HHS Public Access

Author manuscript

Anal Bioanal Chem. Author manuscript; available in PMC 2017 August 23.

Published in final edited form as:

Anal Bioanal Chem. 2017 February ; 409(4): 931–937. doi:10.1007/s00216-016-9933-x.

Quantification of Urinary Mono-hydroxylated Metabolites of Polycyclic Aromatic Hydrocarbons by on-line Solid Phase Extraction-High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Human exposure to polycyclic aromatic hydrocarbons (PAHs) can be assessed through monitoring of urinary mono-hydroxylated PAHs (OH-PAHs). Gas chromatography (GC) has been widely used to separate OH-PAHs before quantification by mass spectrometry in biomonitoring studies. However, because GC requires derivatization, it can be time consuming. We developed an on-line solid phase extraction coupled to isotope dilution-high performance liquid chromatography-tandem mass spectrometry (on-line-SPE-HPLC-MS/MS) method for the quantification in urine of 1-OH-naphthalene, 2-OH-naphthalene, 2-OH-fluorene, 3-OH-fluorene, 1-OH-phenanthrene, the sum of 2-OH and 3-OH-phenanthrene, 4-OH-phenanthrene, and 1-OH-pyrene. The method, which employed a 96-well plate platform and on-line SPE, showed good sensitivity (i.e., limits of detection ranged from 0.007 to 0.09 ng/mL) and used only 100 μL of urine. Accuracy, calculated from the recovery percentage at three spiking levels, varied from 94% to 113%, depending on the analyte. The inter- and intra-day precision, calculated from 20 repeated measurements of two quality control materials, varied from 5.2% to 16.7%. Adequate method performance was also confirmed by acceptable recovery (83–102%) of two NIST standard reference materials (3672 and 3673). This high-throughput online-SPE-HPLC-MS/MS method can be applied in large scale epidemiological studies.

Keywords

polycyclic aromatic hydrocarbons (PAHs); OH-PAHs; exposure; HPLC-MS/MS; urine

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Compliance with Ethical Standards

The authors declare that they have no conflict of interest. This research involved human participants. The Centers for Disease Control and Prevention (CDC) Institutional Review Board approved the anonymous collection of urine, and waived informed consent under 45 CFR46.116 (d).

Disclaimer: The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention (CDC). Use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or the US Department of Health and Human *Ser*vices. The authors declare they have no actual or potential competing financial interests. The authors complied with all needed research requirements regarding human subjects.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants linked to a variety of adverse health effects (1–3). Humans may be exposed to PAHs through occupation, such as in work involving diesel fuels and coal tars (4, 5), as well as through diet and other lifestyle activities (e.g., smoking) (6–8). The urinary concentrations of PAH metabolites, specifically mono-hydroxylated PAHs (OH-PAHs), have been used as biomarkers of human exposure to PAHs (7, 9, 10).

The pioneering quantification of OH-PAHs in urine was conducted by high performance liquid chromatography (HPLC) coupled with fluorescence detection (4). Thanks in part to technology advances of the last few decades, isotope dilution gas chromatography-mass spectrometry (GC-MS) became widely used for the determination of urinary OH-PAHs (11–16). GC-MS showed improved accuracy, sensitivity, and precision, mainly because of the high specificity of mass spectrometry detection. However, sample preparation involved derivatization and solvent evaporation steps, and was, therefore, labor intensive and time consuming. More recently, HPLC-tandem MS (HPLC-MS/MS), introduced to measure OH-PAHs (17–26), eliminated the derivatization step and yet maintained the high specificity of mass spectrometry. Still, a relatively large volume of urine, e.g., 2–5 mL, was required in HPLC-MS/MS methods, and automated sample preparation was not fully applied (20, 21). These conditions have limited HPLC-MS from application in large scale biomonitoring studies when matrix volume is often limited.

For the present study, we developed a fully automatic on-line solid phase extraction coupled with isotope dilution-high performance liquid chromatography-tandem mass spectrometry (on-line SPE HPLC-MS/MS) method for the accurate and reliable measurement of nine OH-PAHs in human urine.

