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Quantitative determination of hydroxy polycylic aromatic hydrocarbons as a biomarker of exposure to carcinogenic polycyclic aromatic hydrocarbons

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ABSTRACT

A high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) method was developed for quantitative analysis of hydroxy polycyclic aromatic hydrocarbons (OH-PAHs). Four hydroxy metabolites of known and suspected carcinogenic PAHs (benzo[*a*]pyrene (B[*a*]P), benz[*a*]anthracene (B[*a*]A), and chrysene (CRY)) were selected as suitable biomarkers of PAH exposure and associated risks to human health. The analytical method included enzymatic deconjugation, liquid – liquid extraction, followed by derivatization with methyl-*N*-(trimethylsilyl) trifluoroacetamide and instrumental analysis. Photo-induced oxidation of target analytes – which has plagued previously published methods – was controlled by a combination of minimizing exposure to light, employing an antioxidant (2-mercaptoethanol) and utilizing a nitrogen atmosphere. Stability investigations also indicated that conjugated forms of the analytes are more stable than the non-conjugated forms. Accuracy and precision of the method were 77.4–101% (<4.9% RSD) in synthetic urine and 92.3–117% (<15% RSD) in human urine, respectively. Method detection limits, determined using eight replicates of low-level spiked human urine, ranged from 13 to 24 pg/mL. The method was successfully applied for analysis of a pooled human urine sample and 78 mouse urine samples collected from mice fed with PAH-contaminated diets. In mouse urine, greater than 94% of each analyte was present in its conjugated form.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed from incomplete combustion of organic matter, and are widely distributed in the ambient environment. Sources of human exposure to PAHs include industrial processes (e.g. petroleum refining), domestic heating, waste incineration, motor vehicle emissions as well as from smoking and dietary sources [1–3]. Approximately 500 individual PAHs have been detected in air, of which 16 are considered a priority by the United States Environmental Protection Agency, owing to their potential adverse health effects in humans [4].

Following exposure, PAHs undergo oxidation by cytochrome P450 enzymes to form hydroxylated PAHs (OH-PAHs). These metabolites may be further biotransformed to reactive electrophiles, which can bind covalently to DNA, leading to carcinogenicity and mutagenicity [5–10]. Deactivation and excretion is facilitated through phase 2 enzymes which produce glucuronide or sulfate conjugates. As the metabolism of PAHs occurs rapidly *in vivo*, exposure characterization relies upon determination of both hydroxylated and conjugated forms of PAH metabolites [11].

OH-PAHs can be analyzed by derivatization gas chromatography high resolution mass spectrometry (GC-HRMS) [12,13], liquid chromatography tandem mass spectrometry (LC–MS/MS) [14–16], or derivatization LC–MS/MS [17–19] but analyte instability remains a frequent and unresolved problem [20–23]. For example, both Whiton et al. [20] and Olmos-Espejel et al. [22] observed significant instability of 3-OH-B[a]P, which prevented determination of this compound with adequate accuracy and precision. During the 2001–2002 and 2003–2004 national health and nutrition exam-

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ination survey (NHANES), less than 50% of hydroxylated PAHs monitored were reported due to unreliable test results for these compounds [21]. Recent NHANES reports continued to include similar analytes [24]. Further investigations into the instability of various OH-PAHs were conducted by Schantz et al. [25] but the problem of analyte losses remains unresolved and low analyte recoveries continue to be reported in recent publications [26]. Some studies have speculated that OH-PAH instability is due to oxidation and have employed antioxidants such as 2-mercaptoethanol (2-ME), for example in measurement of OH-PAHs in fish bile [27]. However, the effectiveness of this antioxidant remains unclear. Tert-butyl hydroquinone has been employed in a similar manner for analysis of OH-PAHs in milk and manure matrices [28,29], resulting in improved recoveries. However, the exact cause of analyte loss remains unclear, as does the relative stability of conjugated versus non-conjugated species, which is particularly relevant for urine matrices.

In this study, hydroxy metabolites of benzo[*a*]pyrene (B[*a*]P), benz[*a*]anthracene (B[*a*]A), and chrysene (CRY), which all contain 'bay regions' that favor production of reactive and potentially carcinogenic metabolites [30–32] were selected as suitable biomarkers of PAH exposure. The objectives of this work were to: 1) develop a method for quantitative analysis of OH-PAHs in urine; 2) investigate the cause of rapid degradation of OH-PAHs during sample handling; including relative stability of conjugated versus non-conjugated OH-PAHs and implications for sample analysis, storage and experimental designs; and 3) implement measures to control analyte instability during analysis and storage in order to produce a reproducible and defensible analytical method suitable for measurements of OH-PAHs in occupational exposure and pharmacokinetic studies.

