



Exploring a new generation of bimetallic magnetic ionic liquids with ultra-low viscosity in microextraction that enable direct coupling with high-performance liquid-chromatography

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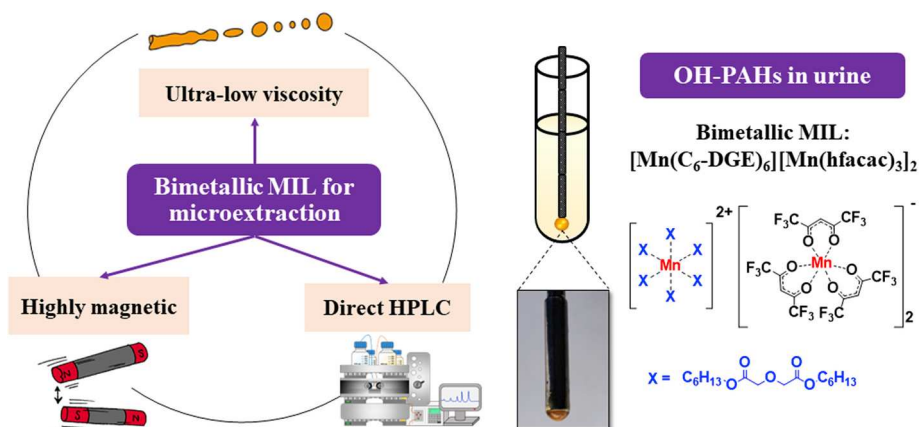
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HIGHLIGHTS

- New generation of bimetallic magnetic ionic liquids for liquid microextraction.
- Bimetallic magnetic ionic liquids possess ultra-low viscosity.
- Microextraction method does not require dispersive solvents or back-extraction.
- Magnetic-based extraction method can be directly coupled to HPLC.
- Microextraction method successfully performed in complex urine samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: The incorporation of bimetallic magnetic ionic liquids (MILs) in microextraction methods is an emerging trend due to the improved magnetic susceptibility offered by these solvents, which relies on the

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Dispersive liquid-liquid microextraction
Liquid chromatography
Monohydroxylated polycyclic aromatic
hydrocarbons
Urine
Magnetic separation

presence of metallic components in both the cation and the anion. This feature favors easy magnetic separation of these solvents in analytical sample preparation strategies. However, reported liquid-phase microextraction methods based on bimetallic MILs still present an important drawback in that the MILs are highly viscous, making a dispersive solvent during the microextraction procedure necessary, while also requiring a tedious back-extraction step prior to the chromatographic analysis.

Results: We propose for the first time a new generation of ultra-low viscosity bimetallic MILs composed of two paramagnetic Mn(II) complexes characterized by their easy usage in dispersive liquid-liquid microextraction (DLLME). The approach does not require dispersive solvent and the MIL-DLLME setup was directly combined with high-performance liquid chromatography (HPLC) and fluorescence detection (FD), without any back-extraction step. The approach was evaluated for the determination of five monohydroxylated polycyclic aromatic hydrocarbons, as carcinogenic biomarkers, in human urine. Optimum conditions of the MIL-DLLME method included the use of a low MIL volume (75 μL), a short extraction time (5 min), and no need of any dispersive solvent neither NaCl. The method presented limits of detection down to 7.50 ng L^{-1} , enrichment factors higher than 17, and provided inter-day relative standard deviation lower than 11%. Analysis of urine samples was successfully performed, with biomarker content found at levels between 0.24 and 7.8 ng mL^{-1} .

Significance: This study represents the first liquid-phase microextraction method using the new generation of low-viscous bimetallic MILs. The proposed MIL-DLLME approach represents 2 important advances with respect to previous methods employing bimetallic MILs: 1) no dispersive solvent is required, and 2) direct injection of the MIL in the HPLC is possible after minor dilution (no back extraction steps are required). Therefore, the microextraction strategy is simple, rapid, and consumes very small amounts of energy.

1. Introduction

Magnetic ionic liquids (MILs) are designer materials derived from ionic liquids (ILs) that exhibit paramagnetic behavior [1]. These solvents possess typical IL chemical structures and are composed entirely of ions while also incorporating paramagnetic components in the anion [2, 3], the cation [4,5], or both components [6,7]. The paramagnetic component can be a metal ion [8], a metal complex [9], and even a radical moiety [10].

MILs also have in common many of the physico-chemical properties of ILs, including negligible vapor pressure at room temperature, the ability to interact with a wide variety of compounds (e.g., ions, non-ionic compounds, and biomolecules), and adequate chemical and thermal stability [11]. In this regard, MILs are considered as tunable materials due to the immense combinations of ions able to generate materials with different properties. Additionally, their intrinsic magnetic behavior can be exploited in order to control or modulate the motion of the material at a macroscopic scale [12] and can also be used to enhance permeability to facilitate the trapping of gas molecules [13]. As a result of this ability to “react” to external magnetic fields, MILs can be included within the group of magneto-responsive and smart materials [14].

With respect to current trends in the design of MILs, recent efforts have been focused on the development of bimagnetic MILs [6,7]. These MILs consist of paramagnetic components in both the anion and the cation, providing significant enhancement in their overall magnetic susceptibility. Two types of bimagnetic MILs have been described until now [6,15]. The first group includes MILs with a radical-based cation combined with a metal-based anion [6] while the second group combines two different metal ions [7,15–18]. Bimetallic MILs are composed by anions consisting of metal ions coordinated with the hexafluoroacetylacetonate (hfacac) ligand combined with different metal-based cations and provide magnetic susceptibilities around four times higher than analogous MILs possessing a single metal ion. Furthermore, they can be designed to have low solubility in water [15].

Given the aforementioned properties, applications of MILs in analytical microextraction strategies have enabled their use in many different types of sample matrices and coupled with various downstream detection modalities [19]. Among all approaches involving MILs, dispersive liquid-liquid microextraction (DLLME) is the most often employed technique [19], mostly due to its overall simplicity and ability to achieve high analyte extraction efficiency.

Most of the MILs that have been employed in DLLME are based on single paramagnetic components [19]. Despite the improved magnetic susceptibility offered by bimagnetic MILs, to the best of our knowledge

only two studies have reported the use of these MILs in DLLME [16,17]. In both cases, the MIL cation was based on a metallic complex with the N,N-dimethyl biguanide ligand with the anion featuring the hfacac ligand coordinated with either Co(II) [16] or Mn(II) [17]. The two DLLME methods clearly benefit from the higher magnetic susceptibility of bimetallic MILs, but also required the use of a dispersive organic solvent in the DLLME procedure given the viscosity of the MIL. The methods needed a back-extraction step to re-extract the analytes from the MIL prior to HPLC analysis making additional vortex and centrifugation steps a necessity, resulting in tedious, lengthy, and solvent-consuming procedures.

This study is the first report describing the use of a MIL from a new generation of ultra-low viscosity bimetallic MILs in a DLLME method that is directly coupled with HPLC. The MIL possessed a viscosity of 63 cP, clearly lower than the typical values of previous bimetallic MILs, that ranged between 9210 and 28310 cP [7]. The bimetallic MIL incorporates two Mn(II)-based complexes with different ligands, namely, the hexyl-substituted diglycolic acid ester (DGE) ligand in the cation, and the hfacac in the anion ($[\text{Mn}(\text{C}_6\text{-DGE})_6][\text{Mn}(\text{hfacac})_3]_2$) [18]. The ultra-low viscosity of the MIL allows it to be easily dispersed during DLLME without requiring any dispersive solvent, and clearly favors its direct compatibility with HPLC. The developed method performs successfully in human urine samples for the determination of monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) as carcinogenic biomarkers, also constituting the first application of a bimetallic MIL for the analysis of complex biological samples.

