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Article

Determination of Urinary PAH Metabolites Using DLLME Hyphenated to Injector Port Silylation and GC–MS-MS

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants and well-known carcinogens. Hydroxy derivatives of PAH are considered as biomarkers of PAH exposure, and there is a need to measure these metabolites at low concentrations. So, a precise and eco-friendly analytical method has been developed for rapid determination of PAH metabolites. For the first time, a new analytical method based on coupling of dispersive liquid–liquid microextraction (DLLME) with auto-injector port silylation (auto-IPS) followed by gas chromatography–tandem mass spectrometry (GC–MS-MS) analysis is reported for the analysis of seven urinary PAH metabolites. Factors affecting DLLME and IPS, such as type and volume of extraction and disperser solvent, pH, ionic strength, injector port temperature, volume of N,O-bis(trimethylsilyl)trifluoroacetamide and type of solvent were investigated. Under optimized conditions, the limit of detection and limit of quantification were found to be in the range of 1–9 and 3–29 ng/mL, respectively. Satisfactory recoveries of metabolites in urine samples in the range of 87–95% were found. The developed method has been successfully applied for the determination of PAH metabolites in urine samples of exposed workers. DLLME–auto-IPS–GC–MS-MS method is time, labor, solvent and reagent saving, which can be routinely used for the analysis of urinary PAH metabolites.

Introduction

Polycyclic aromatic hydrocarbons (PAH), a group of several hundred organic compounds, are believed to be one of the most significant and ubiquitous environmental pollutants produced by incomplete combustion of anthropogenic, industrial and non-industrial sources, e.g., fossil fuels, cigarette smoke, coal fires utilities, vehicle exhaust, steel plant, wood burning ovens etc. (1–3). The carcinogenicity of PAH is well documented by several researchers in animal models. Occupational exposure to PAH is responsible for skin, lung, bladder and gastrointestinal cancers in humans (4–7). Non-polar PAH are metabolized to polar hydroxyl derivatives by a mixed-function oxidase system in the presence of molecular oxygen and nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate (8, 9). Hydroxyl derivatives of PAH for instance 1-hydroxypyrene (1-OHP) are considered as general biomarker for the assessment of PAH exposure (10–12).

There are several reports available in literature for the analysis of PAH metabolites using high-performance liquid chromatography (HPLC). However, gas chromatography–mass spectrometry (GC–MS), still remain the method of choice for researchers, due to its high selectivity, good sensitivity, high separation efficiency and capabilities of definite identification by matching with spectral libraries available for the instrument (13–19). GC–MS-based methods for the determination of polar PAH metabolites necessitate derivatization to convert them into non-polar and volatile derivatives that are acquiescent to GC–MS analysis. Silylation is the most commonly used derivatization technique for conversion of polar PAH metabolites into non-polar derivatives (20–22). However, the major drawbacks associated with silylation are (i) it requires strictly anhydrous conditions for the derivatization, as both reagent as well as derivatives are prone to hydrolysis, (ii) the reaction time needed for silylation is lengthy ranging from 30 to 60 min and (iii) elevated reaction temperature is obligatory for the completion of reaction (i.e., 60°C).

In 1976, Rasmussen has proposed an online derivatization technique, i.e., injector port silylation (IPS), which overcomes the limitations of traditional in-vial silylation (23). IPS process involves a gaseous phase reaction between a silylating reagent and polar analyte, which occur inside the hot GC or GC–MS injection port. It is an almost instantaneous reaction completed within seconds inside the hot GC injection port, which also reduces the possibilities of degradation of derivatives as their exposure to environment is negligible. Extra experimental apparatus such as heater and reaction vials are not requisite in IPS as reagent and analyte are simultaneously or tandem injected inside the GC–MS injection port. Additionally, the required amount of derivatizing reagent and sample is greatly reduced. The reaction efficiency of on-line derivatization is also improved when compared with off-line derivatization which subsequently enhances the detector sensitivity and accuracy of quantification (24).

Sample preparation techniques, such as liquid–liquid extraction (LLE) and conventional solid-phase extraction (SPE), are widely used for the extraction of PAH metabolites from various samples (20, 25–27). However, LLE and SPE, both require large amount of organic solvents, which are perilous to human health and environment. Solid-phase microextraction (SPME), a solvent-free microextraction technique is also reported for the extraction of PAH metabolites. Though, a lengthy extraction time of 45 min to overnight extraction was required by these SPME methods, which make them unfeasible for routine analysis of PAH metabolites (21–22, 28). Recently, dispersive liquid–liquid microextraction (DLLME) has emerged as a potential microextraction technique having advantages of being rapid, eco-friendly, economic and simple. Since its invention DLLME has been extensively applied for the extraction of various organic

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analytes from different matrices of complex nature (29–36). Wang *et al.* reported a method combining supercritical water extraction with DLLME for the extraction of PAH metabolites from sediments and analyzed by GC–MS. However, the final drop obtained after DLLME was subjected to in-vial silylation which consumed 60 min for derivatizing of polar PAH metabolites with N-(*tert*-butyldimethylsilyl)-N-methyl-trifluoroacetamide (MTBSTFA) at 60°C (37).

