listed by the US-EPA and most of their respective OH-PAHs. Analyses revealed that almost all PAHs were detectable in blank sample (Table 2). Surprisingly, the levels of naphthalene, phenanthrene and fluorene were so high compared to the other PAHs that even if the animals received 3 intraperitoneal administrations per week for 4 weeks, no differences were observed between control and treated groups. Concomitantly, 1-OH and 2-OH-naphthalenes, 2-OH and 9-OH-fluorenes and 1-OH-phenanthrene were also encountered in the same blank brain samples. These results are in accordance with previous observations that showed the presence of 1-OH- and



**Figure 6.** Recovery of PAHs and OH-PAHs in brain of PAH-treated rats (0.01 to 1 mg/kg, 28 days, ip). Results are expressed as a mean  $\pm$  SEM. \* $P < 0.05$  and \*\* $p < 0.01$  statistically significant differences from control animals (Dunn's procedure for post hoc comparisons).  $^0$ : Values determined below the LOQ and above the LOD. OH-napt: OH-naphthalene. OH-fluo: OH-fluorene. OH-phe: OH-phenanthrene. OH-chry: OH-chrysene. B[a]A: benz[a]anthracene. B[b]F: benzo[b]fluoranthene. B[k]F: benzo[k]fluoranthene. B[a]P: benzo[a]pyrene. I[1,2,3-c,d]P: indeno[1,2,3-c,d]perylene. Dib[a,h]A: dibenzo[a,h]anthracene. B[g,h,i]P: benzo[g,h,i]perylene. OH-B[a]A: OH-benz[a]anthracene. OH-B[b]F: OH-benzo[b]fluoranthene. OH-B[k]F: OH-benzo[k]fluoranthene. OH-B[a]P: OH-benzo[a]pyrene.

2-OH-naphthalenes, 2-OH- and 9-OH-fluorenes and 2-OH-, 3-OH- and 9-OH-phenanthrenes in human hair exposed to environmental levels of PAHs.<sup>40</sup> All rats received special feed selected for its low or undetectable levels of PAHs (Carfil Quality, Turnhout, Belgium). In addition, water, feed and oil were tested to confirm that all these matrices were free of PAH down to a detection limit of 1 ng/g of fat matter and 10 ng/L of water. Nevertheless, with such precautions, levels of most of the PAHs and 5 OH-PAHs in the control rats are still above the detection limits given in Materials and Methods (Table S-1 in the Supporting Information and Figure 6). Therefore, it is sustainable that the animals have been exposed to PAHs at the supplier. The presence of PAHs and OH-PAHs in the brain of rats used only for analytical development and that were sacrificed just after their arrival in our institute (and without any feed from our side) confirm our hypothesis. Regarding the 4 PAHs (acenaphthylene, anthracene, B[k]F and B[b]F) not detected in the control group, a subacute exposure to PAHs clearly demonstrated a dose-response relationship for 3 of them (Figure 6 and Table S-1). Moreover, analytical blanks (i.e., without matrix) submitted to the whole extraction, purification and derivatization processes did not give any signal for either all PAHs or OH-PAHs investigated. Together, these results prove that the levels are not caused by any contamination during the analytical procedure.

I[1,2,3-c,d]P, Dib[a,h]A and B[g,h,i]P seem to be only slightly bioavailable for the organism and/or weakly bioaccumulated in the brain. No appearance of their respective metabolites analyzed was noticed whatever the dose administrated. The absence of these OH-PAHs could also be explained by lower sensitivity of the method compared to the other metabolites. For 7 PAHs and 8 OH-PAHs, a significant increase in their concentration in brain was observed in a dose-dependent manner (Table S-1 in the Supporting Information, Figure 6). Among the 12 OH-B[a]Ps evaluated, only 3-OH-B[a]P was detected at the highest dose (1 mg/kg PAHs of bw, 28 days, ip). Investigations performed on mouse brain exposed to B[a]P (0.02 to 200 mg/kg of body weight, 10 days, ip) also showed the presence of OH-B[a]P at a dose of 2 mg/kg with 3-OH-B[a]P as the most abundant analyte detected.<sup>18</sup> These results are in accordance with those observed in our study and can be explained by the induction of CYP450s that promote the formation of metabolites. Stable concentrations of the mother compound for all the period of exposure followed by an appearance of metabolites are classically observed in the case of reiterated administration (3 times/week for 28 days) of drugs known to induce the metabolism.<sup>51</sup> Doses of 0.1 and 1 mg/kg appear as thresholds at which the metabolism starts to be induced, which explains why a meaningful dose-response relationship cannot be observed. The doses of PAHs used in our

