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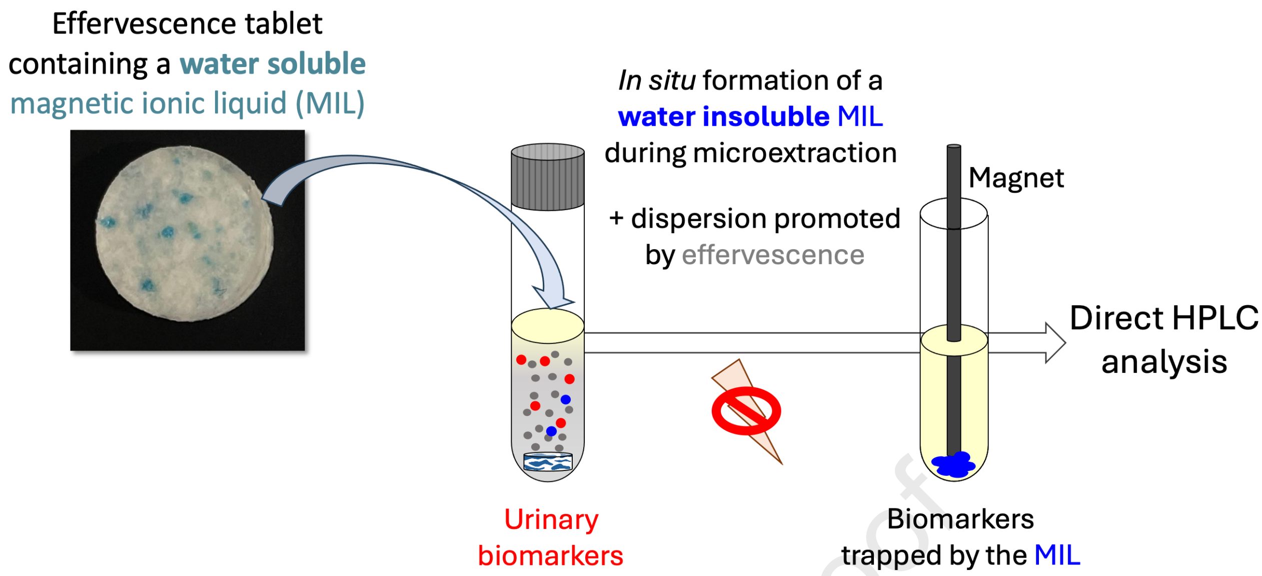
Effervescence tablet
containing a water soluble
magnetic ionic liquid (MIL)
In situ formation of a
water insoluble MIL
during microextraction
+ dispersion promoted
by effervescence
Magnet
Direct HPLC
analysis
Urinary
biomarkers
Biomarkers
trapped by the MIL

# Effervescence tablets based on magnetic ionic liquids as simple microdevices for the *in situ* dispersive liquid-liquid microextraction of urinary biomarkers

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## Abstract

__Background__: Magnetic ionic liquids (MILs) have been explored in dispersive liquid-liquid microextraction (DLLME). Their usage allows to substitute centrifugation and/or filtration steps by a quick magnetic separation. Besides, effervescence-assisted DLLME is one of the most known options to improve the dispersion of the extractant in the sample, while allowing to avoid the consumption of external energy during dispersion. Despite these interesting features, only one study incorporates MILs containing the tetrachloroferrate anion in effervescence tablets. These MILs are highly viscous and liquid at room temperature, thus compromising the stability of the tablets when used as extraction microdevices in effervescence-assisted DLLME, and only allowing their use in the conventional MIL-DLLME mode.

__Results__: A new class of effervescence tablets containing a Ni(II)-based MIL, that is solid at room temperature, is here proposed. This type of tablets permits their use, for first time, in the *in situ* DLLME mode, occurring through the transformation of a water-soluble MIL into a water-insoluble MIL microdroplet. This way, the tablet formulation included: the MIL, the metathesis reagent lithium bis[(trifluoromethyl)sulfonyl]imide, $NaH_2PO_4$ and $K_2CO_3$ as effervescence precursors salts, and $Na_2SO_4$ as salting-out and desiccating agent. The method is combined with high-performance liquid-chromatography and both fluorescence and ultraviolet detection, for the determination of monohydroxylated polycyclic aromatic hydrocarbons (OH-PAHs) and benzophenones (BPs), as biomarkers in urine. The method simply involved the addition of the effervescence tablet to the sample, thus taken place simultaneously the effervescence process and the metathesis reaction, without requiring any external energy consumption. The method presented limits of detection down to 10 $ng \cdot L^{-1}$ for OH-PAHs and to 0.60 $\mu g \cdot L^{-1}$ for BPs, inter-day relative standard deviations lower than 17%, and average relative recoveries of 94% in urine. The determined OH-PAHs contents in urine were between 0.40 and 16 $\mu g \cdot L^{-1}$, and between 17.8 and 334 $\mu g \cdot L^{-1}$ for BPs.

<u>*Significance*</u>: We have developed the first MIL-based effervescence tablets that are completely solid, thus improving the stability and robustness of these microdevices with respect to previously reported tablets involving MILs, while permitting to perform into the *in situ* DLLME mode (thus gaining in extraction efficiency). This approach including the MIL-based effervescence tablets constitutes an alternative *on-site* platform for the analysis of urine, as satisfactory precision, accuracy, and sensitivity are achieved despite not involving any external energy input within the analytical sample preparation setup. This method also constitutes the first application of MIL-based effervescence tablets for bioanalysis.

***Keywords:*** Effervescence tablets, Magnetic ionic liquids; *In situ* dispersive liquid-liquid microextraction; *On-site* microextraction; Urine; Magnetic separation

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## 1. Introduction

Magnetic ionic liquids (MILs) are materials derived from ionic liquids (ILs) that present inherent paramagnetic properties [1,2] because of the incorporation of metallic components [3–5] and/or radical moieties [6,7]. MILs possess the common structure of ILs, formed by combining bulky and asymmetric organic cations with inorganic or organic anions [8]. They also present most of the physicochemical properties of ILs, such as an impressive solvation ability for compounds of different nature, high thermal and chemical stability, and low to negligible vapor pressure at room temperature [8].

There is a wide range of possibilities for preparing MILs depending on the type and/or location of their paramagnetic component(s) [1,9]. Among these possibilities, the most conventional MILs are based on the tetrachloroferrate(III) anion ($[FeCl_4]^-$) [10]. However, in the last years, more rational designs have emerged to increase the stability of the MILs while ensuring lower viscosity and adequate magnetic susceptibility [4,5,11].

The magneto-responsive properties of MILs have been widely exploited in analytical (micro)extraction strategies [2,9], with dispersive liquid-liquid microextraction (DLLME) as the most widely used. Indeed, the usage of MILs is one of the most extended tools to speed up the DLLME procedure, as the isolation of the MIL (containing the extracted analytes) can be simply accomplished with the aid of an external magnet, thus avoiding non-sustainable and time-consuming centrifugation and/or filtration steps [9].

Recently, MILs based on metal cations have expanded their applicability due to the *in situ* DLLME mode [12]. In this approach, the water non-soluble MIL used as extraction solvent is generated *in situ* during the microextraction. This strategy facilitates MIL handling and allows for higher extraction efficiencies than the conventional MIL-DLLME approach using other types of MILs [12].

Despite the clear success of MILs in both conventional and *in situ* MIL-DLLME modes, several challenges still need to be addressed when using these magnetic solvents, mostly related to their high viscosity, which may hinder their effective dispersion in the sample. A popular DLLME option for improving the dispersion of the extractant in the sample without requiring any external energy is the effervescence-assisted DLLME [13]. This approach bases on the *in situ* formation of $CO_2$ bubbles during the microextraction, as a product of the effervescence reaction between two chemicals: one acting as proton donor agent, and other as a $CO_2$ source salt. Furthermore, this DLLME sub-mode offers the possibility to develop *on-site* microextraction strategies, as the reagents required to promote the effervescence can be compressed to form tablets which, if properly designed, can be used as portable extraction microdevices [13].

Many effervescence-assisted DLLME methods involving non-magnetic ILs have been reported in the last decade [14–18]. However, if a magnetic retrieval is required after the effervescence, to avoid centrifugation and/or filtration, it is fair to recognize that there is a requirement for $Fe_3O_4$ magnetic nanoparticles (MNPs) [14,17,18].

In the light of this background, the use of MILs as components of the tablets could clearly involve an advantage, as the magnetic separation can be accomplished without requiring the further incorporation of MNPs, thus simplifying the preparation of the tablet. Despite this interesting feature offered by MILs, there is only one study that reports their integration in effervescence tablets [19]. That application used a MIL containing $[FeCl_4]^-$, which may suffer hydrolysis at basic pH values [20], while being highly viscous and liquid at room temperature, which may compromise the stability of the tablet. Moreover, the MIL is not soluble in water, and thus its use is limited to the conventional MIL-DLLME mode.