The limitations of in-vial silylation, SPE and LLE are lengthy reaction time, need of elevated reaction temperature and moisture-free conditions; consumption of a large amount of toxic organic solvents and reagents can be overcome by combining DLLME with IPS (38–40). In this approach, the sedimented phase obtained after DLLME is subjected to IPS, thus, avoids the requirement of lengthy reaction time and tedious derivatization procedure. Keeping these common drawbacks in mind, we have developed for the first time, a novel combination of DLLME and IPS for the simultaneous extraction, preconcentration and derivatization of urinary PAH metabolites such as 1-napthol (NAP), 9-phenanthrol (PHN), 1-hydroxypyrene (1-OHP), 2-hydroxyfluorene (2-HF), 3-hydroxyfluorene (3-HF), 9-hydroxyfluorene (9-HF) and 6-hydroxychrysene (OH-CHRY) followed by GC–MS-MS analysis.

Experimental

Reagents and materials

Pure PAH metabolites viz., NAP, PHN, 1-OHP, 2-HF, 3-HF, 9-HF and bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + TMCS; 99:1; *v*/*v*) were procured from Sigma (St Louis, MO, USA) and OH-CHRY were procured from Accustandard (New Haven, USA). Acetone (ACE), acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), chloroform (CF), trichloroethylene (TCE), tetrachloroethylene (TeCE), chlorobenzene (CB), dichloromethane (DCM), *n*-hexane (HEX) and ethyl acetate (EA) were purchased from Merck (Darmstadt, Germany). Ultrapure water was produced by the Milli-Q water purification system (Millipore, Badford, MA, USA). Individual stock solution of each PAH metabolite was prepared at a concentration of 1 mg/mL in ACN. A mixture of standard PAH metabolites were prepared by combining individual stock solutions to form a working standard of 0.1 mg/mL. All stock and standard solutions were stored at 4°C. All chemicals and reagents used in this study were of analytical grade unless otherwise stated.

DLLME procedure

Initially, urine sample (10 mL) was deconjugated with 1 mL of 5N HCl for 30 min at 70°C followed by dilution of deconjugated urine sample with equal volume of ultrapure water. The pH of urine sample was adjusted to 6 with the help of 5 N NaOH. Five milliliter of this urine sample was taken in a 15 mL centrifuge tube. To this, a mixture of disperser solvent (300 μL of EtOH) and extraction solvent (100 μL of TCE) was rapidly injected in order to form a cloudy solution followed by centrifugation at 5000 rpm for 5 min to settle down the high-density extraction solvent to the bottom of the centrifuge tube. The sedimented phase of TCE thus formed was collected by using 100 μL syringe (Hamilton, USA) and the collected phase was transferred into a GC–MS autosampler vial which was equipped with an insert of 200 μL capacity. The sample was then subjected to auto-IPS.

Auto-IPS–GC–MS-MS analysis

GC–MS-MS analysis of PAH metabolites were performed on Thermo Scientific Trace GC Ultra gas chromatograph with a TriPlus autosampler (used for auto-IPS) coupled to TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Scientific, FL, USA). The TriPlus autosampler was operated in internal standard mode and BSTFA + TMCS (99:1, *v*/*v*) were kept at internal standard position (i.e., 151) in autosampler tray. The injection of 2 μL of BSTFA + TMCS (99:1, *v*/*v*) and 2 μL of sample was performed in a single instance in programmed temperature vaporization splitless (PTV-SL) mode starting from 50°C (for 0.15 min) which was increased to 300°C at a rate of 1.8°C/s. Initial splitless time of 2 min was used followed by a split of 50 mL/min. Separation was carried out on TG-5MS capillary column (30 m length × 0.25 mm I.D. × 0.25 μm film thickness of 5% phenyl and 95% methylpolysiloxane). Flow rate of carrier gas (helium) was maintained at 1 mL/min, GC oven temperature was started from 100°C (hold for 2 min), increased to 210°C at a rate of 15°C/min and finally reached to 280°C (hold for 5 min) at a rate of 10°C/min. Transfer line temperature and ion source temperature were kept at 290 and 220°C, respectively. The mass spectrometer was operated in the positive electron ionization (+EI) mode at an electron energy of 70 eV. A solvent delay of 6 min was used. Initially, to confirm the derivatization of PAH metabolites, full scan mode was used in the mass range of 50–450 amu. After confirmation, the PAH metabolites were subjected to product ion scan, which facilitated the selection of selected reaction monitoring (SRM) transitions, which were further used for quantification of PAH metabolites. The SRM transitions, collision energies, time windows and retention times of each PAH metabolite and molecular weight before and after derivatization were shown in Table I.

Collection of urine samples from PAH-exposed workers

Urine samples were collected from tobacco users in polypropylene bottles in the health camp organized near Lucknow (India) and stored at −80°C until analysis. All the volunteers participated in the camp are healthy, and a prior consent was taken from the subjects before collecting the samples as per the ethics committee guidelines.

