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Automated solid-phase extraction method for measuring urinary polycyclic aromatic hydrocarbon metabolites in human biomonitoring using isotope-dilution gas chromatography high-resolution mass spectrometry

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Abstract

In order to perform comprehensive epidemiological studies where multiple metabolites of several PAHs are measured and compared in low-dose urine samples, fast and robust methods are needed to measure many analytes in the same sample. We have modified a previous method used for measuring polycyclic aromatic hydrocarbon (PAH) metabolites by automating the solid-phase extraction (SPE) and including an additional eight metabolites. We also added seven new carbon-13 labeled standards, which improves the use of isotope-dilution calibration. Our method included enzyme hydrolysis, automated SPE and derivatization with a silylating reagent followed by gas chromatography (GC), coupled with high-resolution mass spectrometry (HRMS). Using this method, we measured 23 metabolites, representing 9 parent PAHs, with detection limits in the low pg/mL range. All steps in the clean-up procedure were optimized individually, resulting in a method that gives good recoveries (69–93%), reproducibility (coefficient of variation for two quality control pools ranged between 4.6 and 17.1%, $N > 156$), and the necessary specificity. We used the method to analyze nearly 3000 urine samples in the fifth National Health and Nutrition Examination Survey (NHANES 2001–2002).

Keywords: PAH; Polycyclic aromatic hydrocarbon; Biomonitoring; Urinary metabolite; Hydroxylated PAH; Method development

1. Introduction

Human exposure to polycyclic aromatic hydrocarbons (PAHs) has long been a concern because of the known health hazards associated with several of these compounds. A number of PAHs have reported carcinogenic or genotoxic potential [1]. PAHs are formed during incomplete combustion of organic material such as coal, oil, gas, wood, or tobacco. Exposure is primarily through inhaling polluted air or tobacco smoke, and through ingesting contaminated and processed food and water [1]. Some studies also indicate that dermal exposure may be a major exposure pathway of some PAHs in occupational exposure settings [2,3].

Following absorption, PAHs are rapidly biotransformed (predominantly by cytochrome P450 mono-oxygenases) to hydroxylated metabolites, which are then further transformed to glucuronide or sulfate conjugates to increase their polarity and aid in urinary excretion [4,5]. Conjugated hydroxylated PAHs (HO-PAHs) are the most abundant and commonly measured metabolites in urine even though other metabolites such as dihydrodiols and DNA adducts also exist [5–8].

Over the years many reports have been published on separation techniques used for measurement of PAHs. Jongeleen et al. pioneered measuring 1-hydroxypyrene by HPLC coupled with fluorescence spectrometry [9]. In their method, urine samples were pretreated with β-glucuronidase/arylsulfatase to cleave off

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the glucuronide/sulfate conjugate prior to separation using solid-phase extraction (SPE), derivatization and analysis. A number of research groups have used this type of methodology with HPLC being the most commonly used method for analysis. However, GC–MS has also been used to analyze HO-PAHs in urine. Grimmer et al. introduced a method where liquid–liquid extraction was followed by derivatization prior to GC–MS analysis [10]. This analytical method is rapid and efficient and achieves good separation of isomeric metabolites but, due to problems with chromatographic separation and peak shape of hydroxylated PAH metabolites derivatization is necessary.

Many of the early methods of measuring of PAHs focused on specific metabolites or selected biomarkers of exposure [10–15]. Several HO-PAHs have been established as good biomarkers of exposure, with 1-hydroxypyrene being the most commonly used [9,14–20]. However, as exposure is most often to complex mixtures of PAHs, more extensive methods covering a number of PAH metabolites are needed to adequately assess exposure to PAH mixtures [21]. Currently, a number of methods have measured metabolites for multiple PAHs [11,22–24]. One of these most extensive methods was developed at the Centers for Disease Control and Prevention (CDC) [24]. CDC used SPE for sample clean-up prior to GC-HRMS analysis of samples from the fourth National Health and Nutrition Examination Survey (NHANES 1999–2000). This method requires a relatively small volume of urine (3 mL) in which trace-level concentrations (parts per trillion) can be detected. In this report we have expanded this method to measure eight additional metabolites, including 3-hydroxybenzo(a)pyrene—one of the main metabolites of benzo(a)pyrene which has a well documented carcinogenic potential [1].