studies are higher than the levels found in the ambient environment. However, they may be relevant in environmental or occupational settings where specific populations are chronically exposed to PAHs released from hazardous waste sites, where chronic intake occurs via food or tobacco, or during occupational exposure. For example, the lowest dose used in our study is similar to the levels of human exposure through ingestion of contaminated food. As mentioned by Menzie (1992),<sup>34</sup> Hattemer-Frey and Travis (1991),<sup>52</sup> and Jacob and Grimmer (1996),<sup>53</sup> and in the International Programme on Chemical Safety 1998 of WHO,<sup>33</sup> the maximum level of contamination in the nonsmoking population is about 12 µg/day, or 0.2 µg/kg/day for a human of 60 kg. In the present study, the highest dose administered is 10 µg/kg/day, i.e. 50 times higher than the background level of contamination observed in the general population. Levels of contamination are much higher in smokers, or heavy consumers of smoked or grilled meat and fish (for review, see IPCS, 1998),<sup>33</sup> and even higher in populations with occupational exposure. The two lowest doses used in our study (0.01 and 0.1 mg/kg) are more relevant with respect to the level of contamination of the latter populations. The 1.0 mg/kg dose corresponds to "pharmacologically" active doses, whereas higher doses (10 and 100 mg/kg) not used in this study correspond to the "toxic" range of PAHs. For example, IPCS (1998)<sup>33</sup> mentions 150 mg/kg/day as the NOAEL (nonobserved adverse effects level) with respect to gastric, hepatic and renal effects. Salamone et al. have reported a LD<sub>50</sub> equal to 250 mg/kg for an acute ip administration of B[a]P.<sup>54</sup> In comparison with our previous studies, the PAH mixture at 1 mg/kg induced signs of overt toxicity (23% of weight loss at the end of the exposure period; data not shown) whereas a subacute exposure to B[a]P at a concentration 200 times higher (200 mg/kg) did not.<sup>18</sup> This study demonstrated a clear toxic effect of PAH mix compared with a single PAH. From an ethical point of view, an additional dose of PAH mix at 10 or 100 mg/kg was not possible without taking the risk of reaching the LD<sub>50</sub>. Therefore, in order to clearly confirm the suitability of the analytical method developed here, Figure S-1 in the Supporting Information is provided, displaying a meaningful dose-response relationship of fluorene and its respective metabolites in rat brain tissue following a chronic exposure of animals to fluorene (60 days by ip) with doses ranging between 1 and 100 mg/kg.

By comparing chromatograms of blank brain samples ( $n = 4$ ), supplemented brain samples (2 or 20 pg/mg of brain tissue) and brain samples from animals exposed to 0.1 mg/kg ( $n = 5$ ) and 1 mg/kg ( $n = 4$ ) (see Figure S), two unknown OH-PAHs were detected at retention times of 17.9 and 18.4 min respectively that corresponded to the transitions of OH-B[a]P, OH-B[k]F and OH-B[b]F. Given that retention times of all OH-B[a]P isomers were identified, these two peaks should correspond to isomers of OH-B[k]F and/or OH-B[b]F. In the same manner, two isomers of OH-fluoranthene and/or OH-pyrene were detected at retention times of 10.7 and 10.95 min corresponding to a precursor ion of  $m/z$  332.5 and a product ion of  $m/z$  275. Finally, given that all OH-phenanthrenes were analyzed, the 3 peaks detected at retention times of 8.7, 8.85, and 9.3 min with transitions of quantification and qualification similar to OH-phenanthrene should correspond to 3 isomers of OH-anthracene. Even if the method allows satisfying separation and specificity for almost 53 OH-PAHs analyzed, 23 isomers are still missing (unavailable from suppliers). It is possible that these unknown OH-PAH isomers were not separated from each other; thus, the amount of

OH-PAHs determined might also include the amounts of other isomeric OH-PAHs currently not available from suppliers. In a biological issue, some of these metabolites may simply not be formed or stable in mammals. Interspecies specificity of CYP450 involved in metabolism of PAHs leads to the formation of different metabolites; for instance rat produces more of the 1-OH-phenanthrene than human.<sup>55</sup>

## CONCLUSIONS

In this study, an efficient extraction-purification method for PAHs and OH-PAHs in brain samples has been developed. Except for B[a]P, this work displays for the first time a validated, highly sensitive, and selective method for the determination of very low concentrations of PAHs and OH-PAHs in rat brain ranging from 0.6 to 29 pg/mg and 0.5 to 30 pg/mg respectively. The performance characteristics, in terms of selectivity, sensitivity and cleanliness of the extracts, indicate that this method is fully accurate and can be used for the analysis of PAHs and OH-PAHs in brain samples. It exhibits satisfactory separation of most of the metabolites under investigation. Furthermore, the suitability of the method for the determination of PAHs and OH-PAHs in rat brain after exposure to PAH mixture was demonstrated. Thus, the selective analytical method developed here provides an important new tool for neurotoxicological studies, thereby creating the possibility to study the neurotoxic effects of a mixture of PAHs in adult and/or young rats.

## ASSOCIATED CONTENT

**S Supporting Information.** Table S-1 detailing PAHs and OH-PAHs measured in brain samples collected from rat treated with a mixture of 16 PAHs (0.01 to 1 mg/kg, 28 days, ip). Results are expressed as median and quartiles (in brackets) of 5 rats per group. Figure S-1 displaying a meaningful dose-response relationship of fluorene and its respective metabolites in rat brain tissue following a chronic exposure of animals to fluorene (60 days by ip) with doses ranging between 1 and 100 mg/kg. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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