Method validation parameters

The limit of detection (LOD) and limit of quantification (LOQ) for all PAH metabolites were calculated with a signal to noise ratio of 3:1 and 3:10, respectively. A 6-point calibration curve for PAH metabolites in the range of LOQ level of each metabolite to 1000 ng/mL were plotted. Repeatability and reproducibility were studied as intra and inter-day precisions ($n = 5$) which were expressed in percent relative standard deviation (%RSD). Recovery studies of PAH metabolites in urine samples were performed in relation to ultrapure water at three different concentration levels of calibration graphs.

Results and discussion

The developed approach of coupling DLLME with IPS helps to improve the extraction and preconcentration of PAH metabolites

Table I
List of GC–MS-MS Parameters of PAH Metabolites

PAH metabolites	Retention time (min)	Precursor ion	Product ion	Collision energy (V)	Time window (min)	MW before derivatization	MW after derivatization
NAP	8.43	185	115	25	5	144	216
		201	185	15			
		216	201	15			
9-HF	10.52	239	165	15	5	182	254
		254	73	20			
		254	165	20			
3-HF	11.68	239	165	15	5	182	254
		254	165	20			
		254	239	20			
2-HF	11.86	239	165	15	5	182	254
		254	165	20			
		254	239	15			
PHN	12.94	235	220	20	5	194	266
		251	235	15			
		266	251	15			
1-OHP	15.90	259	244	20	5	218	290
		275	259	25			
		290	275	15			
OH-CHRY	17.94	285	270	20	5	244	316
		301	285	15			
		316	301	15			

MW, molecular weight.

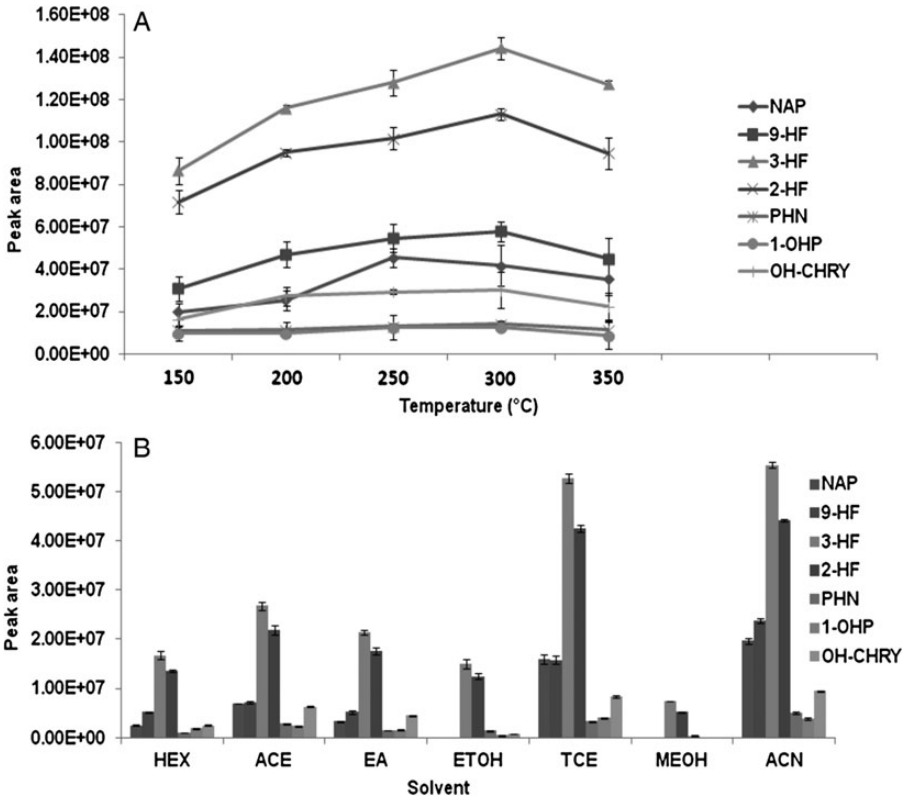

Figure 1. Optimization of IPS parameters at a concentration of 500 ng/mL of PAH metabolites, (A) effect of injector port temperature on IPS of PAH metabolites (B) effect of solvent in which PAH metabolites are dissolved. All the analysis were performed in triplicate.

in urine samples. DLLME helps in the effective extraction of PAH metabolites by utilizing microlitre of the solvent while IPS overcomes the various disadvantages of in-vial silylation. Factors affecting the performance of DLLME and IPS were optimized for the simultaneous extraction and derivatization of seven PAH metabolites followed by GC–MS-MS analysis.

Optimization of IPS parameters

Since, IPS takes place inside the injector port of GC–MS, the temperature of injector port can be regarded as reaction temperature for IPS. Therefore, the effect of injector port temperature on the yield of IPS was investigated in the range of 150–350°C (at an interval of 50°C). From Figure 1A, it is clearly visible that as the temperature of injector port raises from 150 to 300°C, the peak areas of all PAH metabolite derivatives also increases, however, beyond 300°C there is a significant decrease in the area of PAH metabolites. This can be explained by the fact that trimethylsilyl (TMS) derivatives of PAH metabolites may get degraded at this high temperature of 350°C, additionally, this temperature is also destructive to the capillary column of GC. Therefore, 300°C was selected as optimum injector port temperature for further experiments.