To further improve the efficiency of our method, we automated SPE by using the RapidTrace® SPE system. A more efficient sorbent was needed because cartridge size (and sorbent volume) is a limiting factor on the RapidTrace®. Over the last few years, a number of companies have developed specific bi-functional polymeric SPE sorbents based on the styrene–divinylbenzene polymer backbone with a variety of proprietary functionalities. Because some of these companies have patented their sorbents, the exact structure of the sorbent is not known. Using a more efficient polymeric sorbent allows a reduction in sorbent volume (and cartridge size), improves extraction efficiency, and results in a cleaner extract. Automating the extraction portion of the method facilitated the high sample throughput, essential for applying the method to large epidemiological studies, such as NHANES. Within maximizing the extraction efficiency, we optimized and validated each step of the method. Consequently, we have developed a high throughput method for analyzing urine for 23 PAH metabolites representing 9 parent PAHs for human urine biomonitoring.

2. Experimental

2.1. Standards, chemicals, and supplies

All solvents used were of analytical grade. Dichloromethane, methanol, and toluene were obtained from Tedia Company Inc. (Fairfield, OH). Hydrochloric acid (30.0–38.0%) of ultra pure grade was purchased from J.T. Baker (Phillipsburg, NJ). Deionized water was purified by 2000™ Solution Water Purification System, Solution Consultants Inc. (Jasper, GA). Ultra-high purity nitrogen was purchased from Airgas South (Chamblee, GA). Sodium acetate and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Sigma–Aldrich (St. Louis, MO). β-Glucuronidase/arylsulfatase enzyme was obtained from Roche Diagnostic (Indianapolis, IN). All native and labeled standards along with the corresponding suppliers are listed in Table 1. Carbon-13 labeled benzo(b)fluoranthene, which was used as a recovery standard, was purchased from AccuStandard Inc. (New Haven, CT).

We tested five different sorbents for optimizing the SPE; four polymeric sorbents and one C_{18} silica-based control sorbent were compared to our previous method [24]. The control sorbent was EnvirElute-PAH (6 cc, 1 g) from Varian Inc. (Palo Alto, CA), which originally was used to perform extraction on a vacuum manifold [24]. The four polymeric sorbents used were Oasis (3 cc, 60 mg) from Waters (Milford, MA), Strata-X (3 cc, 60 mg), Strata-XC (3 cc, 60 mg) both from Phenomenex (Torrance, CA), and Focus (3 cc, 50 mg) from Varian Inc. (Palo Alto, CA).

2.2. Sample preparation

Urine samples (3 mL) were aliquoted into test tubes (16 mm × 100 mm). Sodium acetate buffer (0.1 M; pH 5.5; 5 mL) was added to each sample to adjust the pH for optimal deconjugation conditions for the enzyme. Samples were spiked with an isotopically C13 labeled internal standard mixture (10 μL, 200 pg/μL), consisting of the 12 currently available hydroxylated PAH labeled standards. Conjugates were hydrolyzed by adding β-glucuronidase/arylsulfatase (10 μL) to the samples followed by incubation (37 °C) for 17–18 h (overnight). Samples were mixed, allowed to equilibrate, then extracted on the RapidTrace® SPE workstation. Cartridges (Focus 60 mg) were preconditioned with methanol (1 mL, 16 mL/min), followed by purified water (1 mL, 16 mL/min). Samples were added to the cartridge at 1 mL/min, rinsed using purified water (1 mL, 10 mL/min), and followed by methanol/sodium acetate buffer (3 mL, 4:6 by volume, pH 5.5, 10 mL/min). The sorbent was dried by applying a constant flow of nitrogen for 10 min to the cartridge and the final extract was eluted with dichloromethane (3 mL, 0.5 mL/min).

The sample extracts were spiked with dodecane (5 μL), evaporated to ~5 μL with a TurboVap LV® evaporator from Caliper Life Sciences (Hopkinton, MA), using a gentle stream of nitrogen (5–10 psi, gradually increasing during evaporation) and a water bath (40 °C), then reconstituted with toluene (20 μL). All samples were subsequently spiked with recovery standard, C13-labeled benzo(b)fluoranthene (BBF) (5 μL, 100 pg/μL) and transferred to amber GC vials with 300 μL fused inserts. The samples were derivatized to their trimethylsilylated derivative, prior to GC/HRMS measurement, by adding MSTFA (10 μL) and incubating (60 °C) for 30 min.

