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RESEARCH PAPER

Magnetic ionic liquids as versatile extraction phases for the rapid determination of estrogens in human urine by dispersive liquid-liquid microextraction coupled with high-performance liquid chromatography-diode array detection

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Abstract

In this study, a rapid and straightforward approach based on magnetic ionic liquids (MIL) as extraction phases and dispersive liquid-liquid microextraction (DLLME) was developed to analyze the hormones estriol, 17-β-estradiol, 17-α-ethynylestradiol, and estrone in human urine samples. This is the first report of an application of manganese-based MILs compatible with HPLC to extract compounds of biological interest from urine samples. The hydrophobic MILs trihexyltetradecylphosphonium tetrachloromanganate (II) ($[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$) and aliquat tetrachloromanganate (II) ($[Aliquat^{+}]_2[MnCl_4^{2-}]$) were employed and the optimized extraction conditions were comprised of 5 mg of MIL ($[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$), 5 μL of methanol (MeOH) as disperser solvent, and an extraction time of 90 s at sample pH 6. The analytical parameters of merit were determined under optimized conditions and very satisfactory results were achieved, with LODs of 2 ng mL^{-1} for all analytes, determination coefficients (R^2) ranging from 0.9949 for 17-β-estradiol to 0.9998 for estrone. In addition, good results of method precision were achieved with the intraday ($n = 3$) varying from 4.7% for 17-β-estradiol to 19.5% for estriol (both at 5 ng mL^{-1}) and interday precision (evaluated at 100 ng mL^{-1}) ranging from 11.4% for estrone to 17.7% for 17-α-ethynylestradiol and analyte relative recovery evaluated in three real samples ranged from 67.5 to 115.6%. The proposed DLLME/MIL-based approach allowed for a reliable, environmentally friendly and high-throughput methodology with no need for a centrifugation step.

Keywords Magnetic ionic liquids · Sample preparation · Urine · Biological samples

Introduction

Steroid sex hormones exhibit very important functions in regulating and developing male and female reproductive organs, secondary sex characteristics, and behavior patterns [1]. In female organisms, some of these compounds, such as estrogens, are widely used to control reproductive cycles and menstrual disorders and also to supplement insufficient natural estrogen levels. In addition, estrogens are known to suppress ovulation and combined with progestogens constitute the basis of oral contraceptives. Moreover, these compounds offer a number of benefits for women including protection against heart attacks and osteoporosis [2]. These molecules can be classified as endogenous estrogens (estriol, 17-β-estradiol, estrone), exogenous estrogens (17-α-ethynylestradiol)

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endogenous progestogens (progesterone) and exogenous progestogens (19-norethisterone and d-(-)-norgestrel) [3]. The determination of these compounds in urine samples is a challenging task and is of great importance for monitoring certain clinical conditions such as ovarian and placental functions [4]. Furthermore, in some cases, high levels of endogenous estrogens in urine samples can be associated with breast cancer [5].

In recent years, the monitoring of estrogens in water and biological samples has mostly been performed by either chromatographic or biological assays, whereas chromatographic methodologies have been largely used due to the possibility of simultaneous determination. The use of high-performance liquid chromatography (HPLC) has generally been preferred because of its versatility and unnecessary derivatization steps [6]. However, in several applications, a sample preparation procedure is needed prior to instrumental analysis.

A sample preparation step is often required in many analytical determinations as this step is crucial for the development of accurate and precise procedures. These strategies allow for the analysis of complex samples and also enable trace determination due to the preconcentration of analytes. Sample preparation techniques with a focus on clinical and biomedical applications generally are performed by using injection of deproteinized or ultrafiltered samples, liquid-liquid extraction, solid-liquid extraction, or solid-phase extraction [4]. In addition, microextraction techniques have also been adopted as valuable tools to analyze complex biological matrices such as urine.

Microextraction techniques offer remarkable advantages over traditional techniques (i.e., liquid-liquid and solid-liquid extractions) including low solvent consumption, rapid analysis, and high enrichment factors. Dispersive liquid-liquid microextraction, developed by Rezaee and coworkers in 2006 [7], is based on a ternary solvent component system consisting of a mixture of extraction solvent (immiscible in aqueous sample) and disperser solvent (miscible in both extraction solvent and aqueous sample). These solvents are rapidly introduced to the sample resulting in the formation of a turbid solution of high surface area. Consequently, equilibration is quickly reached and rapid extraction can be achieved, substantially increasing the throughput of the analytical methodology [8–10].

One of main variables that influences the extraction efficiency in liquid-phase extraction/microextraction techniques is the extraction solvent. Chlorinated solvents, including chloroform, chlorobenzene, and carbon tetrachloride, are often used as classical extraction phases in dispersive liquid-liquid microextraction (DLLME) approaches [11–16]. Besides the satisfactory analytical performance achieved when these solvents are employed, important drawbacks with regard the high toxicity and high volatilities of these chemicals represent a risk to the analyst and, eventually, also lead to reproducibility issues. Therefore, the use of ionic liquids (ILs) as extraction phases in DLLME offers a number of advantages over traditional organic solvents. Studies involving IL as extraction phases in DLLME have been reported for the determination of pyrethroid pesticides [17], aromatic compounds [18], insecticides [19], pharmaceuticals [20], and DNA [21] among other organic or inorganic analytes in different matrices.