2. Experimental

2.1. Chemicals, solutions, reagents, and materials

The reagents and the procedure for the preparation the MIL are detailed in [Procedure S1](#) of the Electronic Supplementary Material (ESM). The target analytes were five monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs), acquired from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Among them, 2-hydroxynaphthalene (2OHnap, 99.9%), 2-hydroxyfluorene (2OHflu, 98%), and 9-hydroxyphenanthrene (9OHphe, 99.9%) were purchased as solid standards, while 2-hydroxyphenanthrene (2OHphe) and 1-hydroxypyrene (1OHpy) were supplied as standard solutions of 10 mg L^{-1} in acetonitrile (ACN). The chemical structures and several physicochemical properties of the OH-PAHs are included in [Table S1](#) of the ESM. Individual standard solutions were prepared in ACN (LC-MS grade Chromasolv™, supplied by Honeywell Riedel-de Haën™, Seelze, Germany) at concentrations of 1050 mg L^{-1} ,

1020 mg L⁻¹, and 1190 mg L⁻¹, for 2OHnap, 2OHflu, and 9OHphe, respectively. Intermediate standard solutions were prepared by mixing the target OH-PAHs in ACN at 1 mg L⁻¹ for 2OHflu, 2OHphe, and 1OHpy, and at 2 mg L⁻¹ for 2OHnap and 9OHphe. Daily working standard solutions were prepared in ultrapure water or diluted urine from these intermediate standard solutions. All standard solutions were stored in a refrigerator at 4 °C and protected from light.

Hydrochloric acid (37%, w/w) was purchased from Honeywell Riedel-de Haën™. Formic acid (98% w/w) and sodium hydroxide (98%) were obtained from Sigma-Aldrich (Steinheim, Germany). Ultrapure water with resistivity of 18.2 MΩ cm was obtained with a Milli-Q water purification system (Watford, United Kingdom). Millipore filters of 0.22 µm were used to filter ACN and ultrapure water for the preparation of the HPLC mobile phase.

Artificial urine was prepared by dissolving the following compounds in 100 mL of ultrapure water: CaCl₂·2H₂O (0.065 g), MgCl₂·6H₂O (0.065 g), NaCl (0.46 g), Na₂SO₄ (0.23 g), Na₃C₆H₈O₇·2H₂O (0.065 g), Na₂C₂O₄ (0.0020 g), KH₂PO₄ (0.42 g), KCl (0.16 g), creatinine (0.11 g) and urea (2.5 g). All of these compounds were purchased from Sigma-Aldrich with purities higher than 99%.

Glass centrifuge tubes of 15 mL (9.5 cm L × 2 cm O.D.) from Pyrex® (Staffordshire, United Kingdom), Eppendorf microtubes of 1.5 mL (Nümbrecht, Germany), and a 100 µL Hamilton syringe (Reno, Nevada, USA), were employed in the microextraction procedure. The magnetic separation was accomplished using rod NdFeB magnets (0.5 cm D × 0.25 cm thick, B = 1.32–1.37 T), which were purchased from Supermagnete (Gottmadingen, Germany).

2.2. Instrumentation and equipment

Characterization of the organic ligands comprising the MIL was carried out with 400 and 600 MHz nuclear magnetic resonance (NMR) spectrometers from Bruker (Billerica, MA, USA). A Thermo Scientific FlashSmart 2000 CHNS/O Combustion Elemental Analyzer (Waltham, MA, USA) was used for elemental analysis. Viscosity measurements were performed with a Brookfield DV1 cone and plate viscometer with a CPA-51Z cone spindle. A magnetic susceptibility balance (MSB, Sherwood Scientific, Cambridge, UK), and a Quantum Design Superconducting Quantum Interference Device (Squid) magnetometer (Mpms XL-7), were required for studying the magnetic properties of the MIL. Thermogravimetric analysis (TGA) was performed using a Netzsch Sta 449 F1 Jupiter thermal analyzer (Selb, Germany).

A vortex agitation system from Heidolph® (Schwabach, Germany) was used in the microextraction procedure. Chromatographic analysis was performed on a 1260 Infinity HPLC system from Agilent Technologies (Santa Clara, CA, USA), equipped with a Rheodyne 7725i injection valve and with a 20 µL loop provided by Supelco. The HPLC was connected to a Waters 474 fluorescence detection (FD) system (Milford, MA, USA) controlled by a Varian Star 800 module interface. The chromatographic column was an InfinityLab Poroshell 120 EC-C18 (100 mm × 4.6 mm × 4.0 µm) supplied by Agilent Technologies and protected by a Pelliguard LC-C18 guard column from Supelco.

The statistical analysis of the experimental design was carried out using the Statgraphics® Centurion XV software. Excel (Microsoft Office, v. 2019) was used for the remaining calculations.

2.3. Procedures

2.3.1. Synthesis and characterization of the bimetallic MIL

The synthesis of the bimetallic MIL was performed following a previously published procedure [18], summarized in Procedure S1 of the ESM. ¹H NMR was used to characterize the DGE ligand, while elemental analysis and TGA were used to characterize the final MIL. The properties of the final MIL were studied through viscosity and magnetic susceptibility measurements. Fig. 1 shows the chemical structure of the bimetallic MIL synthesized.

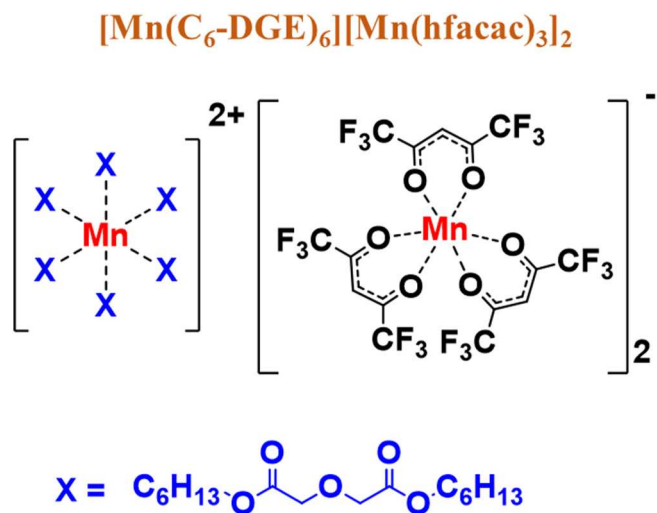


Fig. 1. Chemical structure of the bimetallic MIL employed in this study.

2.3.2. HPLC-FD method in presence of the bimetallic MIL

Chromatographic separation of target OH-PAHs in the presence of the bimetallic MIL was achieved by using a mobile phase formed by ACN and ultrapure water acidified with 0.02% (v/v) formic acid. The flow rate was kept constant at 1.0 mL min⁻¹. Gradient elution started at 50% (v/v) of ACN, and was maintained for 6 min. Then, 100% (v/v) of ACN was reached in 4 min and was kept for 5 additional mins.

The fluorescence program was carefully optimized to obtain the maximum sensitivity for the detection of analytes without any interfering signals coming from the bimetallic MIL. Table S2 of the ESM shows the optimum excitation (λ_{ex}) and emission (λ_{em}) wavelengths for each analyte, as well as the obtained retention times when using the gradient elution program described above.

2.3.3. Urine samples collection, hydrolysis, and pretreatment

Urine samples were collected in the early morning from different healthy volunteers including a smoker female, a non-smoker female, and a non-smoker male, and an informed consent was given to each urine donor. They were stored at -80 °C until their analysis. Samples were discharged after experiments.

The study was approved by the ethical committee board and written informed consent was obtained from all participants (CHUC B1947). This study was conducted in accordance with the Declaration of Helsinki.

The non-smoker female urine (non-subjected to hydrolysis) was selected for the validation of the method via matrix-matched external calibration.