Table 1
Measured metabolites, their abbreviations, parent PAHs, and suppliers for native and istopically labeled standards

Parent PAH	Native standard	Abbreviation	Supplier	
			Native	Labeled
Naphthalene	1-Hydroxynaphthalene	1-NAP	Sigma-Aldrich[a]	In-house[b]
	2-Hydroxynaphthalene	2-NAP	Sigma-Aldrich[a]	N/A
Fluorene	9-Hydroxyfluorene	9-FLUOR	Sigma-Aldrich[a]	Los Alamos[c]
	3-Hydroxyfluorene	3-FLUOR	Sigma-Aldrich[a]	Los Alamos[c]
	2-Hydroxyfluorene	2-FLUOR	Sigma-Aldrich[a]	Los Alamos[c]
Phenanthrene	4-Hydroxyphenanthrene	4-PHEN	Promochem[d]	N/A
	9-Hydroxyphenanthrene	9-PHEN	Promochem[d]	N/A
	3-Hydroxyphenanthrene	3-PHEN	Dr. Ehrenstorfer[e]	CIL[g]
	1-Hydroxyphenanthrene	1-PHEN	Promochem[d]	N/A
	2-Hydroxyphenanthrene	2-PHEN	Dr. Ehrenstorfer[e]	Los Alamos[c]
Benzo(c)phenanthrene	1-Hydroxybenzo(c)phenanthrene	1-BCP	MRI[f]	N/A
	2- Hydroxybenzo(c)phenanthrene	2-BCP	MRI[f]	N/A
	3- Hydroxybenzo(c)phenanthrene	3-BCP	MRI[f]	Chemsyn[h]
Pyrene	1-Hydroxypyrene	1-PYR	MRI[f]	Chemsyn[h]
Benz(a)anthracene	1-Hydroxybenz(a)anthracene	1-BAA	MRI[f]	CIL[g]
	3-Hydroxybenz(a)anthracene	3-BAA	MRI[f]	N/A
	9-Hydroxybenz(a)anthracene	9-BAA	MRI[f]	N/A
Chrysene	4-Hydroxychrysene	4-CHRY	MRI[f]	N/A
	6-Hydroxychrysene	6-CHRY	AccuStandard[i]	CIL[g]
	3-Hydroxychrysene	3-CHRY	MRI[f]	Chemsyn[h]
	1-Hydroxychrysene	1-CHRY	MRI[f]	N/A
	2-Hydroxychrysene	2-CHRY	MRI[f]	N/A
Benzo(a)pyrene	3-Hydroxybenzo(a)pyrene	3-BAP	MRI[f]	N/A

[a] Sigma-Aldrich, St. Louis, MO.
[b] Centers for Disease Control and Prevention, Atlanta, GA.
[c] Los Alamos National Laboratory, Los Alamos, NM.
[d] Promochem, Teddington, United Kingdom.
[e] Dr. Ehrenstorfer, Augsburg, Germany.
[f] Midwest Reseach Institute (MRI), Kansas City, MO.
[g] Cambridge Isotpe Laboratories (CIL), Andover, MA.
[h] Chemsyn Laboratories, Lenexa, KS.
[i] AccuStandard Inc., New Haven, CT.

2.3. Method optimization

The methodology was optimized in several different aspects. Firstly, deconjugation was optimized both for the amount of enzyme added to each sample and also the incubation time of samples spiked with deconjugation enzyme. Enzyme volume was optimized by adding different amounts of β-glucuronidase/arylsulfatase prior to incubation, extraction and analysis. Deconjugation time was optimized by adding β-glucuronidase/arylsulfatase (10 μl) to spiked urine samples and incubating for 0–24 h at 37 °C prior to clean-up and analysis. Secondly, the extraction step was optimized by examining several different factors. These included type of sorbent, sorbent volume, wash solution, elution solvent/volume and sample volume. Four polymeric sorbent, with different functionalities and compositions were tested and compared with EnvirElute-PAH, which is a silica-based C18 sorbent used in our previous method [24]. The four polymeric sorbents were first tested according to each manufacturers recommended method by loading spiked samples and calculating recovery of each metabolite. They were then compared using different wash solutions and eluants. Initially, the sorbents were tested to see how each retained the target metabolites while elution solvents were evaluated only in regards to how efficiently they eluted analytes, without taking urine matrix into consideration. Analytes were eluted off the Strata-X, Strata-XC and Oasis cartridges using dichloromethane:methanol (70:30, v/v) while the Focus cartridges were eluted with acetonitrile:methanol:acetic acid (60:40:0.1, v/v/v) as recommended by the supplier. For wash solution optimization, both acid and neutral wash solutions were tested with different percentages of methanol. Several different solvents were tested as eluants for urine samples on the chosen sorbent, which included toluene, acetonitrile, dichloromethane and methanol. Finally, the evaporation step was improved by adding dodecane as a "keeper" to limit loss of higher volatility analytes.