The aim of this study is the design of a new type of effervescence tablets containing a MIL that is solid at room temperature, thus ensuring the stability and robustness of the resulting tablets without requiring the formation of any composite with the MIL and/or neither involving MNPs. The MIL is composed of a cation featuring Ni(II) coordinated with four *N*-alkylimidazole ligands (specifically, 1-butylimidazole), and chloride as anion: $[Ni(C_4IM)_4]Cl_2$ [4]. As this MIL is also water-soluble, it allows to expand for the first time the use of MIL-based effervescence tablets to the *in situ* MIL-DLLME mode. To ensure that sub-mode of DLLME, the metathesis reagent lithium bis[(trifluoromethyl)sulfonyl]imide ($LiNTf_2$) is also incorporated in the tablet formulation, together with $Na_2SO_4$ as desiccating agent, and $NaH_2PO_4$ and $K_2CO_3$ as effervescence precursors salts. The tablets are used in an *on-site* effervescence-assisted *in situ* MIL-DLLME method, and therefore the effervescence process (to ensure dispersion of the MIL without requiring any external energy input) and the metathesis reaction (to permit the transformation of the MIL into a water-insoluble solvent for the *in situ* mode) are simultaneously accomplished.

The method is shifted to the determination of two groups of metabolites in urine, specifically five monohydroxylated metabolites of polycyclic aromatic hydrocarbons (OH-PAHs) and four benzophenones (BPs). OH-PAHs are biomarkers of the human exposure to PAHs, which are well-known carcinogenic compounds [21], whereas BPs are endocrine-disrupting compounds, and their presence in urine can be related with the excessive use of personal care products containing these compounds (given their use as UV filters) [22]. Urine is a convenient biofluid for the determination of biomarkers, given its non-invasive character (less harmful for patients) and its availability (it is possible to easily obtain higher volumes of urine than other non-invasive biofluids like saliva). In this sense, the method proposed in this study constitutes the first application of MIL-based effervescence tablets to the analysis of complex biological samples.

## 2. Experimental

### 2.1. Chemicals and materials

The chemical structures and several physicochemical properties of the target biomarkers are included in Table S1 of the Electronic Supplementary Material (ESM). With respect to OH-PAHs, they were acquired from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 2-Hydroxyphenanthrene (2OHphe) and 1-hydroxypyrene (1OHpy) were supplied as standard solutions of 10 mg·$L^{-1}$ in acetonitrile (ACN). 2-Hydroxynapthalene (2OHnap, 99.9%), 2-hydroxyfluorene (2OHflu, 98%), and 9-hydroxyphenanthrene (9OHphe, 99.9%), were purchased as solid standards. Individual standard solutions of the 3 solids OH-PAHs were prepared in ACN, specifically at 1050 mg·$L^{-1}$ for 2OHnap, 1020 mg·$L^{-1}$ for 2OHflu, and 1190 mg·$L^{-1}$ for 9OHphe. Two intermediate standard solutions containing all the OH-PAHs were prepared in ACN, one containing 1 mg $L^{-1}$ of 2OHflu, 2OHphe, and 1OHpy, and 2 mg $L^{-1}$ of 2OHnap and 9OHphe; and the second one containing 0.1 mg $L^{-1}$ of 2OHflu, 2OHphe, and 1OHpy, and 0.2 mg $L^{-1}$ of 2OHnap and 9OHphe.

With respect to BPs, 2,4-dihydroxybenzophenone (BP1, 98%), 2,2′-dihydroxy-4-methoxybenzophenone (BP8, 98%), benzophenone (BP, 99%), and 2-hydroxy-4-methoxybenzophenone (BP3, 98%), were purchased from Sigma-Aldrich (San Luis, MO, USA) as solid standards. Individual standard solutions of the BPs were prepared in ACN at concentrations of 1000 mg·$L^{-1}$ for BP1, 1019 mg·$L^{-1}$ for BP8, 2046 mg·$L^{-1}$ for BP, and

1180 mg·L$^{-1}$ for BP3. Two intermediate standard solutions were prepared by having together all BPs in ACN at 100 mg·L$^{-1}$ and 10 mg·L$^{-1}$, respectively.

Working standard solutions containing both OH-PAHs and BPs were prepared daily by dilution of the corresponding intermediate solutions in ultrapure water or in diluted urine samples, depending on the specific study. All the individual and intermediate standard solutions were kept in a refrigerator at 4ºC and protected from light.

1-Butylimidazole (98%) and $NiCl_2$ (98%), both supplied by Sigma-Aldrich, were used for the preparation of the $[Ni(C_4IM)_4]Cl_2$ MIL. $NaH_2PO_4$ (≥99.0%) and $K_2CO_3$ (≥99.0%) were used as $CO_2$ precursors during the effervescence-assisted microextraction, and both salts were purchased from Sigma-Aldrich. Anhydrous $Na_2SO_4$ (≥99.0%) was supplied by Scharlau (Barcelona, Spain). The metathesis reagent $LiNTf_2$ was purchased from Sigma-Aldrich.

HCl (37%, v/v) was supplied by Honeywell Fluka™ (Seetze, Germany), and used to prepare an aqueous solution of 0.5 M HCl. Acetone (≥99.8%) LC grade and formic acid LC-MS LiChropur™ (98–100%) were supplied by Sigma-Aldrich. ACN HiPerSolv Chromanorm® LC grade was provided by VWR (Llinars del Vallés, Spain). Ultrapure water, with a resistivity of 18.2 MΩ cm, was produced by a Purelab® flex water purification system from Elga LabWater (Lane End, UK). 0.22 µm Millipore filters supplied by Sigma-Aldrich were used to filter the chromatographic mobile phases.

The microextraction method was performed in Pyrex® (Staffordshire, UK) centrifuge tubes of 15 mL (9.5 cm L × 2 cm O.D.). Eppendorf™ (Hamburg, Germany) polypropylene microtubes of 1.5 mL were used to dilute the MIL microdroplet prior to injection. The magnetic separation was accomplished using rod NdFeB magnets (0.5 cm D × 0.25 cm thick, B = 1.32–1.37 T) supplied by Supermagnete (Gottmadingen, Germany).

## 2.2. Instrumentation and equipment

A RV 10 digital rotary evaporator with temperature control purchased from Ika® (Staufen, Germany), in combination with a vacuum pump VP 2 Autoyac from Vacuubrand (Wertheim, Germany), was used to remove the solvent used for the synthesis of the MIL. The MIL was dried in a Vaciotem-TV vacuum oven supplied by J.P. Selecta®. The Fourier-transform infrared (FT-IR) spectrum of the MIL was obtained using a Bruker Optics ALPHA-E spectrometer with a universal Zn-Se attenuated total reflection (ATR) accessory. A manual hydraulic press machine from Specac® (Orpington, UK) was used for the preparation of the effervescence tablets containing MILs.

A 1260 Infinity HPLC system from Agilent Technologies (Santa Clara, CA, USA) was used for the chromatographic separation of the analytes. The HPLC was coupled to different detectors in tandem: a Varian (Palo Alto, CA, USA) ProStar 330 UV detector for the quantification of BPs, and a Waters™ (Milford, MA, USA) 474 FD system used for the determination of OH-PAHs. Manual injections in the HPLC were performed using a Rheodyne 7725i injection valve with a 20 μL loop supplied by Supelco (Bellefonte, PA, USA).

The chromatographic separation was accomplished in an InfinityLab Poroshell 120 EC-$C_{18}$ column (100 mm × 4.6 mm × 4.0 μm) purchased from Agilent Technologies and protected by a LC-$C_{18}$ guard column from Phenomenex (Torrance, CA, USA). The elution gradient was performed with a mobile phase formed by ACN and ultrapure water at a constant flow rate of 1.0 mL·min$^{-1}$. The water was acidified with 0.02% (v/v) of formic acid. The optimum gradient started at 50% (v/v) of ACN, keeping this composition for 5 min, and then it was increased during 4 min until reaching 100% (v/v). Finally, this percentage was maintained for 5 min. The detection of the analytes was simultaneously accomplished in the UV and FD systems. Table S2 of the ESM shows the retention times of the analytes obtained with the optimum elution gradient, and the detection conditions selected to ensure the maximum sensitivity.

## 2.3. Procedures

### *2.3.1. Synthesis of the MIL*

The $[Ni(C_4IM)_4]Cl_2$ MIL was prepared following the highly simple protocol described in the literature [4] with slight modifications. Briefly, the synthesis of one batch of the MIL required $NiCl_2$ (4.0 mmol, 0.52 g) and 1-butylimidazole (16 mmol, 1.9 g), which were mixed in deionized water (4.0 mL). The reaction was performed at room temperature, overnight, and under constant stirring. Three batches of MIL were simultaneously prepared under these synthetic conditions, and the final three products were mixed before removing the solvent. Then, the water was removed in a rotary evaporator (60ºC, $1.5 \cdot 10^4$ $N \cdot m^{-2}$) and the final solid product (obtained from the mixture of the three MIL batches) was dried overnight in a vacuum oven (60ºC, $4.0 \cdot 10^4$ $N \cdot m^{-2}$). Under these conditions, ~4 g of MIL were obtained. This amount permits the development of up to 88 extractions when using the optimum amount of MIL (see Section 2.3.2) under the microextraction conditions (see Section 2.3.4). The reaction to prepare the MIL is summarized in Figure S1 of the ESM. Besides, Figure S2 of the ESM includes the FT-IR spectrum of the MIL synthesized.

### *2.3.2. Preparation of effervescence tablets containing a solid MIL*

Effervescence tablets were prepared by directly mixing the solid $[Ni(C_4IM)_4]Cl_2$ MIL with the following salts: $NaH_2PO_4$ as proton donor agent, $K_2CO_3$ as $CO_2$ source salt,

$LiNTf_2$ as metathesis reagent, and $Na_2SO_4$ acting as both salting-out and desiccating agent. In all cases, the salts and the MIL were dried at 50ºC in a vacuum oven and then kept in a desiccator before preparing the tablet.