A subclass of ILs named magnetic ionic liquids (MILs) has gained special attention in analytical procedures. These compounds are produced by incorporating a paramagnetic (generally a metal or lanthanide ions) component in either the cation or anion, and they possess similar physicochemical properties to conventional ILs. However, MILs exhibit a strong response to external magnetic fields [22]. The synthesis and application of these materials have been reported using different ions including iron [23], manganese [24], cobalt [25], and gadolinium [24]. Very recently, manganese-based MILs were reported as extraction solvents for DLLME [26]. In this approach, a small amount of MIL combined with a disperser solvent was introduced into an aqueous solution and, following extraction, the extraction phase was retrieved with the aid of a magnetic rod. This approach has important analytical features including its simplicity and speed, lacking need of a centrifugation step, and high stability of the hydrophobic MILs in aqueous solution.

Related to the determination of estrogens in urine samples, some analytical methodologies have been developed based on solid-phase extraction [27], ultrasound-assisted cloud point extraction [28], vortex-assisted dispersive liquid-liquid microextraction [29], bar adsorptive microextraction [3], conventional dispersive liquid-liquid microextraction [30], and also stir bar sorptive extraction [6].

This work reports a straightforward DLLME/MIL-based analytical methodology for the rapid extraction and determination of estriol, estrone, 17-β-estradiol, and 17-α-ethynilestradiol in human urine using the HPLC-compatible MILs trihexyltetradecylphosphonium tetrachloromanganate (II) ($[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$) and aliquat tetrachloromanganate (II) ($[Aliquat^{+}]_2[MnCl_4^{2-}]$), followed by separation/detection with HPLC-DAD. Optimization of the parameters that affect DLLME was performed by univariate and multivariate approaches, and the determination of the analytical parameters of merit for each analyte was carried out under optimal conditions. Very few studies have been conducted using these materials in microextraction techniques and, according to our knowledge, a DLLME/MIL-based procedure coupled with chromatographic separation has not previously been explored for the analysis of complex biological samples such as urine.

Experimental

Reagents and materials

The reagents trihexyltetradecylphosphonium chloride ($[P_{6,6,6,14}^{+}][Cl^{-}]$) (97.7%), manganese (II) chloride

tetrahydrate ($MnCl_2 \cdot 4H_2O$) (98.0%), and Aliquat® 336 (average molecule weight 442.00) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solid standards of estriol (≥ 97%), β-estradiol (≥ 98%), estrone (≥ 99%), and 17α-ethynylestradiol were also purchased from Sigma-Aldrich. The chemical structure of each analyte, as well as some physicochemical properties, is shown in Table 1.

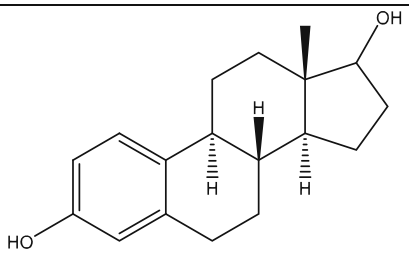
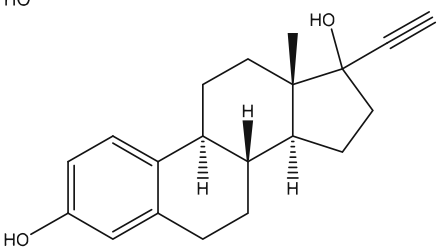
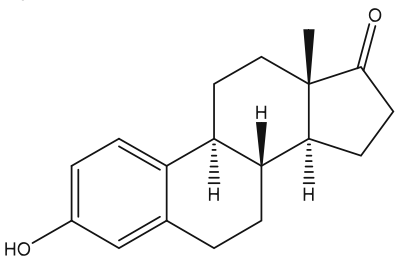
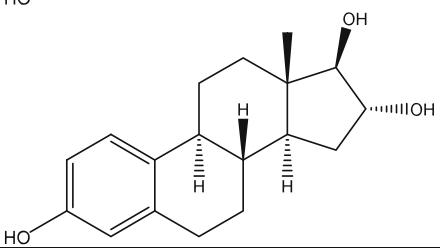
Acetonitrile (ACN) methanol (MeOH) and acetone HPLC-grade were supplied by JT Baker (Mallinkrodt, USA). Ultrapure water (18.2 MΩcm) was obtained from a Mega Purity system (Billerica, USA), and hydrochloric acid and sodium chloride were obtained from Vetec (Rio de Janeiro, Brazil).

A stock solution containing the analytes at a concentration of 250 mg L^{-1} for each compound was prepared by dissolving the appropriate mass of each solid standard in MeOH. Working solutions at concentration of 1 and 0.1 mg L^{-1} were obtained by diluting the stock solution with MeOH. A neodymium magnetic rod ($B = 0.66$ T) was purchased from Ima Shop (Florianópolis, Brazil).