2.4. GC-HRMS conditions

All samples were analyzed on a MAT 95 high-resolution mass spectrometer, by Thermo Finnigan (Bremen, Germany) in electron impact ionization (EI) mode, interfaced with a 6890

Gas Chromatograph (GC), Agilent Technologies (Atlanta, GA). One microliter of the sample extract was injected in splitless mode with an inlet temperature of 270 °C. The GC column used was a 30 m × 0.25 mm 5% phenyl dimethylsiloxane column (DB-5) from Agilent Technologies (Atlanta, GA) with a 0.25 μm film thickness. The column was under a constant flow of 1 mL/min helium. The initial oven temperature of 95 °C was held for 2 min, then ramped at 15 °C/min to 160 °C, followed by a ramp of 10 °C/min to 295 °C, and finally ramped at 40 °C/min to 320 °C and held for 7 min. The transfer line temperature was constant at 270 °C. A full-scan analysis of the derivatized standard mixture was completed to determine the two most abundant ions for routine selective ion monitoring (SIM). Samples were analyzed at a resolution of 10,000 in SIM mode where the acquisition time was divided into six time-windows to maximize sensitivity. The ion source temperature was 250 °C. Compounds with corresponding C13 labeled standards were quantified by C13 isotope dilution quantification using relative response ratios generated from a predetermined calibration curve. Compounds that did not have a corresponding C13-labeled standard were quantified by comparing to the closest structurally similar metabolite. Table 2 lists molecular ions and M-15 ions (as confirmation ions) for each analyte and corresponding C13 labeled standard. The instrument sensitivity was checked daily by injecting 2,3,7,8-tetrachlorobenzo-*p*-dioxin (2,3,7,8-TCDD, 1 μL, 20 pg/mL) and evaluating the signal-to-noise (S/N) ratio, using a minimum of 3/1.

2.5. Standard preparation

A mixture of 23 target compounds was prepared in acetonitrile at a concentration of 1 ng/μL. This solution was then diluted in toluene to five levels ranging from 750 to 1.5 pg/μL. The 12 labeled compounds were added to each of these calibration standards at a concentration of 100 pg/μL. To prepare the calibration curve, 20 μL of each of the five calibration standards was derivatized with MSTFA (10 μL) for 30 min at 60 °C and analyzed by GC/HRMS. Multiple ($n > 7$) analyses at each calibration level were used to construct the calibration curves. Continuing calibration was monitored daily by running a random calibration standard from the calibration curve with each set of samples.

2.6. Biological samples

Quality control material consisted of anonymously collected urine from multiple volunteers that was pooled, diluted (4:1, v/v) with purified water, and filtered through a 0.45 μm SuporCap-100 Capsule (Pall Corp., Ann Arbor, MI. The urine was divided into two pools; QC low and QC high, spiked with 23 unlabeled, unconjugated free hydroxylated PAH standards at 300 and 900 pg/μL, respectively. The pools were mixed at room temperature overnight and subsequently aliquoted (3 mL) into 15 mL 16 mm × 100 mm glass culture tubes and stored at −70 °C until used.

Routine analysis samples are collected in plastic collection cups and aliquoted into cryovials, then frozen until analysis. No pretreatment of these samples is completed prior to analysis.

3. Results and discussion

3.1. Method optimization

After primary biotransformation (phase I) of PAHs to HO-PAHs, metabolites are rapidly transformed to glucuronide or sulfate conjugates (phase II) to facilitate urinary excretion. For GC–MS measurement of HO-PAH metabolites in urine, the

Table 2
Optimized molecular ions, with corresponding M-15 control ions (in order of ascending retention time), and corresponding isotopically labeled standard