2. Experimental

2.1. Standards and reagents

All solvents were HPLC grade. The native analytes 9-OH-benzo[*a*]pyrene (>99%; 9-OH-B[a]P), 3-OH-benz[*a*]anthracene (97%; 3-OH-B[a]A), 3-OH-chrysene (>99%; 3-OH-CRY), pyrene-1-sulfate, potassium salt, $^{13}C_6$-3-OH-chrysene >95%; $^{13}C_6$-3-OH-CRY,- and $^{13}C_6$-1-OH-benz[*a*]anthracene (>99%), ($^{13}C_6$-1-OH-B[*a*]A) were obtained from MRI Global Chemical Carcinogen Repository (Kansas City, Missouri, USA). 3-OH-benzo[*a*]pyrene (95%; (3-OH-B[*a*]P) was obtained from Toronto Research Chemicals Inc. (TRC; Ontario, Canada). 1-OH-naphthalene (>99%; 1-OH-NAP) and 1-OH-pyrene (98%; 1-OH-PYR) were obtained from Sigma-Aldrich, Saint Louis, MO, USA. $^2H_{11}$-3-OH-Benzo[*a*]pyrene (95%; $^2H_{11}$-3-OH-B[*aa*]P) was obtained from Toronto Research Chemicals Inc. (TRC), Ontario, Canada. $^{13}C_{12}$-4′-OH-3,3′,4,5′-TetraCB(>98%), $^{13}C_6$-1-OH−naphthalene (>98%; $^{13}C_6$-1-OH−NAP), and $^{13}C_6$-1-OH-pyrene- (>98%) $^{13}C_6$-1-OH-PYR, were obtained from Cambridge Isotope laboratories Inc., And over, MA, USA. $^{13}C_{12}$-PCB-81 (>98%) was obtained from Wellington Laboratories, Guelph, ON, Canada. 1-naphthyl-β-D-glucuronide sodium salt was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). ß-Glucuronidase from *Helix pomatia* type H-1, partially purified powder, ≥300,000 units/g solid was obtained from Sigma Aldrich (St. Louis, MO, USA). *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and the antioxidants 2-mercaptoethanol and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of standard solutions

All glassware including gas chromatography (GC) microvials were silanized by soaking in 5% dimethyldichlorosilane in heptane for 15 min. All native and isotopically-labeled standard solutions were prepared in the dark to minimize photo-induced degradation. In addition, 30 μL of 2-ME was added to solutions (not more than 10 mL volumes) to minimize oxidation of OH-PAHs. Native and surrogate standard solutions were prepared in acetonitrile to ensure quick mixing with urine or aqueous matrices. Prepared solutions were kept in amber containers and wrapped with foil to minimize exposure to light. The derivatization control and recovery standard solution were prepared in toluene. All standard solutions were stored at −80 °C.

A series of eight calibration solutions containing all native and labeled standards (Table S1) were derivatized and used to establish the initial calibration of the instrument. The concentration of native analytes varied from 0.25 to 1000 ng/mL, while the concentrations of surrogates, derivatization standards and recovery standards remained constant at 200, 50, and 50 ng/mL, respectively. Calibration solutions were prepared in toluene and were stored at −80 °C for a maximum of three weeks prior to use.

2.3. Sample collection

Synthetic urine was prepared as described in Gustafsson and Uzqueda [33]. A brief description of preparation of synthetic urine is provided in Table S3. Pooled human urine samples were collected from volunteers. Mouse urine samples were collected as part of a larger PAH exposure experiment conducted at Ricerca Biosciences (Concord, Ohio, USA). Briefly, mice were divided into 5 groups: Group 1 (controls) were administered a diet consisting of uncontaminated soil (i.e. <0.17 μg/g CRY, B[*a*]A, and B[*a*]P) mixed with feed. Groups 2, 3, and 4 were administered diets consisting of feed mixed with 5, 10, or 20% contaminated soil, respectively, which was obtained from a clay pigeon target military range site. The soil contained 253 μg/g CRY, 230 μg/g B[*a*]A, and 253 μg/g B[*a*]P. The mice in Group 5 were fed a diet consisting of feed mixed with site soil extract to match the 10% soil group. Urine samples were collected following 7 days of exposure for all groups.