IPS is a gaseous phase reaction which takes place inside the GC–MS injector port. The analytes during the reaction are first derivatized with BSTFA followed by vaporization. Therefore, there is a significant effect of solvent in which analytes were dissolved. We have tested five different solvents for the IPS of PAH metabolites. These are EtOH, MeOH, ACE, EA, TCE and ACN. From Figure 1B, it is clear that those organic solvents which are not containing any oxygen atom in their structures such as TCE and ACN are giving maximum derivatization yield. In further experiment, the amount of BSTFA, was varied from 0.5 to 3 μL at an interval of 0.5 μL to get the optimum amount of BSTFA which is needed for complete derivatization of PAH metabolites. Results shown that 2 μL of BSTFA exhibited maximum peak response for all TMS derivatives of PAH metabolites (data not shown).

Selection of extraction solvent and its volume

The extraction solvent should fulfill the following conditions, i.e., (i) the density of extraction solvent should be higher than water, (ii) extraction solvent should be capable of extracting compounds of interest, (iii) it should have low solubility in water and (iv) it should exert good chromatographic behavior. Based on these requirements, the five most commonly used extraction solvents viz., DCM, TCE, CB, TeCE and CF were screened for maximum extraction efficiency of DLLME. To find out best extraction solvent among five tested solvents, a set of experiment was conducted in which 5 mL of ultrapure water was fortified with 0.5 μg/mL of PAH metabolites. Five different mixtures of extraction and disperser solvents were prepared keeping the amount of disperser solvent (acetone) constant at 500 μL and extraction solvent (DCM, TCE, TeCE, CB and CF) at 100 μL. Results show that maximum extraction efficiency of DLLME for all PAH metabolites were obtained when TCE was used as an extraction solvent. TCE has also shown almost comparable derivatization efficiency to ACN for IPS of PAH metabolites (Figure 2A). Therefore, TCE was used