Analyte	Labeled internal standard	Molecular ion	M-15 ion	Labeled molecular ion	Labeled M-15 ion
1-Hydroxynaphthalene	1-NAP	216.0970	201.0736	222.1172	207.0938
2-Hydroxynaphthalene	1-NAP	216.0970	201.0736	222.1172	207.0938
9-Hydroxyfluorene	9-FLUOR	254.1127	239.0893	260.1329	245.1095
3-Hydroxyfluorene	3-FLUOR	254.1127	239.0893	260.1329	245.1095
2-Hydroxyfluorene	2-FLUOR	254.1127	239.0893	260.1329	245.1095
4-Hydroxyphenanthrene	3-PHEN	266.1127	251.0893	272.1329	257.1095
9-Hydroxyphenanthrene	3-PHEN	266.1127	251.0893	272.1329	257.1095
3-Hydroxyphenanthrene	3-PHEN	266.1127	251.0893	272.1329	257.1095
1-Hydroxyphenanthrene	3-PHEN	266.1127	251.0893	272.1329	257.1095
2-Hydroxyphenanthrene	2-PHEN	266.1127	251.0893	272.1329	257.1095
1-Hydroxybenzo(c)phenanthrene	3-BCP	316.1283	301.1049	322.1485	307.1251
2- Hydroxybenzo(c)phenanthrene	3-BCP	316.1283	301.1049	322.1485	307.1251
3- Hydroxybenzo(c)phenanthrene	3-BCP	316.1283	301.1049	322.1485	307.1251
1-Hydroxypyrene	1-PYR	290.1127	275.0893	296.1329	281.1095
1-Hydroxybenz(a)anthracene	1-BAA	316.1283	301.1049	322.1485	307.1251
3-9- Hydroxybenz(a)anthracene	1-BAA	316.1283	301.1049	322.1485	307.1251
4-Hydroxychrysene	3-CHRY	316.1283	301.1049	322.1485	307.1251
6-Hydroxychrysene	6-CHRY	316.1283	301.1049	322.1485	307.1251
3-Hydroxychrysene	3-CHRY	316.1283	301.1049	322.1485	307.1251
1-Hydroxychrysene	3-CHRY	316.1283	301.1049	322.1485	307.1251
2-Hydroxychrysene	3-CHRY	316.1283	301.1049	322.1485	322.1485
3-Hydroxybenzo(a)pyrene	3-CHRY	340.1283	325.1049	343.1380	328.1146

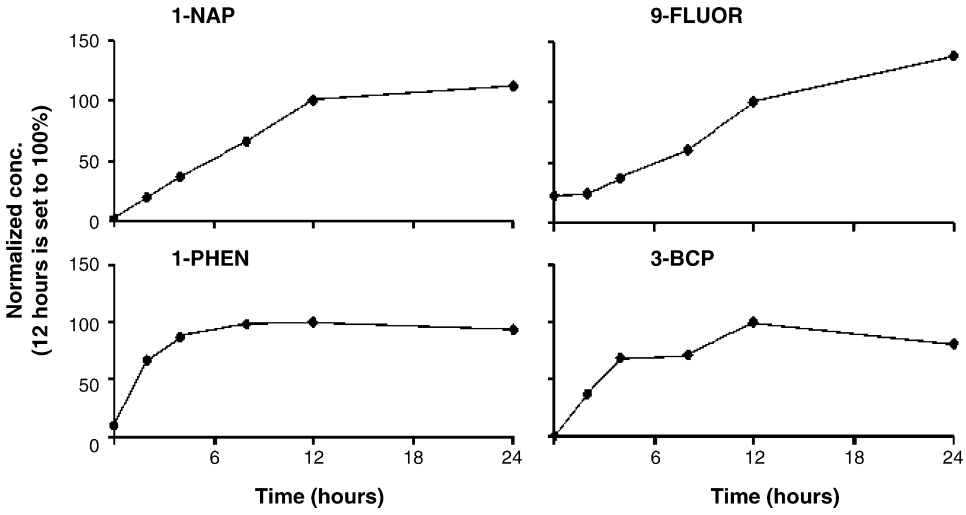

Fig. 1. Deconjugation time optimization, samples are normalized using concentrations at 12 h as reference value.

phase II-metabolites must be deconjugated back to the hydroxylated form before extraction. To determine the percentage of conjugated compounds in human samples, smoker's urine (which is known to have higher concentrations of most analytes) was used. Two measurements were made on the same sample: one with deconjugation step (total PAH metabolites; i.e., nonconjugated and conjugated were measured) and one without deconjugation step (only free PAH metabolites were measured). The difference between the two measurements represents the conjugated PAH metabolites in urine. The percentages of conjugated compounds found in the smokers urine ranged between 93 and 100%, confirming the relative importance of conjugate metabolites of PAHs in urine.