2.4. Sample preparation

Sample processing was conducted in a dark room with limited yellow light. Urine samples (up to 4 mL) were placed in centrifuge tubes and spiked with 30 μL of 2-ME. The samples were then spiked with surrogate standard solution. The laboratory blank was spiked with deconjugation control standards containing 100 ng 1-naphthyl-β-D-glucuronide and 70 ng pyrene-1-sulfate potassium salt. All unknowns and Quality Control (QC) samples were spiked with 1 mL of enzyme solution (1 mg/mL β-glucuronidase enzyme prepared in a 1 M sodium acetate buffer at pH 5.5) [12]. The headspace over each sample was purged with nitrogen, sealed with Teflon tape, and incubated in the dark at 37 ± 2 °C. After 17 h, the samples were extracted in the dark with 4 mL of pentane using a Thermolyne Speci-mix test tube rocker, and then centrifuged at 2800 rpm for 20 min. The supernatant was removed and the extraction was repeated twice, after which the pentane layers were combined and dried by passing the extract through a Pasture pipette filled with anhydrous sodium sulfate. A 20 μL dodecane keeper solvent was added and the extracts were reduced to 20 μL under a gentle stream of nitrogen in a Turbovap evaporator (Caliper Life Sciences MA, USA) that was maintained at 6 psi and 40 °C. Extracts were subsequently reconstituted with 140 μL of a 10:65:65 of 2-mercaptoethanol: acetonitrile: toluene solution and transferred to GC microvial. The resulting extract was spiked with derivatization control standard and recovery standard solutions. The mixture was finally spiked with 20 μL of MSTFA derivatization

agent (final volume 200 μL), capped tightly and derivatized at 60 °C for 30 min, prior to analysis by HRGC-HRMS.

2.5. Instrumental analysis

HRGC/HRMS analysis was carried out using an AutoSpec Ultima HRMS equipped with a HP 6890 GC, a CTC auto-sampler, and an Alpha data system running on Micromass Opus software. A DB-17 capillary chromatography column (30 m, 0.25 mm i.d., 0.25 μm film thickness) was coupled directly to the MS source. The carrier gas was helium and the GC was operated at a constant head pressure of 200 kPa. The head pressure was adjusted as the GC column aged to maintain the retention times listed in Table S4. Immediately prior to running samples, the mass spectrometer was tuned to a static mass resolution of at least 10,000 and operated in the electron impact (EI) ionization using voltage selected ion mode (V-SIR) with perfluorokerosene (PFK) lock masses, acquiring the ions listed in Table S1. GC operating conditions included the following oven temperature program for analyte separation: initial temperature 95 °C, held for 2 min, then ramp at a rate of 15 °C/min to 160 °C; ramp at a rate of 10 °C/min to 230 °C and hold for 5 min, ramp at a rate of 23 °C/min to 320 °C and hold for 7.8 min. Injection temperature and interface temperatures were set at 270 °C and source temperature was set at 250 °C.

2.6. Quality control

An initial assessment of laboratory background levels using four replicates of synthetic urine revealed an absence of target analytes above method detection limits (Table 8). Subsequently, a laboratory blank was included in each analysis batch to monitor laboratory background levels. A spiked human urine sample was also included in each analysis batch to monitor ongoing method accuracy. A suite of deuterium and ^{13}C-labeled surrogates were spiked to monitor sample-to-sample analyte recovery. To monitor the efficiency of enzymatic deconjugation, a solution containing 1-naphthyl-β-D-glucuronide sodium salt and pyrene-1-sulfate potassium salt (100 and 70 ng respectively) was also spiked into each laboratory blank. Similarly, a labeled hydroxy PCB, $^{13}C_{12}$-4′-OH-3,3′,4,5′-tetraCB,(10 ng) was spiked into each sample prior to derivatization in order to monitor completeness of the reaction on a sample-to-sample basis. A labeled $^{13}C_{12}$-PCB 81 (10 ng) was spiked into each sample to ensure complete sample injection into the instrument, and quantify surrogate recoveries. Perfluorokerosene (PFK) lock masses (Table S4) were acquired to monitor signal suppression and enhancement in each acquisition function. Instrument stability and accuracy of injection were verified by analysis of $^{13}C_{12}$ labeled PCB 81 that was spiked into each extract just prior to instrumental analysis. In each analysis bracket, solvent blanks were analyzed to monitor and quantify sample-to-sample carryover. A calibration verification solution (at the mid-point of the calibration) was analyzed at the beginning of the analysis and again every 12 h to verify calibration and monitor instrument drift. The criteria used for positive identification of target analytes, surrogate, derivatization and recovery standards are provided in SI.

