Determination of ten monohydroxylated polycyclic aromatic hydrocarbons by liquid–liquid extraction and liquid chromatography/tandem mass spectrometry

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ABSTRACT

The aim of this study is to develop and validate an analytical method for the quantitation of ten urinary monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) through high pressure liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). After enzymatic deconjugation, urine samples were extracted by liquid–liquid extraction (LLE) and OH-PAHs were analyzed by HPLC/MS/MS operated in negative electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. LLE was conducted with the solvent mixture of pentane and toluene, which reduced the matrix interferences and enhanced the method sensitivity significantly. Deuterated and ^{13}C-labeled analogs are used as internal standards. Calibration curves of all target analytes shows favorable linearity within the concentration range of 5.9–15,000.0 ng/L for different OH-PAHs with the regression coefficients above 0.993. The limits of detection (LODs) in pooled urine ranged from 1.72 to 17.47 ng/L, which were much lower than those obtained by a gas chromatography/high resolution mass spectrometry (GC/HRMS) method. The method shows satisfactory accuracy and precision when analyzing three different levels of OH-PAHs spiked in pooled urine. Except for 1-hydroxynaphthalene, recoveries of other OH-PAHs were in the range of $100 \pm 20\%$ with a variation coefficient of less than 13%. The measurement of OH-PAHs from a QC sample of the Centers for Disease Control and Prevention (CDC) generated results close to the values measured by CDC. This method has been successfully employed in the California Biomonitoring Program.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), as ubiquitous environmental contaminants, are released from incomplete combustion of organic matter. Wood burning fireplaces, tobacco smoke, vehicles exhaust, asphalt road and roofing process and power plants operation are the sources of environmental PAHs [1]. Environmental PAH exposure occurs primarily through the inhalation of smoke and through consumption of barbecue, roasting, broiling or cooked foods [1,2]. Occupational exposure can occur from the contaminated workplace or industrial source, including aluminum and steel manufacture [3]. Human long term exposure to PAHs could lead to skin, lung and bladder cancer [4–7], as well as interference with endocrine function [8,9], and very recent studies also indicated that some PAHs metabolites have strong correlation with atherosclerosis and cardiovascular diseases [10–12].

PAHs are metabolized by cytochrome *P*450 enzymes in human and animals, and excreted in urine. The major metabolites are monohydroxylated PAHs (OH-PAHs) [13] and dihydrodiols [14]. Since a number of PAHs are carcinogenic, there have been of great interests in developing methods for the determination of body burden and the assessment of human exposure. 3-Hydroxybenzo(*a*)pyrene, one metabolite of benzo[*a*]pyrene with the highest carcinogenicity in PAH group, mainly excreted through feces instead of urine and hence could not be easily determined in urine [15]. The metabolite of pyrene, 1-hydroxypyrene (1-OHP) is the most widely used biomarker in numerous studies [15–20]. However, since ratios of various PAHs may vary depending on the source and personal enzymatic capacity, concentration profiles of multiple OH-PAHs biomarkers are necessary and required to assess the environmental exposure risk. Currently, the metabolites of naphthalene, fluorene and phenanthrene, which have two or three benzene rings, are commonly used as biomarkers to comprehensively assess the exposure level and environmental risk, and these metabolites include 2-hydroxynaphthalene (2-OHN), 2-hydroxyfluorene (2-OHF), 9-hydroxyphenanthrene (9-OHPhe) and 3-hydroxyphenanthrene (3-OHPhe) [21–25].

Urinary OH-PAHs, especially for 1-OHP, have been widely determined by high performance liquid chromatography (HPLC) with fluorescence detection (FD) [21,22] due to its high sensitivity on FD and higher concentration in urine. The advantage of this method is that HPLC-FD is cost saving and easily available in most laboratories. The disadvantages are that it is not specific and selective for OH-PAHs and hence the method precision and accuracy are unsatisfactory. In other words, the method is prone to chemical interference. In addition, for those PAH metabolites with lower concentration and poor sensitivity on FD, such as 4-hydroxyphenanthrene (4-OHPhe), it is difficult to detect them with HPLC-FD method [21,22]. Gas chromatography/low resolution mass spectrometry (GC/MS) and gas chromatography/high resolution mass spectrometry (GC/HRMS) were utilized to determine urinary OH-PAHs [26–29]. The GC columns provide better separation of isomers than HPLC columns and could effectively reduce the baseline interference [27,29], then resulting in more accurate quantification and limits of detection (LODs) than those from HPLC methods. However, it is more expensive and time consuming since GC/MS method requires derivatization of OH-PAHs. In addition, instrumental maintenance for GC/HRMS is expensive and it could be a burden for many research laboratories.

Recently, high pressure liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) has been used to measure OH-PAHs. HPLC/MS/MS in the multiple reaction monitoring (MRM) mode is much more specific than HPLC-FD method [30–32]. With proper clean-up and pretreatment of urine sample, it is possible to reduce the matrix effects and reach the LODs comparable to the method based on GC/HRMS. Liquid–liquid extraction (LLE) and solid phase extraction (SPE) methods are reported for the extraction and clean-up of urinary OH-PAHs [21,22,27,29,32] at trace levels. SPE was simple and reliable, and has been widely used in the pretreatment of environmental urine and blood samples. However, we noticed that SPE cleanup is not good enough to measure extremely low level of OH-PAHs, due to matrix interferences. To quantitate lower levels of analytes accurately in those samples, it is very important to reduce matrix interference as possible. LLE technique, as a routine sample preparation method, proved to be very efficient and useful for the extraction and clean-up of OH-PAHs in urine [29].

