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Quasi-targeted analysis of hydroxylation-related metabolites of polycyclic aromatic hydrocarbons in human urine by liquid chromatography–mass spectrometry

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ABSTRACT

Metabolite identification is crucial for revealing metabolic pathways and comprehensive potential toxicities of polycyclic aromatic hydrocarbons (PAHs) in human body. In this work, a quasi-targeted analysis strategy was proposed for metabolite identification of monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in human urine using liquid chromatography triple quadruple mass spectrometry (LC–QqQ-MS/MS) combined with liquid chromatography high resolution mass spectrometry (LC–HRMS). Potential metabolites of OH-PAHs were preliminarily screened out by LC–QqQ-MS/MS in association with filtering in a self-constructed information list of possible metabolites, followed by further identification and confirmation with LC–HRMS. The developed method can provide more reliable and systematic results compared with traditional untargeted analysis using LC–HRMS. In addition, data processing for LC–HRMS analysis were greatly simplified. This quasi-targeted analysis method was successfully applied to identifying phase I and phase II metabolites of OH-PAHs in human urine. Five metabolites of hydroxynaphthalene, seven of hydroxyfluorene, four of hydroxyphenanthrene, and three of hydroxypyrene were tentatively identified. Metabolic pathways of PAHs in human body were putatively revealed based on the identified metabolites. The experimental results will be valuable for investigating the metabolic processes of PAHs in human body, and the quasi-targeted analysis strategy can be expanded to the metabolite identification and profiling of other compounds in vivo.

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1. Introduction

Liquid chromatography–mass spectrometry technologies (LC–MS) have been developed rapidly in the last decades [1–3], which has greatly facilitated metabolite identification and profiling for drugs in drug discovery and development industries [4,5]. In addition, these technologies have also become mainstream tools for the emerging metabolomics [6–9]. Recently, they have been applied to identifying and profiling metabolites and transformation products of environmental pollutants [10–12], promoting studies concerning in vivo and in vitro metabolism/transformation of pollutants.

LC coupled with triple quadruple mass spectrometry (LC–QqQ-MS/MS) operated in multiple-reaction monitoring (MRM) mode is commonly used for targeted analysis. Moreover, it can also be used for tentatively untargeted metabolite identification of xenobiotic compounds via precursor ion scan (PIS), product ion scan, neutral loss scan (NLS), and MRM modes [13]. Some metabolites, e.g., phase II metabolites, can generate chromatographic peaks in MRM channels set for monitoring their parent compounds [14,15]. In addition, mass-to-charge (m/z) values of precursor ions of potential metabolites, usually quasi-molecular ions can be obtained in PIS mode. Furthermore, NLS is also a useful approach for confirming metabo-

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lites, particularly for phase II metabolites such as conjugates of glucuronide, sulfate and glutathione [16]. By means of these scan modes and referring to predicted in vivo metabolic logics of xenobiotics, potential metabolites can be preliminarily screened out. However, accuracy and reliability of the identification could not be guaranteed due to the low mass-resolving power of LC–QqQ-MS/MS.

LC equipped with high resolution mass spectrometry (LC–HRMS), e.g., LC quadrupole time-of-flight MS (LC–QTOF-MS) and LC–Q-Orbitrap-MS, operated in full scan mode has been widely used for untargeted metabolite identification due to its accurate mass determination [13,17]. However, data mining processes are complex and time-consuming, let alone potential false positives [18,19]. Fortunately, combinatorial utilization of LC–QqQ-MS/MS and LC–HRMS can be a promising solution for metabolite identification due to the integrated technical advantages and avoided limitations [20–22].

Polycyclic aromatic hydrocarbons (PAHs) are a group of the most widespread pollutants in the environment. Once entering human body, PAHs are possibly subjected to successive phase I metabolic biotransformations including oxidation, hydroxylation, and hydration, which are catalyzed by cytochrome P450 dependent monooxygenases, giving rise to a variety of epoxy derivatives [23,24]. *In vivo* hydrolysis of PAHs occurs via catalysis of hydrolase [25], producing a variety of dihydrodiol epoxides which are considered to be carcinogenic due to their ability to form DNA adducts through covalent bonds [26,27]. Some of the phase I metabolites, such as monohydroxylated PAHs (OH-PAHs) have been frequently detected in human urine and hair [28–30]. Urinary OH-PAHs, particularly 1-hydroxypyrene (1-OHP), have been used as biomarkers to indicate human exposure to PAHs [31–34]. Therefore, investigation of urinary OH-PAHs has attracted increasing attentions in environmental studies [35,36]. OH-PAHs may further undergo phase I metabolisms (e.g., oxidation and hydration) and phase II metabolisms (such as glucuronidation and sulfation), generating various phase I and phase II metabolites, e.g., polyhydroxylated PAHs, PAH dihydrodiols, and glucuronide conjugates and sulfate derivatives of OH-PAHs. Thus, a variety of both phase I and phase II metabolites of OH-PAHs may be concurrent in human body due to complex *i*n vivo metabolic reactions [23]. Identification and profiling of metabolites of OH-PAHs are therefore of critical importance to explore and elucidate metabolic pathways of PAHs in human body. Previous studies have revealed presence of some metabolites of OH-PAHs, such as glucuronides and sulfates in human urine [37–42]. However, detailed identification and profiling of OH-PAHs metabolites in human body have not been reported yet.

In this work, we conducted a study using quasi-targeted analysis for tentative metabolite identification and profiling of OH-PAHs in human urine. Potential metabolites of OH-PAHs were preliminarily screened out by LC–QqQ-MS/MS combined with filtering in an information list of possibly existent in vivo metabolites of OH-PAHs, and further confirmed by targeted identification approach using LC–QTOF-MS and LC–Q-Orbitrap-MS. A variety of metabolites of OH-PAHs were identified and the corresponding metabolic pathways were subsequently elucidated. The results will be valuable in elucidation and interpretation of in vivo metabolism of PAHs in organisms.