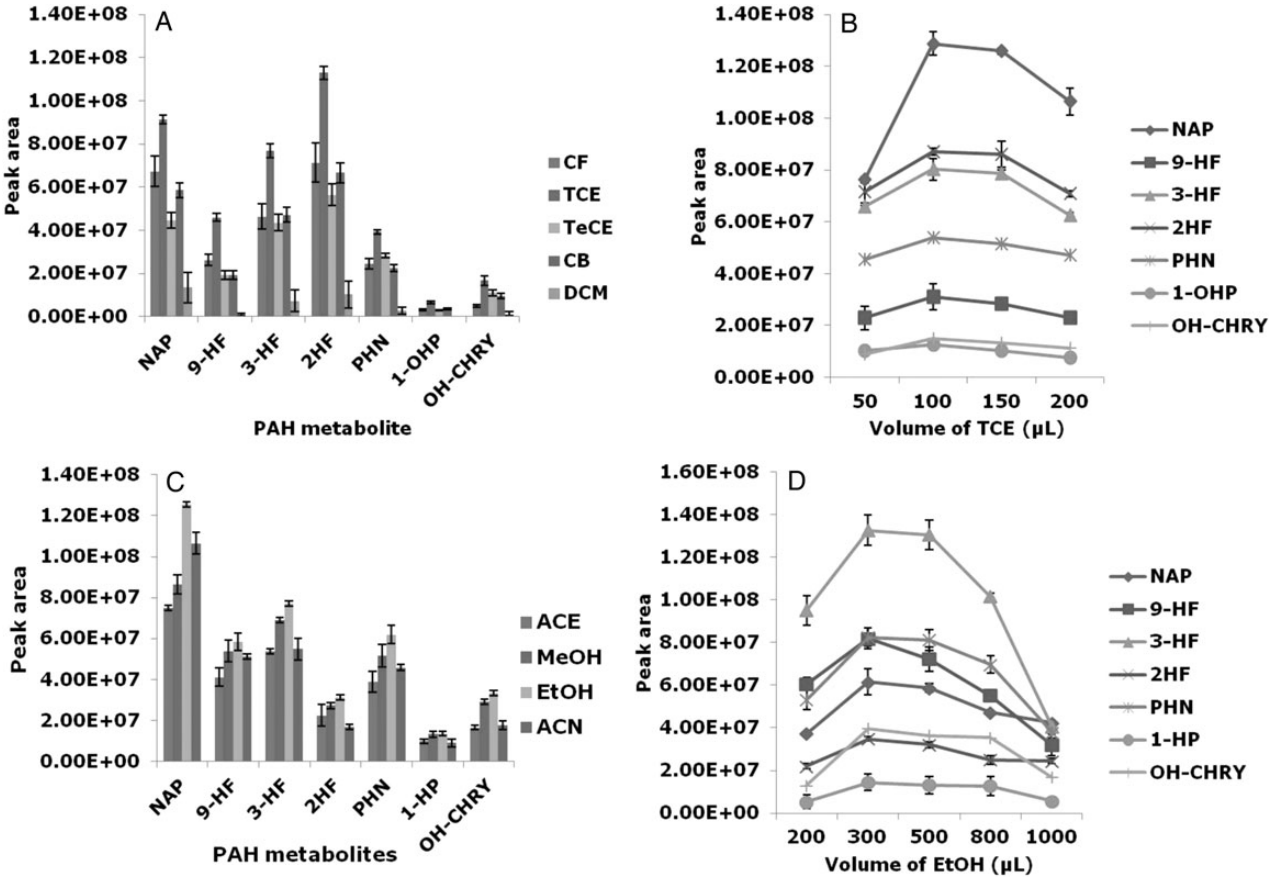

Figure 2. Optimization of DLLME parameters at a concentration of 500 ng/mL of PAH metabolites, (A) selection of extraction solvent, (B) effect of volume of TCE, (C) selection of disperser solvent and (D) effect of volume of EtOH. All the analysis were performed in triplicate.

as extraction solvent for DLLME in further experiments. In next experiment, volume of TCE was optimized. For this, a set of experiment was conducted in which variable volume of TCE in the range of 50–200 μL at an interval of 50 μL was used keeping the volume of acetone constant at 500 μL. Figure 2B shows that peak areas of all PAH metabolites were increased up to 100 μL of TCE and tend to decrease beyond this volume. The decrease in the peak areas of PAH metabolites were due to the dilution of sedimented phase with increased volume of TCE. The approximate volume of sedimented phase obtained is 60 μL as measured by using 100 μL syringe.

Selection of disperser solvent and its volume

As compulsory, the disperser solvent should be miscible in both extraction solvent and aqueous phase. The role of disperser solvent is to disperse the extraction solvent throughout the aqueous phase as fine droplets and to form a cloudy solution (water/disperser solvent/extraction solvent). On the formation of cloudy solution, the contact between extraction solvent and aqueous phase is infinitely larger, which helps in achieving equilibrium very quickly, that results in the reduction of extraction time, enhancing the enrichment factors and extraction efficiency. Based on these conditions, the four most commonly used

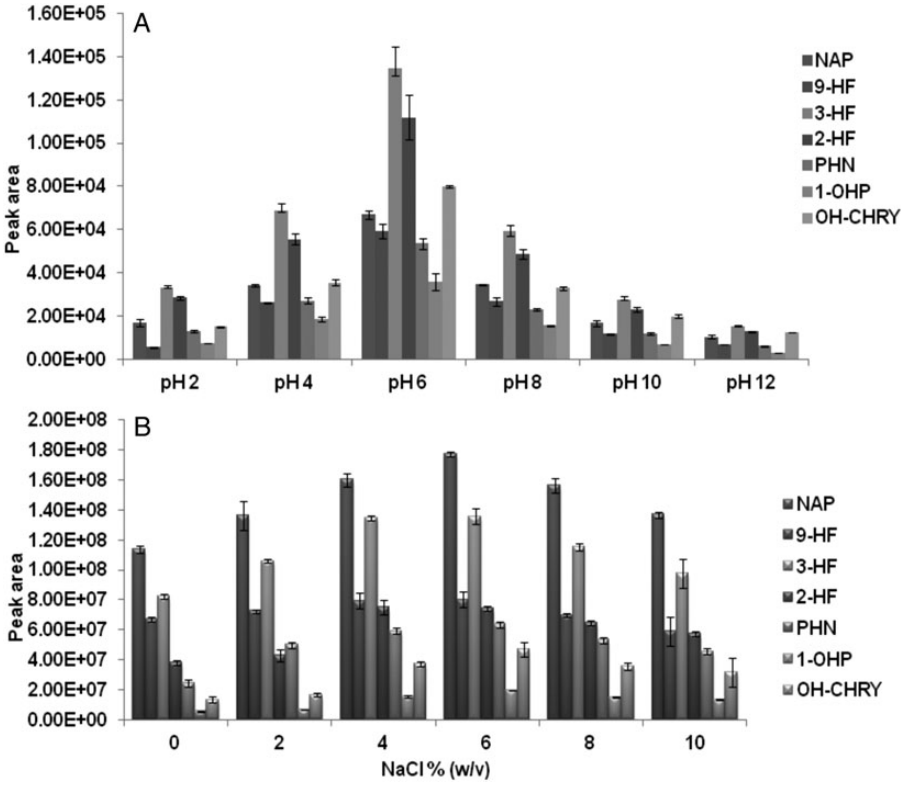

Figure 3. Effect of (A) pH, and (B) ionic strength on DLLME at a concentration of 500 ng/mL of PAH metabolites. All the analysis were performed in triplicate.