3. Results and discussion

3.1. Analyte instability

Rapid degradation or instability of OH-PAHs have been observed in a number of previous studies [20,22,23]. At the initial stages of the current study, it was observed that the concentration of the analytes changed significantly from one test to another, often with very low recoveries for the 3-OH-B[*a*]P, 9-OH-B[*a*]P and $^{2}H_{11}$-3-OH-B[*a*]P. Low recoveries were unrelated to analyte volatility, based on comparison of analyte loss at various speeds of nitrogen blow down and to varying extents of extract dryness (data not shown). In addition, the greatest losses were observed for the heaviest analytes (3-OH-B[*a*]P and 9-OH-B[*a*]P) suggesting that volatility was not the source of analyte loss. Consequently, a systematic investigation into the source(s) of analyte loss was carried out.

3.1.1. Effect of light on analyte stability

To determine if analyte losses were related to photo-induced reactions, spiked solutions were prepared in both toluene and water and placed in clear and amber vials (Table 1). The vials were stored for 14 days at ambient temperature (20 °C) close to a window that received direct sunlight. The lowest mean analyte recoveries (<2%) were observed for solutions prepared with reagent water and stored in clear vials, while the highest mean recoveries (83.5–109%) were observed for solutions prepared with toluene and stored in amber vials. Collectively these data indicate that photolytic decomposition can be reduced by preparing solutions in toluene as opposed to water, with storage in amber glass away from sunlight. Of note was that the individual OH-PAHs displayed varying degrees of instability, 3-OH-B[*a*]P and 9-OH-B[*a*]P displaying consistently lower recoveries than 3-OH-CRY.

3.1.2. Effect of temperature on analyte stability

To study the effect of temperature on analyte stability, target analytes were spiked into amber vials and stored at ambient temperature (20 °C), −20 °C, or −80 °C, for a period of 14 days (Table 2). In toluene, the results did not show major differences between the samples stored at the ambient temperature from those stored at −20 or −80 °C. In HPLC water samples, degradation was observed at all temperatures, however samples stored at −80 °C displayed slightly lower recovery for 3-OH-CRY and 3-OH-B[*a*]A. Overall, this observation indicated that temperature alone was not a major factor in explaining the observed analyte instabilities during storage, an observation that has been made previously [34].

3.1.3. Oxidation as a cause of analyte loss

A freshly prepared standard, spiked into reagent water and processed through the procedure without a nitrogen atmosphere and antioxidant resulted in an almost complete loss (6% recovery) of 3-OH-B[*a*]P (Table 3). To determine if photolytic oxidation was the mechanism of analyte loss, spiked HPLC water samples were analyzed in the presence and absence of 30 μL of 2-ME, an antioxidant. The presence of 2-ME or BHT added at two points in the analytical procedure (prior to sample spiking and enzymatic deconjugation, and during final extract reduction and derivatization) and processing in nitrogen atmosphere improved recovery (from 6% to 37–50%) for 3-OH-B[*a*]P. In addition, spiked urine samples without antioxidant and processed in nitrogen atmosphere showed similar recovery to the HPLC water samples spiked with an antioxidant. Given that urine contains some natural antioxidants [35,36] the observed similarities between the spiked urine and the HPLC water spiked with antioxidant were not surprising. In a previous study [34], improved stability of OH-PAHs in fish bile relative to reagent water was attributed to the presence of antioxidants in the former matrix. This observation highlighted that care should be taken in comparing results obtained in reagent waters to those of urine sample or other matrices. In addition, the results indicated that oxidation was the probable mechanism of analyte loss.

To determine the effect of antioxidants on the analytes during storage, toluene and HPLC water solutions containing the analytes were stored in clear and amber vials (Table 2 and 4). The results showed that small amounts of antioxidant did not improve recovery of the analytes in HPLC water samples stored in clear vials.

Unequivocal evidence for oxidation as a cause for analyte instability is identification of oxidation products. To investigate the

Table 1
Effect of exposure to light on OH-PAH analytes stored in spiked toluene and HPLC water samples using clear and amber vials at ambient temperature (+20 °C).*

Compound	Spiked amount(pg/mL)	Clear glass storage		Amber glass storage	
		In toluene %rec.	In HPLC water %rec.	In toluene %rec.	In HPLC water %rec.
3-OH-CRY	101,000	48.1	1.18	109	63.5
3-OH-B[*a*]P	101,000	0.3	ND	96.9	27.0
9-OH-B[*a*]P	102,000	0.7	ND	83.5	54.1
3-OH-B[*a*]A	100,000	80.4	ND	98.0	58.8

* Each value represents mean of a duplicate measurement. Sample volumes = 1 mL.