Instrumental

An Agilent 1260 Infinity HPLC system (Santa Clara, CA, USA) equipped with a quaternary pump and diode array detector (DAD) was used for the separation/detection of the analytes. The HPLC was equipped with a Rheodyne manual injector containing a loop of 20 μL and the separation was performed using a Phenomenex Gemini-NX C18 column (5.0 μm, 4.6 mm × 250 mm). The gradient method initially applied 65% of mobile phase A (water) and 35% of mobile phase B (ACN). The percentage of mobile phase B was linearly increased from 35 to 70% over 6 up to 10 min, then returning to the initial condition. The total flow rate of mobile phase was kept constant at 1 mL min^{-1}. All analytes were detected at a wavelength of 200 nm and the chromatographic

Table 1 Chemical structure and physicochemical properties of the analytes examined in this study

Analyte	Chemical structure	pKa	log Kow
17-β-estradiol		10.71 [34]	3.94 [6]
17-α-ethynylestradiol		10.46 [35]	4.12 [6]
Estrone		10.34 [35]	3.43 [6]
Estriol		10.38 [35]	2.45 [36]

data were analyzed by Agilent OpenLab CDS System from Agilent Technologies. In addition, a Varian Cary-50 UV-Vis (Varian Inc., Palo Alto, USA) spectrophotometer was used to perform the characterization of the MILs.

Synthesis and characterization of the magnetic ionic liquids

The magnetic ionic liquids were synthesized according to previously described procedures [26, 34]. Briefly, for the synthesis of $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$, a mass of $[P_{6,6,6,14}^{+}][Cl^{-}]$ (1 equiv.) was mixed with $MnCl_2{\cdot}4H_2O$ (0.5 equiv.) in dichloromethane, and this mixture was allowed to react for 24 h at room temperature under constant stirring. For the synthesis of $([Aliquat^{+}]_2[MnCl_4^{2-}]$, a mass of Aliquat®336 (1 equiv.) was mixed with $MnCl_2{\cdot}4H_2O$ (0.5 equiv.) in dichloromethane and the same reaction conditions were followed. The obtained products were dried overnight at 70 °C in a vacuum oven. Characterization of the MILs was performed by UV-Vis spectroscopy and the results are in accordance with those previously reported in the literature [26] with a characteristic absorption associated to the incorporation of manganese in the IL structure (region from 425 to 465 nm). These experimental data are contained in the Electronic Supplementary Material (ESM, Figs. S1-S4).

Experimental DLLME/MIL-based procedure

Briefly, under optimized conditions, a mixture comprised of a disperser solvent (5 μL of MeOH) and the MIL (5 mg of $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$) was inserted in the diluted urine samples with pH adjusted to 6, and a manual shaking step was carried out to facilitate the formation of microdroplets in the solution. Following, an extraction time of 90 s, the MIL was withdrawn with a rod magnet and the extraction phase containing the enriched analytes was desorbed in 20 μL of acetonitrile prior to injection in the HPLC-DAD system; therefore, the total volume injected in the loop of the HPLC system was slightly higher than 20 μL. It can be observed that a centrifugation step was not necessary due to the ease of retrieving the liquid microdroplets by the rod magnet. Therefore, this configuration offers a rapid magnetic-based approach to enhance sample throughput for DLLME. An overview of the experimental procedure used for the extractions in urine samples is shown in Fig. 1.

Sample collection

The urine samples were collected from volunteers who participated as authors of this study ranging in age from 26 to 29 years old (one man and one woman). In addition, a urine sample was collected from a 30-week pregnant woman (29 years old) who previously agreed to participate in this research, with no discomfort or risks related to the procedures for voluntary donation. The samples were collected in PTFE flasks, properly sealed, and stored at 4 °C. The samples were kept at room temperature (22 °C) for 30 min before the analysis.

Optimization of the DLLME/MIL-based procedure

Optimization of the extraction conditions was carried out by multi- and univariate approaches using the $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ MIL. Statistica 8.0 (STATSOFT, USA) was used for data treatment in multivariate approaches. For all optimizations, 75 μL of blank urine samples were spiked with the analytes at a concentration of 400 ng mL^{-1} for each compound and diluted up to 3 mL with ultrapure water. Firstly, the disperser solvent was optimized based on a simplex-centroid design using the solvents ACN, MeOH, and acetone. A ternary surface was obtained using the geometric means of the chromatographic peak areas for all analytes.

Following optimization of desorption solvent, a full-factorial design was performed to examine the influence of the variables extraction time, volume of disperser solvent, MIL mass, and NaCl concentration. In this step, the same quantities of urine, ultrapure water, and the concentration of the analytes used to optimize the disperser solvent were employed. A table containing all the experiments performed with the full-factorial design is shown in the ESM (Table S1). In the last part of the optimization, univariate approaches were adopted to determine more precisely the ideal conditions for extraction time and sample pH.

Results and discussion

Optimization of the extraction conditions

Firstly, the optimization of disperser solvent for the DLLME/MIL-based procedure was carried out using ACN, MeOH, and acetone with all analysis being performed at a concentration of 400 ng mL^{-1} for each compound. Herein, the extractions were performed using 20 mg of MIL, 5 μL of disperser solvent, an extraction time of 120 s. The desorption was carried out in 20 μL of ACN, followed by injection in the HPLC-DAD system. A ternary surface shown in Fig. 2 was obtained using the geometric means of the chromatographic peak areas for all analytes.