The efficiency of deconjugation was found to be dependant on the time of the incubation (Fig. 1) and deconjugation enzyme volume. An increased volume β-glucuronidase/arylsulfatase (10 μL), compared to the previous method, was needed for complete deconjugation (data not shown). As for the deconjugation time, some compounds (e.g., 1-NAP) showed increasing concentrations up to 24 h, and others showed a decrease after 8 h (e.g., 3-FLUOR). Most compounds reached a maximum at 12 h followed by a subsequent and gradual decrease. An incubation time between 17 and 18 h was chosen as the optimum with most analytes reaching a maximum concentration at this time. The incubation demonstrated consistent concentrations in our quality control materials, which included more than 160 samples that were measured over a 4-month period. With optimal deconjugation, maximal efficiency can be achieved in reducing the PAH metabolites back to hydroxylated form, after which they can be extracted through SPE.

Of the four new sorbents that were studied, the best results were achieved using the Focus sorbent packed in 3 cc cartridges (60 mg) (Fig. 2). This cartridge is not a standard format but was used because the three other comparison sorbents were all commercially available in this size.

Any SPE sorbent may retain co-extracted substances from the urine matrix that could interfere with target compound quantification. Therefore, a well-optimized wash step is required to obtain clean extracts. Higher proportions of methanol will produce cleaner extracts but may increase the risk of eluting the target analytes from the cartridge. Therefore, wash optimization experiments were designed to find the maximum amount of methanol that could be used without decreasing recoveries of the target analytes. We determined that up to 50% methanol could be used without a significant decrease in recoveries (Fig. 3). To guarantee minimal loss we used a 40% methanol/sodium acetate buffer (pH 5.5).

In the same way that optimal wash solution is required for clean extracts, the extraction solvent needed to be carefully chosen, not only for achieving clean extracts, but also to minimize losses during evaporation. For extraction solvent optimization, therefore, the goal was to find a solvent with high volatility that would elute all of the compounds using the smallest possible volume and keep the evaporation step and short as possible to minimize loss of the compounds. After testing different solvents the optimum solvent was found to be dichloromethane (3 mL), which provided the adequate volatility with the most eluting potential (data not shown).

To further minimize losses during the evaporation step dodecane (5 μL) was added to the extracts as a "keeper" and instead of evaporating to dryness, the extracts were concentrated to this "keeper" volume in a water bath under a gentle stream of nitrogen. Fig. 4 shows the differences in recovery when extracts are allowed to evaporate to dryness with and without adding dode-

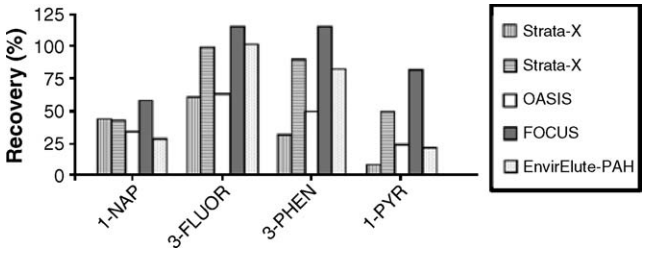

Fig. 2. Sorbent choice optimization, four new sorbents were tested and compared to the previously used EnvirElute-PAH sorbent.