EXPERIMENTAL SECTION

Materials and Methods

All solvents were HPLC grade, and chemicals were reagent grade. We purchased acetonitrile, ethanol, 0.1% formic acid in water, methanol, water, and ammonium fluoride from Thermo Fisher Scientific (Waltham, MA, USA); ascorbic acid, sodium acetate, and Helix pomatia β-glucuronidase type H-1 (β-glucuronidase ≥300,000 units/g, sulfatase ≥10,000 units/g) from Sigma-Aldrich (St. Louis, MO, USA). We obtained 1-hydroxynaphthalene (1-OH-NAP), 2-hydroxynaphthalene (2-OH-NAP), 2-hydroxyfluorene (2-OH-FLU), 3-hydroxyfluorene (3-OH-FLU), 1-hydroxyphenanthrene (1-OH-PHE), 2-hydroxyphenanthrene, 3-hydroxyphenanthrene, 4-hydroxyphenanthrene (4-OH-PHE), 1-hydroxypyrene (1-OH-PYR), and their corresponding ^{13}C-labeled internal standards (IS, listed in Table 1) from Cambridge Isotope Laboratories (Andover, MA, USA).

We purchased smokers' urine samples from BioreclamationIVT (Westbury, NY, USA). We also collected urine anonymously in 2015 from non-smoker adult volunteers with no documented occupational exposure to PAHs in Atlanta, GA. We obtained two Standard Reference Materials® (SRMs), SRM 3672 (smoker urine) and SRM 3673 (non-smoker

urine), from the US National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). All urine specimens were stored upon collection or arrival at −70 °C until use.

Appropriate safety control measures (including engineering, administrative, and personal protective equipment) were used for all procedures based on a site-specific risk assessment that identified physical, health, and procedural hazards.

Preparation of standard stock solutions and quality control materials—We prepared the stock solutions of individual analytical standard in ethanol. Standards with all nine OH-PAHs were generated by serial dilution of the individual stock in 40% ethanol/60% water. The final concentrations of the mixed stock standards ranged from 0.08 – 200 ng/mL (1-OH-NAP and 2-OH-NAP) and 0.005 – 50 ng/mL (all other analytes). The standard solutions were aliquoted into 2 mL silanized amber glass vials and stored at 4 °C until use. The internal standard solution with ^{13}C-labeled analytes was prepared in water with 0.2% acetonitrile so that a 50 μL spike would result in approximate concentrations of 32 ng/mL (^{13}C-1-OH-NAP and ^{13}C-2-OH-NAP) or 8 ng/mL (other ^{13}C-labeled analytes). Internal standards were aliquoted into 15 mL amber glass vials and stored at −70 °C.

Two levels of quality control (QC) materials, QC low (QCL) and QC high (QCH), were prepared by pooling urines from smokers and non-smokers. The QC concentrations were fortified, as needed, with native target compounds to encompass the ranges described for the U.S. general population (27). All QC materials were stored in 4 mL amber glass vials at −70 °C until used. The stability of spiked material stored at −70 °C has been previously evaluated for up to one year (data not shown), and no obvious degradation of OH-PAHs was observed. The QC materials stored at −70 °C for more than one year will be re-evaluated for their stability.

Sample Preparation—Urine samples were thawed and mixed at room temperature. QC samples, reagent blanks, and standards were processed the same way as urine samples, going through all of the sample preparation steps. Sample preparation was automatically conducted on a Perkin-Elmer Staccato® System (controlled by the Perkin Elmer iLink and Maestro software) (Waltham, MA, USA). The robotic system included six main components: Sciclone G3/G3T, Fluidx CESD-24PRO decapper, Hettich Rotanta 460 centrifuge, ThermoScienfic ALPS 3000 sealer, IVD Inheco Incubator shaker DWP, and Mitsubishi robotic arm. We programmed this system to aliquot urine samples, standards, QCs and reagent blanks (100 μL) into a 96-well plate (Corning, NY, USA), and subsequently add ascorbic acid solution (20 μL, ~12.5 mg/mL), internal standards solution (50 μL), and sodium acetate buffer (50 μL, ~1 mol/L, pH 5.5) containing ~10 mg/mL β-glucuronidase/arylsulfatase. The accuracy and precision of automatic aliquating by Sciclone robotic system was previously evaluated, and good aliquoting accuracy (recovery rate from 95% to 105%) and precision (CV<3 %) were achieved from current procedures. Details regarding enzymatic de-conjugation were previously described (16). The robotic system then sealed and transferred the plate for overnight incubation at 37±2 °C. After enzymatic hydrolysis, the robotic system automatically added methanol (175 μL) to all sample wells, mixed the solution, resealed the plate, and centrifuged for ~15 minutes at 5000 rpm (5900 rcf). Finally, the robotic system transferred 200 μL of the supernatant in each well to a new

96-well plate, and added 350 μL of water to each well before on-line SPE-HPLC-MS/MS analysis. Non-spiked and spiked synthetic urine (28) used for determining the limit of detection (LOD) was prepared as study samples.