According to Fig. 2, the chromatographic responses exhibited poor results using only ACN and acetone as disperser solvents; however, the mixture comprised of acetone and MeOH showed better performance when the MeOH/acetone ratio was higher than 75:25 (*v*/*v*), with very satisfactory responses being obtained when the methanol was increased to

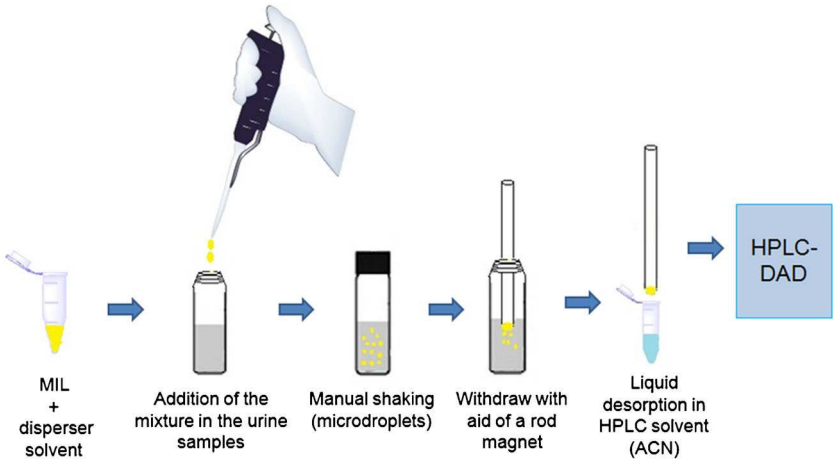


Fig. 1 An overview of the extraction procedure using DLLME/MIL-based approach

100%. Therefore, methanol was chosen for subsequent experiments.

Full-factorial design

A number of variables can influence the DLLME procedure. In this study, a full-factorial design (2^4) with 16 experiments was performed to assess the influence of extraction time (10–120 s), volume of disperser solvent (5–20 μL), MIL mass (5–20 mg), and NaCl concentration (0–30% (*w*/*v*)). All extractions were performed with diluted urine samples spiked with each analyte at 400 ng mL^{-1} and methanol acting as disperser solvent (previously optimized). A Pareto chart containing the effects of the variables and the interactions is shown in Fig. 3.

According to the Pareto chart in Fig. 3, some variables and interactions provided significant influence on the extraction procedure at the 95% confidence level. In the case of extraction time, this variable exhibited significant effect with a positive value indicating that longer extraction times allowed for higher chromatographic responses. On the other hand, NaCl concentration and MeOH volume also exhibited significant influence; however, the negative effects emphasized that higher chromatographic responses were obtained using lower volumes of MeOH and low concentration of NaCl. Based on these results, the lowest value for NaCl concentration (0%) and MeOH volume (5 μL) was chosen as optimal conditions for these variables. Even presenting a positive effect, the MIL mass did not significantly influence the chromatographic response. This is explained because most of the analytes exhibit relatively large partition coefficients in the hydrophobic media (see Table 1); in this case, the chromatographic responses obtained with extractions using low MIL mass

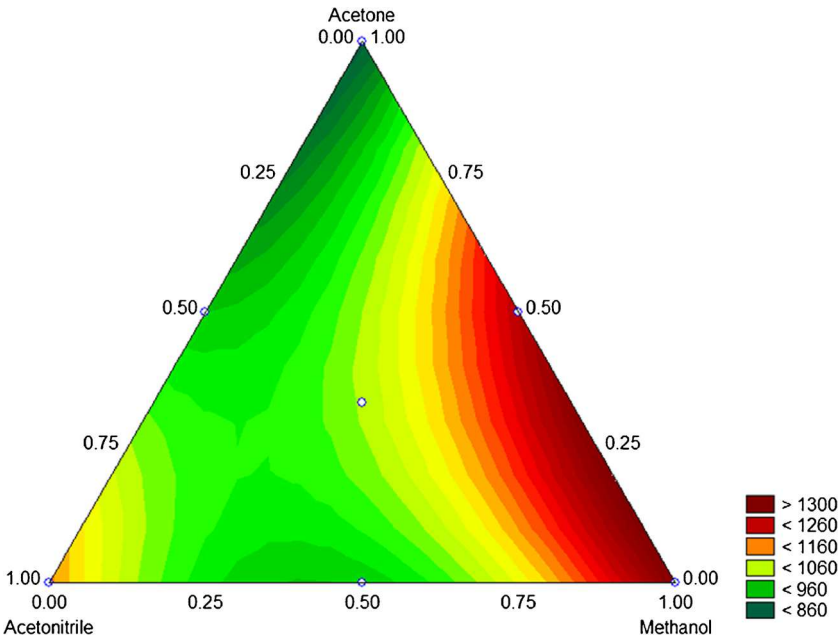


Fig. 2 Triangular surface obtained from a simplex-centroid design with extractions performed in diluted urine samples (1:40 *v*/*v*) spiked with 400 ng mL^{-1} of each analyte. The experiments were performed using 20 mg of $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$, 5 μL of disperser solvent, and 120 s of extraction time and the desorption was carried out in 20 μL of acetonitrile

