Brazilian Journal of Analytical Chemistry
Pre-publication – Accepted after peer review
doi: 10.30744/brjac.2179-3425.AR-69-2024

BrJAC

ARTICLE

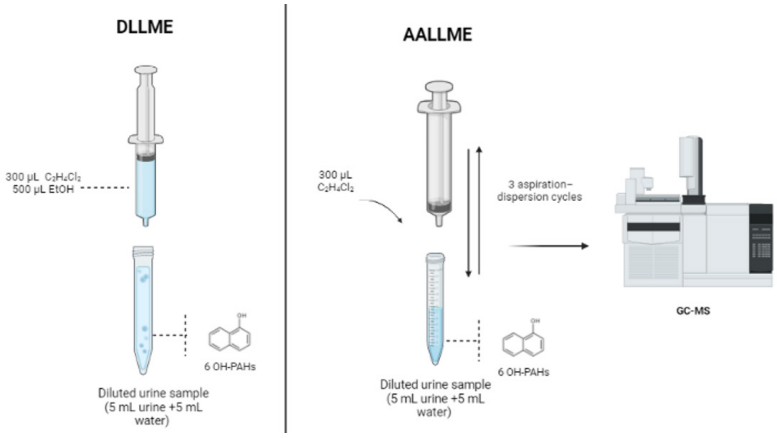
Air-assisted Liquid-Liquid Microextraction Procedure to access Mono-Hydroxylated Polycyclic Aromatic Hydrocarbons Metabolites in Human Urine Samples

Marília Cristina Oliveira Souza[1], João Carlos Jacinto Souza[2], Jonas Carneiro Cruz[2], Bruno Alves Rocha[3]*, Fernando Barbosa Jr[2]

[1]Departamento de Ciências Biomoleculares, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo. Av. do Café s/nº, 14040-903, Ribeirão Preto, SP, Brazil

[2]Laboratório de Toxicologia Analítica e Sistêmica, Departamento de Análises Clínicas, Toxicologia e Ciências dos Alimentos, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/nº, 14040-903, Ribeirão Preto, SP, Brazil

[3]Instituto de Química, Universidade Federal de Alfenas, 37130-001, Alfenas, MG, Brazil



In this study, the air-assisted liquid-liquid microextraction (AALLME) and the dispersive liquid-liquid microextraction (DLLME) techniques were compared in determining six mono-hydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) in human urine samples by gas chromatography coupled to mass spectrometry (GC-MS). The parameters (type and volume of extraction and dispersive solvent, extraction cycles, sample pH, and ionic strength) that influence the extraction efficiency in the proposed procedures were investigated and optimized. After optimization, both methods were compared in terms of extraction efficiency. Although both AALLME (16-83%) and DLLME (15-92%) provided comparable extraction efficiencies, AALLME outperformed DLLME in determining four in six OH-HPAs. In addition, the technique used less organic solvent as it dismissed the dispersive solvent. Therefore, the developed AALLME method was chosen to analyze figures of merit, showing adequate linearity ($r^2 > 0.99$) ranging from 0.72-1.92 (LOQ) to 20 ng mL^{-1}. LODs ranging from 0.24 to 0.69 ng mL^{-1} were obtained. Precision (3.1 to 14.9%) and accuracy (7.8 to 13.3%) were assessed at three concentration levels (3.0, 9.0 and 15.0 ng mL^{-1}). The new method was applied to 20 human urine samples, randomly collected from

Cite: Souza, M. C. O.; Souza, J. C. J.; Cruz, J. C.; Rocha, B. A.; Barbosa Jr, F. Air-assisted Liquid-Liquid Microextraction Procedure to access Mono-Hydroxylated Polycyclic Aromatic Hydrocarbons Metabolites in Human Urine Samples. *Braz. J. Anal. Chem.* (Forthcoming). http://dx.doi.org/10.30744/brjac.2179-3425.AR-69-2024

Submitted May 31, 2024, Resubmitted July 23, 2024, 2nd time Resubmitted September 4, 2024, Accepted September 10, 2024, Available online October, 2024.

This article was submitted to the BrJAC special issue celebrating the achievement of a Journal Impact Factor.

healthy Brazilian adults, to confirm its applicability. Finally, the proposed and validated AALLME method was considered simple, fast, and convenient, making it a straightforward strategy for large-scale biomonitoring studies.

Keywords: microextraction, OH-PAHs, urinary biomarkers, GC-MS, human biomonitoring

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are omnipresent contaminants with a high potential to impact human health.[1] Because these compounds may have carcinogenic, mutagenic, and teratogenic effects public concerns of their widespread presence have emerged recently.[2,3]

Human exposure to PAHs is frequently monitored by determining its urinary metabolites, mainly excreted as hydroxylated-PAHs (OH-PAHs) glucuronide conjugates.[1,3] A sample preparation technique is essential to isolate and concentrate target compounds from urine, enhancing the detectability of OH-PAHs present in low concentrations.[4,5] Conventional sample preparation procedures, such as liquid–liquid extraction and solid-phase extraction, are predominantly employed for the quantification of OH-PAH concentrations in urine samples.[4–6] However, these procedures have been replaced with microextraction techniques to overcome the limitations of classical techniques.[7–13]

One microextraction technique frequently used in sample preparation of bioanalytical methods is dispersive liquid-liquid microextraction. DLLME is a sample preparation procedure involving a water-insoluble extractive solvent, and a disperser solvent (miscible with both the extractive solvent and the sample).[14,15] In DLLME, these two solvents are quickly injected into the sample, causing a rapid turbidity known as cloud point formation. This phenomenon increases the contact area between the extractive solvent and the sample, facilitating the partitioning of the analyte into the organic phase. The sample is then centrifuged to deposit the microdroplets, and the sedimented phase is removed and transferred for further analysis. However, adding a dispersive solvent may promote the relocation of the analytes into the aqueous phase, reducing extraction efficiency.[14,16,17]

To overcome this limitation, Farajzadeh and Mogaddam proposed an update of the DLLME technique, known as air-assisted liquid–liquid microextraction (AALLME).[16,18] In this procedure, both the aqueous and extraction solvents are drawn into a syringe and pushed out repeatedly. This process increases the surface contact between the sample and the organic layer, accelerating mass transfer, shortening analysis time, and increasing extraction efficiency. This approach reduces organic solvent consumption, being suitable for extracting most organic compounds in different matrices, such as urine. The increased surface contact area contributes to higher extraction efficiency compared to DLLME or vortexing alone, making AALLME a distinct and more efficient microextraction method.[16,18]

In this study, a new AALLME procedure (using conventional solvents) was developed and compared with DLLME to determine metabolites of PAHs in human urine (the best strategy for human exposure assessment) by gas chromatography coupled with mass spectrometry (GC-MS). The AALLME-GC-MS method, characterized by its procedural simplicity and outstanding analytical performance, presents a highly favorable alternative for the comprehensive monitoring of OH-HPAs across numerous urine samples. Finally, the proposed approach was applied to determine the OH-HPAs in 20 urine samples from healthy Brazilians.

MATERIALS AND METHODS

Chemical and Reagents

The analytical standards for six OH-HPAs included 1-hydroxy-naphthalene (1OH-NAP), 2-hydroxy-naphthalene (2OH-NAP), 2-hydroxy-fluorene (2OH-FLU), 9-hydroxy-fluorene (9OH-FLU), 9-hydroxy-phenanthrene (9OH-PHE), and 1-hydroxy-pyrene (1OH-PYR). Both the native standards and the internal standard (IS), 1-hydroxy-naphthalene-d7 (1OH-NAP-*d*7), were obtained from Toronto Research Chemicals® (North York, ON, Canada), Accustandard® (New Haven, CT, USA), and Sigma-Aldrich®

(St Louis, MO, USA). Individual stock solutions were prepared in methanol and stored in amber glass vials at -20 °C. Working solutions were prepared via serial dilution with acetonitrile and stored in amber glass vials at 4 °C until analysis. The derivatizing reagent, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), was obtained from Sigma-Aldrich® (St Louis, MO, USA).