Online SPE-HPLC-MS/MS—The on-line SPE-HPLC-MS/MS system consisted of a Sciex 5500 or 6500 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with an electrospray ion source and controlled by AB Sciex Analyst^{TM} software, and one Agilent 1260 pump and one degasser (Santa Clara, CA, USA), and an on-line SPE Spark Holland system (Glassboro, NJ, USA) controlled by the Sparklink® software (iChrom Symbiosis system).

After injection (300 μL), the sample was loaded onto an Oasis WAX on-line SPE cartridge with 0.1% formic acid in water (1.5 mL), and the cartridge was washed with acetonitrile/methanol/water (~0.4 mL, 1/1/2, v/v/v). The target analytes were eluted with methanol (350 μL), and focused on a Chromolith HighResolution RP-18 endcapped guard column (5×4.6 mm, Merck KGaA, Darmstadt, Germany) with the initial HPLC gradient.

We separated the target analytes on a pair of Chromolith HighResolution RP-18 endcapped HPLC columns (100×4.6 mm, Merck KGaA) by a programmed HPLC gradient (Table 2). The mobile phases were water with 0.1 mM ammonium fluoride (A) and methanol with 0.1 mM ammonium fluoride (B).

The ionspray voltage and source temperature were −3.0 kV and 500 °C, respectively. Curtain gas, ion source gas 1, ion source gas 2, and collision gas were 35 psi, 50 psi, 70 psi, and 9 psi, respectively. The representative decluster, entrance and exit potentials were −120 V, −3 V and −12 V, respectively. We quantified OH-PAHs by selected reaction monitoring in the negative ion mode by the ion transitions listed in Table 1, and optimized the collision energies for all ion transitions (Table 1).

Data Analysis—We used Analyst (version 1.6.2, Sciex, Foster City, CA, USA) and MultiQuant (version 3.0, Sciex, Foster City, CA, USA) for data processing. We defined quality control limits, and evaluated analytical runs using SAS (version 9.3, SAS Institute Inc.; Cary, NC, USA) with a multi-rule quality control approach (29).

Results and discussion

OH-PAHs were enriched and extracted from the urine matrix by on-line SPE. We separated the target analytes on a pair of monolithic RP-18 column by using a gradient of water and methanol, including ammonium fluoride (0.1 mM) in both mobile phases. We used ammonium fluoride to improve method sensitivity in the negative ion mode (30). An example of LC-MS/MS selected ion chromatogram is shown in Figure 1. Within 27 mins, we were able to separate several pair of isomers: 2-OH-NAP, 1-OH-NAP, 3-OH-FLU, 2-OH-FLU, 1-OH-PHE, 4-OH-PHE, and 1-OH-PYR, but 2-OH-PHE and 3-OH-PHE were eluted together so we had to measure these two analytes as a sum (Σ2,3-OH-PHE). 9-hydroxyphenanthrene and 3-hydroxyfluoranthene could also be separated from the other target metabolites (data not shown).

Method accuracy was assessed by repeated analyses (n=7) of synthetic urine spiked with the target analytes at three spiking concentrations. Accuracy, expressed as a percentage of recovery, was 105–113% (level 1), 94–100% (level 2), and 98–102% (level 3), depending on the analyte (Table 3). Furthermore, accuracy was evaluated by analyzing two NIST SRMs, SRM 3672 and SRM 3673. The calculated OH-PAHs concentrations were in good agreement with the certified concentrations (31), and accuracy ranged from 83 to 102%, depending on the analyte (Table 3).

We determined the method precision from repeated measurements of low and high QC pools by following the CLSI protocol EP5-A2 (32) on 51 different days (two results from each of two daily runs) over a period of 8 months that involved multiple analysts. The relative standard deviations (RSDs), which reflect the within- and between-run variability, ranged from 3.2% to 12.1% (within-run) and 4.8% to 13.0% (within and between runs) for all analytes (Table 4).