Different ratios were tested, and the optimum composition of the tablet comprises 44.0 mg of MIL, 38.0 mg of $LiNTf_2$, 150 mg of $NaH_2PO_4$, 157 mg of $K_2CO_3$, and 650 mg of $Na_2SO_4$. All the salts were accurately weighted and grinded in a mortar until achieving a powder mixture. After that, the powder was compressed in a hydraulic press machine at 0.4 MPa for 30 s. All the effervescence tablets were stored in a desiccator at room temperature prior to their use.

***2.3.3. Collection and pretreatment of urine samples***

Urine samples were collected in the early morning from healthy volunteers with different genders and smoking habits (two non-smoker females, one smoker female, and one smoker male). All the urine samples were stored at -80ºC until their analysis and were discharged after the experiments.

The urine from a healthy non-smoker female (*a priori* free of the target biomarkers and non-hydrolyzed) was selected for the optimization and validation of the analytical method through a matrix-matched calibration approach. The remaining urine samples (provided by two females and one male) were subjected to an enzymatic hydrolysis to form the hydroxylated metabolites of PAHs. The hydrolysis procedure was performed according to the enzyme manufacturer instructions. Briefly, 2 mL of acetic acid/sodium acetate buffer solution at pH 5 were added to 15 mL of urine and were mixed with 50 μL of the β-glucoronidase/arylsulfatase enzyme. Finally, the samples were incubated for 24 h at 37ºC.

Both hydrolyzed and non-hydrolyzed urine samples required a dilution with ultrapure water prior to the analysis. Thus, under optimum conditions, the solution subjected to the microextraction method had a 14% (v/v) of urine.

### *2.3.4. Effervescence-assisted in situ MIL-DLLME*

All variables exerting an influence in the microextraction method were properly evaluated. The optimum conditions for the *in situ* MIL-DLLME method are schematically shown in Figure 1. A volume of 910 µL of urine (hydrolyzed or non-hydrolyzed, depending on the experiment) was diluted with 5.0 mL of ultrapure water in a 15 mL glass centrifuge tube. The diluted sample was conditioned at 40ºC for 1 min. The pH of the solution was adjusted to pH 5 by adding 0.5 M HCl. Then, 400 µL of acetone were added as auxiliary solvent to ensure the formation of the MIL microdroplet [12]. Under these conditions, the final volume of the diluted urine standard solution (or diluted urine sample) was 6.5 mL, with a total content of 14% (v/v) of urine.

The extraction step was simply performed by adding the effervescence tablet (see Section 2.3.2) to the tube. Immediately, $CO_2$ bubbles were released from the tablet, thus enhancing the dispersion of the water-soluble MIL (with the $Cl^-$ anion) through the sample/standard solution without the assistance of any external force. The complete disintegration of the tablet within the sample/standard solution took place in ~1 min. Then, the extraction vial was equilibrated during 13 min. During this time, the metathesis reaction between the MIL and liberated $LiNTf_2$ was accomplished, leading to the *in situ* formation of a water-insoluble MIL (with the $[NTf_2]^-$ anion) containing the extracted and preconcentrated analytes. This MIL droplet was collected with a rod NdFeB magnet directly introduced into the tube. Finally, the MIL microdroplet (~ 25 µL) was diluted up to 400 μL with ACN and directly injected into the HPLC-UV/FD system. A video showing the procedure is included within the supplementary material.

## 3. Results and discussion

### 3.1. Components of the effervescence tablet based on a solid MIL

The $[Ni(C_4IM)_4]Cl_2$ MIL was selected for the development of the effervescence tablets based on MILs as it possesses two key features. Firstly, this MIL is solid at room temperature [4], and therefore, it can be easily mixed with the remaining components of the effervescence tablet without any risk of losing the material during tableting. Moreover, this MIL is soluble in water and therefore it can be *in situ* transformed during the microextraction into an aqueous-non-soluble MIL (this latter MIL being liquid in nature) [12].

In this sense, two chemical reactions are going to take place during the effervescence-based *in situ* microextraction: *(i)* a metathesis reaction with anion exchange of the MIL (transforming the water-soluble MIL into a water insoluble one), and *(ii)* an effervescence reaction (forming $CO_2$ bubbles thus ensuring proper dispersion of the MIL into the solution while promoting the microextraction performance).

$LiNTf_2$ was added to the tablet to ensure the metathesis reaction of the MIL, following previous studies [12]. Therefore, the reaction promoted (Eq. (1)) was used to exchange the $Cl^-$ of the water-soluble MIL by the $[NTf_2]^-$ anion of the salt, thus generating a water-insoluble MIL.

$$[Ni(C_4IM)_4]Cl_2 + 2\ LiNTf_2 \rightarrow [Ni(C_4IM)_4][NTf_2]_2 + 2\ LiCl \qquad \text{Eq. (1)}$$

With respect to the salts required to perform the effervescence reaction, citric acid and $NaH_2PO_4$ were selected as possible proton donor agents, while $K_2CO_3$ and $KHCO_3$

were studied as possible $CO_2$ sources. These salts have been widely used as effervescence precursors in previous studies [17–19]. Therefore, four types of effervescence tablets including the MIL, $LiNTf_2$, and the different possible combinations of the effervescence salts, were prepared. Aiming to use a miniaturized amount of MIL, 30.0 mg of this material were weighted for the preparation of these initial tablets, together with the required amount of $LiNTf_2$ (26.0 mg) according to the stoichiometry of the metathesis reaction (1:2, MIL:$LiNTf_2$, see Eq. (1)). The amounts of the effervescence salts were calculated to produce the same amount of $CO_2$ (2.25 mmol) for comparative purposes. This amount was fixed based on previous experiments, which demonstrated that it was enough to ensure adequate effervescence for ~5.0 mL of sample. Table 1 includes the formulations of the different tablets prepared.

The tablets containing $KHCO_3$ rapidly absorbed water from the ambient, and thus were discharged given their instability. On the other hand, the tablets containing $K_2CO_3$ combined with both citric acid and $NaH_2PO_4$ (see Table 1) were successfully prepared, manipulated, and stored, and thus they were selected for optimizing the microextraction method.

It was observed that tablets with $K_2CO_3$ and citric acid generated big $CO_2$ bubbles when added to 5.0 mL of ultrapure water. In fact, an abrupt release of $CO_2$ and tablet disintegration occurred in a short period of time (~10 s). On contrary, the bubbles formed when using the tablet based on $NaH_2PO_4$ were smaller and tablet disintegration occurred within a relatively longer period (~1 min). After the disintegration of the tablet formed by citric acid and $K_2CO_3$, the time required for the formation of the water insoluble MIL microdroplet was too long under a microextraction perspective (~30 min). However, when the tablet containing $NaH_2PO_4$ and $K_2CO_3$ was used, the MIL microdroplet started to form in the aqueous solution after ~7 min of equilibration time. For this reason, $NaH_2PO_4$ was selected as proton donor agent.

With respect to the $LiNTf_2$ salt required in the tablet, it is important to consider that it has a hygroscopic character, and the anion can even undergo decomposition in aqueous media [23,24]. To prevent this issue, anhydrous $Na_2SO_4$ was added as desiccating agent to the tablets. Thus, 750 mg of this salt were compressed together with the MIL, the $LiNTf_2$, and the effervescence salts. It was clearly observed that the tablets including $Na_2SO_4$ were more stable and robust than analogous tablets that did not include this salt. These tables with $Na_2SO_4$ could be maintained in the desiccator for 4 days keeping a similar performance (in terms of extraction efficiency) than those freshly prepared. In addition, the tablet weight variation when storing the tablets after 24 h was 0.024%, a value that is lower than that obtained for the already reported effervescence tablets with a liquid MIL (0.42%) [19], thus showing improvement in the stability of the tablets by the incorporation of a solid MIL. Figure 2 shows the visual aspect of different effervescence tablets containing this solid MIL, prepared with different amounts of the salts involved.

## 3.2. Preliminary studies using the effervescence tablets for the *in situ* MIL-DLLME method

### *3.2.1. Coupling the MIL with RP-HPLC-UV/FD*

The effervescence tablets containing the $[Ni(C_4IM)_4]Cl_2$ MIL were evaluated in an effervescence-assisted *in situ* MIL-DLLME microextraction strategy, with the purpose of extracting and preconcentrating a group of biomarkers from human urine. The microextraction method is directly combined to reversed phase (RP)-HPLC together with

UV detection tandem FD for the determination of BPs and OH-PAHs, respectively. With this purpose, the $[Ni(C_4IM)_4][NTf_2]_2$ MIL microdroplet obtained after the extraction (*a priori* containing the extracted and preconcentrated analytes) should be directly injected into the HPLC system only performing a minor dilution with ACN. Aiming to ensure such direct compatibility, several considerations were followed [12].

First, an end-capped $C_{18}$ stationary phase was used to reduce the number of free silanol groups, thus avoiding irreversible interactions between the cation of the MIL and the stationary phase. Second, the mobile phase selected was formed by ACN and an aqueous solution containing formic acid (0.02%, v/v), thus ensuring an acidic pH in the mobile phase. Furthermore, the solubility of the MIL (mostly related to the nature of the $[NTf_2]^-$ anion) in the entire range of mobile phase compositions within the HPLC elution gradient needs to be ensured. As a minimum of 50% (v/v) of ACN was necessary to avoid the precipitation of the MIL, the chromatographic separation of the analytes was optimized using an elution gradient starting at this percentage.

Besides, the detection conditions for each group of biomarkers were separately optimized using both FD and UV. These conditions were adjusted to obtain the maximum sensitivity while simultaneously avoiding any interfering signal from the MIL (considering that that absorption and possible fluorescence were expected for this MIL given the structure of the cation [12]).