2. Experimental

2.1. Chemicals and materials

Authentic standards 2-hydroxynaphthalene (2-OHN, purity: 99%), 3-hydroxyfluorene (3-OHF), 2-hydroxyfluorene (2-OHF, purity: 98%), 9-hydroxyfluorene (9-OHF, purity: 96%), 9-hydroxyphenanthrene (9-OHPhe), and 1-hydroxypyrene (1-OHP, purity: 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Hydroxyphenanthrene (1-OHPhe, purity: 99%), 2-hydroxyphenanthrene (2-OHPhe, purity: 99.6%), and 4-hydroxyphenanthrene (4-OHPhe, 50 μg/mL in acetonitrile) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). 1-Hydroxynaphthalene (1-OHN, purity: 99%) and 3-hydroxyphenanthrene (3-OHPhe, purity: 98%, 50.0 μg/mL in toluene) were purchased from Fluka (St. Louis, MO, USA) and Cambridge Isotope Lab (Andover, MA, USA), respectively. Structures of the chemicals are illustrated in Fig. S-1 and the physicochemical properties are listed in Table S-1. HPLC-grade methanol (MeOH) and acetonitrile (ACN) were purchased from Merck Crop. (Darmstadt, Germany). Ammonium acetate (NH_4Ac) was of HPLC grade and purchased from J&K Scientific Ltd. (Beijing, China). Ultrapure water (electrical resistivity of 18.2 MΩ cm) was prepared with a Millipore water purification system (Millipore Corporation, Bellerica, MA, USA).

Stock solutions of the OH-PAHs were individually prepared in ACN with concentrations from 1.2 to 3.6 mg/mL. A neat solution containing all the OH-PAH standards was prepared with 0.5 mL of MeOH/H_2O (1:1, v/v) and used as the working solution for peak identification and referencing. All standard solutions were kept in a freezer at −20 °C before use.

2.2. Urine samples

Urine samples were collected from occupational exposure populations in two coke plants in Qujing, Yunnan Province, China and non-occupational exposure farmers living in the vicinity of the coke plants. The famers were expectedly exposed to PAHs stemming from biomass burning and coke plants. One hundred coke plant workers including 72 males and 28 females with ages of 36.0 ± 11.0 years were recruited for urine collection. In addition, 14 male and 11 female famers were recruited with ages of 34.9 ± 9.5 years. Other personal and life style information such as living address, job responsibilities in coke plants, cooking styles, smoking, alcohol drinking, and dietary habits have been provided in detail previously [43].

Recruited persons were required to avoid from consuming baked/deep-fried food for three days prior to urine collection in order to exclude additional PAHs exposure. Samplings were conducted in December 2012. Urine samples were collected in polypropylene bottles sealed with screw caps and immediately frozen with dry ice before transport to the laboratory where the urines were stored at −80 °C prior to treatment.

All the urine samples were firstly subjected to quantitative analysis of OH-PAHs with commercially available standards as described in detail previously [43]. Five urine samples that contained relatively high concentrations of the OH-PAHs were subsequently selected and pooled as a composite sample for metabolite identification and profiling in this study. Average concentrations of the quantified OH-PAHs in these five urine samples were 1.32–19.20 ng/mL as shown in Table S-2.

2.3. Sample treatment

Aliquot 800 μL of the composite urine sample was placed into a 2-mL polypropylene tube followed by addition of 800-μL MeOH. The sample was vortex-mixed for 5 min and then subjected to centrifugation at 15800*g* and 4 °C for 30 min using a Thermo Multifuge X3R centrifuger (Thermo-Fisher Scientific, Waltham, USA). After centrifugation, 800 μL of the supernatant was transferred to a clean amber glass vial and subjected to instrumental analysis.

The urine sample was not treated with enzymatic hydrolysis using β-glucuronidase and arylsulfatase in order to keep all

potential metabolites of OH-PAHs (e.g., glucuronide and sulfate metabolites) unchanged prior to instrumental analysis.

2.4. Instrumental analysis

The analyzing procedure was illustrated in Fig. S-2. Untargeted metabolites were preliminarily screened out by LC–QqQ-MS/MS. Parent ions of the preliminarily screened metabolites were then filtered in a self-constructed information list of possible metabolites. The metabolites were finally identified and confirmed using LC–QTOF-HRMS and LC–Q-Orbitrap-HRMS. The LC–QqQ-MS/MS system consisted of an Agilent 1290 ultra-high performance LC (UPLC, Agilent Technologies, Palo Alto, USA) and an API-4000 Q-Trap mass spectrometry (Applied Biosystems, Forster City, CA, USA) interfaced with an electrospray ionization (ESI) source. The LC–QTOF-HRMS system consisted of an Acquity™ Waters UPLC and a Synapt Q-TOF mass spectrometer (Waters, Manchester, UK) with an ESI source. The LC–Q-Orbitrap-HRMS system was comprised of a Dionex LC and a Q-Exactive-MS/MS (Thermo-Fisher Scientific, Waltham, USA).

For all the LC–MS systems, C_{18} reversed-phase chromatographic columns and negative ESI sources were applied. The detailed working conditions and parameters of the LC–MS systems used for metabolite identification are provided in Table 1. The PIS parameters of LC–QqQ-MS/MS for preliminarily screening metabolites are detailed in Table 2.

3. Results and discussion

3.1. Information list construction of theoretically possible metabolites

Theoretical metabolism reactions include phase I (e.g., oxidation and hydration) and phase II (conjugation) biotransformations which can result in mass shifts (i.e., molecular weight changes) between the metabolites and their parent compounds. Based on mass shift values caused by possible metabolic biotransformations, possible metabolites of OH-PAHs can be predicted using theoretically calculated *m*/*z* values of precursor ions. An information list of possible OH-PAHs metabolites containing *m*/*z* values of precursor ions can thus be constructed as shown in Table S-5.

3.2. Metabolite preliminary screening and filtering

Metabolites usually share same characteristic product ions with their parent compounds, finding characteristic product ions of parent compounds is therefore helpful to screen their metabolites. In negative ESI mode, all the investigated OH-PAH standards gave rise to deprotonated precursor ions ($[M-H]^-$). The characteristic product ions of the OH-PAHs were then generated by the precursor ions ($[M-H]^-$) with the loss of a CO group (Figs. S-3 and S-4). Detailed elucidation of the mass spectrometric fragmental pathways of OHN is provided in the Supplementary information.

Metabolites preliminary screening for OH-PAHs was achieved using LC-QqQ-MS/MS in multiple scan modes including MRM, PIS and NLS. A number of unknown peaks were found in MRM transition channels of the OH-PAHs (Fig. 1 and Fig. S-5). Phase II metabolites were reported to be possibly dissociated in ESI source, giving rise to the product ions identical to the precursor ions of their parent compounds [14,15]. In addition, isomers can generate chromatographic peaks in the same MRM channel or co-elute from columns [36]. Therefore, the unknown chromatographic peaks might be generated by metabolites or unknown isomers of the OH-PAHs.

PIS was conducted twice in sequence using the precursor ion and the characteristic product ion of each OH-PAH as the fixed

Table 1
Working conditions and parameters of the three LC–MS systems for metabolite identification of OH-PAHs.