Table 2
Effect of temperature on OH-PAH solutions in toluene and HPLC water in storage.*

Compound	Spiked amount (pg/mL)	Toluene Amber glass, at +20 °C	Toluene Amber glass at −20 °C	Toluene Amber Glass at −80 °C	HPLC water Amber glass, at +20 °C	HPLC water Amber glass, at −80 °C
		%rec.	%rec.	%rec.	%rec.	%rec.
3-OH-CRY	101,000	109	110	110	63.5	31.1
3-OH-B[*a*]P	101,000	96.9	115	113	27.0	28.8
9-OH-B[*a*]P	102,000	83.5	77.7	80.5	54.1	35.0
3-OH-B[*a*]A	100,000	98.0	97.2	102	58.8	29.5

* Each value represent mean of a duplicate measurement. Sample volumes = 1 mL.

Table 3
Effect of antioxidant (AO) on analyte recovery during sample processing.*

Compound	No AO in spiked HPLC water	2-ME in HPLC water	BHT in HPLC water	No AO in Urine	No AO, No N_2 in urine
3-OH-CRY	68.1%	65.0%	59.4%	64.5%	53.4%
3-OH-B[*a*]P	5.91%	37.4%	50.3%	47.1%	7.1%
9-OH-B[*a*]P	53.1%	65.7%	57.6%	62.6%	38.1%
3-OH-B[*a*]A	66.8%	64.5%	59.5%	64.3%	55.9%

* All samples processes with limited light. Each value represents mean of a duplicate measurement.

Table 4
Effect of antioxidant type on OH-PAH solutions in storage.*

Compound	Spiked amount /sample (pg/mL)	Toluene Amber glass −80 °C storage with 2-ME	Toluene Amber glass −80 °C storage with BHT	HPLC water at ambient in clear vial No AO	HPLC water at ambient in clear vial with 2-ME	HPLC water at ambient in clear vial with BHT
		%rec.	%rec.	%rec.	%rec.	%rec.
3-OH-CRY	101,000	110	105	1.18	1.81	9.68
3-OH-B[*a*]P	101,000	113	100	ND	ND	ND
9-OH-B[*a*]P	102,000	81.6	144	ND	0.06	0.15
3-OH-B[*a*]A	100,000	100	105	ND	0.58	6.95

* Each value represents mean of a duplicate measurement. Sample volumes = 1 mL.

existence of oxidation products, duplicate solutions of 3-OH-B[a]P and $^2H_{11}$-3-OH-B[a]P (each at 2 μg/mL) were prepared in HPLC water without antioxidants. The samples were extracted with pentane, derivatized and HRMS Full Scan conducted on DB-17 eluents of the extract. The results were investigated to see if some of the known oxidation products of 3-OH-B[*a*]P such as dihydroxy B[*a*]Ps was present in the reaction mixture. A peak that is characteristic of trimethylsilyl (TMS) derivatives of dihydroxy B[*a*]Ps with *m*/*z* 428.1628 was observed at 27:23 min for the native 3-OH-B[*a*]P and with *m*/*z* 438.224 was observed at 27:18 min for the deuterium labeled $^2H_{11}$-3-OH-B[*a*]P analogue. The reaction mixture contained a large peak made up of multiple reaction products. The existence of TMS derivatives of dihydroxy B[*a*]Ps further supports the hypothesis that photo induced oxidation was the cause of the observed instabilities of the analytes.

3.1.4. Relative stability of conjugated and unconjugated analytes

In order to assess total PAH exposure it was important that our method could accurately measure both conjugated and unconjugated OH-PAHs in urine, and furthermore, that the relative proportion of these species were conserved over the entire sample preparation procedure. To investigate the relative stability of conjugated and unconjugated OH-PAHs, urine samples collected from mice fed with a PAH contaminated diet were used. First, the total concentration (i.e. conjugated + non-conjugated) and non-conjugated forms of each OH-PAH were determined in each urine sample with and without enzymatic deconjugation (Table 5). A portion of the same mouse urine sample was collected in duplicate and stored in a clear vial for 14 days at ambient conditions following enzymatic deconjugation. A second portion of the mouse urine sample was also stored in clear vial and at ambient temperature without deconjugation for 14 days. The results indicated that in mouse urine most (>94%) of the analytes were present in conjugated forms of the analytes. The non-conjugated forms were generally less than 6%. The results from the storage study showed that <20% of each analyte was recovered in the sample that was stored after deconjugation whereas 55–75% of analytes were recovered in samples stored for 14 days without deconjugation. This

Table 5
Composition of rat urine sample in terms of conjugated and non-conjugated analytes. Relative stability of conjugated and non-conjugated analytes.*

Compound	Total urine conc.	Percentage of non-conjugated analytes	Deconjugated and stored sample	Stored without deconjugation
	pg/mL	% of total	% recovery	% Recovery
3-OH-CRY	434,000	1.67	19.9	75.6
3-OH-B[*a*]P	1,703,000	1.11	6.48	55.4
9-OH-B[*a*]P	884,000	1.63	3.40	56.4
3-OH-B[*a*]A	253,000	5.11	15.6	64.8

observation means that, compared to the non-conjugated forms of the analytes the conjugated forms were more stable against photo-induced oxidation. Based on these results, it is important to expedite analytical processes once urine samples have undergone deconjugation so as to minimize analyte instabilities.