The remaining urine samples were subjected to enzymatic hydrolysis. PAHs are metabolized in the human body through hydroxylation, which is enzymatically catalyzed. This way, PAHs are not urinary excreted as they originally entered the organism but as hydroxylated derivatives (OH-PAH compounds). Therefore, the monitorization of the levels of exposure to the PAHs through urine analysis require an enzymatic hydrolysis of the urine to obtain these OH-PAHs derivatives [20]. The hydrolysis was performed following the enzyme manufacturer recommendations. Briefly, 15 mL of urine were mixed with 2 mL of acetic acid/sodium acetate buffer solution at pH 5. Subsequently, 50 µL of the β -glucuronidase/arylsulfatase enzyme were added, and the sample was incubated during 24 h at 37 °C.

All urine samples (hydrolyzed or non-hydrolyzed) were diluted before DLLME. Optimum dilution (20%, v/v) required 2 mL of urine sample mixed with 8 mL of ultrapure water.

2.3.4. MIL-DLLME

The optimum MIL-DLLME procedure is shown in Fig. 2. Under optimum conditions, 10 mL of 20% (v/v) diluted urine (an artificial urine standard, non-hydrolyzed real urine, or hydrolyzed real urine, depending on the experiment) were added to a 15 mL glass centrifuge tube. Then, 75 μ L of the bimetallic MIL were added, followed by vortex at 1000 rpm for 5 min. During this step, small microdroplets of MIL were dispersed through the sample/standard solution, promoting the interaction between the MIL and the OH-PAHs. Afterwards, magnetic separation was performed by introducing a rod NdFeB magnet in the tube. This magnetic recovery of the MIL was instantaneous ($\sim$ 1–2 s). Finally, the MIL microdroplet (20 ± 5 μ L, $n = 5$) was diluted up to 250 μ L with ACN, and directly injected into the HPLC-FD system.

3. Results and discussion

3.1. Ensuring a direct coupling of the bimetallic MIL with HPLC-FD

The bimetallic $[\text{Mn}(\text{C}_6\text{-DGE})_6][\text{Mn}(\text{hfacac})_3]_2$ MIL (see Fig. 1) was synthesized and properly characterized (see Fig. S1 and Fig. S2 of the ESM) to ensure an ultra-low value of viscosity (63.2 cP) [18]. This MIL also exhibits high magnetic susceptibility ($10.42 \mu_B$) [18] due to the presence of two paramagnetic centers in its chemical structure. Armed with these two desirable characteristics for DLLME applications (simple dispersion due to the low viscosity, and lacking requirement of dispersive solvents) together with easy coupling with HPLC (avoiding back-extraction steps), a complex bioanalytical application was pursued. Thus, five OH-PAHs were selected as representative target analytes. Monitoring of urinary levels for these compounds is of high interest, as they are considered biomarkers in human exposure to PAHs, which are well-known persistent contaminants with strong carcinogenic effects [20]. Due to the native fluorescence of the target OH-PAHs, HPLC-FD was selected as sensitive analytical technique.

To ensure a successful direct coupling of the bimetallic MIL (used as extraction solvent in DLLME) with the HPLC, the following aspects must be fulfilled: (1) the bimetallic MIL must be soluble in all mobile phase compositions within the HPLC gradient elution program; (2) irreversible MIL retention in the HPLC system must be avoided, and (3) as the MIL has native fluorescence, the separation must be optimized to avoid signals coming from the bimetallic MIL that interfere in the detection of the selected OH-PAHs.

A mobile phase based on a polar organic solvent (ACN or methanol) and ultrapure water containing 0.02% (v/v) of formic acid was selected for the study, together with an end-capped RP-C₁₈ column. The acidic pH of the mobile phase and the end-capped nature of the column aids in decreasing the possibility of irreversible interactions between the metallic groups of the MIL and the free silanol groups of the chromatographic column [4,19,21]. As previously described, another important factor to consider is the solubility of the bimetallic MIL in the mobile phase. Indeed, other MILs, comprising the C₆-DGE ligand in the cation and the hfacac ligand in the anion, only differing in the metal, specifically Co(II) and Ni(II) [18], were also tested to evaluate their suitability for this application. Both presented high requirements of methanol or ACN content in the HPLC mobile phase to ensure proper solubilization, higher than 70% (v/v), thus limiting HPLC applications for them. With respect to the bimetallic MIL with Mn(II) selected in this study, it was verified that ACN/ultrapure water mixtures were more soluble with the MIL than methanol/ultrapure water mixtures. The minimum percentage of ACN in the mobile phase required for complete solubilization of the injected MIL (MIL that was previously diluted at least 1:1 v/v in ACN) was 45% (v/v). Given these values, a gradient elution program starting at ACN percentages higher than 45% (v/v) was carefully optimized in order to achieve separation of the five OH-PAHs. The optimum gradient was set as described in section 2.3.2, and achieved proper separation of the OH-PAHs in only 9 min. The MIL was completely soluble in both the initial (50/50, ACN/water, v/v) and the final (100/0, ACN/water, v/v) mobile phase compositions, and no pressure problems or any other issues were observed throughout the study, and during the further utilization of this HPLC and/or HPLC column.

Finally, the FD conditions were selected to maximize the detection of the target OH-PAHs without any interference of the MIL. A 50% (w/v) MIL solution in ACN was first directly injected in the HPLC-FD using the optimized elution gradient to verify the background signals derived from the material. The bimetallic MIL produced background signals mostly at the end of the chromatographic run (around 7 min, see Fig. 3 (A)). In contrast, Fig. 3 (B) shows the injection of a standard of the five OH-PAHs in ACN at 60 $\mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and 120 $\mu\text{g L}^{-1}$ for 2OHnap and 9OHphe. By comparing both chromatograms, it can be observed that the background signals provided by the bimetallic MIL did not interfere with the separation and detection of the OH-PAHs using HPLC-FD.

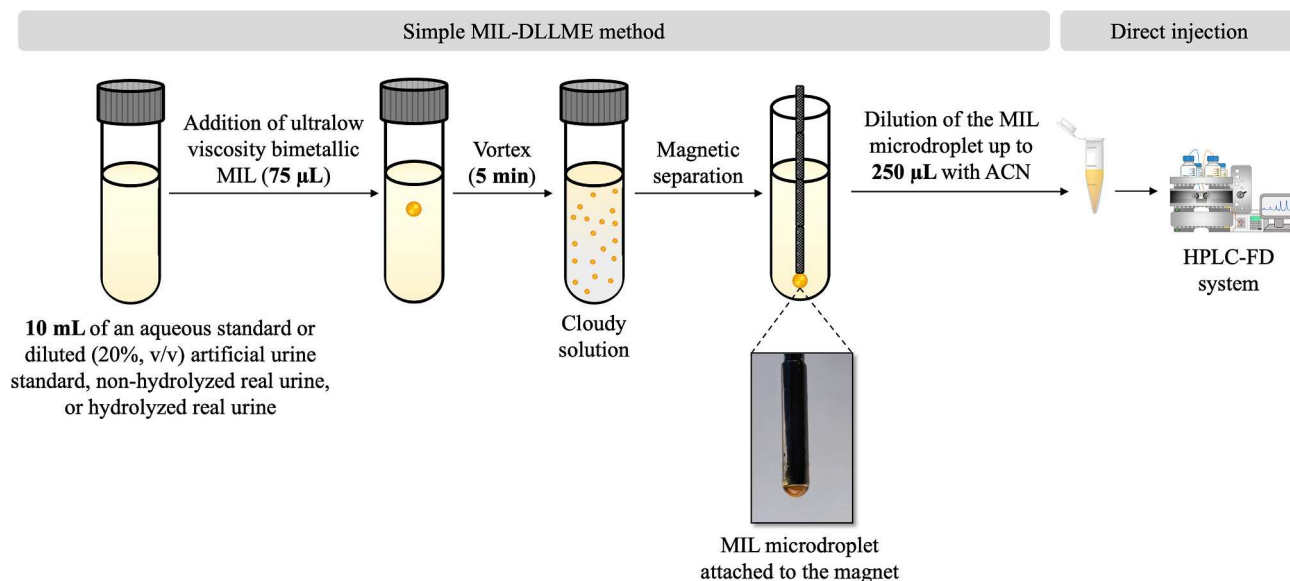


Fig. 2. Scheme of the MIL-DLLME procedure performed under optimum conditions, together with the visual aspect of the MIL once trapped by the magnet after the microextraction strategy.