LC–MS type	LC condition				MS condition	
	Column specification & temperature	Elution program	Mobile phase & flow rate	Injection volume	Source parameter	Scan mode
LC–QqQ- MS/MS	Atlatis® dC18 column (2.1 × 100 mm, 3 μm, Waters, Milford, MA, USA) and Zorbax Eclipse Plus C18 column (2.1 × 100 mm, 1.8 μm, Agilent, USA); temperature: 40 °C.	0–2 min: 5% → 15% B, 2–3 min: 15% → 40% B, 3–12 min: 40% → 80% B, 12–13 min: 80%–90% B, 13–15 min: 90% B, 15–15.1 min: 90% → 5% B, 15.1–18 min: 5% B.	A: H_2O with 2 mM NH_4Ac, B: ACN; flow rate: 320 μL/min.	2 μL/ 15 μL	ESI−; curtain gas: 25 psi; collision induced dissociation: medium; ionspray voltage: −4500 V; temperature: 450 °C; gas 1: 40 psi; gas 2: 45 psi; interface heater: on	MRM (Table S-3); PIS (Table 2); NLS (Table S-4).
UPLC– QTOF-MS	Waters HSS T3 UPLC column (2.1 × 100 mm, 1.8 μm); temperature: 30 °C.		A: H_2O with 2 mM NH_4Ac, B: ACN; flow rate: 300 μL/min.	5 μL	ESI−; capillary voltage: −2.5 KV; cone voltage: 50 V; desolvation temperature: 400 °C; ion source temperature: 100 °C; desolvation gas: 600 L/h; cone gas: 50 L/h; data acquisition mode: centroid; scan time: 0.2 s; TOF acquisition mode: V mode.	Full scan (mass range: 100–1000 u); MS/MS scan (mass range: 100–600 u, collision energy: 30 eV).
LC–Q-Orbitrap-MS	Hypersil Gold C18 column (2.1 × 100 mm, 1.9 μm, Thermo-Fisher Scientific); temperature: 30 °C.		A: H_2O, B: ACN; flow rate: 300 μL/min.	5 μL	HESI-II−; capillary voltage: −3500 V; auxiliary gas pressure: 10 arb; Heater temperature: 350 °C.	Full scan (mass range: 100–600 u, mass resolution (full width at half maximum): 70000); target-MS/MS scan (mass resolution: 140000).

Table 2
Precursor ion scan (PIS) parameters of LC–QqQ-MS/MS for preliminary identification of metabolites of OH-PAHs.

Parent compound	Sequence of PIS	Fixed ion (m/z)	Scan mass range (m/z)	Declustering potential (V)	Collision energy (eV)	Dwell time (ms)
OHN	First	115	100–500	−80	−36	300
	Second	143	100–500	−80	−20	300
OHF	First-I	153	100–500	−70	−35	300
	First-II	180	175–550	−60	−35	300
	Second	181	175–550	−60	−20	300
OHPhe	First	165	160–550	−60	−35	300
	Second	193	190–570	−60	−20	300
OHP	First	189	180–580	−80	−43	300
	Second	217	210–580	−80	−20	300

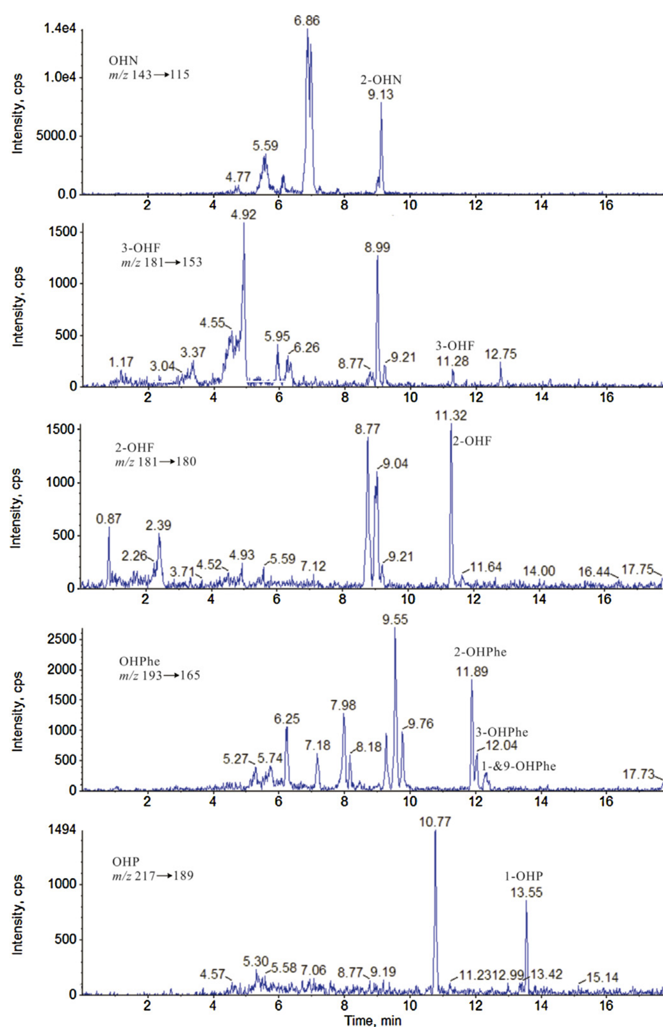

Fig. 1. Representative MRM chromatograms of the quantified OH-PAHs in the human urine sample prepared without enzymolysis treatment. The unmarked chromatographic peaks are possibly corresponding to metabolites or unknown isomers of the quantified OH-PAHs.

ions, respectively to scan metabolites. The total ion chromatograms (TICs) of PIS in human urine are shown in Fig. S-6. In these TICs, a large number of peaks were present, some of which possibly corresponded to metabolites of OH-PAHs.

Some glucuronidation metabolites, sulfate conjugates and *N*-acetylcysteine conjugates of OH-PAHs could be preliminarily identified via NLS with the fixed m/z values of 64, 80, 129, and 176 as shown in Table S-6.

As demonstrated in Table S-7, 83 metabolites were preliminarily screened out, including 16 metabolites of hydroxynaphthalene, 23 of hydroxyfluorene, 38 of hydroxyphenanthrene, and six of hydroxypyrene. Possible structures of the identified metabolites are provided in Table S-8, of which the isomers were not differentiated. The isomers with identical chemical formula were categorized as one type, and therefore the preliminarily identified 83 metabolites were classified into 33 types.