In this study, we used LLE for sample preparation and LC/MS/MS in negative electrospray ionization (ESI) and MRM mode to determine ten OH-PAHs, including 2-OHN, 1-hydroxynaphthalene (1-OHN), 2-OHF, 3-hydroxyfluorene (3-OHF), 1-, 2-, 3-, 4-, 9-hydroxyphenanthrene (1-, 2-, 3-, 4-, 9-OHPhe) and 1-OHP (structure shown in Fig. 1). We found that our method is greatly improved in efficiency and LODs compared with the previous reported methods [21,22,26,31,33]. Deuterated and ^{13}C-labeled analogs are used as internal standards. LLE with a mixture of pentane and toluene significantly improved the method sensitivity compared with using SPE. LC/MS/MS method overcame the disadvantages of GC/MS. The method was fully validated and used for studies by the California Environmental Contaminants Biomonitoring Program. To our best knowledge, it is the first time to report to use LLE and LC/MS/MS to measure urinary OH-PAHs with higher sensitivity.

2. Experiment

2.1. Materials

2-OHN (purity 99%) and 3-OHF were purchased from Sigma (St. Louis, MO, USA). 2-OHF (purity 98%), 9-OHPhe and 1-OHP (purity 98%) were purchased from Aldrich (St. Louis, MO, USA). 1-OHN was purchased from Fluka (purity 99%, St. Louis, MO, USA). 1-OHPhe (purity 99%), 2-OHPhe (purity 99.6%) and 4-OHPhe (50 μg/mL in acetonitrile) were purchased from Dr. Ehrenstorfer

(Augsburg, Germany). 3-OHPhe (purity 98%, 50.0 μg/mL in toluene) and ^{13}C$_6$-3-OHPhe (purity 95%, 50.0 μg/mL in acetonitrile) were purchased from Cambridge Isotope Lab (Andover, MA, USA). D$_8$-2-OHN and D$_9$-1-OHP were purchased from C-D-N Isotope Inc. (Quebec, Canada). D$_9$-2-OHF was obtained from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA). β-Glucuronidase/arylsulfatase from Helix pomatia was from Sigma (St. Louis, MO, USA). Pentane and toluene are purchased from Sigma (St. Louis, MO, USA). Methanol (LC–MS Chromasolv®, ≥99.9%) was obtained from Fluka (St. Louis, MO, USA). Water (Chromosolv plus for HPLC grade), sodium acetate and silver nitrate (HPLC grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glacial acetic acid (HPLC grade) was from VWR International Ltd. (Leicestershire, England). All other reagents were of analytical grade and used without further purification. Zorbax RRHD Eclipse plus C18 column (100 mm × 2.1 mm, 1.8 μm) was from Agilent (Santa Clara, CA, USA).

2.2. Standard preparation, calibration curve and QA/QC

Stock solutions of all individual target and isotope labeled standards were first prepared in acetonitrile or toluene, respectively. Then the exact volumes of stock solutions were measured and diluted in acetonitrile to prepare a mixed native standard solution and a mixed labeled standard solution. The final concentrations were in the range of 30–300 μg/L for natives and 20–300 μg/L for internal standards, respectively. Due to the ubiquitous presence of OH-PAHs in urine samples and lack of OH-PAHs free urine blank, calibration standard solutions were prepared in the range of 5.9–15,000.0 ng/L in 2 mL pooled urine samples, which were pre-diluted ten times by adding an exact volume of native stock solution mixture. Water and urine samples without spiked native and isotope internal standards were used to examine whether the buffer and urine had matrix interferences and background contamination. A ten time diluted urine sample spiked with the internal standards was measured as the method blank. Both standards and blanks, including water blank, urine blank and method blank, were prepared fresh on the day of use. A set of three level of quality control materials (QC sample), including samples with high (1500–7500 ng/L), medium (750–3750 ng/L) and low concentrations (150–1500 ng/L), were prepared in our lab by spiking 10 times diluted pooled urine with calculated volumes of stock solution mixture. 100 μL internal standard mixture solutions were added into urine sample, native standards, blank samples, and QA/QC samples within the concentrations of 1–15 μg/L.