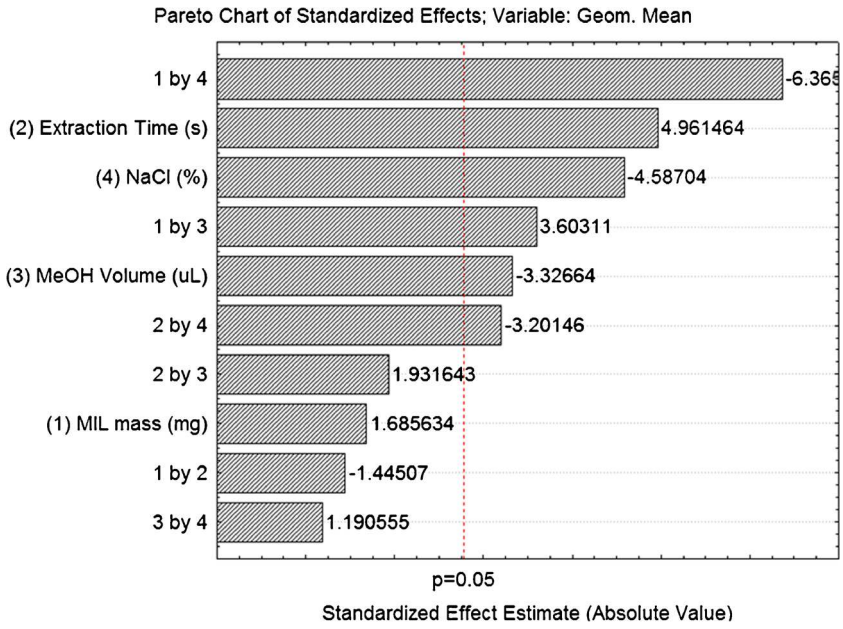


Fig. 3 Pareto chart obtained for the variables extraction time, NaCl concentration, MeOH volume, and MIL mass. Analyses were performed in diluted urine samples (1:40 *v/v*) spiked with 400 ng mL^{-1} of each analyte. Desorption was performed in 20 μL of acetonitrile

were statistically similar than results using higher amounts of extraction phase. Therefore, 5 mg of MIL were used for further experiments.

With regard to extraction time, this variable exhibited a significant positive influence on the chromatographic responses according to the full-factorial design previously discussed. Therefore, a more detailed optimization of the extraction times at 60, 90, 120, 150, and 180 s was carried out using the optimized extraction conditions previously determined for the other variables; the normalized chromatographic peak areas were used with results shown in a bar graph of Fig. 4. All analyses were performed in triplicate.

According to the bar graph of Fig. 4, 60 s of extraction were not enough to reach an equilibrium condition. However, by increasing the extraction time to 90 s, it can be observed that this time provided the highest peak area for all analytes. Therefore, an extraction time of 90 s was chosen for further experiments. Short extraction times offer an important advantage associated to DLLME-based procedures since equilibrium is quickly reached due to a large surface area formed between the extraction phase and the sample matrix.

pH evaluation

The sample pH can exhibit significant influence on the extraction efficiency of the analytes. The partitioning of target compounds from the sample matrix to the extraction solvent strongly depends on the chemical structure of the analytes, and generally, higher extraction efficiencies are obtained for analytes in the non-ionized form compared with those compounds in ionized form [35]. In this study, pH optimization was performed at three different pH values (4, 6, and 10) and the results obtained are shown in Fig. 5, with all the analyses performed in triplicate.

According to Fig. 5, the best results were obtained for all analytes at pH 6. The chromatographic response significantly decreased by modifying the pH to 10, possibly due to a fraction of the analytes being converted to ionized forms and,

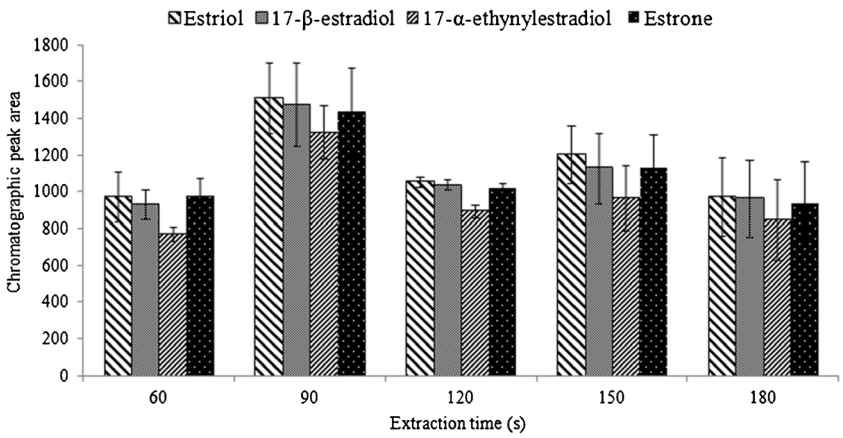


Fig. 4 Univariate optimization of extraction time for DLLME/MIL-based approach using 5 μL of MeOH as disperser solvent, 5 mg of $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ as extraction solvent, 0% of NaCl, and 400 ng mL^{-1} of each analyte

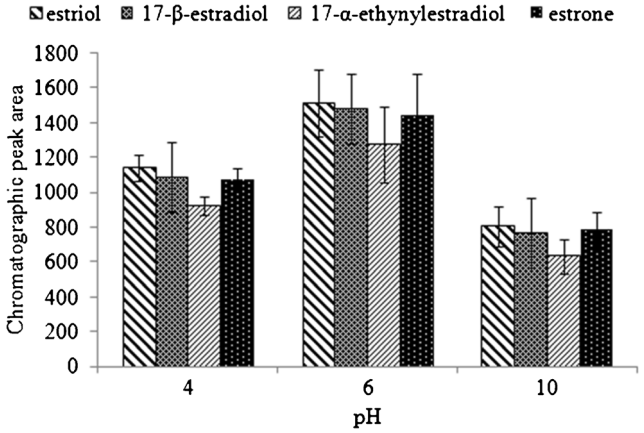


Fig. 5 pH evaluation using 90 s of extraction time, 5 μL of MeOH as disperser solvent, 5 mg of $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ as extraction solvent, 0% of NaCl, and 400 ng mL^{-1} of each analyte

consequently, increasing the solubility of these compounds in aqueous media.

Extraction efficiency comparison

The extraction efficiencies obtained with the $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ and $[Aliquat^{+}]_2[MnCl_4^{2-}]$ MILs as extraction solvents were compared. In this step, diluted urine samples spiked with each analyte at concentration of 100 ng mL^{-1} were subjected to extractions under the optimized conditions previously determined. The chromatographic peak areas for each analyte were monitored and a bar graph comparing these two extraction solvents is shown in Fig. 6. It is worth mentioning that extractions using the $[Aliquat^{+}]_2[MnCl_4^{2-}]$ MIL exhibited a peak at the same retention time of the last analyte (estrone) and, because of this drawback, this analyte was not included in this comparison.

Besides the issue caused by an interfering peak at the same retention time than estrone, $[Aliquat^{+}]_2[MnCl_4^{2-}]$ exhibited an extraction efficiency comparable with $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ for the extraction of estriol. However, related to other analytes, $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ showed higher extraction efficiencies and, moreover, the chromatographic background was lower than that obtained using $[Aliquat^{+}]_2[MnCl_4^{2-}]$. Based on these results, $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ was chosen for subsequent studies involving the determination of analytical parameters of merit.

Determination of the analytical parameters of merit and application in real samples

The determination of analytical figures of merit was carried out by using a calibration curve performed directly in diluted urine samples (40-fold). In this step, all optimized conditions previously established were adopted, and calibration curves containing six spiked concentrations for each analyte were

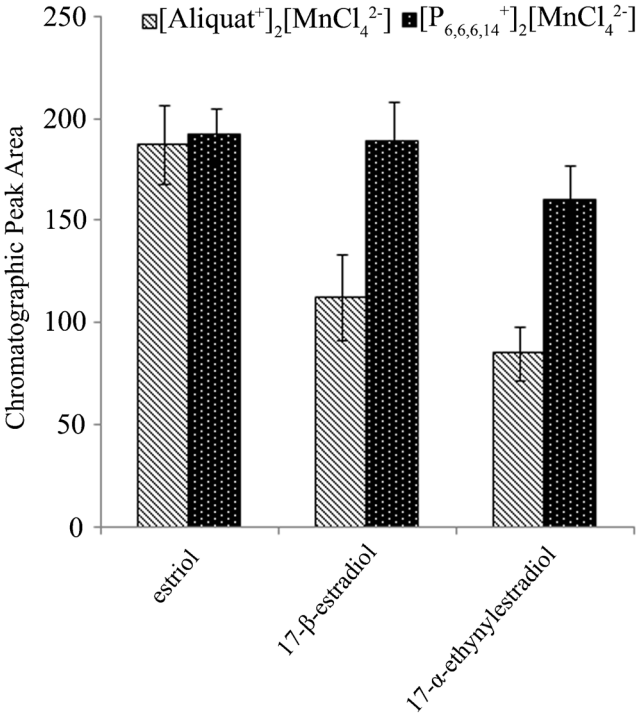


Fig. 6 Extraction efficiency comparison of $[Aliquat^{+}]_2[MnCl_4^{2-}]$ and $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$, using the optimized extraction conditions and 100 ng mL^{-1} of each analyte

performed to assess the analytical parameters: linear range, determination coefficient (R^2), limit of detection (LOD), limit of quantification (LOQ), and precision (inter- and intraday).

In addition, real urine samples collected from volunteers were diluted and used to evaluate the applicability and accuracy of the methodology proposed through analyte relative recoveries. In this step, a urine sample collected from a pregnant woman was included to examine the possible detection of the compounds studied. Moreover, the enrichment factors obtained for each analyte using the DLLME/MIL-based approach were obtained as well.

The analytical parameters of merit were determined using the experimental conditions previously optimized. Herein, the limits of detection (LOD) were calculated by reducing the analyte concentration to obtain a signal-to-noise ratio of 3:1, and limits of quantification (LOQ) were adopted as the first concentration level of the linear range for each analyte, with a signal-to-noise ratio of approximately 10:1. The analytical figures of merit are shown in Table 2.

According to Table 2, LOD and LOQ for all analytes were 2 and 5 ng mL^{-1}, respectively. Good linearity was obtained with determination coefficients (R^2) ranging from 0.9949 for 17-β-estradiol to 0.9998 for estrone. Very satisfactory results of method precision were achieved with intraday ($n = 3$) values varying from 4.7% for 17-β-estradiol to 19.5% for estriol both at 5 ng mL^{-1} and interday precision (evaluated at 100 ng mL^{-1}) obtained in 3 days and ranging from 11.4% for estrone to 17.7% for 17-α-ethynylestradiol.