LC–QqQ-MS/MS generally provides higher sensitivity than LC–HRMS (particularly LC–QTOF-MS) in full scan mode [19]. Furthermore, potential metabolites can be readily found by LC–QqQ-MS/MS in PIS mode in combination with other scan modes. As a result, potential metabolites can be limited to a certain number, which is favorable to further targeted screening by LC–HRMS due to the enhanced analysis efficiency and significant simplification of data processing. In addition, preliminary metabolite identification with LC–QqQ-MS/MS may be helpful to reduce false-positive results caused by interferences in LC–HRMS analysis.

3.3. LC–HRMS screening

3.3.1. LC–QTOF-MS screening

Among the 33 types of preliminarily identified metabolites, 16 types were identified by post-targeted screening with LC-QTOF-MS in full scan mode. These 16 types of metabolites were further subjected to identification with pre-targeted screening using LC–QTOF-MS in MS/MS scan mode, and 15 metabolites were further confirmed. The mass spectra and chemical information (e.g., names, retention times, structures and mass accuracies) of the 15 metabolites are provided in Fig. 2 and Table 3, respectively.

Number and types of the metabolites confirmed by LC–QTOF-MS were significantly lower than those of the preliminarily identified metabolites by LC–QqQ-MS. This can be primarily attributable to lower mass-resolution capability of LC–QqQ-MS which might result in some false positives. On the other hand, some metabolites detected by LC–QqQ-MS at low signals may be undetectable on LC–QTOF-MS due to its obviously lower sensitivity than LC–QqQ-MS.

3.3.2. LC–Q-Orbitrap-MS screening

Similar to the results achieved by LC–QTOF-MS, 16 out of the 33 types of the preliminarily identified metabolites were screened out using LC–Q-Orbitrap-MS in full scan mode (Fig. S-7). These 16 types of metabolites were further scanned using LC–Q-Orbitrap-MS in MS/MS mode. Ten metabolites of OH-PAHs were identified with high confidence level based on the detected accurate masses of the precursor ions and product ions together with mass spectrometric fragmental pathways (Fig. 3). Among the 10 identified metabolites, four compounds, i.e., dihydroxyfluorene glucuronide, dihydroxyfluorene sulfate, dihydroxyphenanthrene sulfate, and hydroxyprene sulfate were identified by LC–Q-Orbitrap-MS only and the rest metabolites were also identified by LC–QTOF-MS as mentioned above (Fig. 3, Table 3).

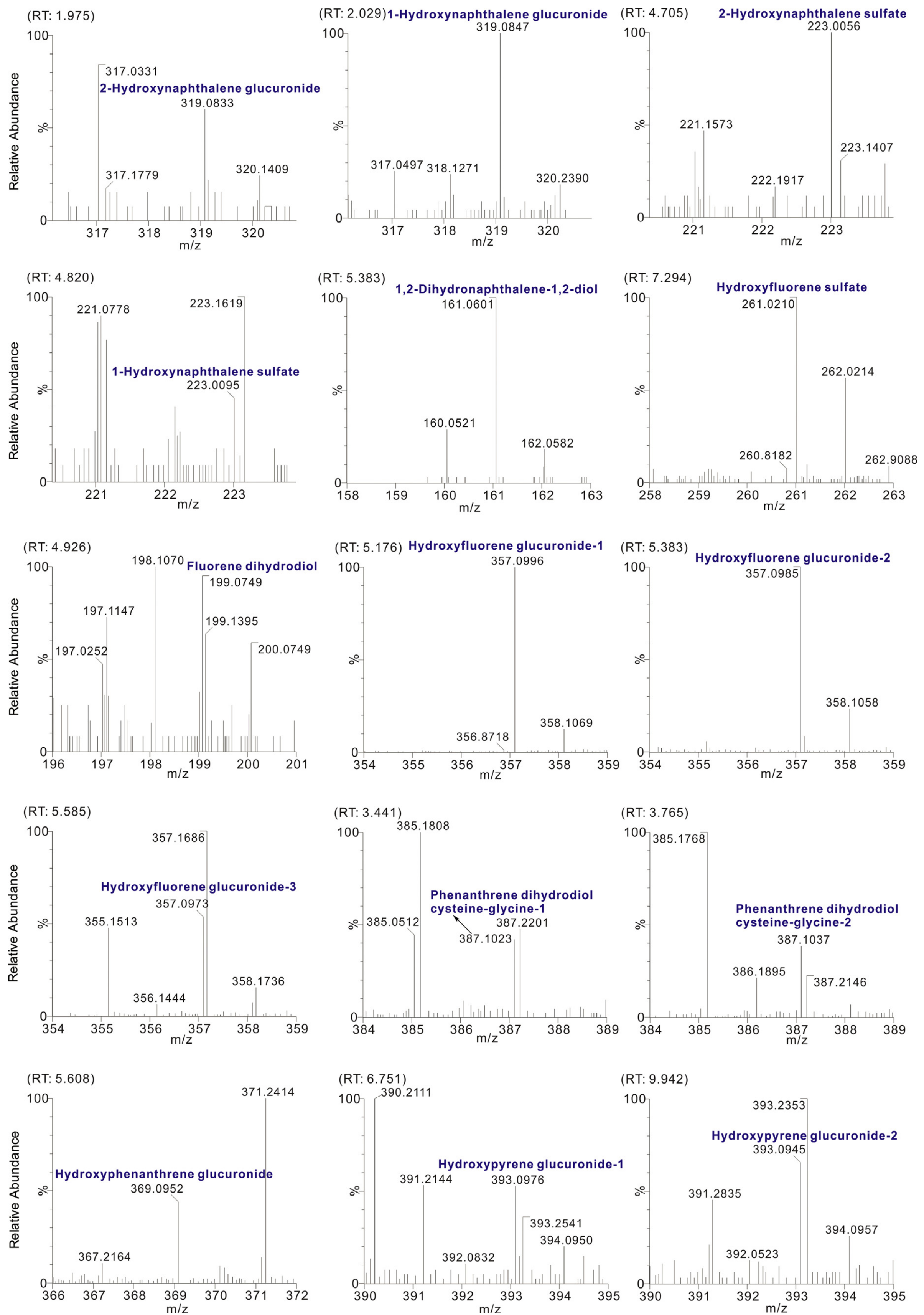

Fig. 2. High-resolution mass spectra of PAH metabolites in human urine identified by UPLC–QTOF-MS.

Table 3
Chemical structures, names and detected mass accuracies of the metabolites of OH-PAHs in human urine identified by UPLC-QTOF-MS.