2.3. Sample preparation

Sample preparation followed the method of Centers for Disease Control and Prevention (CDC), but modified slightly to adjust for equipment differences [29]. 10 μL β-glucuronidase/arylsulfatase enzyme and 3 mL, 0.1 mol/L acetate acid and sodium acetate (HAC–NaAC) buffers were added to the 2.0 mL aliquot of urine sample to adjust the pH to 5.5, and then incubated at 37 °C overnight. The target analytes were extracted twice through automated Gilson 215 liquid handler (Gilson Inc., Middleton, WI, USA) with 5 mL pentane and toluene (volume ratio = 80:20). The two extractions were combined. Then, they were washed and cleaned with 1 m/L of 1 mol/L silver nitrate solution to reduce the interference of sulfur metabolites of PAHs [29]. After 5 min centrifuge, the upper layer solution was taken out and evaporated at 37 °C by gentle nitrogen (lower than 5 psi). When near dryness (5–10 μL left), the residues are redissolved in vials with 40 μL methanol and kept in freezer at −20 °C until analysis.
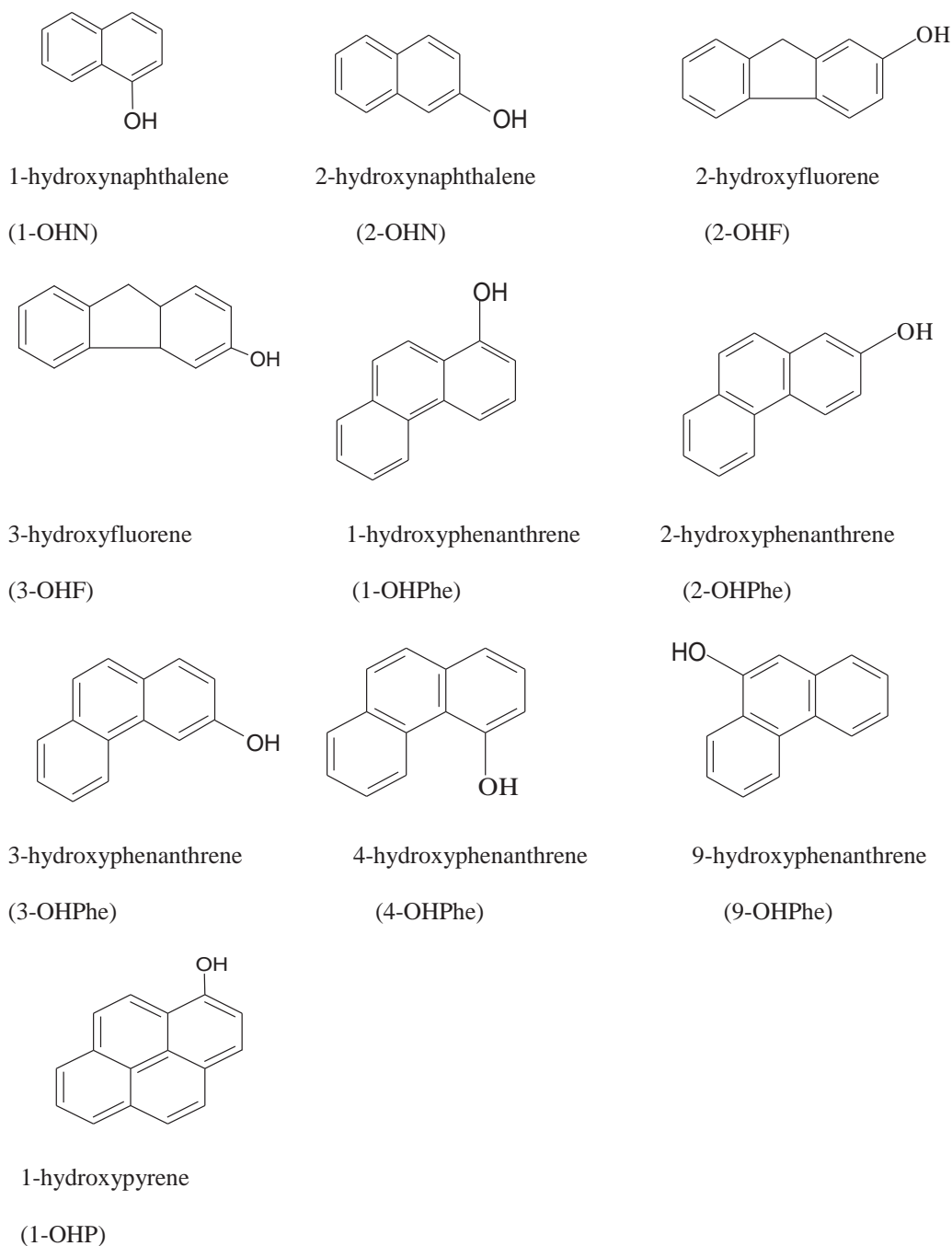
1-hydroxynaphthalene

(1-OHN)

2-hydroxynaphthalene

(2-OHN)

2-hydroxyfluorene

(2-OHF)

3-hydroxyfluorene

(3-OHF)

1-hydroxyphenanthrene

(1-OHPhe)

2-hydroxyphenanthrene

(2-OHPhe)

3-hydroxyphenanthrene

(3-OHPhe)

4-hydroxyphenanthrene

(4-OHPhe)

9-hydroxyphenanthrene

(9-OHPhe)

1-hydroxypyrene

(1-OHP)

Fig. 1. Chemical structures for OH-PAHs.

2.4. Instrumental analysis

All samples are analyzed with an Agilent 6460 Triple Quadrupole LC/MS system (Santa Clara, CA, USA), coupled with an Ultra HPLC 1290, G4220A Infinity Binary Pump, G1316C Infinity TCC, G4226A Infinity Sampler. The mass spectrometer parameters for each of the analytes and corresponding isotope labeled standards are shown in Table 1. The nitrogen gas temperature of source was held at 300 °C with a flow rate of 10 L/min. The nebulizer was 45 psi and the sheath gas temperature was kept at 300 °C with a flow rate of 11 L/min. The nozzle voltages were set at 500 eV for negative ESI modes. The capillary voltage was set at 2000 V for negative mode.

Zorbax RRHD Eclipse plus C18 column (100 mm × 2.1 mm, 1.8 μm) was used as the analysis column. The mobile phases are

water and methanol, respectively. The flow rate is set at 0.4 mL/min and the column temperature is held at 40 °C. The gradient elution program is initialized with 40% methanol, increased to 80% methanol in 10 min, then continually increased to 99% methanol in 1 min and held on for 2 min to wash the column and then allowed column equilibrium in 3 min for next run. The highest column pressure limit was set at 1200 bar.