Table 2 Analytical parameters of merit for the DLLME/MIL-based procedure using the $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ MIL

Analyte	Linear range (ng mL^{-1})	Slope ± error	LOD[a] (ng mL^{-1})	LOQ[b] (ng mL^{-1})	R^2	Precision (intraday)[c], $n = 3$ (%)			Precision (interday)[c], $n = 9$ (%)
						5 ng mL^{-1}	100 ng mL^{-1}	500 ng mL^{-1}	100 ng mL^{-1}
Estriol	5–500	2.1112 ± 0.041	2	5	0.9985	19.5	13.3	11.7	15.4
17-β-Estradiol	5–500	1.9086 ± 0.068	2	5	0.9949	4.7	17.7	8.3	16.9
17-α-Ethynylestradiol	5–500	1.6185 ± 0.024	2	5	0.9991	14.9	13.3	5.7	17.7
Estrone	5–500	1.9606 ± 0.012	2	5	0.9998	13.4	12.9	7.5	11.4

[a] LOD was calculated by evaluating signal-to-noise ratio (3:1)

[b] LOQ adopted as the first concentration level of the linear range

[c] Intraday and interday precisions were calculated based on relative standard deviation (RSD)

Chromatograms obtained with an extraction from a blank urine sample and from a urine sample spiked with 100 ng mL^{-1} of each analyte using the DLLME/MIL-based procedure with $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ as extraction phase are shown in Fig. 7.

In order to evaluate the applicability of the proposed methodology, diluted urine samples collected from volunteers that previously accepted to contribute to this study were analyzed. Sample A was obtained from a volunteer with no consumption of oral estrogen-based contraceptives, sample B was collected from a female volunteer with regular ingestion of low-dose birth control pills (ingestion of 0.03 mg of 17-α-ethynylestradiol/day), and sample C was obtained from a 30-week pregnant woman. Sample C was evaluated due to the fact that some of the hormones studied can be excreted by the urine in the pregnancy period. Moreover, aliquots of these samples were subjected to analyte relative recovery evaluation at three spiked concentrations (5, 100, and 500 ng mL^{-1}). The results regarding the relative recovery and RSD for these determinations are shown in Table 3.

Based on Table 3, the method achieved satisfactory results for accuracy and precision, with analyte relative recoveries varying from 67.5 to 115.6% and RSD ranging from 3.3 to 19.9. Non-spiked urine samples (A, B, and C) were also examined and the chromatographic peaks of the analytes were not detected in samples A and B. Interestingly, for sample B, the chromatographic peak corresponding to 17-α-ethynylestradiol was not detected; this behavior is possible due to the fact that this compound should be kept in the organism for at least 24 h to ensure the efficiency of the pills; therefore, a very low content is excreted by the urine throughout the day and the results have shown that this content was lower than the LOD. Furthermore, 17-α-ethynylestradiol also can undergo different metabolic pathways and form secondary compounds which can reduce the excreted amount of this analyte. In sample C, a peak corresponding to estriol was detected; however, the chromatographic response for this analyte was lower than the LOQ. The presence of this compound is possibly associated to the pregnancy of the volunteer, which influences the release of this compound in the urine [36]. Therefore, the proposed DLLME/MIL-based approach offers a reliable alternative to be used as an analytical tool to evaluate the target analytes in complex biological matrices such as the urine.

In addition, an estimation of enrichment factors (EF) for the analytes was performed. The concentrations obtained for the

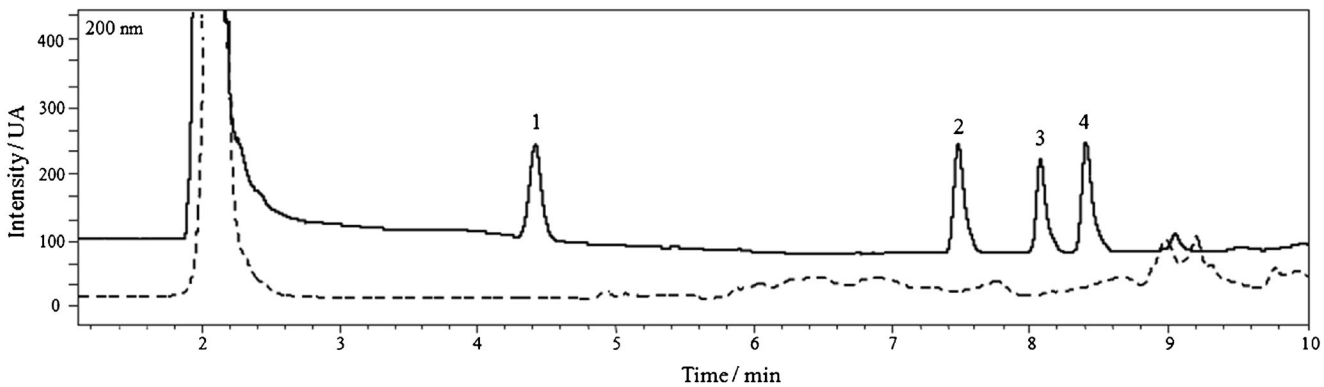


Fig. 7 Chromatograms obtained from a diluted blank urine (dashed line) and from a diluted urine sample spiked with 100 ng mL^{-1} of each analyte (solid line) using the DLLME/MIL-based optimized extraction conditions and 5 mg of $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ as extraction phase. Elution order: 1, estriol; 2, 17-β-estradiol; 3, 17-α-ethynylestradiol; and 4, estrone