Figure 3 (A) and 3 (B) show representative chromatograms of standards of the OH-PAHs and the BPs, respectively, dissolved in ACN using the optimum elution gradient described in Section 2.2 and the UV/FD conditions detailed in Table S2 of the ESM. As it can be observed, the chromatographic separation of both groups of biomarkers was simultaneously achieved in less than 9 min. Additional studies were also performed to test possible interferences of the MIL in the chromatographic calibration (they will be shown in further sections, when describing the microextraction method with the MIL), allowing to conclude that it was possible to perform calibrations with standards in ACN, without the needing of mimicking the final phase injected in the HPLC (MIL plus ACN).

Table S3 of the ESM includes the quality analytical parameters of the chromatographic method performed with standards of the biomarkers in ACN (without any microextraction and preconcentration step). The determination coefficients ($R^2$) were higher than 0.998 in all cases and adequate inter-day reproducibility was also attained.

***3.2.2. Screening study in microextraction with MIL-based effervescence tablets***

The initial studies involved the usage of the tablets with aqueous standards of the target analytes. The composition used for the tablets was: 30.0 mg of MIL, 26.0 mg of $LiNTf_2$, 297 mg of $NaH_2PO_4$, 311 mg of $K_2CO_3$, and 750 mg of $Na_2SO_4$, as above commented (Section 3.1). The initial aqueous standard volume was set at 5.0 mL, as volumes between 5 and 10 mL are quite common in microextraction strategies [12]. The concentration of the aqueous standard subjected to the microextraction method was 9.6 $\mu g \cdot L^{-1}$ for 2OHnap and 9OHphe, 4.8 $\mu g \cdot L^{-1}$ for the remaining OH-PAHs, and 80 $\mu g \cdot L^{-1}$ for all the BPs. Besides, the pH of the standard was adjusted to pH 5, aiming to ensure that all the analytes were on their neutral form (see $pK_a$ values in Table S1 of the ESM) and also to guarantee the stability of the metal complex of the MIL (it is stable at pH values below 7 [25]). Furthermore, 300 µL of acetone were preliminary used, as it was required for the formation of the MIL microdroplet in previous studies [12].

Under these preliminary conditions, $CO_2$ bubbles were quicky released upon addition of the tablet, and its complete disintegration was achieved in only 1 min of effervescence. After this short time, the solution got immediately turbid, likely due to the formation of the water insoluble $[Ni(C_4IM)_4][NTf_2]_2$ MIL. The metathesis reaction was let to perform for 15 min as equilibration time. Then, the obtained MIL microdroplet (~12 µL) was easily recovered with a rod magnet, transferred to an Eppendorf tube, diluted to 400 µL with ACN to reduce its viscosity while ensuring minimum solvent-mismatch, and injected in the HPLC-UV/FD. Under these conditions, the maximum enrichment factor ($EF_{max}$) obtained with this method (not yet optimized), was 13.

Figure S3 (A) and S3 (B) of the ESM show the HPLC-FD and HPLC-UV chromatograms obtained after the injection of the diluted MIL microdroplet. The MIL did not interfere in the separation of both groups of biomarkers, and it only presented signals at the beginning of both HPLC-FD and HPLC-UV chromatograms, right before the elution of the first analyte. The preliminary preconcentration can be easily verified comparing the chromatograms of Figure S3 (C) and S3 (D) of the ESM, showing standards in ACN at concentrations ~ 3 times higher than the concentration of the aqueous standard subjected to the microextraction method (for the latter the diluted MIL microdroplet is injected in the HPLC).

Given the intended applicability of the effervescence tablets for the analysis of urine, the effect of this complex biological matrix on the extraction performance of the method was also studied. The urine coming from a healthy non-smoker female volunteer (*a priori* free of the target biomarkers) was used for these experiments. 5.0 mL of such urine (without any dilution) were subjected to the microextraction method under the conditions above described, and the MIL microdroplet was not formed, likely because the metathesis reaction did not perform in the pure urine matrix. Therefore, different dilutions of the urine with ultrapure water were tested to assess the maximum content of urine that allowed the proper formation of the MIL microdroplet. It was observed that the metathesis reaction performed when the content of urine in the solution did not exceed 18% (v/v).

The extraction was performed in triplicate with standard solutions of diluted urine and the following urine contents: 9.0% (v/v), 14% (v/v), and 18% (v/v), and also in ultrapure water, for comparative purposes. Percentages lower than 9.0% (v/v) were not tested to avoid excessive losses in preconcentration due to the dilution. Figure S4 of the ESM shows that the extraction efficiencies decreased when incorporating real urine in the solution with respect to those obtained in ultrapure water. Among the different urine percentages tested, there were not significant differences between 9.0% (v/v) and 14% (v/v), while there was a slight decrease in the peak areas when using 18% (v/v). Considering these results, and aiming to overcome the strong matrix effect observed, a diluted urine standard solution with a urine content of 14% (v/v) was selected as matrix to perform the further optimization and validation of the method.

### 3.3. Optimization of the *in situ* MIL-DLLME method

Different variables that may exert an influence in the extraction performance of the *in situ* MIL-DLLME method were assessed. Firstly, some parameters were optimized using a factor-by-factor approach: the type and concentration of the salting-out agent, the requirement of an auxiliary solvent and its volume, and the sample volume. The remaining parameters, including the equilibration time, and those related with the effervescence tablet composition (i.e., the amount of MIL and $LiNTf_2$, and the amount of the effervescence salts), were optimized through an experimental design.

In all cases, the values of two variables were fixed: a pH of 5 for the diluted urine standard solution; and 400 µL as the volume of the final extract to be injected in the HPLC (the MIL microdroplet dissolved in ACN). The response variable used during all the optimization was the chromatographic peak area of each analyte, and all the experiments were performed with diluted urine standard solutions from a healthy non-smoker female, with a urine content of 14% (v/v).

### *3.3.1. Effect of type and concentration of salting-out agent*

The desiccating agent, incorporated into the tablets to ensure robustness and stability (above commented in Section 3.1), could also act as a salting-out agent, given the high charge density of the anion $SO_4^{2-}$, and thus it could have a positive effect on the extraction efficiency. Thus, the effect of the concentration of $Na_2SO_4$ was assessed. The extraction efficiency obtained with tablets not containing this salting-out agent was also evaluated with comparative purposes. Other possible salting-out agents were not tested, aiming to reduce the number of salts in the effervescence tablets formulation.

As it can be observed in Figure S5 of the ESM, the addition of 10% (w/v) of $Na_2SO_4$ had a positive effect in the extraction efficiencies. The use of 15% (w/v) involved a decrease in the efficiency, likely due to an increase in the viscosity of the solution, also leading to a reduction of the MIL microdroplet volume. A higher percentage (20%, v/v) was also tested, but in this case the complete disintegration of the tablet in the diluted urine standard was not even possible, given the high total salt content. The incorporation of $Na_2SO_4$ was beneficial as both drying agent and salting-out agent, but its concentration would not surpass 10% (w/v), and thus this percentage was selected as optimum for the remaining experiments.

### *3.3.2. Effect of type and volume of auxiliary solvent*

Certain DLLME procedures do not require the use of an auxiliary solvent to improve the dispersion of the extraction solvent in the sample [17,26]. However, in this study this solvent was necessary, as the MIL droplet was not even formed without it, which agrees with other studies for this MIL [12]. In view of these results, different types of solvents were evaluated: acetone, ACN, and methanol. Methanol was quickly discarded as a white precipitated was observed. With respect to acetone and ACN, as it can be observed in Figure S6 (A) of the ESM, higher extraction efficiencies were obtained with acetone. Indeed, the obtained peak areas were 1.5–2 times higher when using this solvent. Given these results, acetone was selected as optimum solvent.

The effect of the acetone volume was also assessed from 200 µL to 500 µL (Figure S6 (B) of the ESM). An increase in the volume of acetone from 200 µL to 400 µL led to a significant increase in the peak areas (up to 3.75 times increase). However, the use of 500 µL is accompanied with a decrease in the extraction efficiency, due to the partial solubilization of either the MIL or the analytes in the sample in such high volume of acetone, thus hindering their partition to the MIL water-non soluble phase. Therefore, 400 µL was the volume of acetone selected as optimum.

***3.3.3. Effect of sample volume***

The sample volume also has a direct effect on the preconcentration achieved with the method. Clearly, higher sample volumes lead to an increase in the maximum enrichment factors. In this MIL-DLLME approach, the sample volume is limited by the effervescence process, as the dispersion promoted by the $CO_2$ bubbles is more efficient when it is performed with lower volumes. Aiming to achieve a compromise solution between the effervescence efficiency and the preconcentration, different sample volumes were assessed.

In general, there was a slight increase (or no significant differences, depending on the analyte) in the peak areas when using 6.5 mL with respect to those obtained for 5.0 mL, as it can be observed in Figure S7 of the ESM. However, the use of higher sample volumes (7.0 and 8.0 mL) led to a decrease in the extraction efficiencies of all the analytes. Therefore, 6.5 mL was selected as sample/standard volume for the remaining experiments. Under these conditions, the $E_{Fmax}$ was 16.