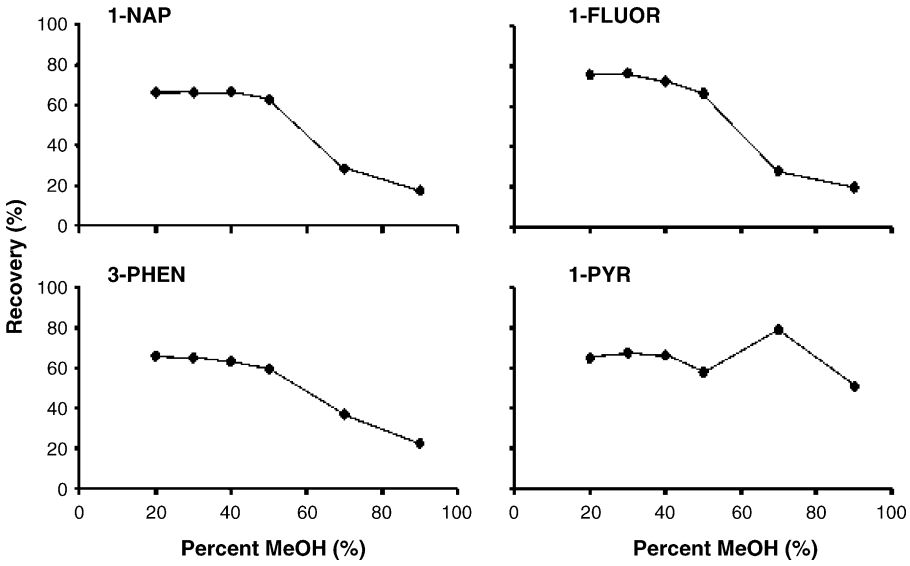

Fig. 3. Wash solution optimization, comparing recoveries of HO-PAHs using different concentrations of methanol in the wash solvent during SPE.

cane. By using a more volatile elution solvent the temperature of the water bath could be lowered to 40 °C. Also, to minimize loss of volatile compounds the nitrogen flow was gradually increased during evaporation starting at a very gentle flow of 5 psi.

In summary, we modified our previous method [24] by automating the SPE procedure, applying a more efficient sorbent, extending the incubation time for maximum deconjugation, adding more deconjugation enzyme, optimizing the SPE parameters, and using a more gentle evaporation. By converting a manual extraction method to an automated SPE system, a more precise and time-efficient method became available for large epidemiological studies. The automated SPE system provides unattended extraction and minimizes manual labor errors, which results in reduced within- and between-day variability.

3.2. Method performance

The standard curves showed good linearity with correlation factors of at least 0.99. In the NHANES study, concentrations of 1- and 2-NAP were sometimes measured up to 60 times higher than the calibration curve. To assure continuous linearity, the calibration curve was extended and linearity was noted to a concentration of 30,000 pg/μL. Recoveries were calculated by adding carbon-13 labeled benzo(b)fluoranthene as a recovery standard to each sample after reconstitution. The ratio of each labeled standard peak area and the recovery standard peak area was then compared to a separately prepared recovery mixture of the 12 labeled standards and benzo(b)fluoranthene in toluene, which was derivatized and analyzed on the GC-HRMS system with each batch of the samples. Average recoveries for measured analytes ranged between 69 and 93%. Method detection limits (MDLs) were calculated as $3S_0$ greater than the average blank concentration where S_0 is the standard deviation at the lowest calibration standard (10 pg/mL). Calculated MDLs were less than 10 pg/mL for all measured metabolites (Table 3).

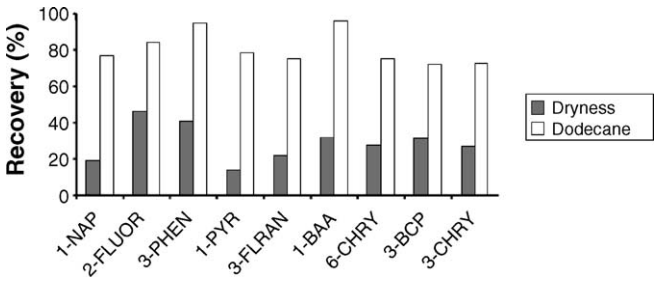

Fig. 4. Evaporation optimization, comparing evaporation of extracts to dryness and adding 5 μL dodecane prior to evaporation.

Table 3
Method performance described as coefficient of variation (CV), LOD and method validation (standard addition)

Analyte	QC-low		QC-high		LOD	Matrix spike		
	CV (%)	*N*	CV (%)	*N*	MDL (pg/mL)	r^2	*b*	QC-low
1-NAP	9.0	200	8.0	199	6.2	0.90	1204	1470
2-NAP	6.9	189	7.6	196	2.4	0.86	1769	2051
9-FLUOR	7.6	180	7.5	202	2.8	0.98	458	521
3-FLUOR	6.2	156	6.8	184	2.0	0.98	508	518
2-FLUOR	6.0	187	5.1	202	3.6	0.99	406	477
4-PHEN	11.7	202	7.6	200	5.7	0.96	324	358
9-PHEN	13.3	197	12.9	201	3.1	0.97	158	159
3-PHEN	4.6	158	6.3	192	3.6	0.98	291	331
1-PHEN	6.5	163	6.3	193	3.5	0.98	297	337
2-PHEN	13.3	158	8.5	170	3.2	0.92	642	519
1-BCP	13.3	200	16.3	199	3.4	0.99	203	297
1-PYR	5.0	196	5.5	202	3.3	0.99	273	304
2-BCP	10.6	202	9.3	183	5.4	0.99	233	300
1-BAA	7.3	201	6.6	199	3.9	0.99	248	279
4-CHRY	9.4	195	8.5	199	2.8	0.99	210	259
6-CHRY	6.7	203	5.7	196	2.4	0.99	237	269
3-BCP	6.2	193	6.2	199	5.4	0.99	255	280
3-CHRY	7.5	202	6.9	199	8.3	0.99	264	317
1-CHRY	11.6	200	7.9	199	5.0	0.98	242	270
3-9-BAA	8.2	185	8.4	198	10.0	0.99	267	267
2-CHRY	9.2	202	8.3	199	5.0	0.99	234	249
3-BAP	17.1	201	15.1	199	10.0	0.97	271	329