Table 3 Analyte relative recovery and RSD for samples A, B, and C, $n = 3$

Analyte	Sample A			Sample B			Sample C		
	5 ng mL^{-1} (%)	100 ng mL^{-1} (%)	500 ng mL^{-1} (%)	5 ng mL^{-1} (%)	100 ng mL^{-1} (%)	500 ng mL^{-1} (%)	5 ng mL^{-1} (%)	100 ng mL^{-1} (%)	500 ng mL^{-1} (%)
Estriol	93.4 ± 6.5	90.2 ± 13.3	105.6 ± 13.9	67.5 ± 5.1	69.3 ± 13.8	85.2 ± 16.6	103.5 ± 15.6	96.7 ± 3.3	103.3 ± 8.5
17-β-Estradiol	101.9 ± 6.7	99.5 ± 19.8	110.1 ± 13.1	115.6 ± 5.9	74.1 ± 14.4	81.8 ± 17.1	83.1 ± 8.7	93.6 ± 11.2	115.1 ± 14.4
17-α-Ethynylestradiol	81.8 ± 15.1	91.5 ± 17.7	111.1 ± 12.8	97.3 ± 13.8	72.2 ± 14.3	70.9 ± 19.2	97.9 ± 19.9	97.1 ± 12.6	114.4 ± 13.0
Estrone	107.5 ± 9.8	94.9 ± 11.4	105.2 ± 11.7	109.1 ± 6.8	68.1 ± 12.7	72.3 ± 12.2	104.3 ± 16.8	90.9 ± 8.2	110.0 ± 10.1

Table 4 Comparison of the analytical features for the DLLME/MIL-based approach with data from literature

Extraction technique	Separation/determination technique	Extraction time	Analytes	Extraction phase	Linear range (ng mL^{-1})	Extraction volume (μL)	Total sample volume (mL)	LOD (ng mL^{-1})	Ref.
SBSE[a]	HPLC-DAD	2–4 h	Estrone 17-β-estradiol 17-α-ethynylestradiol	PDMS	5–50	126	30	1	[6]
UACPE[b]	HPLC-DAD	60 min	Estrone 17-β-estradiol	Tergitol TMN-6	5–1000	500	10	0.1–0.2	[28]
DLLME	HPLC-UV	60 s	17-β-Estradiol	CCl_4	1000–250,000	600	2	250	[30]
DLLME/MIL	HPLC-DAD	90 s	Estrone 17-β-estradiol 17-α-ethynylestradiol estriol	$[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$	5–500	≈ 8	3	2	This study

[a] Stir bar sorptive extraction

[b] Ultrasound-assisted cloud point extraction

analytes from the direct injection of a diluted urine sample (C_{sample}) were compared to the concentrations achieved with a sample extracted by $[P_{6,6,6,14}^{+}]_2[MnCl_4^{2-}]$ (C_{MIL}), using a concentration of 500 ng mL^{-1} for each analyte under optimized extraction conditions. Equation (1) was used to evaluate the EFs for DLLME/MIL-based approach.

$$EF = \frac{C_{MIL}}{C_{sample}} \quad (1)$$

Using Eq. (1), EFs corresponding to 33.4, 28.9, 29.5, and 30.9 were obtained for estriol, 17-β-estradiol, 17-α-ethynylestradiol, and estrone, respectively. According to these results, the DLLME/MIL-based procedure provided significant enrichment of the analytes from urine samples, with the chromatographic responses being enhanced around 30 times for all analytes, indicating a very satisfactory preconcentration capacity of the MIL-based extraction phase. Furthermore, absolute recoveries higher than 70% were achieved for all analytes emphasizing the high extraction efficiency of the DLLME-based procedure.

Moreover, a comparison of this study with other studies previously reported in the literature for the determination of the studied compounds in urine samples was carried out. Selected analytical features of each analytical methodology including the microextraction technique, volume of solvents, linear range, extraction time, and limits of detection are included in Table 4.

Based on Table 4, the method proposed in this study provided important analytical features compared with previously reported methods. The use of very small amount of extraction phase (≈ 8 μL of MIL) and very short extraction time (90 s) with no centrifugation step are all very attractive in emphasizing the environmentally friendly and high-throughput aspects of this powerful analytical methodology in analyzing biological matrices.

Conclusions

A DLLME/MIL-based method coupled with HPLC-DAD was optimized and successfully applied for the first time to determine hormones from urine samples. This report extends the applicability of these very promising extraction solvents to biological and more complex matrices, providing a rapid and reliable alternative to the conventional solvents generally used in DLLME approaches. This straightforward methodology allowed for extractions to be performed in a very short time (90 s) without the need for a centrifugation step. In addition, chromatographic separations were conducted within 10 min allowing for high-throughput analysis and LODs comparable to previously reported data using other microextraction techniques requiring longer extraction times. In addition, very satisfactory analytical performance was obtained regarding precision (intra- and interday assays), analyte relative recoveries, and linearity of the calibration curves. The physicochemical properties of MILs including the hydrophobicity, negligible vapor pressure, tunable chemical structure, and the ease of retrieving the MIL with a rod magnet offer a formidable tool to be explored as attractive extraction solvents in a number of extraction/microextraction techniques. Moreover, further studies focusing on automation can improve the high-throughput and automated aspects of extraction methodologies using these stable and efficient extraction phases.

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Compliance with ethical standards This article contains studies involving human urine samples with informed consent from all volunteers that contributed to this research.

Conflict of interest The authors declare that they have no conflicts of interest.

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