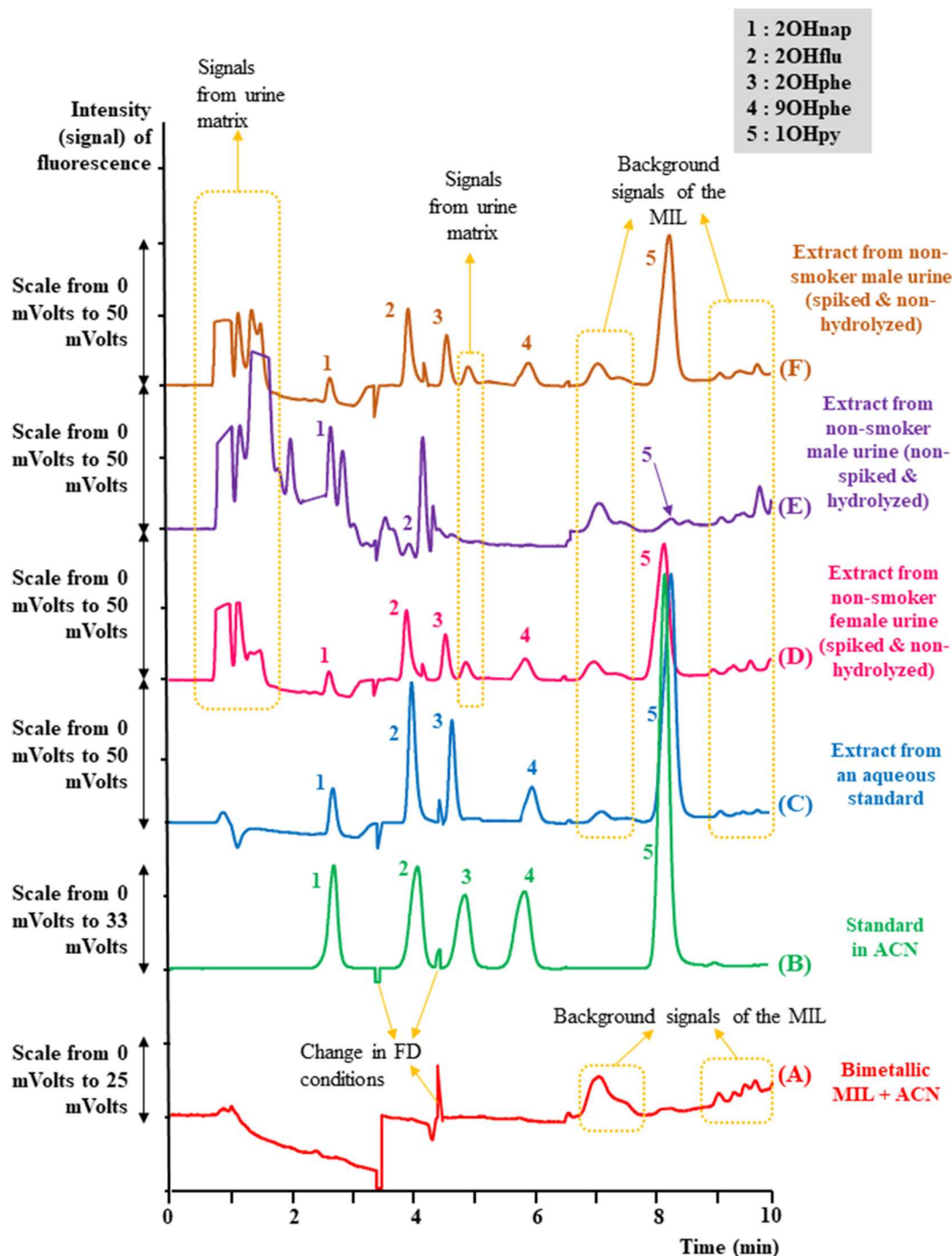


Fig. 3. Representative chromatograms of the injection of: (A) the bimetallic MIL dissolved in ACN; (B) a standard of the OH-PAHs dissolved in ACN (concentrations: $60 \mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and $120 \mu\text{g L}^{-1}$ for 2OHnap and 9OHphe); (C) a diluted MIL microdroplet (1:13, v/v) obtained after subjecting an aqueous standard containing the OH-PAHs (concentrations: $1.5 \mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and $3.0 \mu\text{g L}^{-1}$ for 2OHnap and 9OHphe) to the entire MIL-DLLME method; (D) a diluted MIL microdroplet (1:13, v/v) obtained after analyzing a real urine spiked with OH-PAHs (the urine corresponded to a non-smoker female and was not subjected to hydrolysis); (E) a diluted MIL microdroplet (1:13, v/v) obtained analyzing a real urine from a non-smoker male (the urine was subjected to hydrolysis prior analysis); and (F) a diluted MIL microdroplet (1:13, v/v) obtained after analyzing a real urine spiked with OH-PAHs (the urine corresponded to a non-smoker male and was not subjected to hydrolysis). The concentration level for the spiked urine samples D & F was $0.30 \mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and $0.60 \mu\text{g L}^{-1}$ for 2OHnap and 9OHphe.

Once the HPLC-FD method was optimized in presence of the bimetallic MIL, the extraction performance of the MIL in DLLME needed to be assessed. In the preliminary approach, the extraction was carried out using standards of OH-PAHs prepared in ultrapure water in order to purely evaluate the extraction capability of the MIL without considering possible interferences originating from the urine matrix. Hence, a

volume of $25 \mu\text{L}$ of MIL was added to 10 mL of an aqueous standard containing the OH-PAHs at $1.5 \mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and $3.0 \mu\text{g L}^{-1}$ for 2OHnap and 9OHphe. After 3 min of vortex, a MIL microdroplet was observed to form at the bottom of the extraction tube. The MIL microdroplet could be easily attached to a rod magnet introduced into the vial, ensuring its quick isolation. The microdroplet (~ 14

μL) was then diluted up to 250 μL with ACN to reduce its viscosity, and directly subjected to HPLC-FD analysis. Fig. 3 (C) shows the chromatogram obtained after injection of the diluted MIL microdroplet. A comparison of this chromatogram with the one shown in Fig. 3 (B) (standard of OH-PAHs in ACN at a concentration 40 times higher than that of the aqueous standard subjected to the MIL-DLLME) reveals preconcentration of the five OH-PAHs in the MIL microdroplet. These preliminary studies demonstrated the feasibility of the MIL-DLLME method to be successfully combined with HPLC-FD for the determination of this group of biomarkers, while also showing compatibility with HPLC.

3.2. Optimization of the MIL-DLLME method for urine analysis

The main variables affecting the extraction performance of the MIL-DLLME method were assessed. The volumes of sample and final extract subjected to HPLC-FD analysis were evaluated to ensure high preconcentration and proper sensitivity. With respect to the volume of sample, it was maintained at 10 mL, intending to use the maximum volume possible without sacrificing miniaturization. Additionally, this is desirable volume for urine samples. The volume of the final extract (the MIL microdroplet diluted with ACN) was fixed to 250 μL , as this was the minimum volume required to obtain a clear and non-viscous solution completely compatible with RP-HPLC. If considering the ratio between the volume of sample and the volume of the final extract, the maximum enrichment factor (E_{Fmax}) possible with this method was found to be 40, which is highly adequate. With this group of fixed parameters, the remaining variables that have an influence in the MIL-DLLME method were properly optimized.