Table II
Method Validation Parameters of PAH Metabolites

PAH metabolites	Linearity (ng/mL)	R^2	Precision[a] ($n = 5$; ng/mL)						LOD (ng/mL)	LOQ (ng/mL)
			Intra-day			Inter-day				
			[b]	500	1000	[b]	500	1000		
NAP	7–1000	0.987	5.2	3.5	3.2	9.5	7.8	6.9	2	7
9-HF	7–1000	0.998	4.9	4.1	2.8	8.6	7.6	6.4	2	7
3-HF	3–1000	0.996	4.5	4.3	3.1	8.0	7.9	6.5	1	3
2-HF	4–1000	0.992	5.4	3.8	2.9	9.6	8.1	7.2	1	4
PHN	12–1000	0.991	5.8	4.2	3.3	6.2	8.4	7.3	4	12
1-OHP	30–1000	0.992	4.1	4.8	3.2	8.1	7.8	7.5	9	29
OH-CHRY	7–1000	0.999	4.8	4.7	3.4	7.0	8.3	7.1	2	7

[a]%RSD.
[b]AT LOQ level of each analyte.

disperser solvents viz., ACE, ACN, EtOH and MeOH were screened for the selection of best disperser solvent. Five milliliter of ultrapure water sample was fortified with 0.5 μg/mL of PAH metabolites and subjected for DLLME with 100 μL of TCE as extraction solvent and 500 μL of disperser solvent (ACE, ACN, EtOH and MeOH). From Figure 2C, it is evident that EtOH shown highest extraction efficiency for DLLME of all PAH metabolites. Therefore, in further experiment, the volume of EtOH was optimized as disperser solvent. For this, a set of experiment were conducted taking the volume of TCE at its optimized value (i.e., 100 μL) and varying the volume of EtOH in the range of 200–1000 μL (200, 300, 500, 800 and 1000 μL). The peak areas of all PAH metabolites were found to be increasing from 200 to 300 μL; however, there was a significant fall in the peak areas of all PAH metabolites beyond a volume of 300 μL (Figure 2D). This can be explained by the fact that, as the volume of disperser solvent increases, solubility of extraction solvent in aqueous phase also increases, which results in the declined extraction efficiency of DLLME. Therefore, EtOH at a volume of 300 μL was selected as disperser solvent in this study.

Table III
Relative Recoveries of PAH Metabolites in Urine Sample ($n = 3$)

PAH metabolites	100 ng/mL (%RSD)	500 ng/mL (%RSD)	1000 ng/mL (%RSD)
NAP	87 (6.3)	89 (3.8)	94 (2.4)
1-HF	89 (5.8)	88 (3.6)	91 (2.8)
3-HF	88 (4.9)	91 (4.1)	93 (3.1)
2-HF	91 (5.5)	94 (3.9)	95 (2.9)
PHN	88 (5.1)	90 (4.5)	93 (3.2)
1-OHP	92 (6.2)	91 (4.1)	94 (2.5)
OH-CHRY	90 (5.6)	92 (4.3)	95 (3.2)

Effect of pH and ionic strength

The pH of aqueous sample is the most critical factor which affects the extraction efficiency of DLLME. We have checked the pH of aqueous phase in the range of 2–12 (i.e., 2, 4, 6, 8, 10 and 12) in order to get optimum pH of maximum extraction efficiency of DLLME. The pH of the aqueous phase was modified with the help of 5 N HCl and 5 N NaOH. From Figure 3A, it is evident that extraction efficiency of DLLME for PAH metabolites were maximum when pH of aqueous phase was maintained at 6. All these PAH metabolites having their pK_a in the range of 9–14 (pK_a values of NAP, 9-HF, 3-HF, 2-HF, PHN, 1-OHP, OH-CHRY are 9.6, 13.9, 10.0, 9.9, 9.4, 9.5, 9.3, respectively). At this pK_a, analytes behave as weak acids in aqueous media due to lower dissociation constant. It might be the reason for obtaining maximum extraction efficiency of PAH metabolites at slightly acidic pH medium.

Addition of soluble salt in the aqueous sample decreases the solubility of analytes and increases the ionic strength of the sample (salting out effect). The salting out effect was checked by adding NaCl in aqueous sample in the range of 0–10% (w/v). Results revealed that peak area of PAH metabolites increased

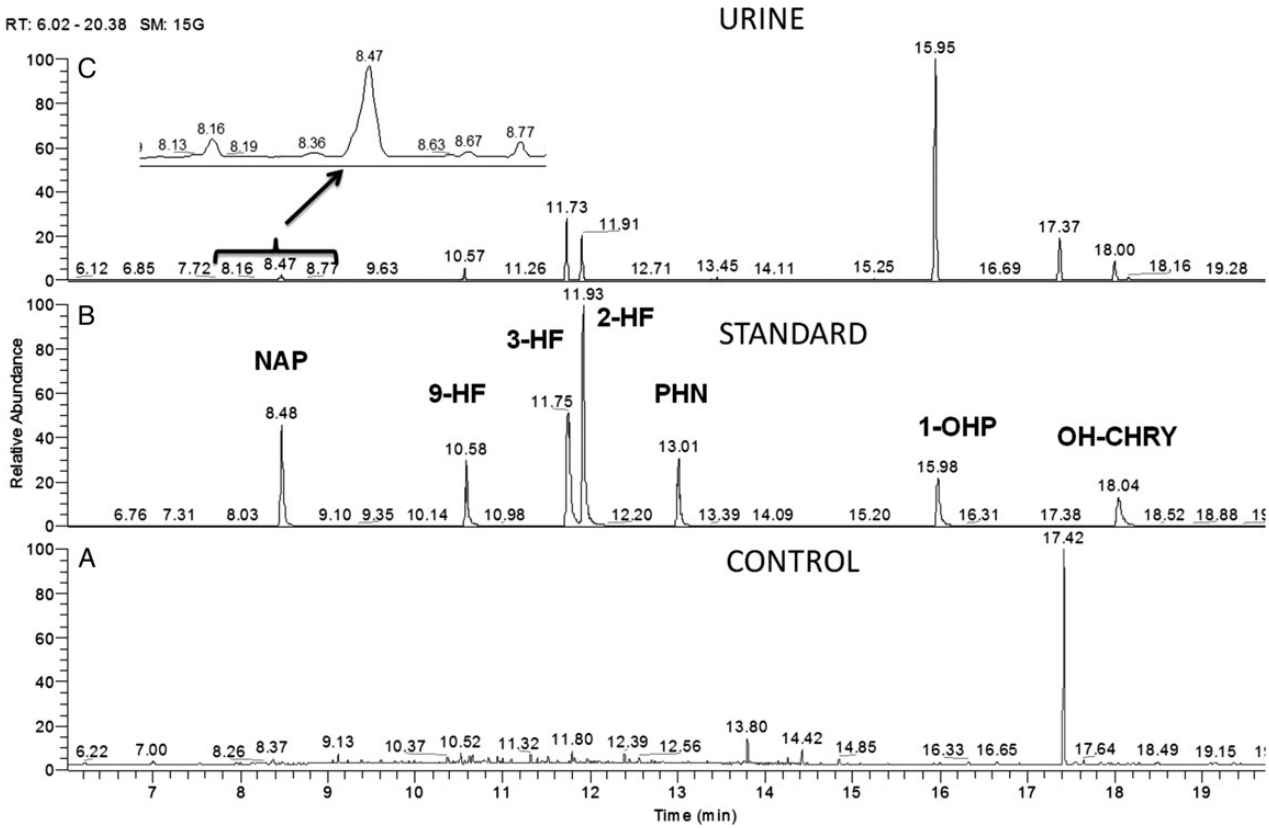

Figure 4. GC–MS-MS chromatograph of PAH metabolites in SRM mode after DLLME–auto-IPS; (A) control urine sample (B) standard at a concentration of 500 ng/mL (C) exposed urine sample.

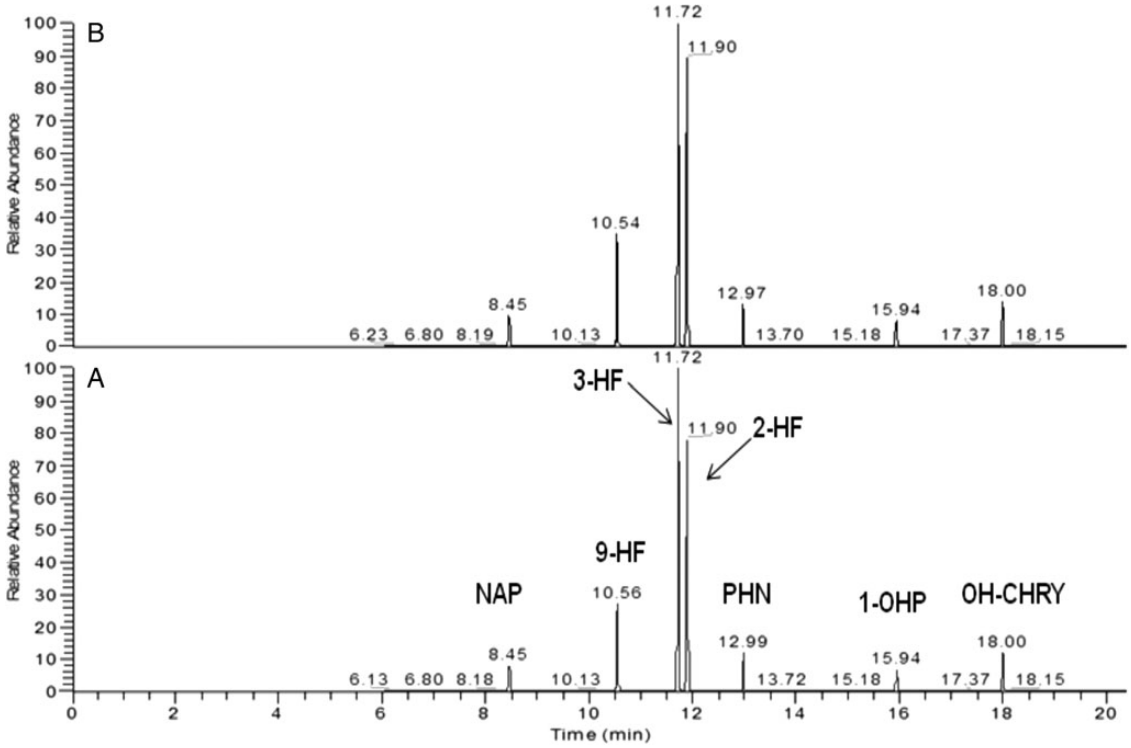

Figure 5. GC–MS-MS chromatograph of PAH metabolites in SRM mode after DLLME–auto-IPS; (A) fortified urine sample (500 ng/mL) and (B) fortified water sample (500 ng/mL).

up to 6% of NaCl and then decreased, which may be due to the increases viscosity of aqueous sample with higher concentration of salt (Figure 3B). Hence, 6% of NaCl was added in 5 mL of sample from further experiments.