Due to the commercial unavailability of internal standards of some target analytes, several hydroxyl metabolite isomers of naphthalene, fluorene and phenanthrene were quantified by only one internal standard (D$_8$-2-OHN, D$_9$-2-OHF, and ^{13}C$_6$-3-OHPhe), respectively (see Table 1). 1- and 9-OHPhe could not be separated chromatographically and were consequently quantified together (and referred to as "1/9-OHPhe"). Data analysis was carried out on

Table 1
The parameters of tandem mass spectrometer and retention time, calibration curve, range, LOD, and LOQ in 10 time diluted pooled urine.

Analyte name	Analyte abbreviation	MRM transitions	Fragmentor (eV)	Collision energy (eV)	Retention Time (min)	Range (ng/L)	LOD (ng/L)	LOQ (ng/L)
2-Hydroxynaphthalene	2-OHN	143.2–115.2	140	25	3.992	39.1–10,000	16.01	39.1
1-Hydroxynaphthalene	1-OHN	143.2–115.2	120	25	3.882	58.6–15,000	17.47	58.6
D_8-2-hydroxynaphthalene	D_8-2-OHN	150.0–122.1	80	20				
2-Hydroxyfluorene	2-OHF	181.1–180.1	148	25	6.170	39.1–10,000	15.79	39.1
3-Hydroxyfluorene	3-OHF	181.1–153.2	137	25	6.172	39.1–10,000	11.01	39.1
D_9-hydroxyfluorene	D_9-2-OHF	190.2–188.2	90	30				
1-/9-Hydroxyphenanthrene	1-/9-OHPhe	192.9–165.1	163	30	7.210	11.7–3000	4.85	11.7
2-Hydroxyphenanthrene	2-OHPhe	192.9–165.1	163	30	6.810	5.9–1500	1.72	5.9
3-Hydroxyphenanthrene	3-OHPhe	192.9–165.1	163	30	6.941	5.9–1500	2.59	5.9
4-Hydroxyphenanthrene	4-OHPhe	192.9–165.1	163	30	7.509	5.9–1500	5.35	5.9
$^{13}C_6$-3-hydroxyphenanthrene	$^{13}C_6$-3-OHPhe	199.2–171.2	160	45				
1-Hydroxypyrene	1-OHP	217.1–189.1	160	35	8.541	23.4–6000	4.85	23.4
D_9-1-hydroxypyrene	D_9-1-OHP	226.2–198.2	195	50				

the MassHunter Workstation Software B 03.01 (Agilent, Santa Clara, CA, USA).

2.5. Validation method

The ten times diluted pooled urine sample was used to validate HPLC/MS/MS. Calibration curve linearity, LOD, method precision and accuracy as well as cross lab QC sample test were evaluated. The LODs for all analytes were calculated as $3S_0$, where S_0 is the standard deviation (SD) of the lowest calibration concentration of ten batch calibration curves with the recoveries between 80% and 120%. The LOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision [34]. The method precision was evaluated by calculating the relative standard deviations (RSDs) of repetitive measurement of the QC materials at three different concentrations (0.75–7.5 μg/L, 0.375–3.75 μg/L and 0.15–1.5 μg/L). More than twenty runs were completed to evaluate the inter- and intra-days deviation. The relative recoveries or the accuracy were calculated in the following way: dividing measured concentrations of the target analytes by the spiked concentration. The method performance (or proficiency tests) was evaluated by analysis of QC samples from CDC. Before daily instrumental analysis, the lowest calibration curve point prepared in urine matrices was analyzed first. Then the response of each analyte and chromatograms were compared with the previous ones to confirm acceptable chromatographic resolution and mass spectral sensitivity. If neither of deviations exceed 15%, the instrumental performance (sensitivity and resolution) is considered acceptable for sample analysis. For the real sample analysis, we required that 15% samples in each batch were analyzed in duplicate and with relative percentage difference of less than 15%.

3. Results and discussion

3.1. Method validation

In GC/MS/MS and LC/MS/MS methods [35,36], ideally two MRM transitions should be monitored and recorded for unique identification. One is for quantification and another is usually for confirmation. But one of the problems of recording two transitions for each analyte in LC/MS/MS method is that the second transition (used for confirmation) is often less sensitive [37]. Furthermore, the second transition of some compounds could not be obtained even with optimized MS parameters. In our study, the second transition was not employed due to its low sensitivity or absence. The chemical stability of the aromatic compounds results in the lack of the second transition. The absence of the second transition would not compromise their unique identification since we used ultra high performance LC pump and column. Ultra HPLC provides

better LC resolution and separation and hence aids in compound identification. The MRM transitions in our method are the same as those reported in other LC/MS/MS method [8,30,31].

Due to high separation efficiency of the micro column packed with micro-particles (1.8 μm) and the employment of high pressure of 700 bars, most target compounds except for 1- and 9-OHPhe got baseline separation within 10 min, as shown in Fig. 2. A good method should be able to not only quantitate accurately, but also be economic in cost and be easy to operate and manage. GC/HRMS method has high peak resolution and low detection limits, however, it is time-consuming due to the requirement of derivatization. In addition, the instrumental operation and maintenance are complex and difficult even for an experienced operator. Previous LC method coupled with a long column (250 mm) with macro-particles (5 μm) needs not only a longer time to analyze and back equilibrium but also a bigger sample injection volume (10–20 μL) to obtain enough response to quantify the compounds [22]. In this study, 10 min analysis time and 2 μL injection volume were employed. Compared with the GC and LC method reported [22,26,29–32], the analysis time is shortened significantly, which not only save the time but also reduce the solvent cost.