3.2.1. Influence of the dispersive solvent and the pH of the sample

The type of dispersive solvent and the pH of the sample were assessed following a one-factor-at-a-time optimization, given the simplicity of these variables. While the purpose is to avoid the use of a dispersive solvent, it is important to demonstrate that the resulting method works adequately when it is not used. Therefore, it was necessary to evaluate if the incorporation of a dispersive solvent could enhance the mass transfer of analytes from the sample to the MIL in the DLLME method. The pH is also an important variable, particularly when dealing with urine samples.

For these first optimization studies, the concentration of the aqueous standards subjected to the MIL-DLLME method was 1.5 $\mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and 3.0 $\mu\text{g L}^{-1}$ for 2OHnap and 9OHphe. The extraction efficiency was monitored by calculating the ratio between the peak areas obtained after the injection of the diluted MIL microdroplet and the peak area obtained when directly injecting in the HPLC-FD a standard of the OH-PAHs in ACN at the concentration that could be achieved if the E_{Fmax} is reached (in other words, 40 times the spiked level: 60 $\mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and 120 $\mu\text{g L}^{-1}$ for 2OHnap and 9OHphe).

With respect to evaluating the need of dispersive solvent, extraction efficiencies obtained in the MIL-DLLME method using ACN as dispersive solvent (300 μL) were compared with those achieved without using any dispersive solvent. In all cases, extractions were performed in triplicate using 25 μL of MIL and 3 min of vortex. It was observed that the addition of ACN as dispersive solvent increased the time required for proper sedimentation of the MIL microdroplet, hindering the procedure in terms of timing. With respect to the obtained MIL microdroplet volume after DLLME, it was lower when adding the dispersive solvent (i.e., $\sim 8 \mu\text{L}$ when using ACN versus $\sim 14 \mu\text{L}$ when no dispersive solvent was added) indicating partial solubilization of the MIL in the aqueous solution due to the ACN. Furthermore, there was no significant improvement in the extraction efficiencies when ACN was used (Fig. S3 of the ESM). With all these considerations, it was clear that the MIL-DLLME method with the ultra-low viscosity MIL not only did not require a dispersive solvent but actually performed better without any dispersive solvent. This not only minimizes organic solvent consumption during the

microextraction stage, but also ensures a simpler procedure.

With respect to the influence of pH of the sample in the performance of the MIL-DLLME method, it relies on the following two aspects: (1) the requirement of having the OH-PAHs in their neutral form (non-ionized) to favor the partitioning to the MIL; and (2) stability of the MIL at different pH values. As observed in Table S1 of the ESM, the OH-PAHs have basic pK_a values ranging from 9.40 to 9.94. In this sense, pH values lower than these values (from 4.0 to 8.0) were studied. The MIL-DLLME method was performed in triplicate using aqueous standards at these pH values, with the abovementioned conditions fixed. No change in the MIL was visually observed at any of the evaluated pH values, and the volume of MIL microdroplet obtained was the same for all the pH values (i.e., $\sim 14 \mu\text{L}$), thus ensuring the stability of the MIL when performing the DLLME method with the studied pH range. Furthermore, there were no significant differences in the extraction efficiencies at the assessed pH range (Fig. S4 of the ESM), so no adjustment of pH was required in further experiments when dealing with urine samples (with common pH values between 4.6 and 8.0). Indeed, the pH of all urine samples utilized in this study, originating from different healthy male and female volunteers, was measured, and had values ranging from 5.0 to 8.0.

3.2.2. Influence of the urine matrix

Although the pH of the urine did not significantly influence the microextraction process, other components of the urine may result in matrix effects given the high complexity of this biological matrix. Thus, before proceeding with the optimization of other variables, the effect of the urine matrix on the extraction performance of the MIL-DLLME method was studied.

A healthy non-smoker female urine was subjected to the MIL-DLLME approach without performing any hydrolysis (to ensure that the sample was free of OH-PAHs) or dilution. Under these conditions, the MIL microdroplet did not form. Thus, the urine was diluted at different proportions with ultrapure water and subsequently tested. The results indicated that the maximum content of urine to ensure the proper formation of the MIL microdroplet was 50% (v/v). Under these conditions, the diluted urine sample was analyzed, and the results confirmed that the sample did not contain any OH-PAH. Subsequently, the urine sample was spiked with the target OH-PAHs, and the extraction efficiency of the MIL-DLLME approach was compared with that obtained with OH-PAHs standards in ultrapure water and in artificial urine (the latter, also diluted to 50% v/v). As was expected given the high complexity of the urine, lower extraction efficiencies were obtained in the real urine sample, thus demonstrating a significant matrix effect (see Fig. 4 (A)). Furthermore, the use of artificial urine was not useful for the optimization, as it did not properly mimic the composition of the real urine despite the high complexity of the urine recipe (see section 2.1). In fact, the extraction efficiencies in artificial urine were ~ 8 times higher than those obtained in real urine.

Aiming to overcome the strong matrix effect observed, non-hydrolyzed real urine was selected as matrix for developing the MIL-DLLME method in order to ensure proper optimization and adequate extraction efficiency. Different dilutions of the real sample were carried out with amounts of urine ranging from 10 to 50% (v/v) being assessed. The value of 50% (v/v) was shown previously as the maximum content of urine allowed to observe the MIL microdroplet. Thus, the purpose was to decrease as much as possible the matrix effect and to minimize the intensity of the background signals of the urine in the chromatogram, while also ensuring enough preconcentration for the proper detection of the OH-PAHs. An amount of urine of 10% (v/v) in the solution was subjected to DLLME and provided the highest extraction efficiencies (Fig. 4 (B)). However, this high dilution unavoidably entailed significant losses in the overall preconcentration and sensitivity of the method. Hence, it was necessary to select a urine amount of 20% (v/v) in the solution that was subjected to DLLME in order to compromise a high preconcentration and decreased matrix effects (to obtain adequate

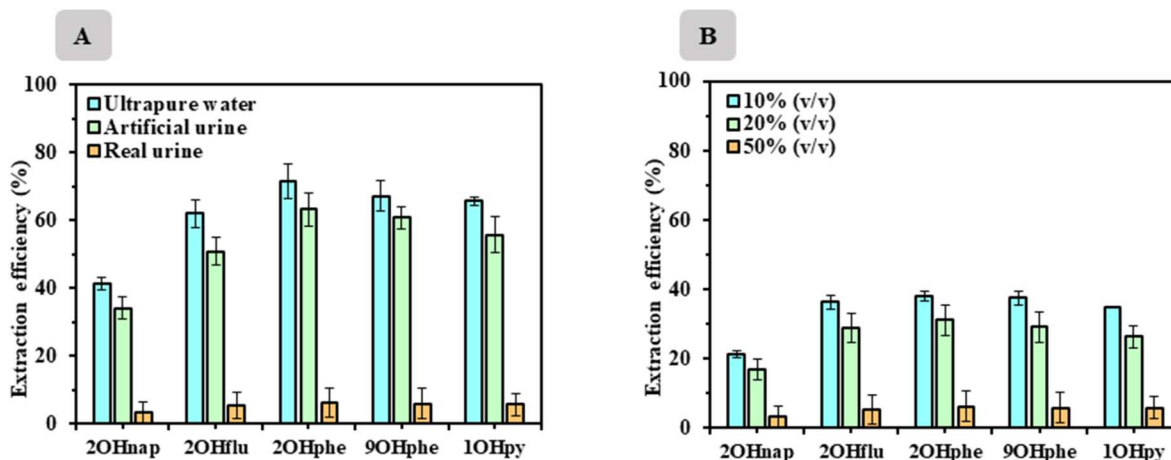


Fig. 4. (A) Extraction efficiencies obtained after performing the non-optimized MIL-DLLME procedure in different matrices. The spiked level was in all the cases $1.5 \mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and $3.0 \mu\text{g L}^{-1}$ for 2OHnap and 9OHphe. Experiments ($n = 3$) required 10 mL of aqueous or diluted urine standard (50%, v/v), 25 μL of MIL, 3 min of vortex time, no addition of dispersive solvent, and no adjustment of pH. (B) Effect of the amount of urine in the solution subjected to the MIL-DLLME procedure. The spiked level was in all the cases $1.5 \mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and $3.0 \mu\text{g L}^{-1}$ for 2OHnap and 9OHphe. Experiments ($n = 3$) required 10 mL of diluted urine standard solution (with amount of urine of 10, 25, or 50%, v/v), 25 μL of MIL, 3 min of vortex time, no addition of dispersive solvent, and no adjustment of pH.

extraction efficiencies).