### *3.3.4. Box-Behnken experimental design*

Three variables were assessed through a Box-Behnken design (BBD): the amount of MIL in the tablet, in mg (variable A), the amount of $NaH_2PO_4$ as proton donor agent for the effervescence, in mg (variable B), and the equilibration time, in min (variable C). The values of other two variables (amount of $LiNTf_2$ as metathesis reagent, in mg; and amount of $K_2CO_3$ as $CO_2$ source salt, in mg) were set depending on the values of variable A and variable B, respectively, given their interdependency. The amount of $LiNTf_2$ was set to maintain the stoichiometry of the metathesis reaction (1:2, MIL:$LiNTf_2$). Regarding the amount of $K_2CO_3$, it was set to have a slight excess of $NaH_2PO_4$ with respect to the theoretical stoichiometry of the effervescence reaction (1.1:1, $NaH_2PO_4$:$K_2CO_3$).

Table S4 of the ESM includes the values of the variables assessed. The amount of MIL was evaluated between 20.0 mg and 60.0 mg (with related amounts of $LiNTf_2$ ranging from 17.0 mg to 52.0 mg). 20.0 mg was the amount of MIL that requires the minimum amount of $LiNTf_2$ that can be accurately weighted for adequate distribution in the tablet (17.0 mg). Amounts higher than 60.0 mg of MIL were not tested to ensure a microextraction method. With respect to the effervescence salts, 150 mg was the minimum amount of $NaH_2PO_4$ evaluated, as lower amounts did not ensure the generation of enough $CO_2$ bubbles for the proper dispersion of the MIL. 500 mg was the maximum amount possible of $NaH_2PO_4$ to properly form the effervescence tablet, given the maximum capacity of the press machine. Thus, the amount of $K_2CO_3$ ranged between 157 mg and 523 mg. With respect to the equilibration time, it was assessed between 7.0 min and 30 min. It was observed that the formation of enough volume of MIL microdroplet that was easy to handle and collect with the magnet required at least ~7.0 min of equilibration time. Besides, times higher than 30 min were not tested, to ensure simplicity.

Figure S8 and Figure S9 of the ESM show the individual response surfaces of each biomarker. With respect to OH-PAHs, similar values of optimum amount of MIL were obtained (ranging from 43.8 mg to 47.5 mg), and the optimum amount of proton donor agent was the same for all the OH-PAHs (150 mg). The optimum equilibration was close to the middle of the assessed range (between 10.7 min and 15.9 min). These results explain the similar profile obtained for the response surfaces of the OH-PAHs (see Figure S8 of the ESM). With respect to the BPs, a similar behavior to that of the OH-PAHs was observed, except for BP1 and BP8, for which lower MIL amounts were optimum (see Figure S9 of the ESM). Table S5 of the ESM includes the main effects and interactions between the variables assessed with the BBD for each of the target analytes. Similar profiles were obtained for most of the analytes, and interactions between all the variables were observed, except for BP1 and BP8, for which there was no interaction between the equilibration time and the amount of $NaH_2PO_4$. To get a better overview, Table S6 of the ESM includes the optimum values according to the desirability function, and Figure 4 shows the overall response surfaces obtained for all analytes. The optimum values were selected according to the desirability function as: 44.0 mg of MIL (which required 38.0 mg of $LiNTf_2$), 150 mg of $NaH_2PO_4$ (requiring 157 mg of $K_2CO_3$), and 13.0 min of equilibration time.

Clearly, the optimum amount of MIL was in the middle of the range assessed, constating that high amounts of MIL were not positive for the extraction procedure, likely due to the difficulty to disperse such high amounts of material in the sample while achieving an efficient interaction with the analytes (especially when no external energy assistance was involved). In any case, a low consumption of MIL is desirable for a microextraction approach. The optimum equilibration time was also in the middle of the range of values assessed, as longer times may provoke losses of the analytes present in the MIL microdroplet due to back-extraction in the aqueous sample and/or lack of stability of the MIL. Furthermore, this time (13.0 min) is highly suitable for a DLLME method. With respect to the amount of effervescence salts, the minimum value was the optimum, probably due to the increase in the viscosity of the solution when high amounts of salts are used, which may hinder the formation of the MIL microdroplet.

***3.3.5. Robustness studies***

Aiming to verify if slight modifications in the optimum conditions of the method could lead to significant changes in the extraction efficiencies achieved, the robustness of the method was assessed. Two representative variables related to the effervescence tablets composition were selected for these studies: the amount of $NaH_2PO_4$, and the amount of $LiNTf_2$. In the case of $NaH_2PO_4$, the studies evaluated if lower amounts than the optimum value could improve the extraction efficiency, as this value was the minimum assessed (150 mg). With respect to the amount of $LiNTf_2$, it was tested if a slight change in the metathesis reaction molar ratio could favor the extraction, given the key role of this metathesis reagent in the MIL microdroplet formation. In this case, an excess of $LiNTf_2$ was studied keeping the amount of MIL at its optimum value obtained in the BBD (i.e., 1:3, MIL:$LiNTf_2$, 57.0 mg of $LiNTf_2$ for 44.0 mg of MIL).

The results obtained in these studies demonstrated that decreasing the amount of $NaH_2PO_4$ to 90 mg did not imply significant differences ($p > 0.05$) in the extraction efficiency with respect to using the optimum amount of 150 mg, except for 1OHpy, BP1 and BP (see Figure S10 and Table S7 of the ESM). Furthermore, the use of an excess of

$LiNTf_2$ led to a decrease in the peak areas (1.5 to 2.0 times lower) when compared with those amounts coming from the theoretical metathesis reaction stoichiometry (see Figure S10 of the ESM). Hence, those values obtained in the optimization studies (150 mg of $NaH_2PO_4$ and 38.0 mg of $LiNTf_2$ for 44.0 mg of MIL) were kept for the subsequent analytical performance of the method.

### 3.4. Analytical performance of the *in situ* MIL-DLLME-HPLC-UV/FD method in urine

Matrix-matched calibration curves were performed using diluted urine standard solutions (with a urine content of 14%, v/v) obtained from a healthy non-smoker female. Table 2 includes the main quality analytical parameters of the overall method. The method presented an adequate linearity ($R^2$ values higher than 0.992) for wide calibration ranges.

With respect to limits of detection (LODs), they were experimentally obtained by decreasing the concentration of the diluted urine standard solutions subjected to the overall method until a signal-to-noise (S/N) ratio of 3 was obtained. Limits of quantifications (LOQs) were estimated by 10/3 times the LODs, and experimentally verified by subjecting standard solutions at the predicted concentrations to the entire method. As it can be observed in Table 2, LODs were at the ng $L^{-1}$ level for the OH-PAHs (ranging from 10.0 ng·$L^{-1}$ to 120 ng·$L^{-1}$), while BPs presented LODs at the µg·$L^{-1}$ level (between 0.60 and 2.0 µg·$L^{-1}$).

The method was assessed in terms of precision and relative recoveries (RRs, %) by using diluted urine standard solutions at two concentration levels belonging to the calibration range, but not used for obtaining the calibration curves (see Table 3). Precision was evaluated with the RSD values obtained in both intra-day and inter-day studies. With respect to the intra-day precision, RSD values ranged from 2.3% to 7.7% for the low concentration level. The intermediate precision was assessed in three non-consecutive days, obtaining RSD values lower than 17% for the low concentration level. According to the Horwitz equation [27], the precision of the method was highly adequate for the concentration levels involved. Indeed, the maximum RSD value for OH-PAHs was 17% (for a concentration of 1.6 $\mu g \cdot L^{-1}$). With respect to BPs, the maximum RSD was 11% for 30 $\mu g \cdot L^{-1}$ (which is highly adequate considering an acceptable RSD value of 32% for 10 $\mu g \cdot L^{-1}$ [27]). In the light of these results, it is important to highlight that the heterogeneous distribution of the MIL and the other salts in the tablets (see Figure 2) did not affect the reproducibility. Indeed, the influence of the tablet composition in the analytical performance seems to be only linked to the amounts of the components involved, which were kept constant in all the tablets.

RRs values were obtained by the ratio between the concentration of the analytes found in the MIL microdroplet extract (obtained using the calibration curves of the entire method, see Table 2), and the initial concentration of the diluted urine standard solution subjected to the method (see Table 3), and expressed as percentage. RRs ranged from 84% to 103% for the low concentration level. It should be noted that these values were also adequate for the assessed concentration levels according to the Horwitz criterium [27].

The enrichment factor (EF) obtained in these experiments were up to 3.5, thus achieving adequate preconcentration for all the biomarkers in urine (Table S8 of the ESM). It should be noted that the MIL-DLLME procedure is a non-exhaustive extraction approach, and that an adequate sensitivity is reached for the application intended.

Figure 3 (C) and 3 (D) shows representative chromatograms after analyzing diluted urine standard solutions at the low concentration level used. As it can be observed, there were only few signals coming from the urine matrix at the beginning of both chromatograms, which did not interfere with the determination of the analytes.

**3.5. Analysis of urine samples using the *in situ* MIL-DLLME-HPLC-UV/FD method**

The developed method was applied for the analysis of urine from three volunteers: two smokers with different genders (one male and one female) and smoking habits, and one non-smoker female.

The samples were first subjected to hydrolysis and then analyzed (see Table 4). All the OH-PAHs were detected in urine samples coming from the smoker individuals, except 2OHnap in the male urine, and 9OHphe, which was not detected in any of the samples. Several of the OH-PAHs could be also quantified: 2OHflu and 1OHpy in the male urine; and 2OHnap, 2OHflu, 2OHphe and 1OHpy in the female urine. The concentrations found were at low $\mu g \cdot L^{-1}$ levels, ranging from $0.40 \pm 0.04$ $\mu g \cdot L^{-1}$ for 1OHpy to $16.0 \pm 0.7$ $\mu g \cdot L^{-1}$ for 2OHnap, the latter being indeed the most abundant PAH metabolite according to previous studies regarding the occurrence of these biomarkers in human urine [23]. With respect to the smoking habits of the individuals, the female volunteer had more smoking frequency than the male volunteer. This was observed by the urinary levels of OH-PAHs found for both individuals, as the female urine presented more OH-PAHs and at higher concentrations than the male urine. It is interesting to highlight that the non-smoker female urine was free of OH-PAHs, which indicated a low to negligible exposure of this individual to these carcinogenic contaminants.