The validation results of the analysis of OH-PAHs were completed over 2 months (shown in Tables 1–3). The previous study, which will be published in another paper, has shown that the standards and samples are stable over 2 months under the same condition. Ideally, analyte free blank matrix should be used for the preparation of calibration standards. Practically, it does not exist. We used an alternative solution, ten times diluted pooled urine, to prepare standard calibration solutions. The 10 times diluted pooled urine has the concentrations of targets lower than the LODs and keep the same urinary matrix effect. Results show that all run batches of the calibration curves had good linearity with weighted $(1/x)$ linear least regression ($r^2 > 0.993$) and the slope deviations below 20% to control the precision and accuracy successfully. Most compounds demonstrated a wider linearity range covering 3–4 orders of magnitude. A relatively narrow but good linearity (23.4–6000 ng/L) was obtained for 1-OHP. In order to avoid the potential carry-over from the highest standard and high QC sample, a methanol blank was run immediately after them. No peaks were found in the methanol blank.

The LODs ranged from 1.72 to 17.47 ng/L, has been improved significantly compared with LC and GC/MS studies reported [22,26,31,32] (Table 4) and were comparable with the other LC/MS/MS methods reported [30,38]. Advantages of GC/HRMS are its high chromatographic resolution capability and low detection limits. In this study, the LOD of 2-OHN, 2-OHF and 3-OHF by LC/MS/MS method are comparable to GC/HRMS method and the LODs of 1-OHN, 2-, 3-, 4-OHPhe and 1-OHP are even multiple times lower than GC/HRMS method. Solid phase extraction (SPE) C-18
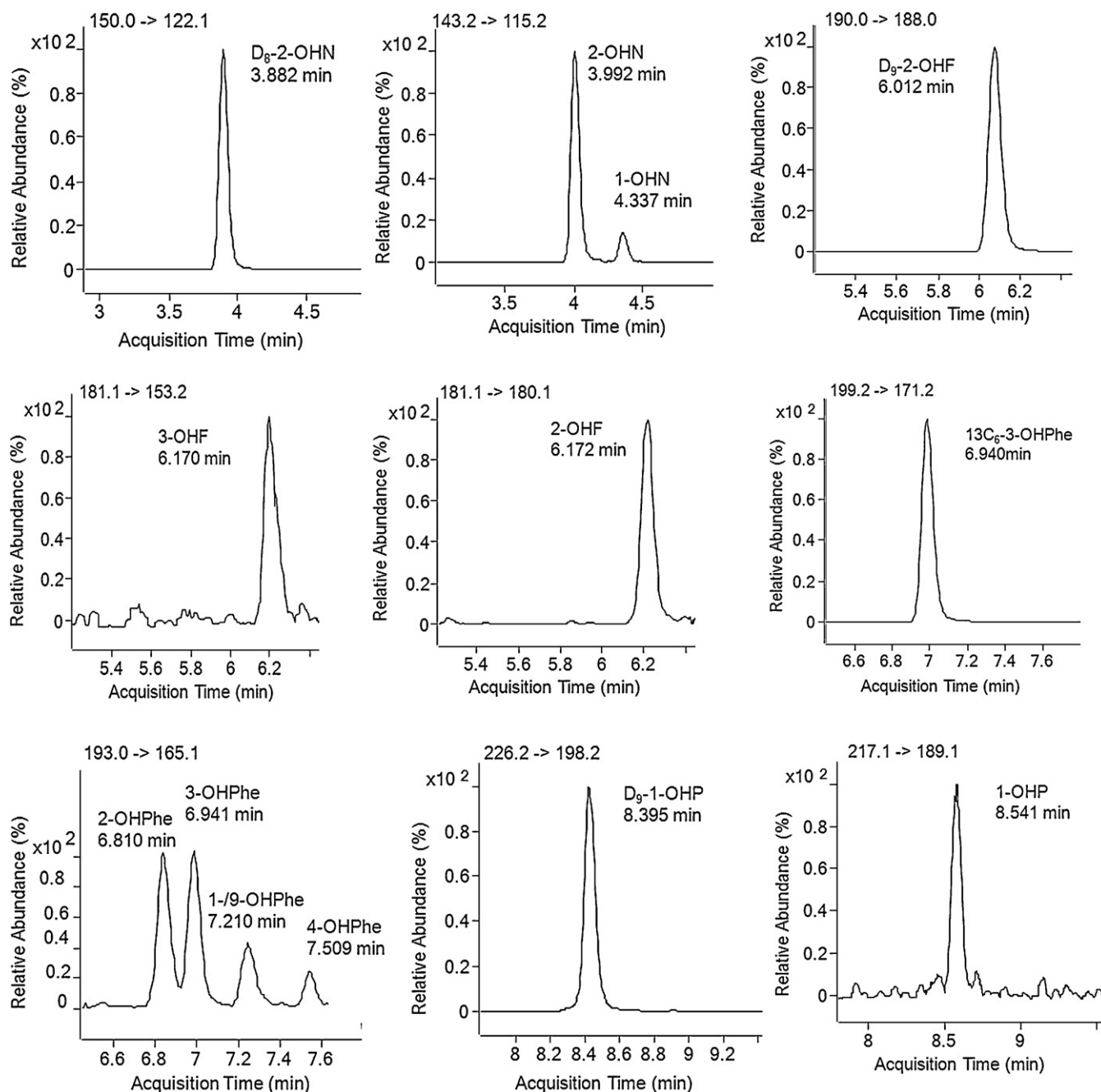
Fig. 2. LC/MS/MS chromatograms from the analysis of standard, showing the detected OH-PAHs and corresponding internal standards. The concentrations were 58.6 ng/L for 2-OHN, 39.1 ng/L for 1-OHN, 2-OHF and 3-OHF, 5.9 ng/L for 2-OHPhe, 3-OHPhe and 4-OHPhe, 11.8 ng/L for 1-/9-OHPhe and 23.4 ng/L for 1-OHP, respectively.

cartridges are widely used to process environmental samples due to their efficiency and convenience, but they could retain many non-polar and weak polar compounds and therefore lack selectivity and specificity to targets. Due to the weak polarity of pentane and toluene and the planar ring structures of toluene, the use of pentane and toluene mixture (80:20, V:V) could be selective and effective to extract urinary OH-PAHs which also have two, three or four co-planar benzene rings. Similar planar ring structure of toluene and analyte selectively enriches OH-PAHs from urine and reduces the matrix effectively. As most targets could be successfully quantified in urine sample of non- or low exposure population, it is a useful and favorable method.

The imprecision (CV) of our method is within 15% and accuracy within $100 \pm 15\%$ of the expected amounts, except at the lowest concentration QC samples, for which $\pm 20\%$ of imprecision is considered acceptable [34]. The precisions of 2-OHN, 2-OHF, 3-OHF, 2-OHPhe, 3-OHPhe and 1-OHP were even lower than 10%. Due to its high volatility, 1-OHN could be easily lost during evaporation procedure, thus generating a poor calibration curve and QC results. It is also the reason why 1-OHN has the poorest precision of 16–23% in our study. In CDC GC/HRMS method, 10 μL dodecane was added as the keeper to prevent the dryness during evaporation [29]. Dodecane is not compatible with ESI in our HPLC–MS/MS study, the samples were evaporated to a volume of 5–10 μL under a gentle nitrogen stream of 3–4 psi.

The recoveries and accuracies ranged from 89.5% to 119.4%, which met the required range of 80–120% stated in the method validation guideline [34]. Moreover, it demonstrated surrogate standards used in the method did not decrease its reliability and accuracy.

Table 2
Method precision and accuracy (*n* = 20) in pooled urine spiked at various concentrations.

Analyte	Added amount (ng/L)	Expected amount (ng/L)	Measured mean (ng/L)	Accuracy (% of expected)	Precision CV (%)
2-OHN	0		13.6		
	1500	1514	1808	119.4	3.3
	3750	3764	4122	109.5	4.4
	7500	7514	7686	102.3	3.2
1-OHN	0		65.1		
	1000	1065	1233	115.8	22.2
	2500	2565	2547	99.3	19.7
	5000	5065	5127	101.2	16.8
2-OHF	0		12.3		
	1000	1012	947	93.5	6.7
	2500	2512	2391	95.2	7.2
	5000	5012	4617	92.1	6.1
3-OHF	0		10.6		
	1000	1011	938	92.8	6.8
	2500	2511	2388	95.1	6.7
	5000	5011	4485	89.5	5.1
1-/9-OHPhe	0		4.1		
	300	304.1	300	98.7	8.5
	750	754.1	688.9	91.4	11.5
	1500	1504	1375	91.4	10.3
2-OHPhe	0		0.9		
	150	150.9	143.8	95.3	8.0
	375	375.9	351.6	93.5	6.6
	750	750.9	692.7	92.2	6.9
3-OHPhe	0		1.8		
	150	151.8	147.2	97.0	3.9
	375	376.8	356.3	94.6	5.3
	750	751.8	724.3	96.3	5.8
4-OHPhe	0		1.4		
	150	151.4	159.4	105.3	11.3
	375	376.4	368.1	97.8	12.6
	750	751.4	742.2	98.8	9.6
1-OHP	0		2.7		
	600	602.7	607.5	100.8	6.7
	1500	1503	1456	96.9	8.1
	3000	3003	2999	99.9	8.7

3.2. Inter-lab tests

The profile measurement of OH-PAHs can only be carried out in limited labs due to its technical challenges. There are no established proficiency test (PT) materials for profile measurement in the world and currently CDC is the only laboratory running this assay. Therefore, CDC QC sample was used to evaluate the method proficiency and accuracy. We took 2 mL CDC QC sample and processed it as the sample preparation afore mentioned. The results were shown in Table 3. CDC did not provide us the acceptable reference range, except for the average value of each target. As the spiked concentrations are lower than 1 µg/L and according to Germany External Quality Assessment Scheme (G-EQUAS) [39], which indicates 30% deviation between different methods and batches is tolerable, hence 30% deviation ranges were used by us in Table 3. In our study, 1-OHN, 2-OHF, 3-OHF, 2-OHPhe and 3-OHPhe are exactly in the range given; 2-OHN and 1-OHP are a slightly lower or higher than the ranges, respectively. 9-OHPhe is not detected in the CDC method, so we only give the quantified value of 1- and 9-OHPhe together in this method. As for 4-OHPhe, the value is distinctively higher than the one given by CDC. The deviation might come from the standard, including the production and preparation, or from the method, such as ion suppression due to the lack of corresponding internal standard [40,41]. In summary, the method of HPLC/MS/MS is accurate and reliable and could be employed in the biomonitoring of real urine samples. As the methods were newly developed, its uncertainty will be evaluated further and assessed through different batches, labs and operators.

3.3. Matrix effects evaluation

Matrix effect, displayed by suppression or enhancement of the ESI-MS/MS signals, is a major challenge in the analysis of biological fluids by LC/MS/MS, and would directly affect the accuracy of analytical results of urinary OH-PAHs. Matrix effects could be avoided, eliminated or reduced through sample clean-up, optimization of chromatographic separation and MS conditions, as well as adding of isotope-labeled internal standards. In our study, matrix effects were evaluated in accordance of FDA (Food and Drug Administration) guideline [34]. The calibration curves were prepared in the same pooled diluted urine and consist of 9 standard points, covering the entire measurement range. In addition, the slopes of calibration curves measured with 10 different lots of urine were of high precision (RSD < 10%). Furthermore, satisfactory intra-day, inter-day precision, accuracy values from all QC samples and inter lab test results between two different methods (HRGC/MS and LC/MS/MS) were the indicators that the matrix effects had been compensated for in our method.

Table 3
Proficiency test results compared with CDC results (unit: ng/L).

	1-OHN	2-OHN	2-OHF	3-OHF	2-OHPhe	3-OHPhe	4-OHPhe	1-OHP	1-/9-OHPhe
CDC value	1528	1661	578.7	476.5	505.2	523.2	503.8	500.3	N.A.
Tolerance range	1070–1987	1162–2159	405.1–752.2	333.6–619.5	353.6–656.8	366.2–680.2	352.7–654.9	350.2–650.1	
Measured in this study	1781	975.6	442.7	377.0	436.1	436.4	921.7	685.6	959.7
Difference %	16.5	−41	−23.5	−20.9	−13.7	−16.6	82.9	37	

Difference % = (measured-CDC value)/CDC value × 100; N.A.: not available.

3.4. Method application

The method was applied to urinary samples collected in a study of the California Environmental Contaminants Biomonitoring Program. Chromatograms of a real urine sample were shown in Fig. 3. Concentrations of OH-PAHs in QC samples were within the established standard deviation range (Table 2). The results were shown in Table 5. Five field blanks were analyzed to detect potential contamination from sample collection, storage and analysis. Though the peaks of some of targets existed in chromatograms, including 2-OHN, 2-OHF, 2-OHPhe and 3-OHPhe, the concentrations were below quantitation limits (B.L.Q.) or LOD and determined insignificant. The detection frequency of 2-OHN, 1-OHN, 2-, 3-, 4- and 1/9-OHPhe were 100%; one sample of 2-OHF and three samples of 1-OHP were B.L.Q. But 12 and 17 samples of 3-OHF were B.L.Q. and could not be detected, respectively. It may be due to the higher LOD of 3-OHF in this method and lower concentration of 3-OHF in authentic urine [31,42].

In order to assess method precision and accuracy for real sample, more than 15% samples were selected randomly to be duplicated. Except for 3-OHF in three urine samples, the relative percent difference (RPD) of all other nine compounds were below or equal to 15%, indicating the good repeatability of this method for real urine sample. Both the low sensitivity of 3-OHF in this method and low concentration in real urine could contribute to poorer repeatability, especially for those samples with low concentrations close to LOQ. It often could not be quantified accurately when concentrations were close to LOD or limit of quantitation (LOQ). Repeatability for 3-OHF is acceptable, those below LOD was labeled as B.L.Q. or N.D. (no detection).

The levels of OH-PAHs in samples we analyzed are comparable to those reported for NHANES (National Health and Nutrition Examination Survey [42]). The concentrations of several compounds, notably 2-OHN, 3-OHF and 1-OHP, were two or three times higher than the means for NHANES. As our samples were collected from subjects who live in an urban area, the higher OH-PAH levels
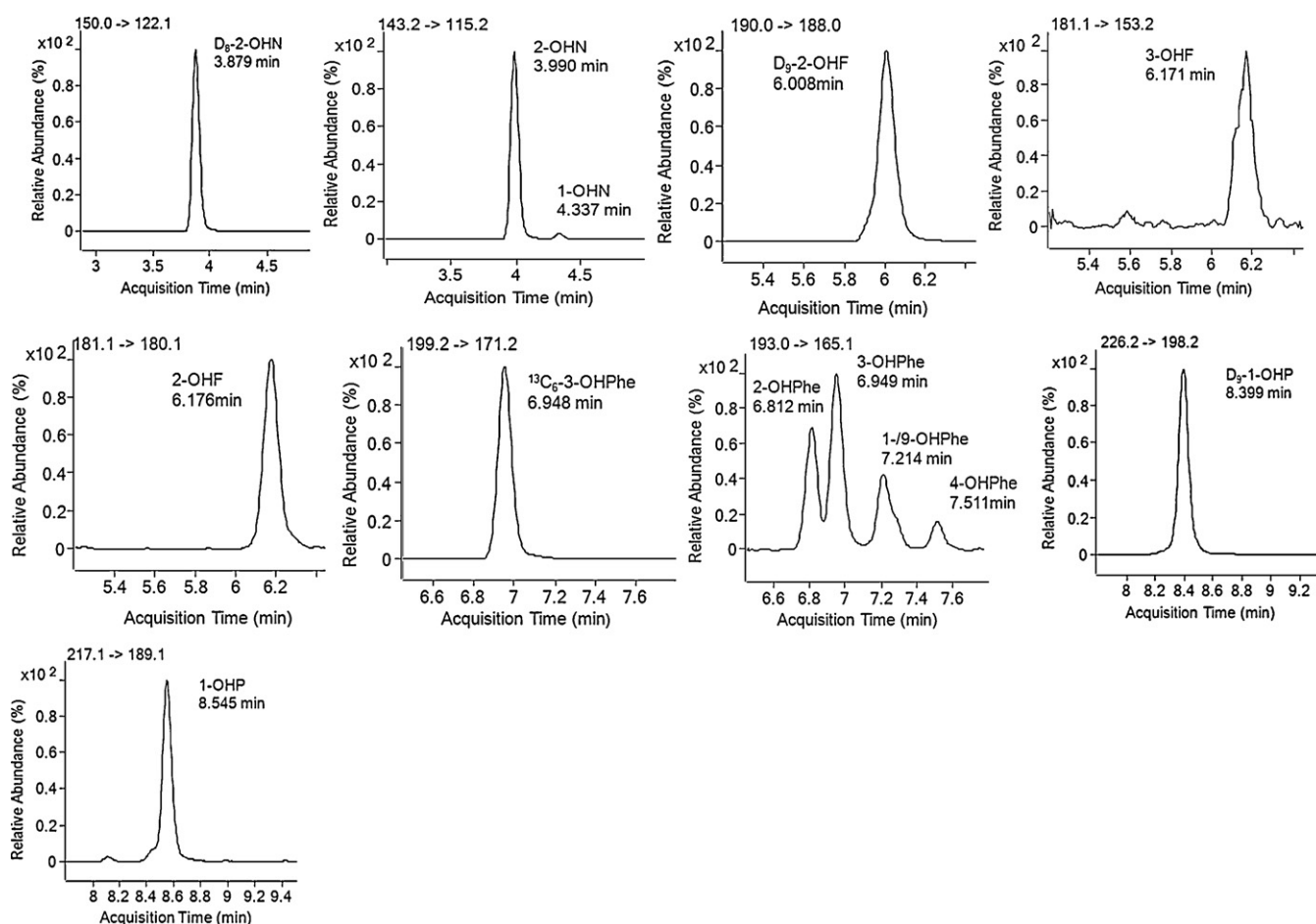

Fig. 3. The chromatograms of a real urine sample. The concentrations were 9201.6 ng/L for 2-OHN, 2589 ng/L for 1-OHN, 187.8 ng/L for 2-OHF, 113.9 ng/L 3-OHF, 17.4 ng/L for 2-OHPhe, 23.7 ng/L for 3-OHPhe, 22.3 ng/L for 4-OHPhe, 76.6 ng/L for 1-/9-OHPhe and 121.1 ng/L for 1-OHP, respectively.

Table 4
The LOD of different method reported (unit: ng/L).

Compound Used method:	This study LC/MS/MS	Romanoff et al. [28] GC/HRMS	Onyemauwa et al. [30][a] LC/MS/MS	Jacob et al. [31][a] LC/MS/MS	Xu et al. [32] LC/MS/MS	Campo et al. [26][a] GC/LRMS	Ramsauer et al. [38] LC/MS/MS
2-OHN	17.47	19	1	500	110	900	10
1-OHN	16.01	35	10	N.A.	430	900	30
2-OHF	15.79	10	5	25	500	100	20
3-OHF	11.01	10	N.A.	N.A.	N.A.	N.A.	
2-OHPhe	1.72	15	N.A.	10	N.A.	100	3
3-OHPhe	2.59	10	N.A.	N.A.	10	200	2
1-/9-OHPhe	4.85	N.A.	10	N.A.	N.A.	500	10
4-OHPhe	5.35	20	5	N.A.	N.A.	100	1
1-OHP	4.85	10	5	25	12	500	7

N.A.: not available.
[a] Limit of quantitation (LOQ).

Table 5
The results of OH-PAHs of 92 California samples in this study and the comparison of levels from NHANES [42] (Unit: ng/L).

Compound	Mean	SD	Median	Range	Numbers of B.L.Q.	Number of N.D.	Mean concentrations reported from NHANES in 2009 [42]
1-OHN	1953.6	4917.3	769.7	73.8–41,996.5	0	0	2680
2-OHN	5991.8	8334.3	3435.0	237.2–61,093.1	0	0	2470
2-OHF	325.3	572.9	175.8	B.L.Q.–383.28	1	0	304
3-OHF	263.8	640.6	77.1	N.D..–4264.0	12	17	134
1/9-OHPhe	305.9	340.7	186.2	25.9–189.8	0	0	N.A.
2-OHPhe	63.7	69.8	44.5	6.1–455.8	0	0	54
3-OHPhe	62.9	103.7	34.8	6.0–795.4	0	0	105
4-OHPhe	77.2	90.6	49.6	6.9–606.6	0	0	25.1
1-OHP	378.1	557.4	222.4	B.L.Q.–4503.8	3	0	89.2

N.A., not available; N.D., non detected; B.L.Q., below quantitation limit.

in urine are understandable. The profile of exposure level and characterization of exposure source will be discussed in other papers.

4. Conclusion

In summary, a highly sensitive and specific LC/MS/MS method coupled with LLE to pretreat urine samples was developed in this study. The measurements of ten OH-PAHs in human urine samples are precise and accurate. The LODs are low or equal to GC/HRMS method reported, which make it possible to detect OH-PAHs concentration in non-occupational exposure or general population, even children's environmental exposure. Applicability of the method to study PAH exposure in pregnant women has been demonstrated, and sensitivity is adequate for determination of ten PAH metabolites in urine without complicate pretreatment.

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