Metabolite No.	Retention time (*min*)	Metabolism type	Name	Proposed chemical structure	Molecular formula	Theoretical exact mass (*u*)	Detected accurate mass (*u*)	Mass error (*ppm*)
OHN metabolites								
OHN-M1	1.975	Glucuronidation	2-Hydroxynaphthalene glucuronide		$C_{16}H_{16}O_7$	319.0818	319.0833	4.7
OHN-M2	2.029	Glucuronidation	1-Hydroxynaphthalene glucuronide		$C_{16}H_{16}O_7$	319.0818	319.0847	9.1
OHN-M3	4.705	Sulfation	2-Hydroxynaphthalene sulfate		$C_{10}H_8O_4S$	223.0065	223.0056	−4.0
OHN-M4	4.820	Sulfation	1-Hydroxynaphthalene sulfate		$C_{10}H_8O_4S$	223.0065	223.0095	13.5
OHN-M5	5.383	Hydration	Naphthalene dihydrodiol		$C_{10}H_{10}O_2$	161.0603	161.0601	−1.2
OHF metabolites								
OHF-M1	7.294	Sulfation	Hydroxyfluorene sulfate		$C_{13}H_{10}O_4S$	261.0222	261.0210	−4.6
OHF-M2	4.926	Hydration	Fluorene dihydrodiol		$C_{13}H_{12}O_2$	199.0759	199.0749	−5.0
OHF-M3	5.176	Glucuronidation	Hydroxyfluorene glucuronide		$C_{19}H_{18}O_7$	357.0974	357.0996	6.2

Table 3 (*Continued*)

Metabolite No.	Retention time (*min*)	Metabolism type	Name	Proposed chemical structure	Molecular formula	Theoretical exact mass (*u*)	Detected accurate mass (*u*)	Mass error (*ppm*)
OHF-M4	5.383	Glucuronidation	Hydroxyfluorene glucuronide		$C_{19}H_{18}O_7$	357.0974	357.0985	3.1
OHF-M5	5.585	Glucuronidation	Hydroxyfluorene glucuronide		$C_{19}H_{18}O_7$	357.0974	357.0973	−0.3
OHPhe metabolites								
OHPhe-M1	3.441	Hydration + cysteine-glycine	Phenanthrene dihydrodiol cysteine-glycine		$C_{19}H_{20}N_2O_5S$	387.1015	387.1023	2.1
OHPhe-M2	3.765	Hydration + cysteine-glycine	Phenanthrene dihydrodiol cysteine-glycine		$C_{19}H_{20}N_2O_5S$	387.1015	387.1037	5.7
OHPhe-M3	5.608	Glucuronidation	Hydroxyphenanthrene glucuronide		$C_{20}H_{18}O_7$	369.0974	369.0952	−6.0
OHP metabolites								
OHP-M1	6.751	Glucuronidation	Hydroxypyrene glucuronide		$C_{22}H_{18}O_7$	393.0974	393.0976	0.5
OHP-M2	9.942	Glucuronidation	Hydroxypyrene glucuronide		$C_{22}H_{18}O_7$	393.0974	393.0945	−7.4

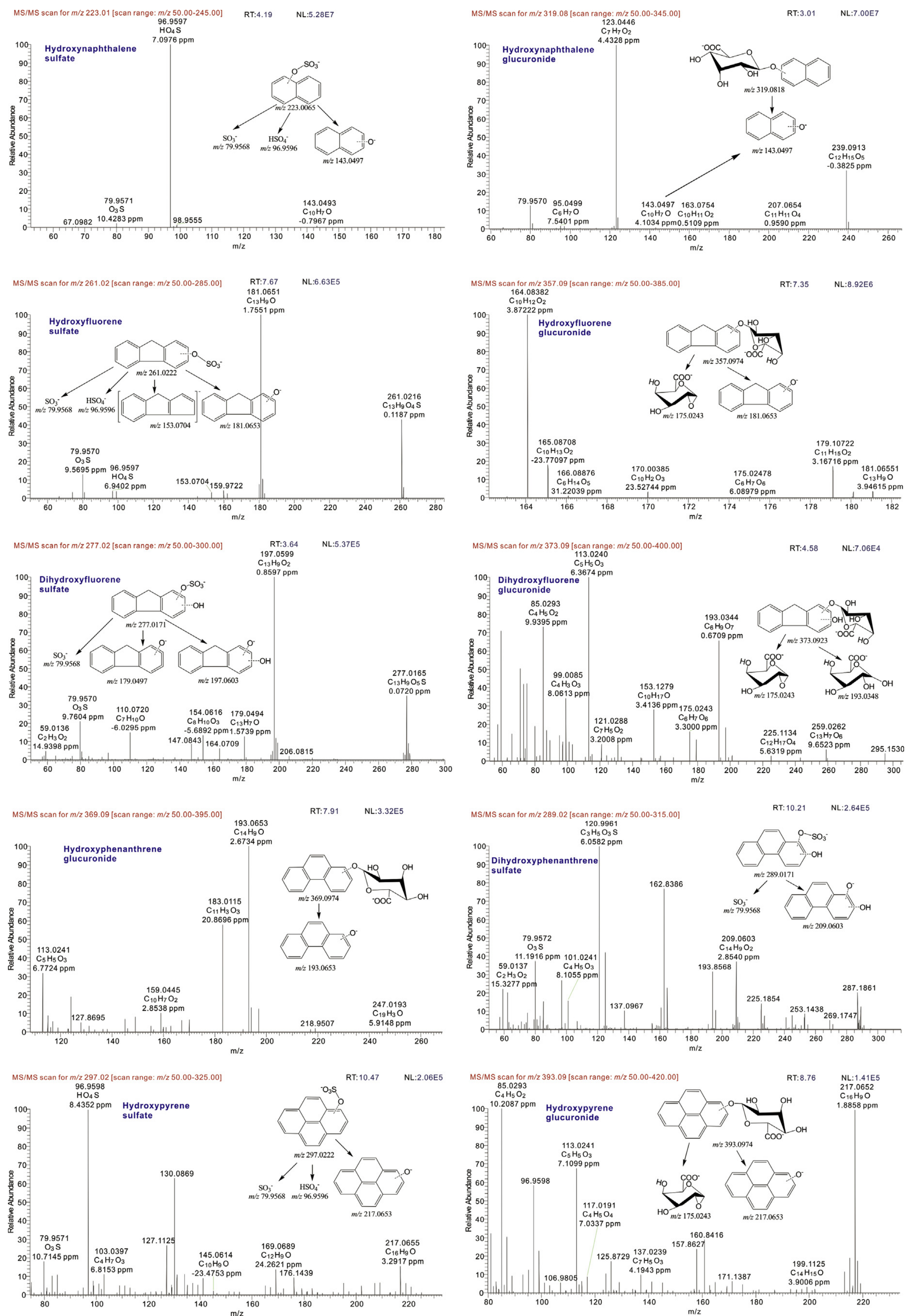

Fig. 3. Mass spectrometric information and fragmental pathways of metabolites of OH-PAHs in human urine identified by LC–Q-Orbitrap-MS in MS/MS mode. Dihydroxyfluorene glucuronide, dihydroxyfluorene sulfate, dihydroxyphenanthrene sulfate and hydroxyprene sulfate were further identified by LC–Q-Orbitrap-MS only.

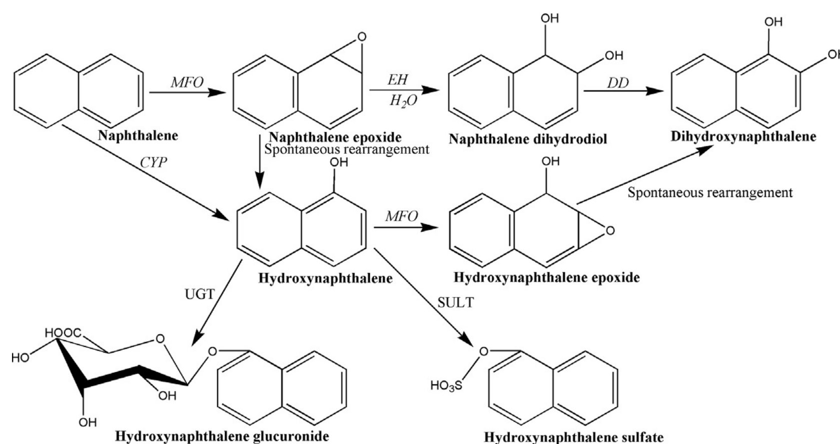

Fig. 4. Proposed possible metabolic pathways of PAHs in human body using naphthalene as an example. MFO: mixed-function oxidase; CYP: cytochrome P450; EH: epoxide hydrolase; DD: dihydrodiol dehydrogenase; UGT: UDP-glucuronosyltransferase; SULT: sulfotransferase.

3.4. Finally identified metabolites of OH-PAHs

Summarily, 19 metabolites have been finally identified, including two phase I and 17 phase II metabolites (Table 3). Phase I metabolites, i.e., naphthalene dihydrodiol and fluorene dihydrodiol could be regarded as plausible metabolites of OHN and OHF, respectively due to their structural similarities. Yet data about the correspondingly direct metabolic pathways are still unavailable. Nevertheless, naphthalene can be in vivo oxidized to naphthalene epoxide with catalysis of mixed-functional oxidase and further transformed to naphthalene dihydrodiol via hydration catalyzed by hydrolases [25], which means that OHN and naphthalene dihydrodiol could coexist in human body. Similar formation mechanisms can be speculated for fluorene dihydrodiol. Fluorene dihydrodiols have more than one isomers, e.g., 2,9-dihydro-1H-fluorene-1,2-diol and 4,9-dihydro-3H-fluorene-3,4-diol. However, only one isomer of fluorene dihydrodiol was detected. Thus the detected fluorene dihydrodiol could not be exactly structurally identified.

Seventeen phase II metabolites included nine glucuronides, six sulfates and two cysteine-glycine conjugates, suggesting glucuronidation and sulfation are the predominant phase II transformation pathways of OH-PAHs in human body.

Specifically, the identified phase II metabolites of OHN included 1-hydroxynaphthalene glucuronide (1-OHN-Glu), 2-hydroxynaphthalene glucuronide (2-OHN-Glu), 1-hydroxynaphthalene sulfate (1-OHN-S) and 2-hydroxynaphthalene sulfate (2-OHN-S) (Table 3). The dipole moments of 1-OHN, 2-OHN, 1-OHN-Glu, 2-OHN-Glu, 1-OHN-S and 2-OHN-S, calculated by ChemBio3D Ultra 12.0 (Chemoffice 2010, CambridgeSoft, Cambridge, MA) and MOPAC 2012 (Stewart Computational Chemistry, CO, USA), were 0.901, 1.460, 2.750, 3.230, 3.133 and 3.659 Debye, respectively. This calculated result confirmed the differences in polarities between the 1-position-substituted and 2-position-substituted naphthalene metabolites, because dipole moments are positively correlated with polarities. Thus, 1-OHN-Glu, 2-OHN-Glu, 1-OHN-S and 2-OHN-S could be structurally identified based on their quantitative structure-(chromatographic)-retention relationships.

Three OHF glucuronidation metabolites were identified, implying that at least three OHF isomers were present in human urine. Theoretically, OHF glucuronides have more than three isomers. Thus, chemical structures of the three isomers could not be exactly elucidated with available information. A sulfation metabolite of OHF and a dihydroxyfluorene sulfate were also found (Fig. 2 and Fig. 3). The sulfation and glucuronidation metabolites of dihydroxyfluorene were identified by LC-Q-Orbitrap-MS as shown in Fig. 3, indicating that fluorene could be metabolized to polyhydroxylated compounds followed by further transformation to glucuronides and sulfates.

Four identified phase II metabolites of phenanthrene included two phenanthrene dihydrodiol cysteine-glycine metabolites, an OHPhe glucuronide and a dihydroxyphenanthrene sulfate. Unfortunately, exact structures of these metabolites could not be elucidated due to absence of reference standards. Presence of the dihydroxyphenanthrene sulfate in human urine (Fig. 3) indicated that phenanthrene could be metabolized to polyhydroxylated entities and subsequently transformed into complex phase II metabolites.

Two OHP glucuronides and one OHP sulfate were identified. Theoretically, OHP glucuronides have three theoretical isomers. Therefore, it could not be affirmed which two isomers were identified and their chemical structures thus could not be ascertained based on the available data. These identified OHP metabolites suggested glucuronides and sulfates would be the major metabolites of OHP in human urine.

3.5. Proposition of metabolic pathways of PAHs in human body

Naphthalene was chosen as an example to elucidate possible metabolic pathways of PAHs in human body on the basis of identified metabolites (Fig. 4). After entering human body, naphthalene could be oxidized to OHN with catalysis of cytochrome P450 enzyme system, or oxidized to naphthalene epoxide through the catalysis of mixed function oxidase. Naphthalene epoxide can be transformed to 1,2-dihydronaphthalene-1,2-diol, and then to 1,2-dihydroxynaphthalene by catalytic oxidation effect of epoxide hydrolase [44], or converted to OHN by spontaneous rearrangement [23]. Naphthalene dihydrodiol epoxide can be transformed to 1,2-dihydroxynaphthalene via spontaneous rearrangement. OHN can be transformed to OHN glucuronide via glucuronidation catalyzed by UDP-glucuronosyltransferase, and also can be sulfated through the catalysis of sulfotransferase [23].

4. Conclusions

Metabolite identification and profiling of OH-PAHs in human urine were tentatively conducted by means of a quasi-targeted analysis method using three different LC–MS systems in multiple scan modes. The metabolites were preliminarily screened out by LC–QqQ-MS/MS in MRM mode, dual PIS mode and/or NLS mode in combination with filtering in an information list of theoretically possible metabolites. The preliminarily identified suspect metabo-

lites were further identified and confirmed by UPLC–QTOF-HRMS and LC–Q-Orbitrap-HRMS in full scan and MS/MS scan modes. In total, 19 metabolites including five of OHN, seven of OHF, four of OHPhe and three of OHP were tentatively identified, covering both phase I and phase II metabolites. Two phase I metabolites, i.e., naphthalene dihydrodiol and fluorene dihydrodiol were found. The major phase II metabolites were glucuronide and sulfate conjugates. Based on the identified metabolites, the metabolic pathways of PAHs in human body were accordingly elucidated. Phase I metabolic processes of naphthalene involved oxidation and hydration, and the major phase II metabolisms were glucuronidation and sulfation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.07.051.

References

[1] S. Eliuk, A.A. Makarov, Evolution of orbitrap mass spectrometry instrumentation, Annu. Rev. Anal. Chem. 8 (2015) 61–80.

[2] M. Holčapek, R. Jirásko, M. Lísa, Recent developments in liquid chromatography–mass spectrometry and related techniques, J. Chromatogr. A 1259 (2012) 3–15.

[3] M.M. Gómez-Ramos, C. Ferrer, O. Malato, A. Agüera, A.R. Fernández-Alba, Liquid chromatography–high-resolution mass spectrometry for pesticide residue analysis in fruit and vegetables: screening and quantitative studies, J. Chromatogr. A 1287 (2013) 24–37.

[4] O. Beckonert, H.C. Keun, T.M. Ebbels, J. Bundy, E. Holmes, J.C. Lindon, J.K. Nicholson, Metabolic profiling metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts, Nat. Protoc. 2 (2007) 2692–2703.

[5] E.J. Want, I.D. Wilson, H. Gika, G. Theodoridis, R.S. Plumb, J. Shockcor, E. Holmes, J.K. Nicholson, Global metabolic profiling procedures for urine using UPLC–MS, Nat. Protoc. 5 (2010) 1005–1018.

[6] J.K. Nicholson, J.C. Lindon, System biology: metabonomics, Nature 455 (2008) 1054–1056.

[7] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Metabonomics: a platform for studying drug toxicity and gene function, Nat. Rev. Drug Discov. 1 (2002) 153–161.

[8] A. Scalbert, L. Brennan, O. Fiehn, T. Hankemeier, B.S. Kristal, B. van Ommen, E. Pujos-Guillot, E. Verheij, D. Wishart, S. Wopereis, Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research, Metabolomics 5 (2009) 435–458.

[9] D.S. Wishart, Applications of metabolomics in drug discovery and development, Drugs in R & D 9 (2008) 307–322.

[10] K. Lu, R.P. Abo, K.A. Schlieper, M.E. Graffam, S. Levine, J.S. Wishnok, A. James, J.A. Swenberg, R. Steven, S.R. Tannenbaum, J.G. Fox, Exposure perturbs the gut microbiome and its metabolic profile in mice: an integrated metagenomics and metabolomics analysis, Environ. Health Perspect. 122 (2014) 284–291.

[11] Y. Picó, D. Barceló, Transformation products of emerging contaminants in the environment and high-resolution mass spectrometry: a new horizon, Anal. Bioanal. Chem. 407 (2015) 6257–6273.

[12] M. Farré, Y. Picó, D. Barceló, Application of ultra-high pressure liquid chromatography linear ion-trap orbitrap to qualitative and quantitative assessment of pesticide residues, J. Chromatogr. A 1328 (2014) 66–79.

[13] J.F. Xiao, B. Zhou, H.W. Ressom, Metabolite identification and quantitation in LC–MS/MS-based metabolomics, TrAC-Trend Anal. Chem. 32 (2012) 1–14.

[14] C. Tang, O.S. Sojinu, Simultaneous determination of caffeic acid phenethyl ester and its metabolite caffeic acid in dog plasma using liquid chromatography tandem mass spectrometry, Talanta 94 (2012) 232–239.

[15] C. Tang, C. Tang, W. Zhan, J. Du, Z. Wang, X. Peng, Strategies for ascertaining the interference of phase II metabolites co-eluting with parent compounds using LC–MS/MS, J. Sep. Sci. 36 (2013) 2584–2592.

[16] K. Levsen, H.M. Schiebel, B. Behnke, R. Dötzer, W. Dreher, M. Elend, H. Thiele, Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview, J. Chromatogr. A 1067 (2005) 55–72.

[17] A. Yang, M. Zang, H. Liu, P. Fan, J. Xing, Metabolite identification of the antimalarial piperaquine in vivo using liquid chromatography-high-resolution mass spectrometry in combination with multiple data-mining tools in tandem, Biomed. Chromatogr. 30 (2016) 1324–1330.

[18] M. Zhu, H. Zhang, W.G. Humphreys1, Drug metabolite profiling and identification by high-resolution mass spectrometry, J. Biol. Chem. 286 (2011) 25419–25425.

[19] K. Thoren, How does high resolution mass spectrometry compare to tandem mass spectrometry, APR. 9 2015//. Source: NACB https://www.aacc.org/community/national-academy-of-clinical-biochemistry/scientific-shorts/2015/how-does-high-resolution-mass-spectrometry-compare-to-tandem-mass-spectrometry (2015) (accessed in July 2016).

[20] A. Masiá, M. Ibáñez, C. Blasco, J.V. Sancho, Y. Picó, F. Hernández, Combined use of liquid chromatography triple quadrupole mass spectrometry and liquid chromatography quadrupole time-of-flight mass spectrometry in systematic screening of pesticides and other contaminants in water samples, Anal. Chim. Acta 761 (2013) 117–127.

[21] S. Grimalt, J.V. Sancho, Ó.J. Pozo, F. Hernández, Quantification, confirmation and screening capability of UHPLC coupled to triple quadrupole and hybrid quadrupole time-of-flight mass spectrometry in pesticide residue analysis, J. Mass Spectrom. 45 (2010) 421–436.

[22] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Quantitative and confirmative performance of liquid chromatography coupled to high-resolution mass spectrometry compared to tandem mass spectrometry, Rapid Commun. Mass Spectrom. 25 (2011) 979–992.

[23] Z. Li, L.C. Romanoff, D.A. Trinidad, N. Hussain, R.S. Jones, E.N. Porter, D.G. Patterson Jr., A. Sjödin, Measurement of urinary monohydroxy polycyclic aromatic hydrocarbons using automated liquid–liquid extraction and gas chromatography/isotope dilution high-resolution mass spectrometry, Anal. Chem. 78 (2006) 5744–5751.

[24] G. Grimmer, J. Jacob, G. Dettbarn, K.W. Naujack, Determination of urinary metabolites of polycyclic aromatic hydrocarbons (PAH) for the risk assessment of PAH-exposed workers, Int. Arch. Occup. Environ. Health 69 (1997) 231–239.

[25] Z. Senturk, Analysis of carcinogenic polycyclic aromatic hydrocarbons (PAHs): an overview of modern electroanalytical techniques and their applications, Curr. Drug Deliv. 10 (2013) 76–91.

[26] A. Toriba, K. Hayakawa, Biomarkers of exposure to polycyclic aromatic hydrocarbons and related compounds, J. Health Sci. 53 (2007) 631–638.

[27] L. Xu, Mass Spectrometric Analysis of Environmentally Relevant Phenolic Compounds, ProQuest, New Jersey, 2007.

[28] O. Adetona, Z. Li, A. Sjödin, L.C. Romanoff, M. Aguilar-Villalobos, L. Needham, D.B. Hall, B.E. Cassidy, L.P. Naeher, Biomonitoring of polycyclic aromatic hydrocarbon exposure in pregnant women in Trujillo, Peru—comparison of different fuel types used for cooking, Environ. Int. 53 (2013) 1–8.

[29] X. Li, R. Zenobi, Use of polyetheretherketone as a material for solid phase extraction of hydroxylated metabolites of polycyclic aromatic hydrocarbons in human urine, Anal. Chem. 85 (2013) 3526–3531.

[30] C. Schummer, B.M. Appenzeller, M. Millet, R. Wennig, Determination of hydroxylated metabolites of polycyclic aromatic hydrocarbons in human hair by gas chromatography–negative chemical ionization mass spectrometry, J. Chromatogr. A 1216 (2009) 6012–6019.

[31] T. Chetiyanukornkul, A. Toriba, T. Kamede, N. Tang, K. Hayakawa, Simultaneous determination of urinary hydroxylated metabolites of naphthalene fluorene, phenanthrene, fluoranthene and pyrene as multiple biomarkers of exposure to polycyclic aromatic hydrocarbons, Anal. Bioanal. Chem. 386 (2006) 712–718.

[32] L. Kuusimäki, Y. Peltonen, P. Mutanen, K. Peltonen, K. Savela, Urinary hydroxy-metabolites of naphthalene, phenanthrene and pyrene as markers of exposure to diesel exhaust, Int. Arch. Occup. Environ. Health 77 (2004) 23–30.

[33] M. Bouchard, C. Viau, Urinary 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons: biological monitoring strategies and methodology for determining biological exposure indices for various work environments, Biomarkers 4 (1999) 159–187.

[34] H. Hemat, J. Wittsiepe, M. Wilhelm, J. Miller, T. Goen, High levels of 1-hydroxypyrene and hydroxyphenanthrenes in urine of children and adults from Afghanistan, J. Exposure Sci. Environ. Epidemiol. 22 (2012) 46–51.

[35] Y. Guo, K. Senthilkumar, H. Alomirah, H.B. Moon, T.B. Minh, M.A. Mohd, H. Nakata, K. Kannan, Concentrations and profiles of urinary polycyclic aromatic hydrocarbon metabolites (OH-PAHs) in several Asian countries, Environ. Sci. Technol. 47 (2013) 2932–2938.

[36] R. Fan, D. Wang, R. Ramage, J. She, Fast and simultaneous determination of urinary 8-hydroxy-2′-deoxyguanosine and ten monohydroxylated polycyclic aromatic hydrocarbons by liquid chromatography/tandem mass spectrometry, Chem. Res. Toxicol. 25 (2012) 491–499.

[37] P. van den Hurk, G.A. Kubiczak, H.J. Lehmler, M.O. James, Hydroxylated polychlorinated biphenyls as inhibitors of the sulfation and glucuronidation of 3-hydroxy-benzo [a] pyrene, Environ. Health Perspect. 110 (2002) 343–348.

[38] X. Li, R. Zenobi, Use of polyetheretherketone as a material for solid phase extraction of hydroxylated metabolites of polycyclic aromatic hydrocarbons in human urine, Anal. Chem. 85 (2013) 3526–3531.

[39] R.A. Wilson, C.J. Seliskar, G. Talaska, W.R. Heineman, Spectroelectrochemical sensing of pyrene metabolites 1-hydroxypyrene and 1-hydroxypyrene-glucuronide, Anal. Chem. 83 (2011) 3725–3729.

[40] L. Luukkanen, J. Mikkola, T. Forsman, P. Taavitsainen, J. Taskinen, E. Elovaara, Glucuronidation of 1-hydroxypyrene by human liver microsomes and human UDP-glucuronosyltransferases UGT1A6, UGT1A7, and UGT1A9: development of a high-sensitivity glucuronidation assay for human tissue, Drug Metab. Dispos. 29 (2001) 1096–1101.

[41] R.B. Gunier, P. Reynolds, S.E. Hurley, S. Yerabati, A. Hertz, P. Strickland, P.L. Horn-Ross, Estimating exposure to polycyclic aromatic hydrocarbons: a comparison of survey, biological monitoring, and geographic information system-based methods, Cancer Epidemiol. Biomarkers Prev. 15 (2006) 1376–1381.

[42] H.W. Hagedorn, G. Scherer, J. Engl, K. Riedel, F. Cheung, G. Errington, J. Shepperd, M. McEwan, Urinary excretion of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in nonsmokers and in smokers of cigarettes with different ISO tar yields, J. Anal. Toxicol. 33 (2009) 301–309.

[43] R. Fan, J. Li, L. Chen, Z. Xu, D. He, Y. Zhou, Y. Zhu, F. Wei, J. Li, Biomass fuels and coke plants are important sources of human exposure to polycyclic aromatic hydrocarbons, benzene and toluene, Environ. Res. 135 (2014) 1–8.

[44] F. Onyemauwa, S.M. Rappaport, J.R. Sobus, D. Gajdošová, R.A. Wu, S. Waidyanatha, Using liquid chromatography–tandem mass spectrometry to quantify monohydroxylated metabolites of polycyclic aromatic hydrocarbons in urine, J. Chromatogr. B 877 (2009) 1117–1125.