With respect to BPs, their presence was only evidenced in the female urine samples, regardless on their smoking habits, likely due to the extended use by these specific individuals of suncreams and/or other personal care products containing UV filters [24]. Hence, all the BPs were detected in the smoker female urine (except BP8), and BP1 and BP were successfully quantified. On the other hand, BP1, BP8, and BP3 could be quantified in the non-smoker female urine, while BP was not detected in this sample. The concentrations of BPs ranged from 17.8 ± 0.3 $\mu g \cdot L^{-1}$ to 334 ± 15 $\mu g \cdot L^{-1}$.

Finally, aiming to assess the RR values and precision in the different urine matrices non-hydrolyzed urine samples coming from the smoker individuals were spiked with the biomarkers at the low concentration level (1.6 $\mu g \cdot L^{-1}$ for 2OHnap and 9OHphe; 0.80 $\mu g \cdot L^{-1}$ for the remaining OH-PAHs; and 30 $\mu g \cdot L^{-1}$ for all the BPs). As it can be observed in Table S9 of the ESM, the method was highly precise in both samples, with intra-day RSD values lower than 11%. OH-PAHs were not detected in non-hydrolyzed urine. For BPs, the RRs were estimated considering both the concentration spiked in the sample and the initial concentration quantified (Table 4). The RRs values ranged from 73% to 157% in the smoker male, and they were between 47.6% and 109% in the smoker female urine (Table S9 of the ESM).

### 3.6. Comparison of the *in situ* MIL-DLLME method with already reported IL- and MIL-DLLME methods

The performance of this method in terms of LODs was similar to other methods reported for the determination of OH-PAHs and BPs in biofluids using HPLC combined with FD and/or UV/DAD detection, respectively [11,28], whereas better sensitivity is obviously obtained when using MS detection [29,30]. In addition, this method was as simple as other magnetic-assisted DLLME approaches for the monitorization of OH-PAHs [11], with the additional advantage of possible *on-site* analysis.

With respect to the energy consumption, it is important to point out that, in general, MIL/IL-DLLME methods, including both effervescence-assisted extraction approaches [16,17] and other non-assisted methods [12,31,32], consumed significant external energy during microextraction (from 0.002 to 0.8 kW·h per extraction, depending on the study, as shown in Table S10 of the ESM). These procedures generally require vortex [12,31] and/or ultrasounds [32] for IL/MIL dispersion, and centrifugation in the case of ILs for their isolation [16,32]. Certain analytical methods, despite of using the effervescence assistance, required additional steps of evaporation [16,17], something that is not needed in the current approach.

Two metrics, one related to sustainability and another one to applicability, were applied to the current method and to those included in Table S10 of the ESM. The sample preparation metric of sustainability (SPMS) tool [33] was applied for assessing sustainability (Figure S11 of the ESM). SPMS was selected because it only evaluates the sample preparation step, while other green metrics, consider the entire analytical method, thus including the sampling and the subsequent analytical technique used for separation and detection/quantification (e.g., chromatography). The proposed method achieved the highest score with SPMS (7.16), followed by the study reporting an *in situ* MIL-DLLME not assisted by effervescence (6.95). The remaining studies obtained lower scores. The main differences justifying such high SPMS score in the method proposed rely on the following parameters: *(1)* the low number of procedural steps, *(2)* the null energy consumption and its high simplicity, as this method only involves three steps: extraction (simple addition of the effervescence tablet to the sample, followed by 1 min of effervescence and 13 min of equilibration time), magnetic separation, and dilution of the MIL microdroplet.

It is important to mention that, despite the SPMS tool does not assess the toxicity of all the reagents involved in the microextraction method (only that of the extractant), the $LiNTf_2$ employed as metathesis reagent has certainly toxicity issues, given the presence of fluor atoms in its structure. However, in any case, the amount of this salt in the tablet formulation is quite low (only 38 mg).

The Blue Applicability Grade Index (BAGI) metric was also applied [34]. As it can be observed in Figure S12 of the ESM, the proposed method achieved the same score (60) than previously reported approaches [12,31]. All the methods have in common two parameters colored in white in the BAGI diagram, related with the degree of automation, as they are non-automated systems, and the extractions can be only performed one-at-a-time. However, it is interesting to highlight that the proposed approach has obtained a dark blue color in the BAGI parameter devoted to the sample preparation, as it can be performed *on-site*.

## 4. Conclusions

A new type of magnetic effervescence tablets was developed. The tablets incorporated, for the first time, a water-soluble MIL composed of a Ni(II) complex as cation and chloride as anion. This MIL is solid at room temperature, allowing to develop the first MIL-based effervescence tablets that are completely solid, thus improving the stability and robustness of these microdevices with respect to previously reported tablets involving MILs.

The tablets were successfully implemented in a simple effervescence-assisted *in situ* MIL-DLLME method for the microextraction and preconcentration of two groups of biomarkers (OH-PAHs and BPs) from human urine. This approach constitutes the first method involving MIL-based effervescence tablets for the analysis of biological fluids. Several features of the method merit to be highlighted: *(1)* the use of a MIL, which allows to avoid centrifugation in the phase separation step; *(2)* the direct incorporation of $LiNTf_2$ into the tablet formulation, which reduces the number of steps of the procedure, as the effervescence and metathesis reactions can be simultaneously accomplished; and *(3)* the avoiding of external energy requirement to perform precise and accurate extractions. The method achieved enough sensitivity for the determination of the target biomarkers in complex urine samples without any type of stirring assistance. Hence, the proposed method can be performed as an *on-site* microextraction approach, which is not possible with previously reported effervescence-assisted DLLME methods that require some kind of external energy within the sample preparation setup.

Ongoing work is undertaken to expand the applicability of these MIL-based effervescence tablets to the analysis of other biofluids, as well as to the incorporation of MILs of different nature to the formulation of the tablets.

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## Figure Captions

**Figure 1** Scheme of the effervescence-assisted *in situ* MIL-DLLME procedure performed under optimum conditions, together with real images of the effervescence tablet, and the magnetic recovery of the MIL microdroplet obtained after the metathesis reaction.

**Figure 2** Photo of the effervescence tablets containing the solid $[Ni(C_4IM)_4]Cl_2$ MIL, $LiNTf_2$, $NaH_2PO_4$, $K_2CO_3$, and $Na_2SO_4$, prepared with different amounts of the components.

**Figure 3** Representative chromatograms of: **(A)** a standard of the OH-PAHs in ACN at 8 $\mu g \cdot L^{-1}$ for 2OHflu, 2OHphe and 1OHpy, and 16 $\mu g \cdot L^{-1}$ for 2OHnap and 9OHphe; **(B)** a standard of the BPs in ACN at 300 $\mu g \cdot L^{-1}$; **(C)** & **(D)** an extract (MIL microdroplet diluted with ACN) obtained after analyzing a spiked female urine with the MIL-based tablet at optimum conditions with the microextraction method. The analytes were not detected in the non-spiked urine. The concentrations of the analytes in the aqueous standard subjected to the entire microextraction method were: 0.8 $\mu g \cdot L^{-1}$ for 2OHflu, 2OHphe and 1OHpy, 1.6 $\mu g \cdot L^{-1}$ for 2OHnap and 9OHphe, and 30 $\mu g \cdot L^{-1}$ for the BPs.

**Figure 4** Overall BBD response surfaces obtained through the multiple response methodology. Each graph has one of the variables set at its optimum value: **(A)** equilibration time; **(B)** amount of $NaH_2PO_4$; **(C)** amount of MIL.

**Table 1**. Formulation of the effervescence tablets prepared with fixed amounts of the MIL and the metathesis reagent, and different combinations of proton donor agents and $CO_2$ source salts, together with the effervescence reactions.

| MIL (amount) | Metathesis reagent (amount) | Proton donor agent (amount)[a] | $CO_2$ source salt (amount)[a] | Molar ratio of the effervescence salts[b] | Effervescence reaction |
|---|---|---|---|---|---|
| $[Ni(C_4IM)_4]Cl_2$ (30.0 mg) | $LiNTf_2$ (26.0 mg) | Citric acid (99.0 mg) | $KHCO_3$ (225 mg) | 1.1:3 | $(C_3H_4OH)\text{-}(COOH)_3 + 3KHCO_3 \longrightarrow (C_3H_4OH)\text{-}(COO)_3K_3 + 3CO_2 + 3H_2O$ |
| $[Ni(C_4IM)_4]Cl_2$ (30.0 mg) | $LiNTf_2$ (26.0 mg) | Citric acid (189 mg) | $K_2CO_3$ (311 mg) | 2.1:3 | $2(C_3H_4OH)\text{-}(COOH)_3 + 3K_2CO_3 \longrightarrow 2(C_3H_4OH)\text{-}(COO)_3K_3 + 3CO_2 + 3H_2O$ |
| $[Ni(C_4IM)_4]Cl_2$ (30.0 mg) | $LiNTf_2$ (26.0 mg) | $NaH_2PO_4$ (148 mg) | $KHCO_3$ (225 mg) | 1.1:2 | $NaH_2PO_4 + 2KHCO_3 \longrightarrow NaK_2PO_4 + 2CO_2 + 2H_2O$ |
| $[Ni(C_4IM)_4]Cl_2$ (30.0 mg) | $LiNTf_2$ (26.0 mg) | $NaH_2PO_4$ (297 mg) | $K_2CO_3$ (311 mg) | 1.1:1 | $NaH_2PO_4 + K_2CO_3 \longrightarrow NaK_2PO_4 + CO_2 + H_2O$ |

[a] Amounts calculated to obtain 2.25 mmol of $CO_2$, using the molar stoichiometry specified in this table.