Because the added analyte, 9-BAA, co-elutes with the structurally similar congener, 3-BAA, these two compounds were measured together, and one total concentration was reported.

We validated the method for accuracy by completing a four-point matrix spike into existing QC low material. All spiked and non-spiked QC materials were run in triplicate. We used linear regressions of the measured concentrations to determine the intercept, which would correspond to the concentration in the QC low pool for an accurate method [25]. The spiked samples showed good linearity for all compounds, and measured intercepts were similar to the measured QC low analyte concentrations, which demonstrates a nonbiased method (Table 3).

To determine the method precision, two QC pools were prepared by spiking filtered diluted urine with the 23 target metabolites at two different concentrations. The reason for filtering and diluting the urine pool was to remove any sediment and particles, which are known to occur in certain urine samples, in order to assure QA/QC pool stability over long periods of time. We measured more than 50 QC low and QC high samples over a 2-month period. Three analysts and two HRMS instruments were used throughout the QC characterization to further monitor method performance. The QC characterization established target concentrations and limits of variation for each of the analytes in both QC pools. During biomonitoring studies, one QC sample at each concentration level along with a water blank were included with each set of 17 unknown samples for quality assurance. QC samples were monitored according to the multi-rule QC control parameters defined by Westgard [26]. After each study, the QC results from sets that passed QA/QC were added to the QC characterization data for subsequent biomonitoring studies. This procedure results in a varying number of QA/QC results for each of the analytes measured. The precision of the method is determined by calculating the coefficient of variation (CV) for the repeated measurements of the two QC pools. For a 5-month period ($N > 157$) the CV ranged between 5 and 17% (Table 3).

The method described herein was used to measure 23 PAH metabolites in the fifth NHANES study, consisting of 2946 urine samples. Only 6% (181 samples) could not be included in the study because of problems with analysis or lack of sample. The most common problem in the methodology was blockage of the cartridge caused by particles in the urine sample. This problem was resolved for some samples by using smaller volumes of urine for clean-up or by running manual SPE. Another problem was "dirty" extracts, which led to loss of lock mass during HRMS analysis. For these samples, the compounds eluting in the window where lock mass was lost were excluded from the reported data. Dirty extracts may be avoided by using a smaller volume of urine for extraction, though this may result in higher MDL and a less sensitive method. Therefore, this option is used only for samples which prove difficult to extract using the pre-described volume of 3 mL.

When samples had concentrations exceeding the linear range of the method, they were re-extracted using smaller volumes of urine and reanalyzed for proper quantification. The time-limiting factor of the method was the run-time on the GC-HRMS, which was 27 min (compared with 21 min in the previous method [24]). The overall maximum capacity for the method was 42 samples per day per instrument.

4. Conclusion

We have developed an analytical method for the quantitative analysis of 23 HO-PAH metabolites in urine utilizing an automated SPE system. We optimized each step in our method, which resulted in a sensitive and precise analytical method that only requires a small urine volume (3 mL). Consequently, a high-throughput precise method is available, which is ideal for analyzing samples for large epidemiological studies. The method was applied to the analyzing almost 3000 samples in the fifth NHANES study, where 6% of the samples were lost because of blockage of the cartridge or "dirty" extracts. Although these problems have a minor effect on the overall performance of the method, this issue needs to be addressed in future method development. Furthermore, true isotopic dilution is not possible for 12 analytes. Adding more carbon-13 labeled standards to the internal standard mixture is a future objective of method development, as well as adding more target analytes of interest.

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