Fig. 3 (D) shows a representative chromatogram from the diluted MIL microdroplet obtained after analysis of the female urine (solution containing 20% (v/v) of such urine) spiked with the OH-PAHs. The background signals from the urine matrix appeared in the first 2 min of the chromatogram and did not interfere with the detection of the target OH-PAHs. Therefore, optimization and subsequent validation of the MIL-DLLME method were directly performed in a healthy non-smoker female urine with a content of 20% (v/v).

3.2.3. Box-Behnken experimental design

Optimization of the remaining variables affecting the MIL-DLLME method (MIL volume, vortex time, and ionic strength of the sample) was accomplished using an experimental design. A Box-Behnken design (BBD) was selected, as it was the approach that allowed optimization with the minimum number of experiments.

Table S3 of the ESM includes the values of the assessed variables for each experiment of the BBD. A total of 15 experiments were conducted with the center point performed in triplicate. The MIL volume (variable A) was evaluated between 25 μL and 75 μL . No more than 75 μL were included in the design, as it was observed that higher volumes of MIL required also higher volumes of ACN to completely dissolve the MIL microdroplet, thus hindering the procedure in terms of preconcentration. With respect to the vortex time (variable B), it was evaluated between 1 min and 5 min. 1 min was selected as the minimum time required to properly disperse the MIL in the sample, while no more than 5 min were assessed to shorten the procedure as much as possible. The ionic strength (variable C) was controlled with NaCl, as % (w/v), added to the sample. It was evaluated given the positive effect that the addition of salt might exert in the extraction efficiency (salting-out effect).

Table S4 of the ESM includes the regression parameters of the polynomial equation obtained in the BBD, using the chromatographic peak areas of each analyte as the response variables. The R^2 statistics ranged between 82.4% and 97.9%, showing a good adjustment of the experimental data to the mathematical model of the BBD. In general, the higher the coefficient associated to a specific variable (or to an interaction between variables), the most significant the effect of this variable (or this interaction) in the response. Thus, according to the values shown in Table S4 of the ESM, the vortex time (coefficient B) was the variable with the highest effect on the peak areas for most OH-PAHs. This agrees with the obtained Pareto graphs and the main effect graphs included in Fig. S5 and Fig. S6 of the ESM, respectively. Indeed, vortex time is the

only variable with a significant and positive effect for the analytes, while the MIL volume is significant only for 2OHflu, 9OHphe, and 1OHpy. It can also be observed that the graphic profile in Fig. S6 of the ESM is similar for the vortex time and all analytes (with increased response at the highest values evaluated), but differences can be observed among OH-PAHs with respect to the MIL volume. On the contrary, the effect of the ionic strength was not statistically significant for any OH-PAH.

Table S5 of the ESM includes the individual optimum conditions for each of the OH-PAHs. All OH-PAHs exhibited the highest response when using a vortex time of 5 min. With respect to the MIL volume, 75 μL was adequate for all, except for 9OHphe (in this case requiring 25 μL). With respect to the ionic strength, there was not a common value, but its effect was negligible according to the smooth profile shown in Fig. S6 and the Pareto chart included in Fig. S5. Fig. S7 of the ESM shows the individual response surfaces obtained in the BBD for each of the OH-PAHs studied. These graphics were plotted by fixing the ionic strength (the non-significant variable) at 0.0% (w/v), thus assessing the effect of the two significant variables on the peak areas of each analyte individually. In general, all of these statistical data showed that the optimum values of the variables were different depending on the analyte, and thus it was necessary to achieve a compromise solution to select the same experimental conditions for all OH-PAHs.

The optimum compromised conditions were obtained by applying the multiple response methodology (see Table S5 of the ESM and Fig. 5). The desirability function was used as a tool to assess the effect of the three variables on a combined response involving simultaneously all OH-PAHs. The optimum conditions from the desirability were adopted as optimum compromise conditions: 75 μL of MIL, 5 min of vortex, and no addition of NaCl (0% w/v). Under these conditions, the average MIL microdroplet volume obtained after the magnetic retrieval was $20 \pm 5 \mu\text{L}$ ($n = 5$).

3.3. Analytical performance of the MIL-DLLME-HPLC-FD method in urine

Matrix-matched calibrations for the entire MIL-DLLME-HPLC-FD method were performed in real urine free of OH-PAHs. Calibrations were accomplished in the same real urine (coming from the same volunteer) that was used during the optimization (i.e., healthy non-smoker female urine that was not subjected to any hydrolysis step, with the solution subjected to DLLME containing 20%, v/v, of such urine). Table 1 includes several quality analytical parameters of the

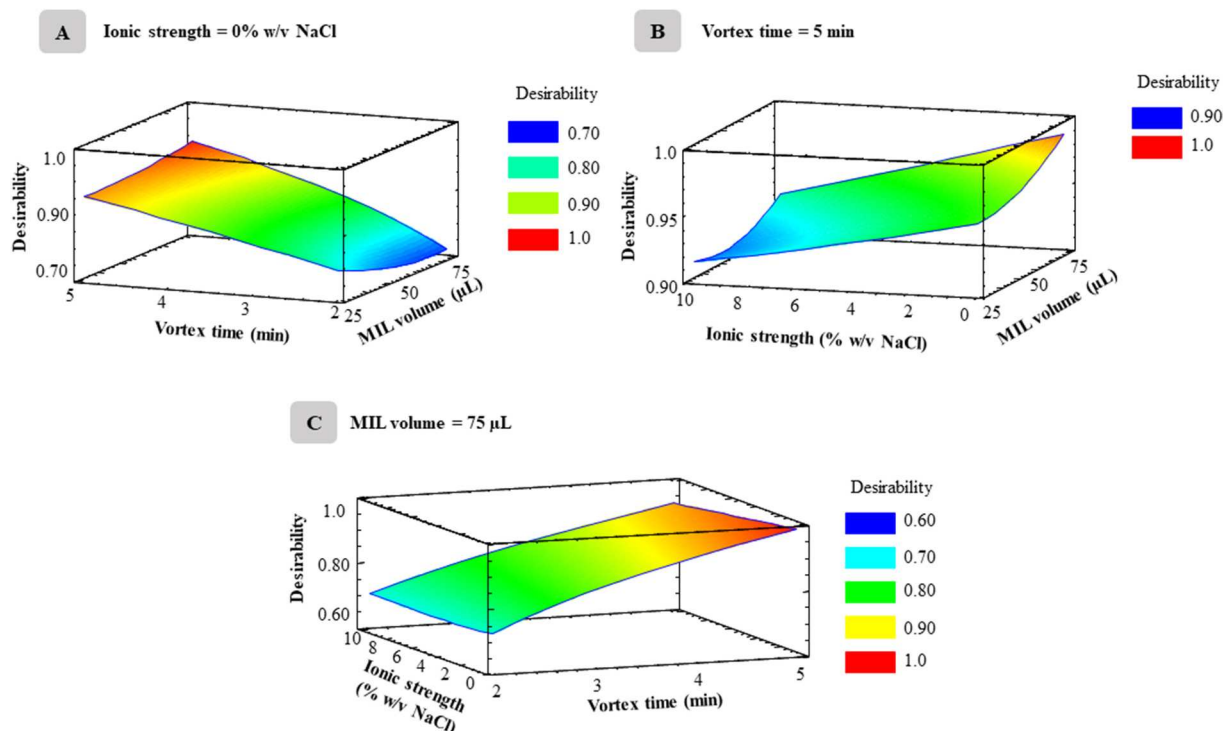


Fig. 5. Overall multiple response surfaces obtained in the Box Behnken experimental design for the set of assessed OH-PAHs. Each graph has one of the variables fixed at its optimum value: (A) ionic strength; (B) vortex time; (C) MIL volume.