[b] Molar ratio (proton donor agent:$CO_2$ source salt) adjusted to have the same excess of proton donor agent in all the tablets with respect to the theoretical stoichiometry of each effervescence reaction.

**Table 2**. Several quality analytical parameters of the *in situ* MIL-DLLME-HPLC-UV/FD method performed in a healthy non-smoker female urine (matrix-matched calibration).

| Analytes | Calibration range (μg·$L^{-1}$) | (Slope ± t·$S_b$[a])·$10^{-3}$ | $R^2$[b] | $S_{y/x}$[c]·$10^{-4}$ | LOD[d] (μg·$L^{-1}$) | LOQ[e] (μg·$L^{-1}$) |
|---|---|---|---|---|---|---|
| ***OH-PAHs*** | | | | | | |
| 2OHnap | 0.40–80 | 47 ± 4 | 0.996 | 10 | 0.12 | 0.40 |
| 2OHflu | 0.20–40 | 131 ± 9 | 0.996 | 12 | 0.050 | 0.17 |
| 2OHphe | 0.20–40 | 105 ± 6 | 0.997 | 8.9 | 0.060 | 0.20 |
| 9OHphe | 0.40–80 | 34 ± 3 | 0.996 | 7.3 | 0.12 | 0.40 |
| 1OHpy | 0.050–40 | 263 ± 23 | 0.994 | 31 | 0.010 | 0.050 |
| ***BPs*** | | | | | | |
| BP1 | 7.00–400 | 1.1 ± 0.1 | 0.996 | 1.2 | 2.0 | 7.00 |
| BP8 | 7.00–400 | 1.2 ± 0.1 | 0.992 | 1.7 | 2.0 | 7.00 |
| BP | 2.00–400 | 2.0 ± 0.3 | 0.993 | 3.3 | 0.60 | 2.00 |
| BP3 | 2.00–400 | 1.7 ± 0.1 | 0.998 | 1.5 | 0.60 | 2.00 |

[a] Uncertainty of the slope within the calibration range (n = 5) for a confidence level of 95% (α = 0.05).
[b] Determination coefficient.
[c] Standard deviation of the residuals.
[d] Limit of detection.
[e] Limit of quantification.

**Table 3**. Analytical performance of the *in situ* MIL-DLLME-HPLC-UV/FD method in terms of precision and relative recovery, using diluted urine standard solutions.

| Analytes | Low concentration level[a] | | | Intermediate concentration level[b] | | |
|---|---|---|---|---|---|---|
| | Intra-day RSD[c] (%) | Intermediate precision RSD[d] (%) | (RR ± SD)[e] (%) | Intra-day RSD[c] (%) | Intermediate precision RSD[d] (%) | (RR ± SD)[e] (%) |
| ***OH-PAHs*** | | | | | | |
| 2OHnap | 7.7 | 8.5 | 90 ± 8 | 4.0 | 4.3 | 85 ± 3 |
| 2OHflu | 8.3 | 9.1 | 91 ± 8 | 1.4 | 1.8 | 86 ± 1 |
| 2OHphe | 4.3 | 4.8 | 84 ± 4 | 3.9 | 4.5 | 85 ± 3 |
| 9OHphe | 4.9 | 17 | 89 ± 5 | 1.4 | 11 | 85 ± 9 |
| 1OHpy | 4.2 | 9.4 | 103 ± 10 | 4.3 | 5.5 | 94 ± 5 |
| ***BPs*** | | | | | | |
| BP1 | 2.6 | 3.1 | 100 ± 6 | 3.3 | 3.7 | 68 ± 3 |
| BP8 | 7.3 | 11 | 103 ± 14 | 4.2 | 5.6 | 69 ± 3 |
| BP | 3.2 | 3.0 | 93 ± 3 | 0.90 | 3.0 | 85 ± 1 |
| BP3 | 5.2 | 13 | 93 ± 12 | 0.70 | 3.1 | 77 ± 1 |

[a] 1.6 $\mu g \cdot L^{-1}$ for 2OHnap and 9OHphe; 0.80 $\mu g \cdot L^{-1}$ for 2OHflu, 2OHphe and 1OHpy; 30 $\mu g \cdot L^{-1}$ for all the BPs.

[b] 16 $\mu g \cdot L^{-1}$ for 2OHnap and 9OHphe; 8.0 $\mu g \cdot L^{-1}$ for 2OHflu, 2OHphe and 1OHpy; 150 $\mu g \cdot L^{-1}$ for all the BPs.

[c] Relative standard deviation for intra-day precision (n = 3).

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

[e] Average relative recovery and standard deviation (n = 9).

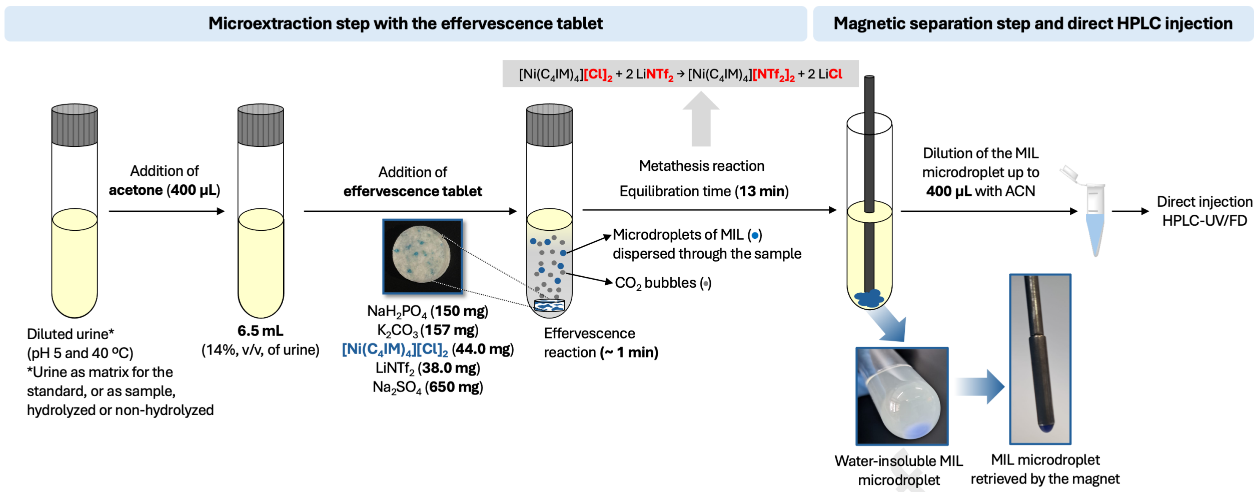
**Table 4**. Analysis of hydrolyzed urine samples using the *in situ* MIL-DLLME-HPLC-UV/FD method.

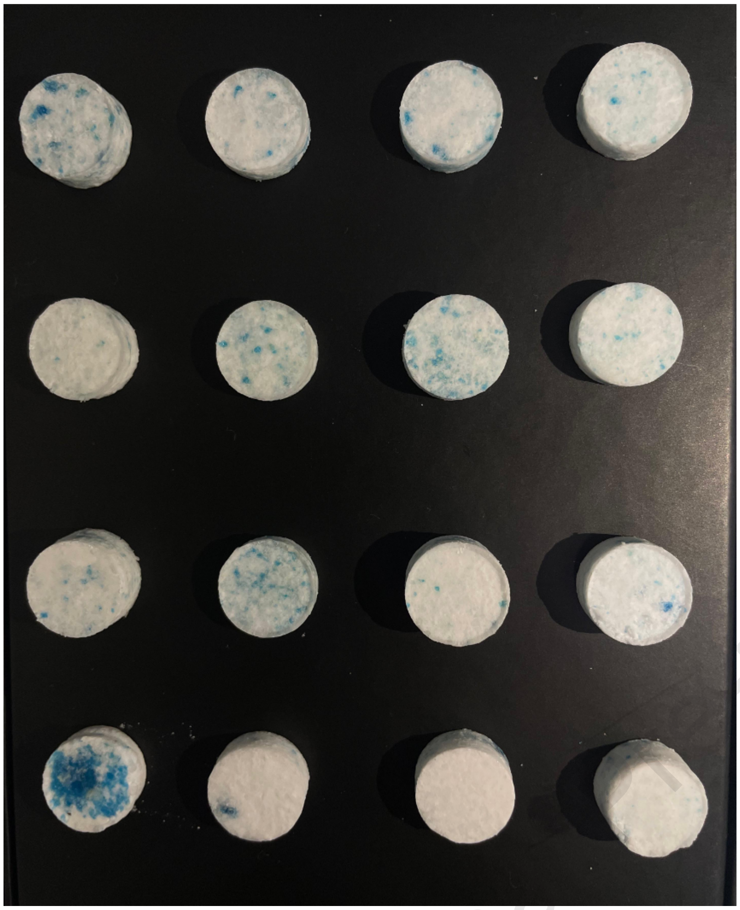
| Analytes | Content[a] ± SD ($\mu g \cdot L^{-1}$) | | |
|---|---|---|---|
| | **Smoker male urine** | **Smoker female urine** | **Non-smoker female urine** |
| ***OH-PAHs*** | | | |
| 2OHnap | <LOD[b] | 16.0 ± 0.7 | <LOD[b] |
| 2OHflu | 1.3 ± 0.2 | 4.6 ± 0.3 | <LOD[b] |
| 2OHphe | <LOQ[c] | 4.6 ± 0.3 | <LOD[b] |
| 9OHphe | <LOD[b] | <LOD[b] | <LOD[b] |
| 1OHpy | 0.40 ± 0.04 | 0.50 ± 0.02 | <LOD[b] |
| ***BPs*** | | | |
| BP1 | <LOD[b] | 126 ± 11 | 331 ± 15 |
| BP8 | <LOD[b] | <LOD[b] | 334 ± 15 |
| BP | <LOD[b] | 17.8 ± 0.3 | <LOD[b] |
| BP3 | <LOD[b] | <LOQ[c] | 38 ± 2 |