The solvents used in extraction procedures, all of HPLC grade, included methanol and chloroform, which were purchased from JT Baker® (Phillipsburg, NJ, USA), as well as ethanol, acetone, isopropanol, acetonitrile, dichloromethane, and dichloroethane, obtained from Sigma-Aldrich® (St. Louis, MO, USA). High-purity deionized water, with a resistivity of 18.2 MΩ·cm, was produced using a Milli-Q water purification system (Millipore RiOs-DITM®, Bedford, MA, USA).

The reagents (analytical grade) employed for the preparation of synthetic urine were obtained from Sigma-Aldrich®). Due to a lack of OH-HPAs free human urine samples, synthetic urine was used for optimization and calibration purposes. This strategy has been previously applied for these purposes in the determination of different contaminants.[19–22] The β-glucuronidase enzyme from *Helix pomatia* (type H-1) in a partially purified powder form (≥100,000 units/g solid), was obtained from Sigma-Aldrich®.

Sample collection and enzymatic hydrolysis

This study was approved by the Research Ethics Committee of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (CAAE: 91124418.2.0000.5403). Twenty urine samples were collected from eight male and twelve female participants, aged 18 to 35 years. Each participant provided approximately 50 mL of urine in polypropylene tubes, which were stored at 5 °C until analysis. Participants were instructed to collect the first urine of the day or with a 4-hour interval between urinations, clean their genitalia with water and soap, and deliver the samples within 1 hour of collection.

Due to the short half-life of PAHs in the human body, they are quickly excreted in urine primarily as glucuronide conjugates. Thus, the metabolites (OH-PAHs) are effective biomarkers for assessing human exposure to PAHs following a hydrolysis step. It is well-established that PAHs are mainly excreted as glucuronide conjugates, with only a small fraction excreted in their free form. Consequently, human urine must undergo an initial hydrolysis step with β-glucuronidase prior to the determination of urinary concentrations of PAHs.[1] Therefore, before the extraction step, 5 mL of each human urine sample was transferred into 15 mL conical tubes and spiked with an internal standard solution (1OH-NAP-*d*7, final concentration: 20 ng mL^{-1}). An amount of 100 µL of 1.0 mol L^{-1} ammonium acetate buffer (pH 5) and β-glucuronidase (2000 units) was added to the samples (overnight incubation at 37 ºC).[23]

Optimization of the AALLME and DLLME conditions

In this assay, 5 mL of synthetic urine was diluted with 5 mL milli-Q water and spiked with a fixed amount of six OH-PAH (1OH-NAP, 2OH-NAP, 9OH-FLU, 2OH-FLU, 9OH-PHE, 1OH-PYR) corresponding to a final concentration of 50 ng mL^{-1}. The extraction efficiencies were evaluated by comparison of peak areas.

For the AALME procedure, the influence of the following parameters was univariately optimized (n=3) to achieve the highest extraction efficiencies: number of extraction cycles (0-10), type (chloroform, dichloromethane, and 1, 2-dichloroethane) and volume of extraction solvent (100-1000 µL), sample pH (2-5.5) and ionic strength (no salt addition - 10% NaCl).

For the DLLME technique, the following conditions were univariately (n=3) optimized: type (chloroform, dichloromethane, and 1,2-dichloroethane) and volume (100-500 µL) of extraction and dispersive solvent (acetone, acetonitrile, ethanol, isopropanol, and methanol; 200-1000 µL), pH (2-5.5) and ionic strength (no salt addition – 10% NaCl).

In the optimized conditions, both techniques were compared according to their extraction efficiencies. The sample volume was fixed at 5 mL, and the OH-PAHs concentration at 50 ng mL^{-1}. The best sample preparation procedure was further analyzed regarding analytical figures of merit and applied to a pilot biomonitoring study.

Optimized AALLME procedure

For the AALLME, 5 mL of the urine samples (after enzymatic hydrolysis) was diluted to 10 mL with ultrapure water (15 mL conical tube), and 25 µL of a methanolic solution containing the IS (1-OH-NAP-*d*7, final concentration: 20 ng mL^{-1}). pH adjustment and salt addition were not necessary. Subsequently, 300 µL of 1,2-dichloroethane (extraction solvent) was injected into the sample, aspirated, and dispensed into it to form a cloudy solution. A Hamilton glass syringe with a stainless-steel needle (1.6 mm of internal diameter) was used. After performing 3 aspiration-dispersion cycles, the mixture was vortex-mixed for 60 seconds and centrifuged for 5 min at 20 ºC (2500 x *g*). The AALLME apparatus was cleaned with methanol and washed with ultra-pure water before extraction to prevent sample contamination. The sedimented phase (160 µL) was transferred to a 2 mL Eppendorf tube using a micropipette, evaporated to dryness, and redissolved with 100 µL of a mixture of derivatizing reagent (BSTFA – 1% TMCS) with acetonitrile (25:75 v/v). After vortexing for 30 seconds, the mixture was transferred to an amber vial and adequately sealed with a crimping tool. Then the analyte's derivations were performed at 60 °C for 45 minutes in a water bath and injected into the GC-MS (2 mL).

Optimized DLLME procedure

In the DLLME procedure, a 5 mL urine sample was diluted with 5 mL of ultrapure water after enzymatic hydrolysis (15 mL conical tube). 25 µL of IS (1-OH-NAP-*d*7 in methanol) was added, resulting in a final concentration of 20 ng mL^{-1} (pH adjustment or salt addition was not necessary). Separately, a mixture of extraction (300 µL of dichloroethane) and dispersive solvents (500 µL of ethanol) was aspirated using a syringe and quickly dispensed (in a single movement) at the sample to form the cloud point. The tubes were vortexed for 45 seconds and centrifuged for 5 minutes at 20 ºC (2500 x *g*). The residue of organic phase (standardized volume of 160 µL) was transferred to 2.0 mL Eppendorf tubes and evaporated to dryness in a vacuum concentrator.

Instrumental analysis

HPA metabolites were measured following enzymatic deconjugation of glucuronidated HPAs. This was succeeded by either AALLME or DLLME microextraction procedures, coupled with electron impact ionization in gas chromatography-mass spectrometry (GC-MS) after derivatization of the OH-PAHs to their trimethylsilylated derivatives, as detailed in the supplementary material.[23]

RESULTS AND DISCUSSION

Optimization of the microextraction techniques

Optimization of the AALLME procedure

The evaluation and selection of the extraction solvent selection were based on the following requirements: low aqueous solubility, ability to be easily dispersed into the aqueous solution during the dispersing stage, and high extraction efficiency.[16] Therefore, the following common classical solvents were investigated: chloroform, dichloromethane, and 1,2-dichloroethane. Figure 1a shows that $C_2H_4Cl_2$ was the most effective solvent to extract the analytes, highlighted by the higher peak areas for most analytes. This result might be explained by the intermediate polarity of 1,2-dichloroethane compared to chloroform and dichloromethane. Similarly, OH-PAHs might be considered compounds with medium polarity due to a hydrophobic chain derived from the unmodified PAH and the hydroxyl group provided by phase I metabolism.[24,25]

The volume of the extraction solvent may largely impact the extraction in AALLME. Generally, the lowest volume of solvent is preferable to ensure the environmentally friendly characteristics of the techniques. However, the volume should be enough to provide efficient extraction to all analytes and availability for further chromatographic analysis.[5,6] The results indicate that the extraction efficiency substantially increased from 200 to 300 µL, and then small increases were obtained for most compounds when 500 µL was used. For more significant volumes, the peak areas of some analytes decreased, which might be explained by the excess of the extractor solvent. In this case, the organic solution may alter the polarity of the aqueous

phase, increasing the analyte's solubility in the sample. Thus, 300 µL of 1,2-dichloroethane was selected as the extraction solvent with adequate extraction for most analytes (Figure 1b).