3.1.5. Comparison of antioxidants

Two antioxidants, BHT and 2-ME were compared for effectiveness in controlling analyte degradation during analysis (Table 3). BHT and 2-ME showed comparable performance in analyte recovery. However, BHT has limited solubility in acetonitrile and toluene and tended to co-elute with the deconjugation control standards, which created large lock mass disturbances in the mass detector. On the other hand, 2-ME (a liquid reagent) is miscible with both aqueous and organic solvents and did not affect the chromatography and mass spectrometric methods. Consequently, 2.ME was selected as the antioxidant of choice. Finally, this reagent was applied to each standard (30 μL per 10 mL reagent), and to each sample (prior to deconjugation of urine samples (30 μL per sample) and prior to the derivatization step (30 μL)).

Although addition of antioxidants was effective in minimizing instability of non-conjugated OH-PAHs for a short period of time, such as the duration of analysis and with repeated use of the antioxidant at multiple steps in the analytical procedure, addition of a small amount of antioxidant was ineffective for controlling instability over an extended storage period, say 14 days (Table 4). Based on these findings we recommend collecting samples for OH-PAHs analysis in amber glass containers, spiking with antioxidants, and then quickly carrying out the sample preparation and analysis, with additional application of antioxidants to avoid analyte degradation.

3.1.6. Stability of derivatized analytes

The stability of derivatized products cannot be assumed to be the same as the parent compounds and needs to be determined independently. In this study, three mouse urine sample extracts and a calibration standard were analyzed. Upon completion of the analysis the derivatized extracts were stored at −80 °C for a period of 55 days. The extracts were taken out of storage and reanalyzed using a freshly prepared calibration standard (Table S5). The results showed that the derivatized analytes were stable over the study period both in urine sample extracts and in a standard solution.

3.2. Method validation

Once analyte stability was understood and controlled with a suitable antioxidant and nitrogen atmosphere as well as through minimum exposure to light, other aspects of the analytical method were investigated. These included, derivatization of the analytes to enable gas chromatographic analysis, controlling enzymatic deconjugation, extraction and cleanup, as well as validation of the final analytical protocol.

3.2.1. Derivatization method

Derivatization with MSTFA has been previously used for analysis of OH-PAHs [12,13], but the efficiency of the derivitization reaction is rarely assessed, or tracked during routine analysis. In the current method we evaluated the robustness of the derivatization protocol to varying temperature, amount of reagent and derivatization time (Figs. 1–3) and found the reaction to be quite insensitive to slight variations in derivatization conditions. We also included a sample-to-sample measurement of completeness of the derivatization reaction. This was accomplished by spiking $^{13}C_{12}$-4′-OH-3,3′,4,5′-TetraCB to each sample prior to derivatization and measuring % derivatization of this compound against the recovery standard $^{13}C_{12}$-PCB-81. During derivatization of 78 samples, we observed six samples with incomplete derivatization, i.e. recovery values of the derivatization control standard were below 60%. In addition, the observed values were <20%. The derivatization control standard enabled a quick diagnosis of the derivatization status of each sample and remedial re-derivatizations were conducted.

3.2.2. Deconjugation control

Two model hydroxy PAHs: 1-OH-NAP-GLU and 1-OH-PYR-SO_4 (100 and 70 ng each respectively) were used to monitor enzymatic hydrolysis of the conjugated analytes on a batch-to-batch level. This was accomplished by spiking these compounds into the laboratory blanks prior to enzymatic hydrolysis. Recoveries of the deconjugated analytes 1-OH-NAP and 1-OH-PYR in laboratory blanks were used as an indication of the deconjugation status of individual sample batches.

3.2.3. Method performance

3.2.3.1. Method accuracy and precision. Method accuracy was demonstrated initially and on an ongoing basis. Initial demonstration of method accuracy involved analysis of replicates of spiked synthetic urine samples. Accuracy (recovery) values of 77.4–101% were achieved with the developed protocol, Table 6. Accuracy was also measured using spiked human urine samples and values of 92.3–117% were achieved. The replicate spiked synthetic urine samples and human urine samples were also used to determine precision (%RSD) of the method. Values of (1.9–4.9%RSDs, n = 5) were measured in the synthetic replicate spikes and 7.4–15% were measured for the human urine samples. Overall, good accuracy and precision were observed for the developed method.