The LOD was determined according to procedures previously described (33) from 60 repeated measurements of non-spiked and spiked synthetic urine analyzed by multiple operators and using four different mass spectrometers (Sciex 5500 or 6500). The LODs ranged from 0.007 ng/mL to 0.09 ng/mL for all analytes (Table 4), indicating the good sensitivity of the method, especially considering the relatively low volume of urine used (100 μL). The method also provided wide dynamic ranges, with upper linearity of the method at 200 ng/mL (2-OH-NAP, 1-OH-NAP); 25 ng/mL (2-OH-FLU); 20 ng/mL (Σ2,3-OH-PHE); and 10 ng/mL (3-OH-FLU, 1-OH-PHE, 4-OH-PHE, 1-OH-PYR).

SPE recovery was calculated as previously described (34). The mean recoveries of three repeated measurements were 67±4% (2-OH-NAP), 67±5% (1-OH-NAP), 81±4% (3-OH-FLU), 64±2% (2-OH-FLU), 63±2% (Σ2,3-OH-PHE), 72±5% (1-OH-PHE), 55±2% (4-OH-PHE), and 49±6% (1-OH-PYR). These recoveries, which are comparable with those reported before using a GC-MS method (16), are adequate for quantitative measurement of OH-PAHs in urine from both smokers and non-smokers. We also evaluated matrix effects using a matrix factor, defined as the ratio of IS peak area in the presence of urine matrix to the IS peak area in the absence of urine matrix (34). The matrix factor, calculated from 2 different QC concentrations of three repeated measurements varied from 63% to 101%, depending on the analyte.

To validate the method, we measured OH-PAHs in 36 non-smokers and 36 self-identified smokers' urine samples (Table 5). Among non-smokers, detection frequencies were 56% for 1-OH-PYR, 14% for 4-OH-PHE, 94% for 3-OH-FLU, and 100% for the other analytes. For smokers, detection frequencies were 97% for 1-OH-PYR, 53% for 4-OH-PHE, and 100% for the rest of analytes. The geometric mean concentrations of OH-PAHs were 2.5–12.4 (average 6.6) times higher in smokers than non-smokers (Table 5). 2- and 3-OH-FLU and 1- and 2-OH-NAP showed the largest concentration differences between smokers and non-smokers.

Compared with our previous GC-MS method (13, 16), the current approach eliminated the derivatization and solvent evaporation steps, simplified the sample processing procedure,

and greatly improved throughput, while using 10 times less urine volume (0.1 vs 1.0 mL). This robust, sensitive, and highly automated on-line SPE-HPLC-MS/MS method is suitable for the quick analysis of human samples in national surveys and other large epidemiological studies to evaluate human exposure to PAHs.

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Novel aspect

We developed a fully automated and high throughput on-line SPE-HPLC-MS/MS method for concurrent quantification of nine urinary OH-PAH metabolites.

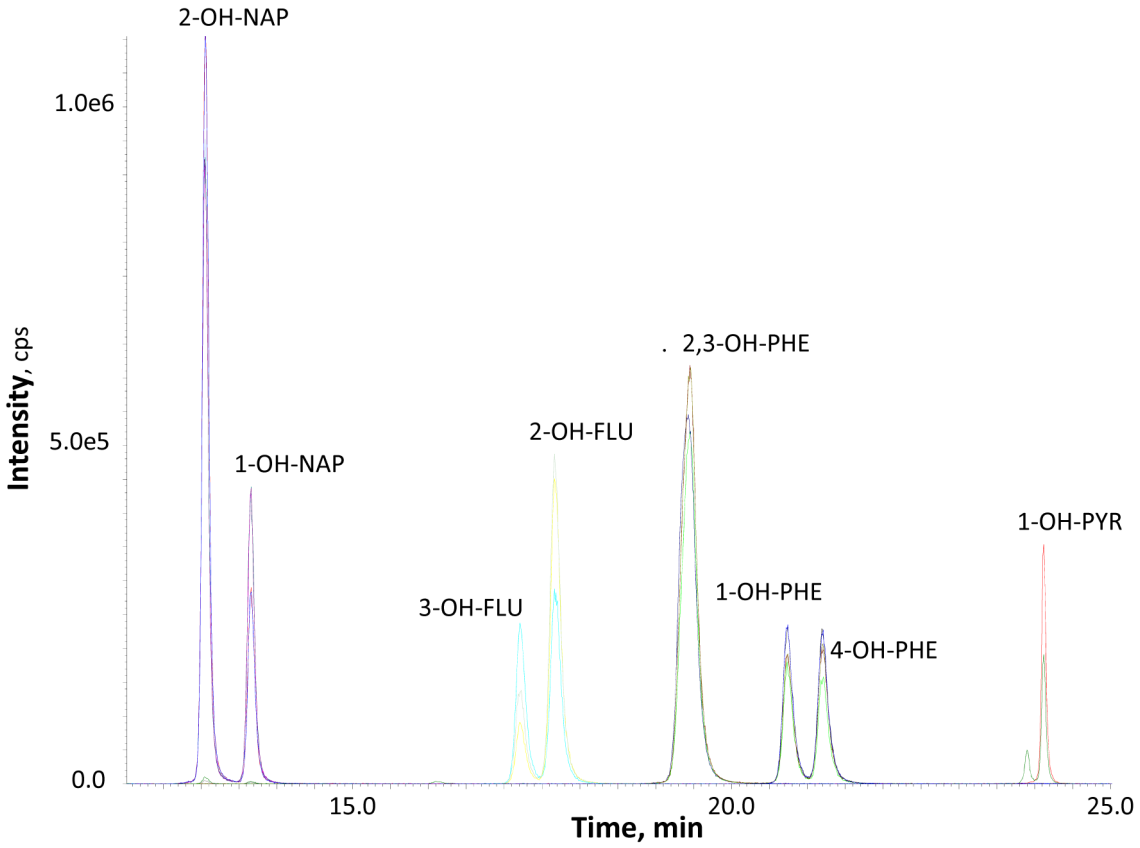

Figure 1. Example Chromatogram

2-OH-NAP and 1-OH-NAP at 10 ng/mL, Σ2,3-OH-PHE at 5 ng/mL, and other analytes at 2.5 ng/mL.

Table 1

Optimized MS parameters for nine hydroxylated PAH metabolites.

No.	Analyte	Abbreviation	Ion Transition (m/z)	IS Ion Transition (m/z)	Collision Energy (eV)	Parent PAH
1	1-hydroxynaphthalene	1-OH-NAP	143→115	149→121	−34	Naphthalene
2	2-hydroxynaphthalene	2-OH-NAP	143→115	149→121	−34	
3	2-hydroxyfluorene	2-OH-FLU	181→180	187→186	−34	Fluorene
4	3-hydroxyfluorene	3-OH-FLU	181→180	187→186	−26	
5	1-hydroxyphenanthrene	1-OH-PHE	193→165	197→168	−38	Phenanthrene
6	2-hydroxyphenanthrene	Σ2,3-OH-PHE*	193→165	199→171	−41	
7	3-hydroxyphenanthrene					
8	4-hydroxyphenanthrene	4-OH-PHE	193→165	197→168	−38	
9	1-hydroxypyrene	1-OH-PYR	217→189	223→195	−45	Pyrene

*2-OH-PHE and 3-OH-PHE are measured together.

Table 2

Optimized LC gradient.

Flow Rate (μL/min)	A%	B%	Time (min)
500	99	1	0.0
500	99	1	3.5
500	40	60	3.9
500	40	60	4.3
800	38	62	5.0
800	32	68	18.0
800	30	70	19.5
1000	15	85	20.0
1000	14	86	21.0
1000	10	90	22.0
1000	5	95	24.0
1000	5	95	24.5
1000	99	1	24.6
1000	99	1	27.0

* A: HPLC grade water with 0.1 mM ammonium fluoride, B: HPLC grade methanol with 0.1 mM ammonium fluoride.

Table 3

Accuracy for the measurement of hydroxylated PAH metabolites.

Analyte	Level -1 (n=7)		Level -2 (n=7)		Level -3 (n=7)		SRM 3672 Smoker urine (n=4)			SRM 3673 non-Smoker urine (n=4)		
	Spiked (ng/mL)	Accuracy	Spiked (ng/mL)	Accuracy	Spiked (ng/mL)	Accuracy	Measured (ng/mL)	NIST Certified (ng/mL)	Accuracy	Measured (ng/mL)	NIST Certified (ng/mL)	Accuracy
2-OH-NAP	0.71	112.8±4.3%	3.85	99.7±1.6%	19.23	99.7±0.8%	8.88±0.17	8.73	101.7±2.0%	1.43±0.07	1.35	106.3±4.8%
1-OH-NAP	0.70	113.3±1.9%	3.83	95.3±1.2%	19.13	98.7±1.2%	36.85±0.68	34.4	107.7±2.7%	207.38±3.94	211.00	98.3±1.87%
3-OH-FLU	0.17	107.2±4.2%	0.91	98.6±2.6%	4.57	98.1±2.0%	0.41±0.02	0.43	96.8±4.7%	0.04±0.01	0.04	112.0±25.6%
2-OH-FLU	0.18	107.4±2.3%	0.96	99.3±1.3%	4.78	100.3±1.4%	0.72±0.04	0.87	82.8±4.6%	0.09±0.01	0.11	83.7±9.3%
Σ2,3-OH-PHE	0.36	113.1±1.8%	1.94	99.4±1.8%	9.68	101.9±1.2%	0.20±0.02	0.21	96.1±9.6%	0.05±0.01	0.05	103.0±18.9%
1-OH-PHE	0.18	109.8±2.8%	0.95	99.4±2.8%	4.75	101.2±2.4%	0.11±0.01	0.14	84.3±7.4%	0.05±0.01	0.05	98.4±20.4%
4-OH-PHE	0.17	108.5±3.2%	0.94	98.3±3.2%	4.7	98.2±1.2%	0.05±0.01	0.05	94.2±20.4%	*	0.01*	*
1-OH-PYR	0.18	105.5±5.0%	0.97	93.6±2.6%	4.86	101.8±2.6%	0.18±0.02	0.17	102.2±11.6%	*	0.03*	*

*Results not reported because NIST certified values are close or below the method LOD for these analytes.

Table 4

Precision, limits of detection (LODs), and upper linearity limits.

Analytes	QCH*			QCL*			LOD (ng/mL)	Upper linearity limit (ng/mL)
	Mean (ng/mL)	Within-run precision (CV%)	Method precision (CV%)	Mean (ng/mL)	Within-run precision (CV%)	Method precision (CV%)		
1-OH-NAP	7.78	3.3%	4.8%	1.22	5.9%	7.4%	0.06	200
2-OH-NAP	8.94	3.2%	5.7%	1.54	4.4%	6.2%	0.09	200
2-OH-FLU	1.06	3.8%	5.4%	0.25	4.3%	5.7%	0.008	25
3-OH-FLU	1.14	3.3%	4.8%	0.28	4.2%	5.1%	0.008	10
1-OH-PHE	0.68	7.2%	8.0%	0.17	8.6%	9.7%	0.009	10
Σ2,3-OH-PHE	1.36	6.5%	7.9%	0.36	7.1%	8.2%	0.01	20
4-OH-PHE	0.54	7.0%	10.5%	0.13	9.5%	12.6%	0.007	10
1-OH-PYR	0.79	12.1%	12.4%	0.40	11.4%	13.0%	0.07	10

*QCH: QC high; QCL: QC low.

Table 5

Geometric mean (GM) and select percentile concentrations (ng/mL) of OH-PAHs in 36 nonsmokers and 36 smokers.

Analyte	NonSmoker (n=36)				Smoker (n=36)				Smoker GM / Nonsmoker GM
	GM	25th	50th	75th	GM	25th	50th	75th	
2-OH-NAP	1.97	1.09	1.90	4.56	11.24	5.91	10.44	21.79	5.72
1-OH-NAP	0.57	0.39	0.57	0.88	6.81	3.19	8.06	15.53	12.00
3-OH-FLU	0.04	0.02	0.04	0.06	0.46	0.20	0.46	1.35	12.37
2-OH-FLU	0.09	0.04	0.08	0.15	0.76	0.38	0.82	1.84	8.53
Σ2,3-OH-PHE	0.08	0.05	0.08	0.12	0.33	0.20	0.32	0.64	4.31
1-OH-PHE	0.05	0.03	0.05	0.08	0.13	0.06	0.11	0.33	2.49
4-OH-PHE	0.01	0.01	0.01	0.02	0.03	0.02	0.04	0.07	2.82
1-OH-PYR	0.08	0.06	0.08	0.12	0.38	0.17	0.38	0.99	4.75