The repeated aspiration/dispersion cycle is the key mechanism in AALLME. Generally, higher extraction cycles are expected to improve the analyte's recovery until the extraction equilibrium.[19,22,26] To optimize this step, the extraction cycles were evaluated from 0 to 10. Figure 1c shows that increasing the extraction cycles from zero to one produced a significant increase in the extraction of all analytes, remaining constant after three cycles. Although the peak area for the analytes using one and three cycles was similar, three extraction cycles were selected for the subsequent experiments as they afforded fewer standard deviations in a short time (approximately 30 s).

Sample pH is also an essential parameter in extraction methods.[5,6,27,28] Therefore, the sample pH solution was evaluated ranging from 2 to 5.5. The extraction efficiency from urine samples at their natural pH of 5.5 was compared to that of samples acidified to pH 2.0 and pH 4.0. No significant differences in extraction efficiency of OH-HPA metabolites were observed. It appears that a pH of 5.5 is sufficient to ensure the neutrality of the studied metabolites (with typical pKa values greater than 9). Therefore, pH adjustment is unnecessary.[25]

The salt addition was also evaluated to investigate if the salting-out effect could decrease the solubility of the analytes (and organic solvents) in the sample solution.[5,6,10] The experiments were performed in the range of 0-10% (w/v, sodium chloride). Overall, adding NaCl did not favor the extraction (Figure 1e). Therefore, further analysis was carried out without controlling ionic strength to minimize the time during the sample preparation procedure.

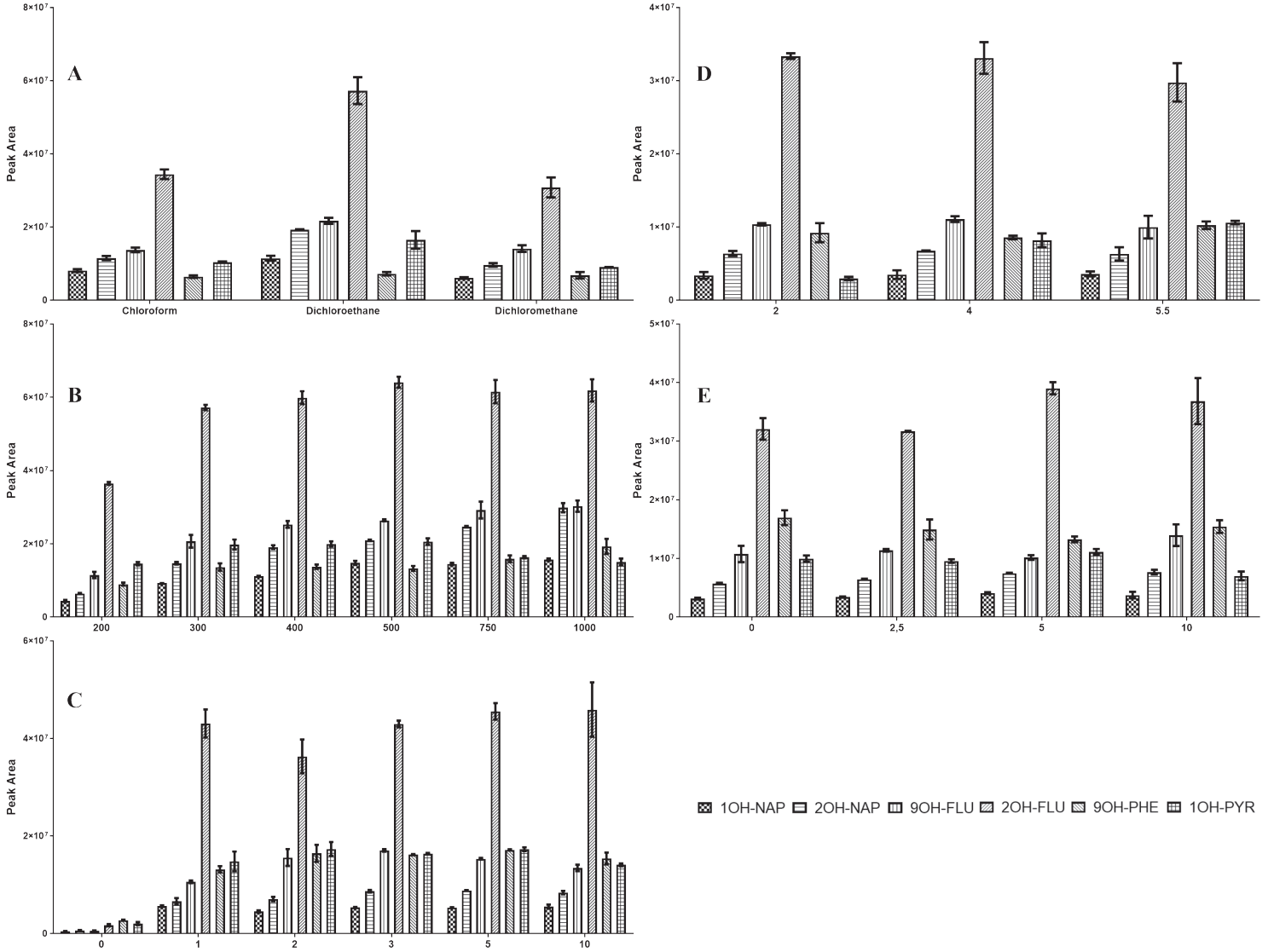


Figure 1. Optimization of the AALLME parameters to extract six OH-PAHs in synthetic urine samples: A) extraction solvent type, B) extraction solvent volume, C) number of extraction cycles, D) sample pH, and E) ionic strength.

Optimization of the DLLME procedure

Similarly, to the experiments conducted for the AALME optimization, the following solvents were evaluated as extraction solvents: chloroform, dichloromethane, and 1,2-dichloroethane in volumes ranging from 100 to 500 µL. Figure 2a illustrates that 1,2-dichloroethane afforded the highest peak areas for most analytes. This result was similar to those for AALME and might also be explained by the intermediate polarity of 1,2-dichloroethane.

For the extraction solvent volume, Figure 2b shows that the lowest extraction efficiency was obtained for 100 µL, possibly due to the ineffective partitioning equilibrium of the analytes between the aqueous phase and the organic phase. For volumes greater than 200 µL, all analytes' extraction efficiency remained almost constant, except for 1OH-PYR, the less polar compound. These results might also be explained by an excess of the extractor solvent in the aqueous sample, which can change the polarity of the solution and favor the portioning equilibrium for more hydrophobic analytes.

Appropriate dispersive solvent selection is essential to ensure adequate extraction efficiency. This solvent must be miscible with the aqueous phase and with the extraction solvent to favor the extractor solvent's dispersion as tiny droplets in the sample (increasing surface area and mass transfer).[17] In this context, the following dispersive solvents were evaluated: acetone, acetonitrile, ethanol, isopropanol, and methanol. As observed in Figure 2c, ethanol afforded the highest peak area for most compounds.

The volume of the dispersive solvent is also an essential parameter in DLLME. The volume should be enough to ensure the adequate formation of the cloud point but the small as possible to not alter the aqueous sample's polarity.[14,15,17,27] Therefore, 200, 300, 500, 750 e 1000 µL were evaluated as the volume of the dispersive solvent. The results showed that 200 µL of the dispersive solvent afforded the lowest extraction efficiency for most compounds (Figure 2d), possibly because of insufficient dispersion of the extraction solvent in the sample (consequently failing to form an adequate cloud point). The difference between the largest volumes tested after 500 µL was insignificant, so this volume was selected for the dispersive solvent.

Similarly to the AALLME procedure, the addition of NaCl (Figure 2e) and sample acidification (Figure 2f) did not improve the extraction efficiency for most analytes. Therefore, sample pH correction and NaCl addition modification were not conducted to reduce analysis time and sample handling.