3.2.3.2. MDL data. Method detection limits (MDLs) were determined following the protocol outlined in Federal Register 40CFR Part 136, Supplementary Material. For this study eight replicates of human urine were fortified with a low level of target analytes (final concentration = 40 pg/mL). The results are provided in Table 7. Calculated MDLs ranged from 13 to 24 pg/mL using 4 mL samples. To reflect variations in detection limits based on the characteristics of the sample matrix, sample specific detection limits (SDL) were also calculated by converting three times the representative noise to concentration following the same procedure used to convert target

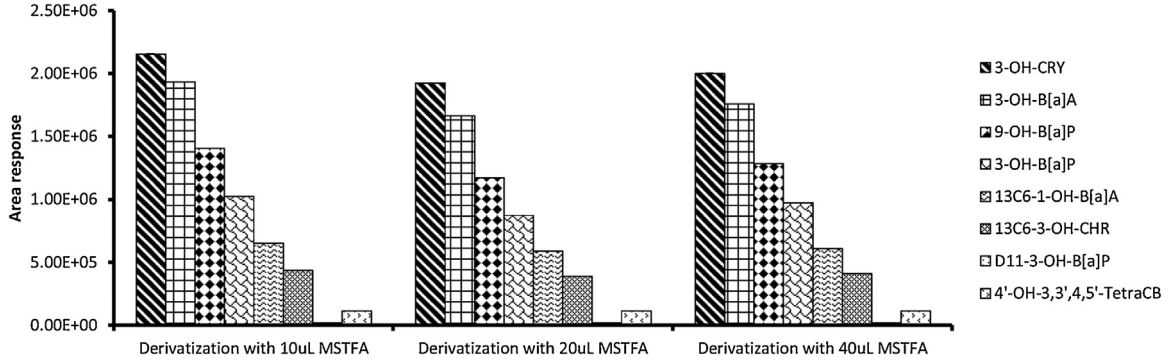

Fig. 1. Effect of derivatization reagent amount on derivatization reaction.

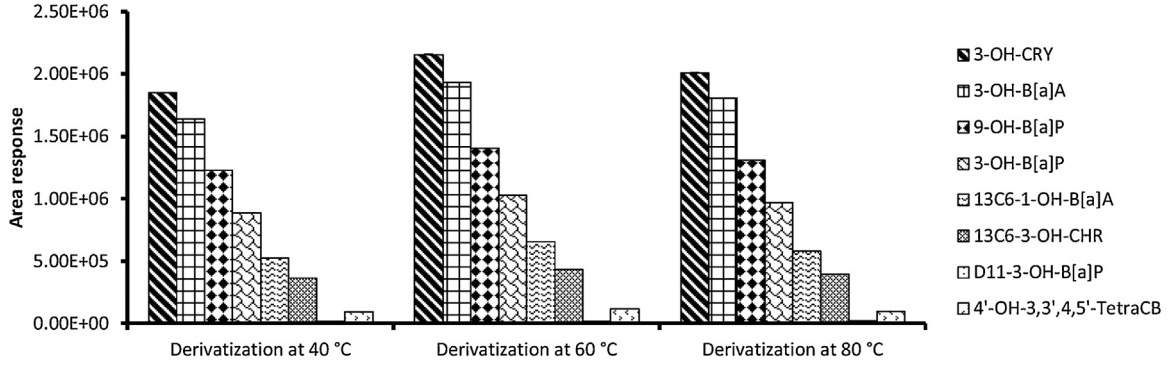

Fig. 2. Effect of derivatization temperature on derivatization reaction.

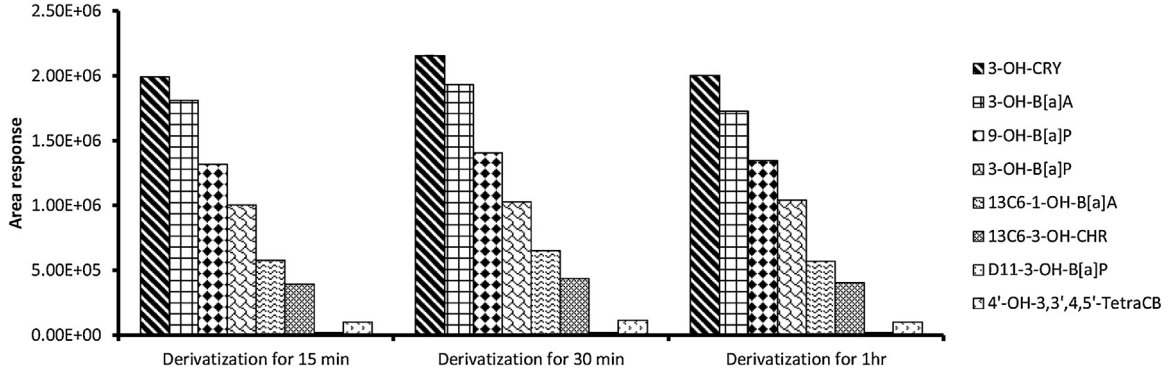

Fig. 3. Effect of derivatization time on derivatization reaction.