Table 1

Several quality analytical parameters of the MIL-DLLME-HPLC-FD method, using a matrix-matched calibration approach and urine from a healthy non-smoker female as matrix (with the optimum established conditions).

OH-PAHs	Calibration range ($\mu\text{g L}^{-1}$)	(Slope $\pm t \cdot S_b^a$) 10^{-5}	R^{2b}	$S_y/x^c \cdot 10^{-5}$	LOD ^d (ng L^{-1})	LOQ ^e (ng L^{-1})
2OHnap	0.20–20	1.2 ± 0.1	0.998	0.4	50.0	200
2OHflu	0.10–5.0	8.0 ± 0.5	0.998	0.8	25.0	100
2OHphe	0.10–10	5.5 ± 0.3	0.999	0.9	25.0	100
9OHphe	0.40–20	1.3 ± 0.1	0.997	0.6	100	300
1OHpy	0.025–2.5	27 ± 1	0.999	0.8	7.50	25.0

^a Standard deviation of the slope within the calibration range ($n = 6$) for a confidence level of 95%.

^b Determination coefficient.

^c Standard deviation of the residuals.

^d Limit of detection.

^e Limit of quantification.

calibrations. All calibration curves showed good linearity, with determination coefficients (R^2) higher than 0.997 for all OH-PAHs. Limits of detection (LODs) and limits of quantification (LOQs) were estimated as 3 and 10 times de signal-to-noise ratio, respectively, and verified experimentally by preparation of urine standard solutions at those levels. LODs ranged from 7.50 ng L^{-1} for 1OHpy to 100 ng L^{-1} for 9OHphe.

The method was also evaluated in terms of precision, extraction efficiency (E_R), enrichment factor (E_F), and relative recovery (RR) using urine standard solutions at two different concentration levels included within the calibration range, but not used for obtaining the calibration curves (see Table 2). The intra-day precision, as relative standard deviation (RSD, %) ranged from 1.0% to 5.8% for the low concentration level (values of 0.3 or $0.6 \mu\text{g L}^{-1}$, depending on the OH-PAH) and from

1.4% to 5.9% for the intermediate concentration level (values of 1.5 or $3.0 \mu\text{g L}^{-1}$, depending on the OH-PAH), depending on the day. With respect to the intermediate precision, it was evaluated during three non-consecutive days, with a maximum RSD value of 11% being obtained for the low concentration level. It is noteworthy that, according to the Horwitz equation [22], a maximum RSD value of 45.3% is allowed for a concentration level of $1 \mu\text{g L}^{-1}$, and even the accepted RSD is 32% at $10 \mu\text{g L}^{-1}$. Considering that the RSD values obtained with the MIL-DLLME-HPLC-FD were much lower than those accepted following the Horwitz criterium, the precision of the method was highly adequate, despite the high complexity of the urine matrix.

With respect to E_R and E_F , they were obtained as described in the Procedure S2 of the ESM. The obtained values are included in Table 2. The results clearly showcase the adequate preconcentration achieved with the MIL-DLLME approach, with E_F values ranging between 19 and 27 for the low concentration level, and from 17 to 24 for the intermediate concentration level. With respect to E_R values, they ranged from 47 to 68% for the low concentration level, and from 42 to 59% for the intermediate concentration level. These values are highly acceptable for a microextraction approach, particularly if considering that the MIL-DLLME method entails a non-exhaustive extraction due to the use of a low volume of MIL with respect to the large volume of sample involved. Indeed, E_R values of 20% are considered adequate for a microextraction method if the method has good precision and enough sensitivity for the intended application [23].

The average RR values obtained in three non-consecutive days are also included in Table 2. Procedure S2 of the ESM includes all details regarding the calculations for obtaining RR. RR values obtained with the MIL-DLLME-HPLC-FD method ranged between 85% and 119% despite the high complexity of the urine matrix.

Compared to previously reported methods devoted to the determination of OH-PAHs in human urine samples, the analytical features of the proposed MIL-DLLME-HPLC-FD method are similar in terms of sensitivity and precision, despite the variety of extraction techniques and extractants used in the literature studies (see Table S6 of the ESM).

Table 2

Analytical performance of the entire MIL-DLLME-HPLC-FD method in terms of precision, extraction efficiency, and relative recovery, using standard solutions of real urine.

OH-PAHs	Low concentration level ^a					Intermediate concentration level ^b				
	Intra-day RSD range ^c (%)	Intermediate precision RSD ^d (%)	E _F ^e	E _R ^f (%)	(RR ± SD) ^g (%)	Intra-day RSD range ^c (%)	Intermediate precision RSD ^d (%)	E _F ^e	E _R ^f (%)	(RR ± SD) ^g (%)
2OHnap	2.8–5.0	11	19	47	94 ± 5	3.1–4.9	4.7	17	42	103 ± 4
2OHflu	4.4–4.7	5.2	26	65	86 ± 9	1.8–4.9	4.6	22	55	100 ± 2
2OHphe	3.0–5.8	6.9	27	68	85 ± 7	1.4–5.4	5.0	22	56	89 ± 2
9OHphe	1.3–5.0	9.0	25	62	110 ± 8	3.6–5.1	6.2	23	57	119 ± 8
1OHpy	1.0–1.6	8.9	25	63	104 ± 2	3.5–5.9	6.7	24	59	116 ± 9

^a 0.30 µg L⁻¹ for 2OHnap and 9OHphe; 0.60 µg L⁻¹ for 2OHflu, 2OHphe and 1OHpy.

^b 1.5 µg L⁻¹ for 2OHnap and 9OHphe; 3.0 µg L⁻¹ for 2OHflu, 2OHphe and 1OHpy.

^c Range (day 1 to day 3) of relative standard deviation for intra-day precision (n = 3).

^d Relative standard deviation for intermediate precision (n = 9, 3 non-consecutive days).

^e Enrichment factor.

^f Extraction efficiency (considering E_{Fmax} = 40).

^g Average relative recovery and standard deviation (n = 9).

All the studies of Table S6 of the ESM reached LODs at the ng·L⁻¹ level, obtained by coupling the extraction technique with liquid chromatography either with tandem mass spectrometry [24,25] or fluorescence detection [26,27]. In all cases, the sensitivity is enough for determining the OH-PAHs at their most common levels found in urine [20]. Furthermore, the precision of the MIL-DLLME-HPLC-FD approach is also in agreement with the RSD values reported by previous studies, ranging from 8.10% [24] to 17.0% [26].

The improved features of the proposed method with respect to the literature articles rely on its short extraction time, simplicity, and extraction efficiency. Indeed, it is important to highlight the short sample preparation time of approximately 6.5 min that was required in the MIL-DLLME method. Reported studies ranged from ~11 min [24] to 19 min [27]. Clearly, in most of those studies, centrifugation is the limiting step in terms of time and in terms of simplicity. In fact, most of the reported methods that separated the liquid extraction solvent by centrifugation also used a Hamilton syringe to isolate the final extraction phase, a procedure that is tedious and requires training. This step is avoided in the proposed method by using the magnetic separation step. Furthermore, this method also provides an impressive average E_R of 61%, almost three times higher than the one reported in a previous study that involves the use of a non-magnetic IL as extractant in DLLME [26].