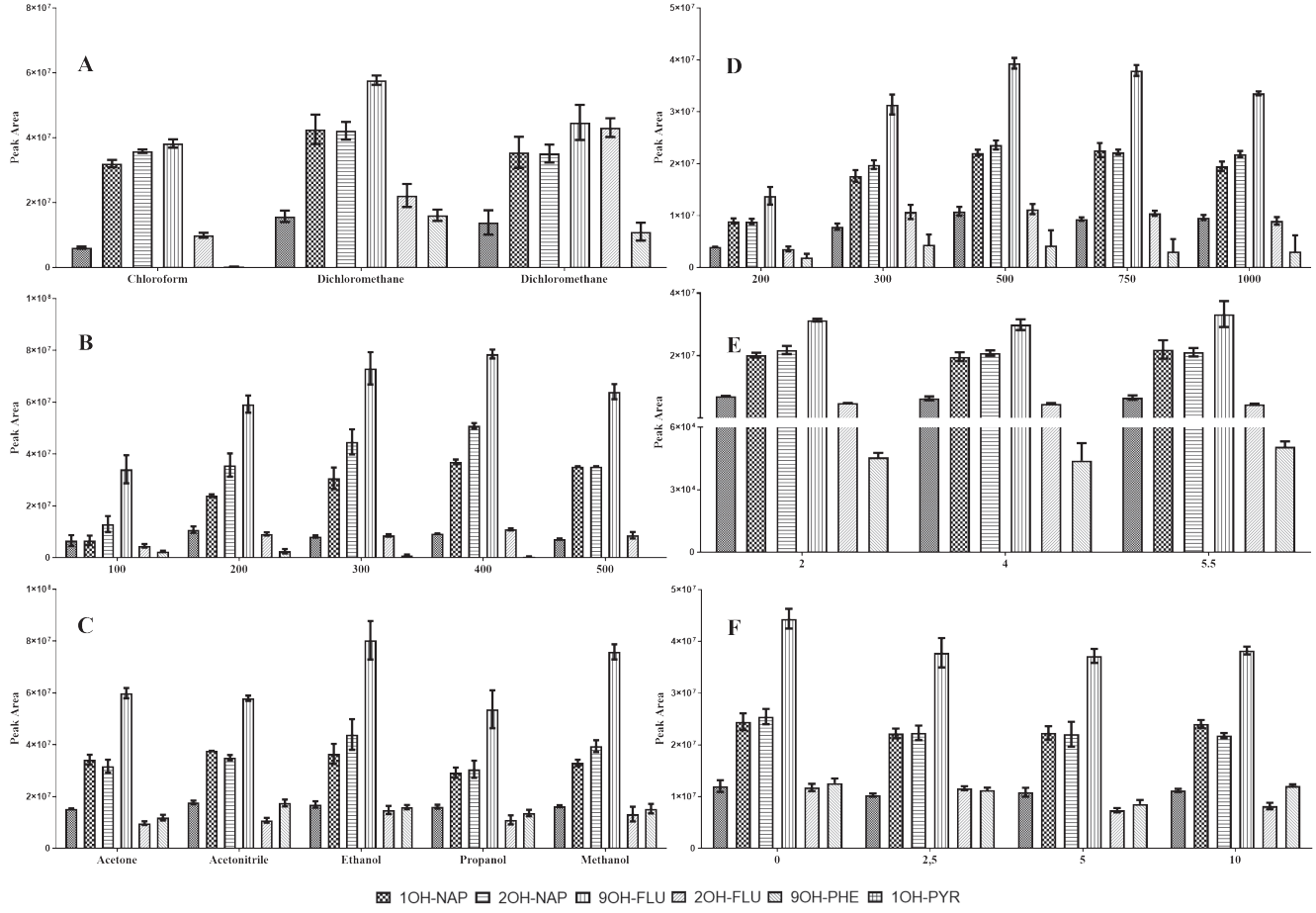


Figure 2. Optimization of the DLLME parameters to extract six OH-PAHs in synthetic urine samples: A) extraction solvente type, B) extraction solvent volume, C) dispersive solvent type, D) dispersive solvent volume E) sample pH, and F) ionic strength.

Comparison between the optimized AALLME and DLLME procedure

In the optimized conditions, both AALLME and DLLME were compared regarding extraction efficiencies (Table S2). In both techniques, 9OH-FLU, 2OH-FLU, 9OH-PHE and 1OH-PYR were the compounds with the highest extraction efficiency. Figure 3 illustrates that both techniques showed similar performances for most analytes, with recovery ratios varying from 16 to 83% and from 15 to 92% for AALLME and DLLME, respectively. In this context, both techniques showed good performance in extracting more hydrophobic compounds, such as 9OH-PHE (containing 14 carbon atoms) and 1OH-PYR (containing 16 carbon atoms) while poor efficiencies were observed for 1OH-NAP and 2OH-NAP (containing 10 carbon atoms). However, the AALLME procedure outperformed DLLME for extracting four in six compounds, using the same volume of extractor solvent (300 µL). In addition, the AALLME technique dismisses dispersive solvent, showing more environmentally friendly characteristics. Therefore, the AALLME technique was selected for validation in a pilot biomonitoring study.

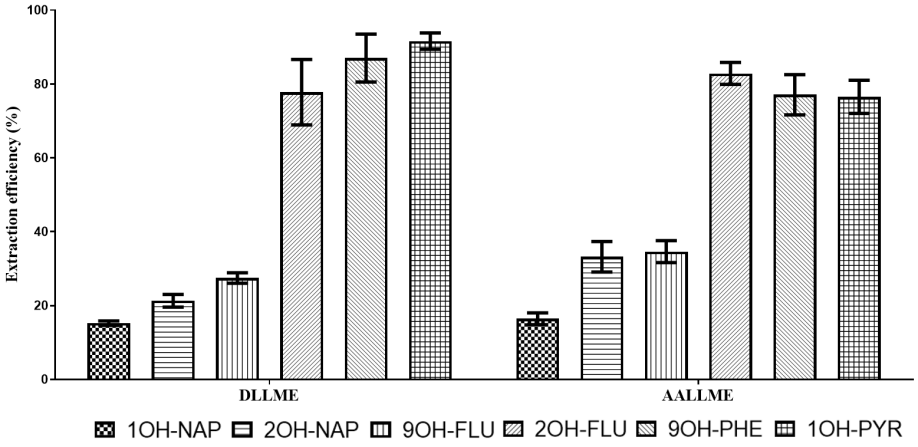


Figure 3. Comparison of the extraction efficiencies between AALLME and DLLME.

Analytical figures of merit of the optimized AALLME procedure

The analytical figures of merit of the proposed procedure were evaluated using synthetic urine samples that mimic natural composition, and under optimized conditions, the method was assessed with a matrix-matched calibration curve. Moreover the proposed method was validated by evaluating linearity, limits of detection and quantification, precision, accuracy, and matrix effect according to the according to the ICH and EMA guidelines on bioanalytical method validation.[29]

Linearity was assessed by a six-concentration (n=3) analytical curve for each OH-PAH plotting the ratio of the analyte to IS (1OH-NAP-*d*7; 20 ng mL^{-1}) peak areas obtained from three independent chromatographic runs. The concentration ranges tested for each analyte were as follows: 1OH-NAP from 1.95 to 20.0 ng mL^{-1}, 2OH-NAP from 0.72 to 20.0 ng mL^{-1}, 9OH-FLU from 1.75 to 20.0 ng mL^{-1}, 2OH-FLU from 2.09 to 20.0 ng mL^{-1}, 9OH-PHE from 1.89 to 20.0 ng mL^{-1}, and 1OH-PYR from 1.75 to 20.0 ng mL^{-1}. As shown in Table I, the optimized AALLME method provided adequate linearity ranging from 0.72-2.09 (LOQ) to 20 ng mL^{-1} with R^2 greater than 0.99 for all OH-PAHs, indicating adequate linearity. Moreover, the linearity was checked using the F test for lack-of-fit, and a p value of 0.05. The linear model for the relationship between concentration and response was considered to be appropriate as no significant lack of fit was observed (Table I).

The limit of detection LOD was defined as the lowest concentration of the analyte in the sample that could be reliably detected and identified with the method, estimated by the signal/noise ratio (three-fold). The LOQ was defined as the lowest concentration of analyte that can be determined with acceptable repeatability and trueness considering the ten-fold signal/noise ratio. The calculated LODs ranged from 0.24 to 0.69.