Table 6
Method accuracy and precision in synthetic and spiked urine samples.

Compound		Synthetic urine			Human urine		
	Spiked Amount (ng/mL)	Unspiked Sample conc. (pg/mL)	Average% recovery (n = 5)	%RSD	Unspiked urine conc. (pg/mL)	Average % recovery (n = 7)	%RSD
3-OH-CRY	5	<10.0	101	1.9	<16.1	101	7.4
3-OH-B[*a*]A	5	<10.0	77.4	1.5	<20.5	107	15
9-OH-B[*a*]P	5	<14.6	89.7	4.9	<23.9	117	10
3-OH-B[*a*]P	5	<20.0	98.5	4.0	<14.9	92.3	14

Table 7
Method detection limits data.

Compound	Spiked amounts (pg/mL)	Unspiked Urine conc. (pg/mL)	Measured average (n = 8) (pg/mL)	STDEV	MDL (pg/mL)
3-OH-CRY	40.2	<13.0	38.5	4.3	13.0
3-OH-B[*a*]A	40.2	<17.2	39.3	5.7	17.2
9-OH-B[*a*]P	40.7	<23.9	29.6	8.0	23.9
3-OH-B[*a*]P	40.2	<15.0	27.0	5.0	14.9

MDL = STDEV × student's *t*-value.

Table 8
Analysis data for mouse urine samples fed with PAH contaminated feed and pooled human urine samples.

Compounds	Mouse urine samples					Pooled human urine		
	Group 1	Group 2	Group 3	Group 4	Group 5	Pooled urine	Pooled urine DUP	Pooled urine TRP
	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL	pg/mL
3-OH-CRY	<271	60,100	102,000	113,000	99,700	<16.1	<20.7	<13.2
3-OH-B[*a*]A	<353	11,800	34,100	39,500	33,100	<20.5	<23.6	<23.0
9-OH-B[*a*]P	282	72,600	256,000	295,000	360,000	<23.9	<23.9	<23.9
3-OH-B[*a*]P	45.9	103,000	363,000	394,000	466,000	<14.9	<14.9	<14.9

Note: Group 1 = Control soil, Group 2 = 5% site soil, Group 3 = 10% site soil, Group 4 = 20% site soil and Group 5 = site soil extract matching 10% soil group. Concentrations represent total of (free + conjugated) analytes. Analyte levels in mouse urine samples (Group 2 −5) were taken from diluted extract analysis data.

peak responses to concentration. The greater of the SDL and MDL was used as a detection qualifier for reporting field sample data.

3.2.3.3. Analysis of human urine and mouse urine samples. Total (free + conjugated) concentrations of OH-PAHs in human and mouse urine are summarized in Table 8. The pooled human urine sample showed no detectable OH-PAH. The mouse urine sample showed varying levels of OH-PAHs corresponding to the PAH levels in the test diet. Given that clay pigeon target fragments contain coal tar pitch as a binding agent and site soil have some target fragments incorporated over time, increasing site soil consumption is expected to coincide with increasing exposure to PAHs. The mouse urine analysis results showed an increase in OH-PAH metabolites that was correlated to increased exposure to the PAH contaminated diet and demonstrated the applicability of the developed method. The developed method was successfully used for analysis of 78 mouse urine samples to study the bioavailability of the parent PAH compounds (data not presented).

4. Conclusions

A HRGC-HRMS method for quantitative analysis of OH-PAHs was developed. The causes of analyte instabilities that affected previously published analytical methods were identified to be related to photo initiated oxidation. Restriction to light through the use of amber containers for sample collection plus sample processing in limited yellow light and addition of antioxidants controlled instabilities during analysis. Studies that are conducted in reagent water need to take into account of the absence of antioxidants which are present in urine matrices. The developed method included controls that monitor completeness of: extraction plus analyte instability, enzymatic deconjugation and derivatization. The method was applied for analysis of synthetic urine, human urine and mouse urine matrices.

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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.2016.05.057.

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