Method validation

The developed DLLME–IPS–GC–MS-MS was validated for its linearity, precision, sensitivity and recoveries in fortified urine samples. A 6-point calibration curve for each PAH metabolite was plotted in the range of LOQ of each analyte to 1000 ng/mL. A good correlation between the peak areas and concentrations of PAH metabolites were obtained, which was in the range of 0.987–0.999 (R^2). The intra and inter-day precisions were expressed as %RSD. Intra-day precision (repeatability) was determined on the same day at three different concentration levels of calibration graphs, i.e., low, middle and high (100, 500 and 1000 ng/mL, $n = 5$). Similarly, inter-day precision (reproducibility) was investigated at same concentration levels for 5 subsequent days. Intra and inter-day precision was found to be in the range of 2.8–5.7 and 6.4–9.7%, respectively. LOD and LOQ were calculated at a signal to noise ratio of 3:1 and 10:1, respectively. The LOD and LOQ of PAH metabolites were found to be in the range of 1–9 and 3–29 ng/mL, respectively, in fortified urine samples (Table II). Relative recoveries in urine samples were calculated with respect to pure PAH metabolites standard fortified in ultrapure water at three different concentration levels, i.e., 100, 500 and 1000 ng/mL (Table III). The recoveries were found to be in the range of 87–95%. A GC–MS-MS chromatogram of TMS derivatives of PAH metabolites, blank and exposed urine samples in SRM mode are shown in Figure 4. Figure 5 depicts the GC–MS-MS chromatograph in SRM mode of PAH metabolites in urine and water samples. This figure illustrates very negligible effect of matrix components of urine on the peak intensity PAH metabolites. Figure S-1 depicts the GC-MS-MS chromatograph based on individual SRM transitions for each PAH metabolite after DLLME-auto-IPS in spiked urine (500 ng/ml).

Table IV
Amount of PAH Metabolites Found in Human Urine Samples (Expressed in μg/mL)

Sample no.	NAP	9-HF	3-HF	2-HF	PHN	1-OHP	OH-CHRY
1	0.319	0.160	0.025	0.064	ND	0.135	ND
2	0.297	0.134	0.030	0.069	ND	0.068	ND
3	0.286	0.147	0.021	0.079	ND	0.104	ND
4	0.264	0.111	0.033	0.0665	ND	0.115	ND
5	0.284	0.111	0.027	0.063	ND	0.1125	ND
6	0.294	0.130	0.033	0.0625	ND	0.149	ND
7	0.284	0.106	0.023	0.049	ND	0.127	ND
8	0.291	0.1245	ND	0.054	ND	0.1385	ND
9	0.286	0.104	ND	0.0445	ND	0.139	ND
10	0.286	0.107	ND	0.048	ND	0.141	ND
11	0.288	0.103	0.024	0.048	ND	0.134	ND
12	0.284	0.103	ND	0.048	ND	0.145	ND
13	ND	ND	ND	ND	ND	0.142	ND
14	0.273	ND	ND	0.044	ND	0.130	ND
15	0.2745	ND	ND	0.042	ND	0.136	ND

ND, not detected.

Application of developed method to human urine samples

The newly developed DLLME–IPS–GC–MS-MS method has been successfully applied for the determination of PAH metabolites in 15 urine samples of exposed workers. Two PAH metabolites, PHN and OH-CHRY, could not be detected in any urine sample.

The amount of PAH metabolites was found to be in the range of 0.02–0.319 μg/mL in all tested urine samples.

Table IV shows the amount of PAH metabolites detected in urine samples of exposed workers. No PAH metabolites were detected in blank urine samples.

Conclusion

In the present communication, a rapid, eco-friendly, economic and sensitive analytical method, based on coupling of DLLME and auto-IPS followed by GC–MS-MS analysis for the determination of urinary PAH metabolites has been reported. Since DLLME is fast, green and simple microextraction method, it overcomes the drawbacks of traditional extraction methods such as SPE and LLE whereas, IPS knock out the disadvantages of conventional in-vial silylation such as requirement of lengthy reaction time, moisture-free conditions and elevated temperatures. The method is well validated and has been successfully applied for the determination of PAH metabolites in urine samples of exposed workers. The developed method may be of immense use for the routine analysis of urinary PAH metabolites by various analytical laboratories.

Supplementary Data

Supplementary data are available at *Journal of Analytical Toxicology* online.

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