Precision and accuracy were assessed within-run (six spiked synthetic urine samples for each concentration on the same day) and between-run (six spiked synthetic urine samples for each concentration for three consecutive days) at three concentration levels, namely 3.0, 9.0, and 15.0 ng mL^{-1}. The precision results were expressed as the relative standard deviation (RSD%), and the accuracy results were expressed as the relative error (RE%) using the following formula: (measured value-nominal value)/ nominal value) × 100. Values lower than 15% were established as the acceptance criteria. The accuracy and precision (Table II) values were within-run 15% of the variation, which agrees with most validation guidelines. Accuracy (RE%) varied from -7.8 to 13.3% for the whitin-run test and from -7.8 to 13.4% for the between-run test. Precision ranged from 3.9 to 14.9% and from 3.1 to 13.1% for the within-run and between-assay investigation.

Matrix effect (ME) was evaluated at the concentration level of 9.0 ng mL^{-1} and expressed as the normalized matrix factor (NMF). NMF was calculated as the ratio of the analytical response (IS-normalized) in the presence of the matrix compared to the peak area (also normalized by the IS) of the same analyte

in an aqueous sample (in the absence of the matrix) multiplied by 100. Values lower than 15% were set as the acceptance criteria. The NMF values varied from 2.7 to 13.1%, showing no significant matrix effect (Table I). Selectivity was evaluated by monitoring the analytical signal of a pool of blank urine samples at the same retention time as the analytes and IS. Acceptance criteria were set as analytical responses less than 20% of the LOQ for the OH-PAHs and 5% for the IS. The absence of detected peaks attested to the method selectivity. Figure 4 shows the chromatographic analysis of the OH-PAHs at the LOQ.

Table I. Linear ranges (ng mL^{-1}), linear equations, coefficients of determination, limits of detection (ng mL^{-1}), and matrix effect for the OH-PAHs in the AALLME-GC-MS method

Analyte	Linear Range	Linear equation	R^2	ANOVA Lack-of-fit		LOD	Matrix effect (NMF)
				F-Value	*p*-Value		
1OH-NAP	1.95 - 20.00	0.1400x – 0.0621	0.9916	1.36	0.423	0.65	13.1
2OH-NAP	0.72 - 20.00	0.1649x + 0.1625	0.9968	2.02	0.236	0.24	2.7
9OH-FLU	1.75 - 20.00	0.1434x + 0.0831	0.9972	0.96	0.185	0.58	7.3
2OH-FLU	2.09 - 20.00	0.4015x – 0.1084	0.9944	1.15	0.365	0.69	9.9
9OH-PHE	1.89 - 20.00	0.1960x + 0.0690	0.9944	2.22	0.335	0.62	14.4
1OH-PYR	1.75 - 20.00	0.1158x – 0.1828	0.9980	1.74	0.520	0.58	11.8

Coefficients of determination (R^2); Limits of detection (LOD); Normalized matrix factor (NMF); F_{tabled} = 3.11.

Table II. Accuracy and precision of the AALLME-GC-MS method

OH-PAHs[a]	Spiked (ng mL^{-1})	Within-run[a]			Between-run[c]		
		Found	Accuracy (%)	Precision (RSD%)[b]	Found	Accuracy (%)	Precision (RSD%)[b]
1OH-NAP	3.0	3.2	6.7	9.5	3.1	3.3	12.5
	9.0	8.6	-4.4	8.6	8.6	-4.4	8.7
	15.0	14.3	-4.7	7.9	14.3	-4.7	9.2
2OH-NAP	3.0	2.8	-6.7	9.2	2.9	-3.3	10.7
	9.0	8.8	-2.2	3.9	8.8	-2.2	3.1
	15.0	15.2	1.3	5.9	15.0	0	6.7
9OH-FLU	3.0	2.9	-3.3	8.2	2.9	-3.3	8.3
	9.0	8.3	-7.8	5.1	8.3	-7.8	5.5
	15.0	15.5	3.3	5.1	15.4	2.7	7.6
2OH-FLU	3.0	3.2	6.7	14.9	3.0	0	12.7
	9.0	9.0	0	4.7	9.1	1.1	10.8
	15.0	17.0	13.3	4.6	16.5	10	6.7

(continued on next page)

Table II. Accuracy and precision of the AALLME-GC-MS method (continuation)

OH-PAHs[a]	Spiked (ng mL^{-1})	Within-run[a]			Between-run[c]		
		Found	Accuracy (%)	Precision (RSD%)[b]	Found	Accuracy (%)	Precision (RSD%)[b]
9OH-PHE	3.0	3.3	10	10.7	3.3	10	13.1
	9.0	10.2	13.3	4.8	10.21	13.4	8.5
	15.0	15.7	4.7	7.9	15.7	4.7	12.0
1OH-PYR	3.0	3.3	10	8.7	3.4	13.3	12.7
	9.0	10.0	11.1	9.2	9.9	10	8.0
	15.0	16.7	11.3	9.3	16.2	8	10.9

[a]Number of replicates = 3; [b]Relative standard deviation; [c]based on three different consecutive days.

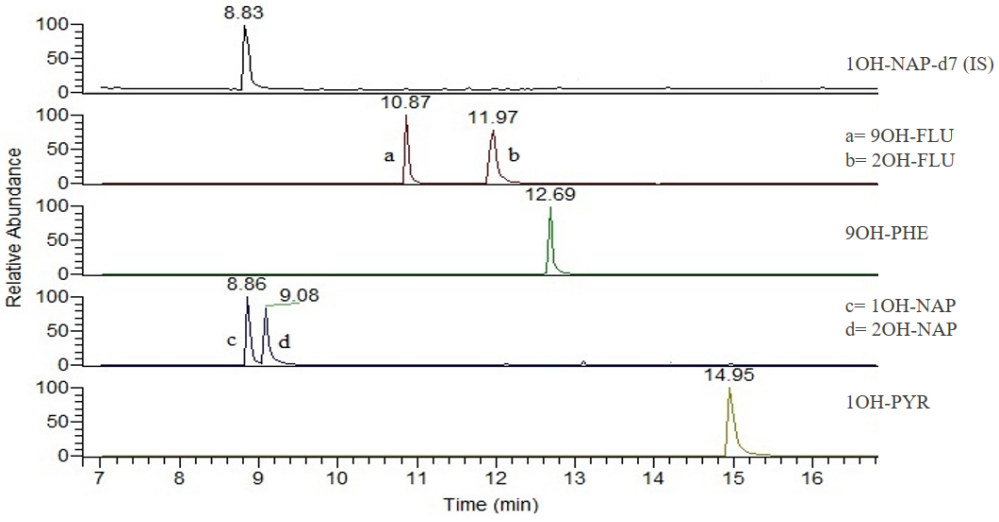


Figure 4. Chromatogram of a synthetic urine sample spiked with the OH-PAHs at LOQ level. IS = internal standard.

Comparison between the proposed procedure and previous publications

Compared to other microextraction procedures (Table III), the proposed AALLME method was fast, simple, economical, and easy to perform. Consequently, the analysis time was substantially shortened (about one minute per sample). However, some limitations are highlighted; first, one of the significant issues of this method was the detectability. In this context, only about 50% of the extractor solvent was recovered, which may affect extraction efficiency. However, the LODs may also be partially explained by the limitations regarding the single MS detector. Therefore, chromatographic systems with greater analytical sensitivity, such as GC-MS/MS or LC-MS/MS might be used to improve detectability. Second, the classical AALLME procedure requires using organic solvents that are hazardous to humans and the environment, such as chlorinated compounds. Finally, although the sample preparation procedure is simple, it requires extensive sample handling, and automatization is still impossible in classical AALLME (Table III).

Compared to conventional sample preparation methods, the AALLME technique reduced the organic solvent consumption, the number of preparation steps, and the total analysis time. Therefore, this approach might be helpful in several studies to replace traditional techniques.

Table III. Comparison of the proposed AALME-GC-MS method with other microextraction procedures to determine OH-HPAs in human urine samples

OH-PAHs	Sample volume (mL)	Sample preparation method	Sorbent/solvent	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Analytical technique	Reference
1OH-NAP	10	d-SPE/ DLLME	Polystyrene/ 750 µL of Choline chloride-3,3-dimethyl butyric acid	5.3	19	GC-MS	9
2OH-NAP				7.2	26		
1OH-PYR				3.6	11		
2OH-FLU				7.2	24		
2OH-NAP	5	LPME	200 µL Octanoic acid	0.076	0.25	LC-FD	7
2OH-FLU				0.018	0.059		
9OH-PHE				0.052	0.17		
1OH-PYR				0.064	0.21		
6OH-CHRY				0.059	0.19		
3OH-PHE	0.5	SPME	C18	0.05	0.10	Direct-MS	12
1OH-NAP,				0.05	0.10		
2OH-FLU				0.05	0.10		
1OH-PYR				0.05	0.10		
6OH-CHRY				0.05	0.10		
2OH-NAP	0.4	In-tube-SPME	Zn/Polypyrrole nanocomposite	0.039	0.130	LC–MS/MS	7
3OH-PHE				0.040	0.133		
1OH-PYR				0.050	0.167		
1OH-NAP	0.8	Automated- MEPS	C18	14.9	49.7	GC-MS	13
2OH-NAP				11.6	38.7		
1OH-NAP				14.6	48.6		
9OH-FLU				10.1	33.6		
2OH-FLU				10.8	36.0		
9OH-PHE				39.6	132.2		

(continued on next page)

Table III. Comparison of the proposed AALME-GC-MS method with other microextraction procedures to determine OH-HPAs in human urine samples (continuation)

OH-PAHs	Sample volume (mL)	Sample preparation method	Sorbent/solvent	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Analytical technique	Reference
1OH-NAP	5	SBSE	Polydimethylsiloxane	0.01	0.002	LC-MS/MS	30
2OH-NAP				0.01	0.003		
2OH-FLU				0.01	0.002		
3OH-FLU				0.01	0.022		
1OH-PHE				0.01	0.003		
2OH-PHE				0.01	0.003		
3OH-PHE				0.01	0.003		
4OH-PHE				0.01	0.003		
9OH-PHE				0.01	0.002		
1OH-PYR				0.01	0.003		
9OHBaP				0.01	0.001		
1OH-NAP	5	DLLME	Tetrachloroethylene (300 µL)	2	7	GC-MS/MS	10
9OH-FLU				2	7		
3OH-FLU				1	3		
2OH-FLU				1	4		
9OH-PHE				4	12		
1OH-PYR				9	29		
6OH-CHRY				2	7		
1OH-NAP	5	AALLME	1, 2-dichloroethane (300 µL)	0.65	1.95	GC-MS	This work
2OH-NAP				0.24	0.72		
9OH-FLU				0.58	1.75		
2OH-FLU				0.69	2.09		
9OH-PHE				0.62	1.89		
1OH-PYR				0.58	1.75		

Dispersive liquid-liquid microextraction (DLLME); Fluorescence detector (FD); Liquid-phase microextraction (LPME); Microextraction by packed Sorbent (MEPS); Solid-phase extraction (SPE); Solid-phase microextraction (SPME); Stir bar sorptive extraction (SBSE).

Application of the AALLME-GC-MS method

The proposed AALLME method was successfully applied to determine six OH-PAHs in the urine of 20 healthy volunteers (Table IV). The observed values agree with other biomonitoring studies reported in the literature.[1,23,31] The differences observed in these ranges might be explained by the degree of exposure and individual metabolism parameters that affect the active compound's absorption, distribution, excretion, and metabolization.[1]

Table IV. Concentrations of OH-HPAs (ng mL^{-1}) in human urine of 20 volunteers

Samples	1OH-NAP	2OH-NAP	9OH-FLU	2OH-FLU	9OH-PHE	1OH-PYR
(Mean)	3.21	4.20	3.27	1.28	4.70	<LOD
(Min – Max)	0.87 – 7.59	1.86 – 7.24	0.58 – 8.58	1.12 – 1.43	3.71 – 5.59	<LOD
Detection rate(%)	45	25	25	10	60	0
(Sample – 1)	<LOD	<LOD	<LOD	1.12	5.02	<LOD
(Sample – 2)	<LOD	<LOD	3.58	<LOD	5.29	<LOD
(Sample – 3)	<LOD	1.89	0.58	<LOD	<LOD	<LOD
(Sample – 4)	1.86	<LOD	<LOD	<LOD	<LOD	<LOD
(Sample – 5)	<LOD	<LOD	<LOD	<LOD	4.73	<LOD
(Sample – 6)	<LOD	<LOD	<LOD	<LOD	4.72	<LOD
(Sample – 7)	<LOD	<LOD	<LOD	<LOD	4.32	<LOD
(Sample – 8)	1.77	<LOD	<LOD	<LOD	<LOD	<LOD
(Sample – 9)	3.62	<LOD	<LOD	1.43	3.72	<LOD
(Sample – 10)	4.04	<LOD	<LOD	<LOD	3.92	<LOD
(Sample – 11)	0.87	<LOD	<LOD	<LOD	3.71	<LOD
(Sample – 12)	<LOD	<LOD	<LOD	<LOD	4.82	<LOD
(Sample – 13)	1.96	3.71	8.58	<LOD	5.17	<LOD
(Sample – 14)	4.82	6.32	2.51	<LOD	<LOD	<LOD
(Sample – 15)	2.32	1.86	<LOD	<LOD	5.43	<LOD
(Sample – 16)	7.59	7.24	<LOD	<LOD	<LOD	<LOD
(Sample – 17)	<LOD	<LOD	<LOD	<LOD	5.59	<LOD
(Sample – 18)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
(Sample – 19)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
(Sample – 20)	<LOD	<LOD	1.11	<LOD	<LOD	<LOD

LOD: limit of detection.

CONCLUSIONS

This manuscript showed that both AALLME and DLLME techniques provided similar extraction efficiencies to determine six mono-hydroxylated PAHs in human urine. Better performances (recoveries around 90%) were achieved for more hydrophobic compounds (such as 1OH-PYR), while more polar analytes (e.g. 1OH-NAP) showed poor recovery ratios (around 15%). However, the ALLME-GC-MS method was slightly superior in determining four from six PAH metabolites and was further investigated regarding analytical figures of merit. In addition, the sample preparation was fast and straightforward and did not require any additional instrumentation to conduct the microextraction procedure. Finally, the method achieved adequate sensitivity and was applied in a pilot biomonitoring study, showing to be an attractive alternative for routine analyses.

Conflicts of interest

The authors declare that they have no known competing financial interests that could have influenced the work reported in this study.

Acknowledgements

This research was supported by the São Paulo Research Foundation (2018/11150-7, 2018/24069-3, 2021/03633-0, 2022/07979-1, 2020/02939-6, 2019/07161-6, and 2022/06443-0) and by the Brazilian National Council for Scientific and Technological Development (406442/2022-3). Thank you for your financial support and for granting us research fellowships.

REFERENCES

(1) Barbosa, F.; Rocha, B. A.; Souza, M. C. O.; Bocato, M. Z.; Azevedo, L. F.; Adeyemi, J. A.; Santana, A.; Campiglia, A. D. Polycyclic Aromatic Hydrocarbons (PAHs): Updated Aspects of Their Determination, Kinetics in the Human Body, and Toxicity. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews* **2023**, *26* (1), 28–65. https://doi.org/10.1080/10937404.2022.2164390

(2) Palazzi, P.; Hardy, E. M.; Appenzeller, B. M. R. Biomonitoring of Children Exposure to Urban Pollution and Environmental Tobacco Smoke with Hair Analysis – A Pilot Study on Children Living in Paris and Yeu Island, France. *Sci. Total Environ.* **2019**, *665*, 864–872. https://doi.org/10.1016/j.scitotenv.2019.02.177

(3) Oliveira, M.; Slezakova, K.; Delerue-Matos, C.; Pereira, M. C.; Morais, S. Children Environmental Exposure to Particulate Matter and Polycyclic Aromatic Hydrocarbons and Biomonitoring in School Environments: A Review on Indoor and Outdoor Exposure Levels, Major Sources and Health Impacts. *Environ. Int.* **2019**, *124*, 180–204. https://doi.org/10.1016/j.envint.2018.12.052

(4) Santos, P. M.; del Nogal Sánchez, M.; Pavón, J. L. P.; Cordero, B. M. Determination of Polycyclic Aromatic Hydrocarbons in Human Biological Samples: A Critical Review. *TrAC - Trends Anal. Chem.* **2019**, *113*, 194–209. https://doi.org/10.1016/j.trac.2019.02.010

(5) Nováková, L.; Vlcková, H. A Review of Current Trends and Advances in Modern Bio-Analytical Methods: Chromatography and Sample Preparation. *Analytica Chimica Acta* **2009**, *656* (1–2), 8–35. https://doi.org/10.1016/j.aca.2009.10.004

(6) Kole, P. L.; Venkatesh, G.; Kotecha, J.; Sheshala, R. Recent Advances in Sample Preparation Techniques for Effective Bioanalytical Methods. *Biomed. Chromatogr.* **2011**, *25* (1), 199–217. https://doi.org/10.1002/bmc.1560

(7) Wang, X.; Sun, Z.; Luo, X.; Wang, K.; Zhang, S.; Ji, Z.; Gao, Y.; You, J. A Novel Switchable Solvent Liquid-Phase Microextraction Technique Based on the Solidification of Floating Organic Droplets: HPLC-FLD Analysis of Polycyclic Aromatic Hydrocarbon Monohydroxy Metabolites in Urine Samples. *New J. Chem.* **2020**, *44* (7), 3038–3044. https://doi.org/10.1039/c9nj05548g

(8) Wang, S. L.; Xu, H. Inorganic–Organic Hybrid Coating Material for the Online in-Tube Solid-Phase Microextraction of Monohydroxy Polycyclic Aromatic Hydrocarbons in Urine. *J. Sep. Sci.* **2016**, *39* (23), 4610–4620. https://doi.org/10.1002/jssc.201600712

(9) Jouyban, A.; Nemati, M.; Farazajdeh, M. A.; Alizadeh Nabil, A. A.; Afshar Mogaddam, M. R. A Polymer-Based Dispersive Solid Phase Extraction Combined with Deep Eutectic Solvent Based-Dispersive Liquid–Liquid Microextraction for the Determination of Four Hydroxylated Polycyclic Aromatic Hydrocarbons from Urine Samples. *J. Sep. Sci.* **2021**, *44* (21), 4025–4036. https://doi.org/10.1002/jssc.202100269

(10) Gupta, M. K.; Jain, R.; Singh, P.; Ch, R.; Mudiam, M. K. R. Determination of Urinary PAH Metabolites Using DLLME Hyphenated to Injector Port Silylation and GC-MS-MS. *J. Anal. Toxicol.* **2015**, *39* (5), 365–373. https://doi.org/10.1093/jat/bkv023

(11) Jinadasa, B. K. K. K.; Monteau, F.; Morais, S. Critical Review of Micro-Extraction Techniques Used in the Determination of Polycyclic Aromatic Hydrocarbons in Biological, Environmental and Food Samples. *Food Addit. Contam.: Part A* **2020**, *37* (6), 1004–1026. https://doi.org/10.1080/19440049.2020.1733103

(12) Yang, B. C.; Fang, S. F.; Wan, X. J.; Luo, Y.; Zhou, J. Y.; Li, Y.; Li, Y. J.; Wang, F.; Huang, O. P. Quantification of Monohydroxylated Polycyclic Aromatic Hydrocarbons in Human Urine Samples Using Solid-Phase Microextraction Coupled with Glass-Capillary Nanoelectrospray Ionization Mass Spectrometry. *Anal. Chim. Acta* **2017**, *973*, 68–74. https://doi.org/10.1016/j.aca.2017.04.009

(13) García-García, S.; Matilla-González, H.; Peña, J.; Nogal Sánchez, M. del; Casas-Ferreira, A. M.; Pérez Pavón, J. L. Determination of Hydroxy Polycyclic Aromatic Hydrocarbons in Human Urine Using Automated Microextraction by Packed Sorbent and Gas Chromatography–Mass Spectrometry. *Int. J. Environ. Res. Public Health* **2022**, *19* (20). https://doi.org/10.3390/ijerph192013089

(14) Rezaee, M.; Yamini, Y.; Faraji, M. Evolution of Dispersive Liquid-Liquid Microextraction Method. *J. Chromatogr. A* **2010**, *1217* (16), 2342–2357. https://doi.org/10.1016/j.chroma.2009.11.088

(15) Rezaee, M.; Assadi, Y.; Milani Hosseini, M. R.; Aghaee, E.; Ahmadi, F.; Berijani, S. Determination of Organic Compounds in Water Using Dispersive Liquid-Liquid Microextraction. *J. Chromatogr. A* **2006**, *1116* (1–2), 1–9. https://doi.org/10.1016/j.chroma.2006.03.007

(16) Farajzadeh, M. A.; Mohebbi, A.; Pazhohan, A.; Nemati, M.; Afshar Mogaddam, M. R. Air–Assisted Liquid–Liquid Microextraction; Principles and Applications with Analytical Instruments. *TrAC - Trends Anal. Chem.* **2020**, *122*. https://doi.org/10.1016/j.trac.2019.115734

(17) Zgoła-Grześkowiak, A.; Grześkowiak, T. Dispersive Liquid-Liquid Microextraction. *TrAC - Trends Anal. Chem.* **2011**, *30* (9), 1382–1399. https://doi.org/10.1016/j.trac.2011.04.014

(18) Farajzadeh, M. A.; Mogaddam, M. R. A. Air-Assisted Liquid-Liquid Microextraction Method as a Novel Microextraction Technique; Application in Extraction and Preconcentration of Phthalate Esters in Aqueous Sample Followed by Gas Chromatography-Flame Ionization Detection. *Anal. Chim. Acta* **2012**, *728*, 31–38. https://doi.org/10.1016/j.aca.2012.03.031

(19) González-Mariño, I.; Casas-Ferreira, A. M.; Nogal Sánchez, M. del; Pérez Pavón, J. L. Use of a Guard Column Coupled to Mass Spectrometry as a Fast Semi-Quantitative Methodology for the Determination of Plasticizer Metabolites in Urine. *J. Chromatogr. A* **2023**, *1690*, 463788. https://doi.org/10.1016/j.chroma.2023.463788

(20) Hinkelmann, K. (Ed). *Design and Analysis of Experiments: Special Designs and Applications*. John Wiley & Sons, Inc. 2012. https://doi.org/10.1002/9781118147634

(21) Gomes, J. M.; Almeida, T. F. A.; da Silva, T. A.; de Lourdes Cardeal, Z.; Menezes, H. C. Saliva Biomonitoring Using LPME-GC/MS Method to Assess Dentistry Exposure to Plasticizers. *Anal. Bioanal. Chem.* **2020**, *412* (28), 7799–7810. https://doi.org/10.1007/s00216-020-02908-x

(22) Rocha, B. A.; Gallimberti, M.; Ximenez, J. P. B. ; Basso, C. G.; Martino-Andrade, A. J.; Koch, H. M.; Calixto, L. A.; Barbosa, F. An Eco-Friendly Sample Preparation Procedure Based on Air-Assisted Liquid-Liquid Microextraction for the Rapid Determination of Phthalate Metabolites in Urine Samples by Liquid Chromatography-Tandem Mass Spectrometry. *Talanta* **2024**, *266*, 124974. https://doi.org/10.1016/j.talanta.2023.124974

(23) Souza, M. C. O.; Rocha, B. A.; Ximenez, J. P. B.; Devóz, P. P.; Santana, A.; Campíglia, A. D.; Barbosa, F. Urinary Levels of Monohydroxylated Polycyclic Aromatic Hydrocarbons in Brazilian Children and Health Risk Assessment: A Human Biomonitoring-Based Study. *Environ. Sci. Pollut. Res.* **2022**, *29* (31), 47298–47309. https://doi.org/10.1007/s11356-022-19212-z
(24) Xing, B.; McGill, W. B.; Dudas, M. J. Sorption of α-Naphthol onto Organic Sorbents Varying in Polarity and Aromaticity. *Chemosphere* **1994**, *28* (1), 145–153. https://doi.org/10.1016/0045-6535(94)90208-9
(25) 1-Naphthol Compound Summary. PubChem / National Library of Medicine. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/1-Naphthol [accessed: January/2024].
(26) Asimakopoulos, A. G.; Xue, J.; De Carvalho, B. P.; Iyer, A.; Abualnaja, K. O.; Yaghmoor, S. S.; Kumosani, T. A.; Kannan, K. Urinary Biomarkers of Exposure to 57 Xenobiotics and Its Association with Oxidative Stress in a Population in Jeddah, Saudi Arabia. *Environ. Res.* **2016**, *150*, 573–581. https://doi.org/10.1016/j.envres.2015.11.029
(27) Rocha, B. A.; Da Costa, B. R. B.; De Albuquerque, N. C. P.; De Oliveira, A. R. M.; Souza, J. M. O.; Al-Tameemi, M.; Campiglia, A. D.; Barbosa, F. A Fast Method for Bisphenol A and Six Analogues (S, F, Z, P, AF, AP) Determination in Urine Samples Based on Dispersive Liquid-Liquid Microextraction and Liquid Chromatography-Tandem Mass Spectrometry. *Talanta* **2016**, *154*, 511–519. https://doi.org/10.1016/j.talanta.2016.03.098
(28) Rocha, B. A.; de Oliveira, S. R.; da Silva, R. M.; Barcelos, G. R. M.; de Oliveira, A. R. M.; Barbosa, F. An Eco-Friendly Sample Preparation Procedure Base on Low-Density Solvent-Based Air-Assisted Liquid-Liquid Microextraction for the Simultaneous Determination of 21 Potential Endocrine Disruptors in Urine Samples by Liquid Chromatography-Tandem Mass Spectro. *Microchem. J.* **2019**, *147*, 207–214. https://doi.org/10.1016/j.microc.2019.03.019
(29) European Medicines Agency (EMA). *ICH M10 on Bioanalytical Method Validation - Scientific Guideline* 2019. Available at: https://www.ema.europa.eu/en/ich-m10-bioanalytical-method-validation-scientific-guideline [accessed: February/2024].
(30) Zhao, G.; Chen, Y.; Wang, S.; Yu, J.; Wang, X.; Xie, F.; Liu, H.; Xie, J. Simultaneous Determination of 11 Monohydroxylated PAHs in Human Urine by Stir Bar Sorptive Extraction and Liquid Chromatography/Tandem Mass Spectrometry. *Talanta* **2013**, *116*, 822–826. https://doi.org/10.1016/j.talanta.2013.07.071
(31) Murawski, A.; Roth, A.; Schwedler, G.; Schmied-Tobies, M. I. H.; Rucic, E.; Pluym, N.; Scherer, M.; Scherer, G.; Conrad, A.; Kolossa-Gehring, M. Polycyclic Aromatic Hydrocarbons (PAH) in Urine of Children and Adolescents in Germany – Human Biomonitoring Results of the German Environmental Survey 2014–2017 (GerES V). *Int. J. Hyg. Environ. Health* **2020**, *226*. https://doi.org/10.1016/j.ijheh.2020.113491

SUPPLEMENTARY MATERIAL

Instrumental analysis

The GC-MS analysis was performed using a gas chromatograph coupled to mass spectrometry-ISQ single quadrupole (Thermo Fisher Scientific®, Waltham, MA, USA). The chromatographic separation of the OH-PAH was carried out on column FS-CAP SLB-5MS from Sigma-Aldrich® (30 m x 0.25 mm x 0.25 µm; 5% diphenyl polysiloxane, 95% dimethyl polysiloxane). The initial temperature of the chromatographic column oven was 100 °C, maintained for 2 minutes, followed by a heating ramp at 15 °C min^{-1} to 210 °C, with a subsequent increase of 20 °C min^{-1} until 280 °C, holding for 4 minutes. The total run time was 17 minutes. Helium was used as carrier gas at 1 mL min^{-1}. The injector temperature was maintained at 280 °C, and the injection volume used was 2 µL, performed in splitless mode with valve opening after 1 minute.

The detection system used was a mass spectrometer operated in electron impact ionization mode (EI). The temperatures of the ionization source and the transfer line to the mass spectrometer were maintained at 300 °C and 280 °C, respectively. The data were acquired using Full Scan mode and the selective ion

monitoring (SIM) of each native standard and labelled internal standard (IS). The mass spectrum (*m/z*) of SIM of all compounds, besides the other instrumental details, is described in Table S1. Data acquisition and quantification were performed using the Thermo Xcalibur™ version 2.2 (Thermo Fisher Scientific®) software.

Table S1. Chromatographic and MS parameters of the OH-PAHs

Analyte	Monitored ion (*m/z*)	Retention time (min)
1OH-NAP	185	8.9
2OH-NAP	216	9.1
2OH-FLU	239	12
9OH-FLU	254	10.8
9OH-PHE	266	12.7
1OH-PYR	290	14.5
1OH-NAP-d7	223	8.9

Table S2. Extraction efficiencies (%) of AALLME and DLLME method

Technique	1OH-NAP	2OH-NAP	2OH-FLU	9OH-FLU	9OH-PHE	1OH-PYR
DLLME	15.2	21.3	27.5	77.8	87.0	91.6
AALLME	16.4	33.2	34.6	82.9	77.1	76.5

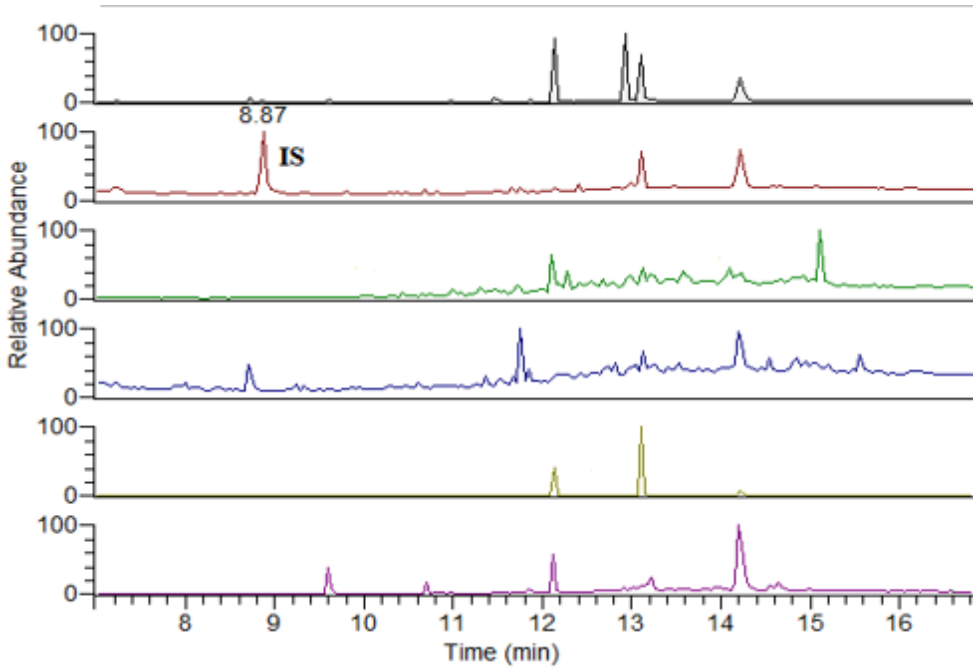


Figure S1. Chromatogram of a blank synthetic urine sample spiked with the internal standard. IS = internal standard.