Finally, the greenness of the proposed MIL-DLLME method was assessed by applying recent metrics, such as the Sample Preparation Metric of Sustainability (SPMS) [28]. Fig. S8 of the ESM includes the SPMS clock diagram of the MIL-DLLME method, together with the diagrams obtained for all reported methods from Table S6 of the ESM, with comparative purposes. Among all assessed studies, the proposed MIL-DLLME approach has obtained the highest score (7.16), and this is despite not having used the greenest material (given the non-degradable character of the MIL due to the presence of metals in its structure). Clearly, the magnetic features of the solvent contribute to this high score in the sustainability of the method. The key parameters justifying the score are: (1) requirement of only 4 steps to perform the method (addition of the MIL to the sample, vortex, magnetic separation, and dilution of the MIL microdroplet); (2) low extraction time (only 5 min); (3) requirement of only a minor dilution prior the chromatographic analysis; and (4) avoiding centrifugation.

Another recent metric was tested, the Blue Applicability Grade Index (BAGI) [29]. As it can be observed in Fig. S9 of the ESM, the MIL-DLLME method achieves the same score (52.5) than several reported studies from Table S6 of the ESM, thus supporting its adequate analytical performance for determining OH-PAHs in urine, while possessing additional advantages conferred by the magnetic properties of the MIL.

3.4. Analysis of urine samples using the optimum MIL-DLLME-HPLC-FD method

The MIL-DLLME-HPLC-FD method was applied to the analysis of other human urine samples (different from that used for the analytical optimization and performance) in a healthy non-smoker male and a healthy smoker female.

Both samples were first subjected to hydrolysis, and then to the entire method to evaluate their content of OH-PAHs. Fig. 3 (E) shows a chromatogram of the diluted MIL microdroplet obtained after the analysis of the hydrolyzed male urine sample. As it can be observed, the chromatographic profile was similar to the one obtained after the analysis of the female urine used for method validation (see Fig. 3 (D)), without any interfering signal from the matrix that may hinder detection of the OH-PAHs. Table 3 shows the results obtained with the analysis of the hydrolyzed urine samples. 2OHnap and 2OHflu were quantified in both samples, 2OHphe was detected in the smoker female urine, and 1OHpy detected in both samples. These results are in agreement with the occurrence of OH-PAHs in human urine samples. Indeed, the

Table 3

Analytical performance of the MIL-DLLME-HPLC-FD method when analyzing non-hydrolyzed urine samples from different individuals, as well as the analysis of hydrolyzed urine samples.

OH-PAHs	Healthy non-smoker male urine			Healthy smoker female urine		
	Hydrolyzed urine	Non-hydrolyzed urine		Hydrolyzed urine	Non-hydrolyzed urine	
	Content ± SD ^a (µg·L ⁻¹)	RSD ^b (%)	RR ± SD ^c (%)	Content ± SD ^a (µg·L ⁻¹)	RSD ^b (%)	RR ± SD ^c (%)
2OHnap	8.50 ± 0.90	3.5	99 ± 3	7.81 ± 0.01	10	98 ± 10
2OHflu	0.24 ± 0.01	3.4	80 ± 3	0.34 ± 0.01	7.3	73 ± 7
2OHphe	<LOD	3.3	69 ± 2	<LOQ	4.6	59 ± 5
9OHphe	<LOD	6.0	101 ± 5	<LOD	13	88 ± 13
1OHpy	<LOQ	6.6	85 ± 5	<LOQ	8.5	69 ± 10

^a Concentration found, together with their standard deviation (n = 3).

^b Intra-day precision, as relative standard deviation (n = 3), for a spiked level of 0.30 µg L⁻¹ for 2OHflu, 2OHphe, and 1OHpy and 0.60 µg L⁻¹ for 2OHnap and 9OHphe.

^c Average relative recovery and its standard deviation (n = 3), for a spiked level of 0.30 µg L⁻¹ for 2OHflu, 2OHphe, and 1OHpy and 0.60 µg L⁻¹ for 2OHnap and 9OHphe.

hydroxylated metabolite of naphthalene has been identified as the most common biomarker in the urine of people with high exposure to PAHs [20], and thus 2OHnap is the analyte with the highest concentration in both female ($7.81 \pm 0.01 \mu\text{g L}^{-1}$) and male ($8.50 \pm 0.90 \mu\text{g L}^{-1}$) urine samples. The results also agree with the smoking habits of the individuals. Smoking individuals are generally exposed more to PAHs and, therefore, higher concentrations of hydroxylated metabolites are expected [20]. It can be observed that the female smoker presented more OH-PAHs than the male non-smoker (with the 2OHphe only detected in the urine sample of the smoker), and in general, higher concentrations of the biomarkers were found in the female smoker sample (see Table 3), except for 2OHnap. Furthermore, it has been reported that males normally present higher levels of OH-PAHs than females [20], which may explain why the concentration of 2OHnap is higher in the male urine than in the female despite their smoking habits. It is also important to consider that the male individual may be a passive smoker, which contributes to his high urinary levels of the OH-PAHs.

In order to study the influence of urine matrices, different from the one used for the matrix-matched calibration in the precision and RR, both non-hydrolyzed non-smoker male and smoker female urine samples were spiked with the OH-PAHs (at $0.30 \mu\text{g L}^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and $0.60 \mu\text{g L}^{-1}$ for 2OHnap and 9OHphe), and subjected to the entire method. Fig. 3 (F) shows a chromatogram obtained after analysis of non-hydrolyzed male urine spiked with OH-PAHs as representative example. There were no differences between this chromatogram and that obtained with the non-smoker female used for the validation (see Fig. 3 (D)). The results of the precision and RR studies are also included in Table 3. The precision was adequate in both samples, with RSD values lower than 13%. With respect to the RR values, they ranged from 69% to 101% for the male non-smoker urine, and between 59% and 98% for the female smoker urine.

4. Conclusions

A new generation of bimetallic MILs was successfully incorporated for the first time in DLLME. The MIL is composed by two metallic complexes of Mn(II), and was characterized by its ultra-low viscosity, improved magnetic susceptibility, and RP-HPLC compatibility. Due to these features, the DLLME approach involving this MIL did not require any dispersive solvent, and the final MIL microdroplet after extraction was directly injected in the HPLC-FD after a minor dilution. These features were not possible in the only two previous DLLME methods involving bimetallic MILs [16,17].

As proof-of-concept, the method was successfully applied for the determination of a group of OH-PAHs in urine. After optimization and validation, the overall MIL-DLLME-HPLC-FD method showed high sensitivity and enrichment factors, high extraction efficiencies, and adequate intermediate precision despite the complexity of the matrix analyzed. The analysis of different urine samples allowed quantification of several OH-PAHs at the most common concentrations that have been reported for these biomarkers in urine, considering the gender and smoking habits of the individuals involved. This approach also constitutes the first application in which a bimetallic MIL is used in the analysis of complex biological samples.

Ongoing work will focus on the incorporation of other MILs from this new generation of solvents within different liquid-phase microextraction techniques, as well as on the design of novel bimetallic MILs with improved properties for the analysis of other relevant biological fluids.

CRediT authorship contribution statement

Raúl González-Martín: Writing – original draft, Methodology, Investigation, Formal analysis. **Sirintorn Jullakan:** Methodology, Investigation, Formal analysis. **María J. Trujillo-Rodríguez:** Writing – review & editing, Supervision, Project administration, Funding

acquisition, Conceptualization. **Nabeel Mujtaba Abbasi:** Methodology, Investigation. **Shashini De Silva:** Methodology, Investigation. **Jared L. Anderson:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Verónica Pino:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aca.2024.342448>.

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