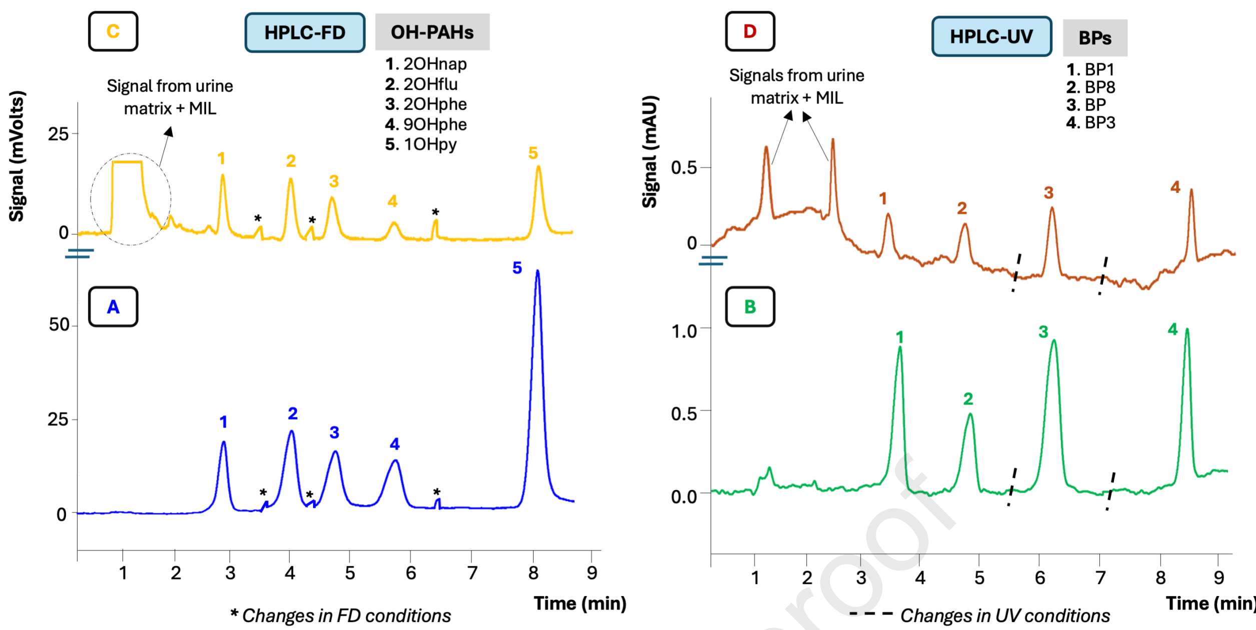
[a] Concentration of the biomarkers found in the urine samples, together with its standard deviation (n = 3).

[b] Non-detected.

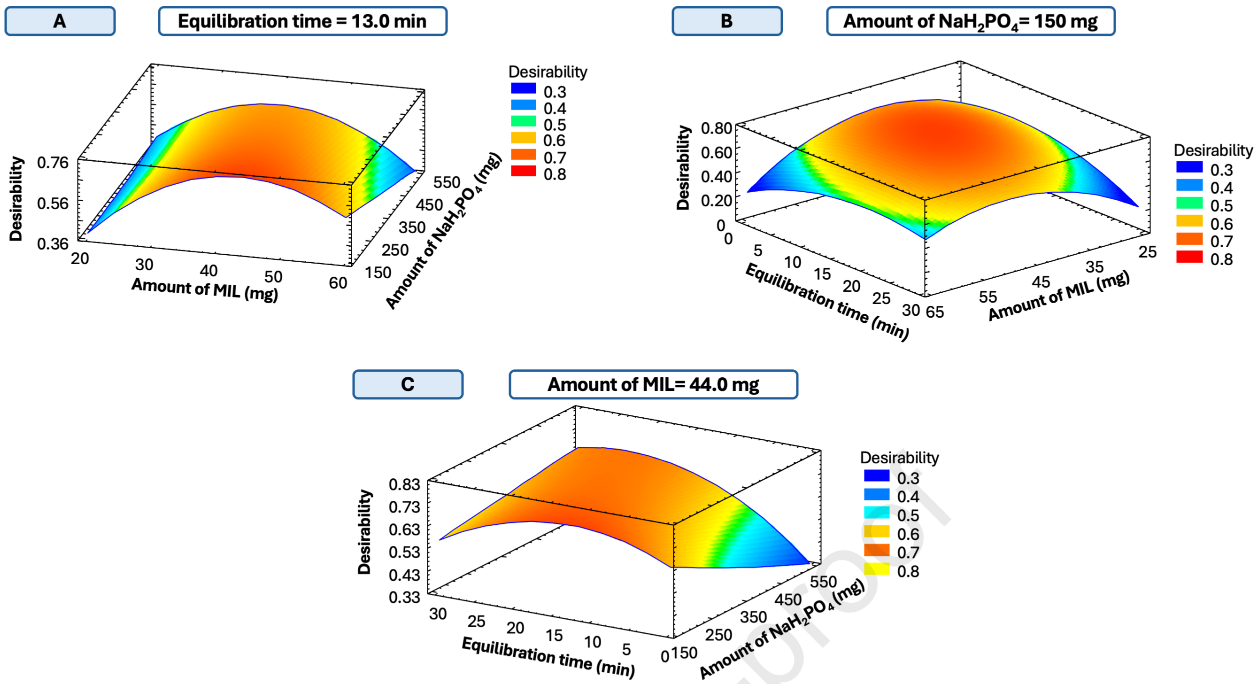
[c] Detected but not quantified (below the LOQ).


Microextraction step with the effervescence tablet
Magnetic separation step and direct HPLC injection
[Ni(C4IM)4][Cl]2 + 2 LiNTf2 → [Ni(C4IM)4][NTf2]2 + 2 LiCl
Addition of acetone (400 µL)
Addition of effervescence tablet
Metathesis reaction
Equilibration time (13 min)
Dilution of the MIL microdroplet up to 400 µL with ACN
Direct injection HPLC-UV/FD
Microdroplets of MIL (●) dispersed through the sample
CO2 bubbles (●)
Diluted urine*
(pH 5 and 40 ºC)
*Urine as matrix for the standard, or as sample, hydrolyzed or non-hydrolyzed
6.5 mL
(14%, v/v, of urine)
NaH2PO4 (150 mg)
K2CO3 (157 mg)
[Ni(C4IM)4][Cl]2 (44.0 mg)
LiNTf2 (38.0 mg)
Na2SO4 (650 mg)
Effervescence reaction (~ 1 min)
Water-insoluble MIL microdroplet
MIL microdroplet retrieved by the magnet





C
HPLC-FD
OH-PAHs
1. 2OHnap
2. 2OHflu
3. 2OHphe
4. 9OHphe
5. 1OHpy
Signal from urine matrix + MIL
Signal (mVolts)
A
Time (min)
* Changes in FD conditions
D
HPLC-UV
BPs
1. BP1
2. BP8
3. BP
4. BP3
Signals from urine matrix + MIL
Signal (mAU)
B
Time (min)
Changes in UV conditions


A
Equilibration time = 13.0 min
Desirability
0.76
0.56
0.36
20
30
40
50
60
Amount of MIL (mg)
150
250
350
450
550
Amount of $NaH_2PO_4$ (mg)
Desirability
0.3
0.4
0.5
0.6
0.7
0.8
B
Amount of $NaH_2PO_4$= 150 mg
Desirability
0.80
0.60
0.40
0.20
0
0
5
10
15
20
25
30
Equilibration time (min)
65
55
45
35
25
Amount of MIL (mg)
Desirability
0.3
0.4
0.5
0.6
0.7
0.8
C
Amount of MIL= 44.0 mg
Desirability
0.83
0.73
0.63
0.53
0.43
0.33
30
25
20
15
10
5
0
Equilibration time (min)
150
250
350
450
550
Amount of $NaH_2PO_4$ (mg)
Desirability
0.3
0.4
0.5
0.6
0.7
0.8

**Highlights**

- A new type of effervescence tablets containing a water-soluble Ni(II)-based MIL
- The MIL is solid at room temperature, improving the stability of the tablets
- The tablet formulation ensures the *in situ* formation of a water-insoluble MIL
- The effervescence promotes dispersion without any external energy assistance
- The method succeeds determining urinary biomarkers with simple magnetic retrieval

**Declaration of interests**

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: