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Electro-assisted liquid phase microextraction for the determination of parabens and their main metabolites in maternal urine and amniotic fluid samples

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ABSTRACT

In this study, an electro assisted liquid phase microextraction (EA-LPME) procedure to determine seven parabens (methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and benzyl paraben) and three of their key hydroxy metabolites (4-hydroxy, 3,4-dihydroxy, and 3,4,5-trihydroxy benzoic acid) was optimised in maternal urine and amniotic fluid obtained at delivery from pregnant women. The samples were analysed by ultra-high-performance liquid chromatography coupled with electrospray ionisation tandem mass spectrometry (UPLC-ESI-MS/MS). PHBs and their metabolites were extracted from a pH 4 donor solution (10 mL) into a pH 13 acceptor solution (50 μL) using 1-octanol as the supported liquid membrane (SLM) and 30 V for 40 min at 400 rpm. Under optimal operational conditions, enrichment factors between 10 and 90 were achieved, and low quantitation limits within 0.022–0.20ng mL^{-1} and 0.025–0.18 ng mL^{-1}, were obtained for amniotic fluid and urine, respectively. The proposed analytical procedure was satisfactorily applied for the determination of target compounds in seven paired maternal urine and amniotic fluid samples to evaluate the possible placental transfer of these compounds from mothers to babies.

1. Introduction

Scientific and technological progress has generated diverse synthetic substances that enhance daily convenience and comfort, but their potential toxicity and environmental persistence pose hazards to both animals and humans [1]. These substances include endocrine disrupting chemicals (EDCs), synthetic compounds that interfere with the hormonal system, causing dysfunction by mimicking natural hormones, blocking their actions, hindering their synthesis, or modifying receptor expression [2].

A particular group of EDCs are parabens (PHBs). Some of them are commonly used as antibacterial and antimicrobial preservatives in everyday consumer products and have come under scrutiny due to increasing research suggesting potential endocrine-disrupting effects. This fact has raised significant concerns in the scientific community regarding the toxicity and risks of ongoing paraben exposure to both animals and humans. Parabens can enter the body through ingestion, dermal absorption, or inhalation, and they can also impact unborn babies and infants through transfer from the placenta [3,4], amniotic fluid [5–7], and breast milk [8]. Due to the growing risks associated with paraben exposure, in 2014, Regulation (EC) no 1004/2014, amending Annex V to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products, banned the use of some parabens in cosmetics including isopropylparaben, isobutylparaben and benzylparaben among others. In parallel, it modified the maximum allowed concentrations of propylparaben and butylparaben [9]. Nowadays, methylparaben and propylparaben are the most employed. Other widely used PHBs are ethylparaben and butylparaben, while isobutylparaben, isopropylparaben, and benzylparaben used is banned.

Several analytical methods have been developed to determine parabens (PHBs) in different biological matrices, including urine and amniotic fluid among others [10]. However, analysing PHBs in these complex matrices is challenging due to their low levels of presence and potential interference from other sample components. Therefore, a sample preparation step is necessary to clean up the sample and concentrate the analytes before instrumental analysis. Sample

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preparation techniques used for the extraction of PHBs include classical extraction techniques such as solid-phase extraction (SPE) [11,12] or liquid–liquid extraction (LLE) [13] as well as most recent sample preparation miniaturised techniques like dispersive liquid–liquid microextraction (DLLME) [14,15], hollow fiber liquid phase microextraction (HF-LPME) [16], electromembrane extraction (EME) [17], and microfluidic platforms [18,19] among others [20]. These miniaturised techniques are preferred due to their cost-effectiveness, speed, environmental friendliness, selectivity, and sensitivity. Overall, the determination of PHBs in biological matrices requires careful sample preparation techniques followed by instrumental analysis using chromatographic or electrophoretic methods coupled with appropriate detectors [10]. These approaches enable the accurate and sensitive measurement of PHBs, despite their low levels and potential interference from other sample components.

LPME and EME are two popular liquid phase microextraction techniques well known by the scientific community [16]. LPME is based on the passive diffusion of analytes from an aqueous donor phase (analytes in their neutral form) to an aqueous acceptor phase (ionised analytes) through a supported liquid membrane, thanks to a pH gradient between the two phases. In EME, the extraction is based on the electromigration of the analytes from the donor phase (ionised analytes) to the acceptor phase (ionised analytes) through the SLM, thanks to an electric field generated between two electrodes placed in each phase [21]. The application of an electric field in EME during extraction has demonstrated to decrease extraction times in the hollow fiber configuration [22]. Electro-assisted hollow fiber liquid phase microextraction (EA-HF-LPME) consists of a combination of EME and LPME in which the extraction process implies a first diffusive step of the analytes to the SLM and then a second step consisting of their migration from the SLM to the acceptor phase, decreasing the extraction times and improving sensitivity due to the introduction of the electric field. This behaviour has been previously described for some parabens and other species [17,23].

In this study, an electro-assisted liquid phase microextraction (EA-LPME) procedure for the simultaneous determination of seven PHBs (methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and benzyl paraben) and three of their main hydroxy metabolites (4-hydroxy 3,4-dihydroxy and 3,4,5-trihydroxy benzoic acid) is proposed. Selected PHBs were extracted using EA-HF-LPME followed by high-performance liquid chromatography-mass spectrometry (HPLC-MS). This approach proved to be a powerful tool for determining trace levels of analytes due to the preconcentration achieved and the high sensitivity of mass spectrometry detection in HPLC-MS. The optimised method was then applied to maternal urine and amniotic fluid samples collected from pregnant women at delivery, as this group is particularly vulnerable to the harmful effects of PHBs, which can potentially pass through the placenta and pose risks to new-borns.

2. Materials and methods

2.1. Chemicals and standard solutions

Target compounds, 4-hydroxy benzoic acid (PHBA) (100 %), methyl 4-hydroxybenzoate (MePHB) (99 %), ethyl 4-hydroxybenzoate (EtPHB) (99 %), propyl 4-hydroxybenzoate (PrPHB) (99 %), butyl 4-hydroxybenzoate (ButPHB) (99 %), isobutyl 4-hydroxybenzoate (iButPHB) (97 %), benzyl 4-hydroxybenzoate (BzPHB) (99 %), 3,4-dihydroxybenzoic acid (3,4-OH-HBA) (97 %) were purchased from Sigma-Aldrich (Madrid, Spain), 3,4,5-trihydroxybenzoic acid (3,4,5-OH-HBA) was from Apollo Scientific (Bredbury, UK), and isopropyl 4-hydroxybenzoate (iPrPHB) (98 %) was from Alfa Aesar, Thermofisher Scientific (Karlsruhe, Germany). The chemical structures and physicochemical properties of each compound can be found in the Supplementary Material (Table S1). Individual stock solutions for each analyte (500 mg L^{-1}) were prepared in methanol (Merk, Darmstadt, Germany) and stored at 4 °C. Stock solutions of 1 M HCl and 1 M NaOH were prepared in Milli-Q Plus water (Millipore, Bedford, MA, USA) by dissolving an adequate amount of concentrated HCl (37 %, Scharlab, Barcelona, Spain) and NaOH pellets (≥99 %, Merck, Darmstadt, Germany), respectively. Daily working solutions (analyte standard solutions for UPLC separation control, donor solutions, and acceptor solutions) were prepared using ultrapure water by adequate dilutions of methanolic stock solutions, 1 M NaOH stock solution, and 1 M HCl stock solution, as the case may be. Organic solvents tested as SLM (1-heptanol (≥99.5 %), 1-octanol (≥99 %), 1-nonanol (≥98 %)) were purchased from Merck (Darmstadt, Germany). Methanol (UltraChromasolv™, Honeywell, Riedel de Haën, LC-MS grade) and formic acid ammonium acetate (LC-MS grade) for LC-MS measurements were purchased from Honeywell Chemicals (Seelze, Germany).

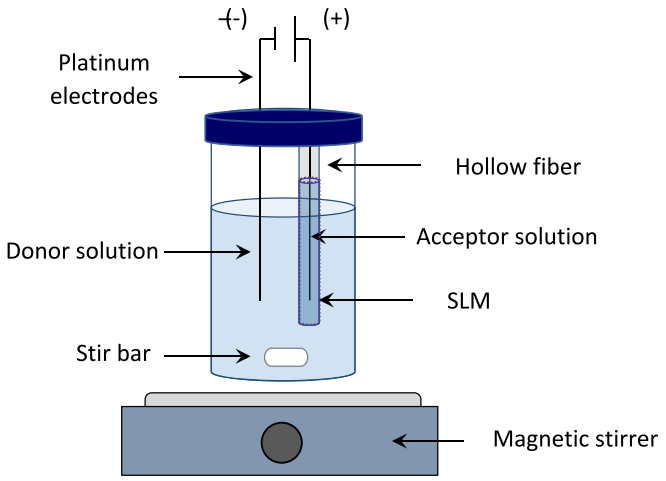

Fig. 1. Scheme of the EA-LPME set-up.

2.2. Real samples

Maternal urine (MU) and amniotic fluid (AF) were collected from pregnant women at delivery. All procedures applied were approved by the Coordinating Committee of Ethics of the Biomedical Investigation of Andalucía (protocol number: 1479-N-20), and informed consent was obtained from each pregnant woman. Maternal urine (125 mL) was collected just after the delivery in the Department of Gynaecology and Obstetrics in two of the University Hospitals in Seville (Spain). Amniotic fluid samples (15 mL) were collected during delivery in patients with complete amniotic membranes and cervical dilation greater than 6 cm to avoid any risk of accelerated labour. Samples were kept in sterile polyethylene containers. The collected biomaterial was frozen (−20 °C) and transported under refrigeration (4 °C) to the laboratory, where it was immediately frozen and stored at −80 °C until extraction.

Maternal urine and amniotic fluid samples were first microfiltered (0.22 μm) and diluted with ultrapure water at a ratio of 1:20 (v/v) and 1:10 (v/v), respectively. Hollow fibers cut into 30 mm pieces were washed with acetone and dried before its use. After that, fibers were sealed at the end and immersed in 1-octanol for 5 s to fill the pores. The excess of organic solvent was carefully wiped off by using a soft tissue. 50 μL of the acceptor phase (0,1 M NaOH aqueous solution) were introduced into the lumen of the fiber using an HPLC syringe. The hollow fiber was immersed into 10 mL sample solution (pH 4) and positive and negative electrodes were placed in the acceptor and donor phases, respectively to carry out the extraction applying 30 V for 40 min at 400 rpm stirring speed. After extraction, 10 μL of the extract were injected into the LC-MS system.

3. Methodology

3.1. Electro-assisted liquid phase microextraction procedure

Electro-assisted liquid phase microextraction (EA-LPME) was performed in a three-phase configuration mode using a classical set-up based on the use of polypropylene hollow fibers (HF) as support for the liquid membrane (HF-EA-LPME). A scheme of the employed device is depicted in Fig. 1. The extraction set-up was conducted similarly to that previously described by Aranda-Merino et al. [24] and involves the following steps: (i) Porous polypropylene hollow fibers (Accurel®PP S6/2, Membrana, Wuppertal, Germany, wall thickness = 450 μm; i.d. = 1800 μm; pore size = 0.2 μm) were cut into 30 mm pieces, washed with acetone (Sharlab S.L., Barcelona, Spain), and air dried. (ii) Each piece was closed at the lower end by mechanical pressure and thermally sealed. (iii) The HF was dipped in the organic solvent for 5 s to form the liquid membrane, and the solvent excess was removed with a medical wipe. (iv) The HF was filled with 50 μL of aqueous acceptor solution (NaOH 100 mM, pH 13) using a 50 μL HPLC syringe (705 LT (Hamilton®), VWR International Eurolab, Barcelona, Spain), and the upper end was assembled in the narrow end of a pipette tip for guiding one of the electrodes into the HF lumen. (v) Finally, the extraction unit was introduced in a glass vial (10 mL, 20-mm inner diameter; VWR International Eurolab, Barcelona, Spain) containing 10 mL of the aqueous donor solution (pH 4, analytes solution). (vi) Two platinum electrodes (0.5 mm, Premion®, 99.997 % (metal basis) ≈ 4.21 g/m, Alfa Aesar, Thermo Fisher Scientific, Germany) were introduced in the corresponding aqueous phases, with negative in the donor and positive in the acceptor, respectively. (vii) Both electrodes were connected to an external laboratory DC power supply (Benchtop Instrument, Pennsylvania, USA) with a programmable voltage in the range of 0–120 V, and the sample solution was agitated using a FB 15107 magnetic stirrer (Fisher Scientific, Pittsburg, PA, USA).

The best extraction efficiency was achieved by applying a potential difference of 30 V for 40 min at a constant stirring rate of 400 rpm. Once the extraction was completed, the acceptor solution was transferred using a microsyringe from the HF to a HPLC microinsert to be injected (10 μL) into the LC-MS system.

3.2. Chromatographic and mass spectrometry conditions

Target compounds were measured using liquid chromatography connected to a triple quadrupole mass spectrometer (Waters Acquity H-Class coupled to a Xevo TQ-XS, Milford, MA, USA) (UPLC-MS/MS) equipped with an electrospray ionisation source (ESI). The analytes were detected in negative ESI mode and multiple reaction monitoring (MRM). The optimisation of MS parameters (precursor ions, collision energy, cone voltage, and quantitation and confirmation transitions) was performed by flow injection analysis at 10 μL min^{-1} for each compound dissolved in the mobile phase. To obtain the precursor ion for each compound, the cone voltage was studied from 5 to 50 V. Then, the collision energy (CE) was optimised from 5 to 50 eV to obtain the two more intense fragment ions, and argon was used as collision gas (99.995 %, Praxair). For each analyte, the two most abundant product ions were selected as quantitation and confirmation ions. The optimal parameters are displayed in Table S2. The desolvation gas (nitrogen) was set at 600 L/h and the cone gas (nitrogen) to 150 L/h; the desolvation temperature was set to 550 °C and the source temperature to 150 °C. The capillary was set to −1.0 kV.

The separation was performed by UPLC with a conditioned autosampler at 10 °C, using a Zorbax Eclipse Plus C18 analytical column (150 mm × 2.1 mm i.d., 3.5 μm particle size) (Agilent Technologies, Santa Clara, CA, USA). The column temperature was maintained at 30 °C. The mobile phase consisted of ammonium formate (5 mM) aqueous solution (solvent A) and methanol (solvent B) under the following gradient elution: 0 min 100 % A, 2.5 min 100 % A, 4 min 30 % A, 14 min 30 % A, returning to initial conditions. Three minutes were waited before injections to re-equilibrate the column. The injection volume was 5 μL. MassLynx™ version 4.2 (Waters, Milford, MA, USA) was used to conduct system setting, data collection, and MS analysis. MS/MS data processing was carried out using the software TargetLynx™ XS (Waters®).

3.3. Calculations

The extraction efficiency of the HF-EA-LPME procedure was evaluated in terms of enrichment factor (EF), which represents the preconcentration level achieved for each analyte under optimal operational conditions. The enrichment factor is defined as the ratio of the final analyte concentration in the acceptor solution, $C_{a,\ final}$, and the initial analyte concentration in the donor solution, $C_{d,\ initial}$. This parameter is calculated according to equation (1):

$$\text{Enrichment factor (EF)} = \frac{C_{a,\ final}}{C_{d,\ initial}} \quad (1)$$

4. Results and discussion

4.1. Operational parameters optimisation for Electro-assisted liquid phase microextraction

4.1.1. Organic solvent selection

Preliminary extraction conditions were selected based on a previous reported EME for some parabens [17]: pH 4 and pH 12 (NaOH 10 mM) as donor and acceptor phase, respectively; 10 mL and 50 μL as donor and acceptor volumes, respectively; 40 V, 40 min extraction and 400 rpm as stirring rate. For all experiments, 1 $mg{\cdot}L^{-1}$ of each analyte was added to the donor solution. According to their lower pK_a values (deprotonation of the hydroxyl group) in Table S1, the target analytes are in their neutral form in the donor solution below that pK_a and negatively charged above that pK_a [25]. Under those preliminary conditions, two experiments were carried out at 0 and 40 V to study the influence of LPME and the EME, respectively. Higher EF were obtained at 40 V although the pH conditions belong to LPME phenomena for MePHB, EtPHB, PrPHB, BuPHB, iPrPHB, iBuPHB and BzPHB. Those mentioned compounds were extracted at 0 V but no enrichment was observed. The application of an electric field improved the mass transfer of the analyte once it reached the membrane thanks to an electrokinetic migration mechanism. For these reasons, the selected conditions described above were set for the study of the organic solvent and new hypotheses will be described in the following sections for the study of the pH of the donor and acceptor phase. When an electrical field is involved in the extraction, the stability of the system is mainly influenced by the electric current generated, which depends on the applied voltage, and the donor and acceptor solution composition and the chemical composition of the liquid membrane [26,27]. Before selecting the organic solvent, some physicochemical criteria should be considered when EME is involved, such as: (i) being poorly water-soluble; (ii) being non-volatile to prevent solvent loss; (iii) having enough permeability to allow mass transfer; and (iv) having sufficient electrical conductivity to ensure a continuous electric field, avoiding power failures [24].

In this study, 1-heptanol, 1-octanol, and 1-nonanol were examined as potential liquid membranes based on previous findings, since particularly long-chain aliphatic alcohols have shown successful molecular interactions with acidic compounds [28]. 1-heptanol showed the lowest efficiency due to its higher polarity and conductivity. 1-nonanol showed higher and lower efficiency compared to heptanol and 1-octanol, respectively. 1-nonanol showed higher viscosity (11.7 cp) and increased the electrical resistance of the SLM, however, 1-octanol showed adequate electrical resistance to allow sufficient mass transfer without compromising the stability of the system. This result agreed with those previously reported for EME of parabens [17,29], where 1-

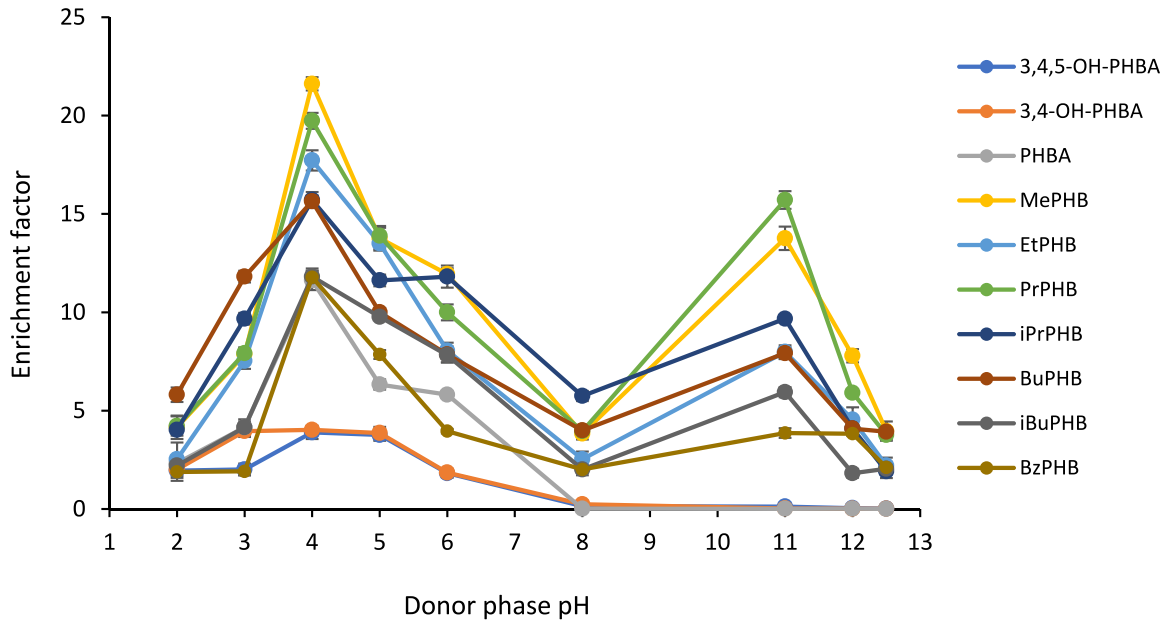

Fig. 2. Effect of donor solution pH. HF-EA-LPME conditions: SLM:1-octanol; pH acceptor solution: 12 (NaOH 10 mM); Agitation speed: 400 rpm; Voltage: 30 V; Extraction time: 40 min; n = 3.

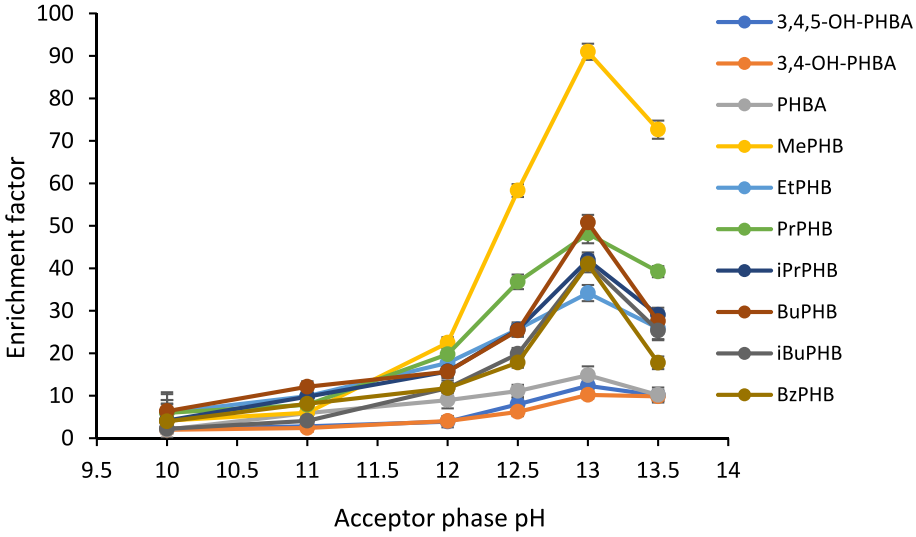

Fig. 3. Effect of acceptor solution pH. HF-EA-LPME conditions: pH of donor solution: 4 (not adjusted); SLM:1-octanol; Agitation speed: 400 rpm; Voltage: 30 V; Extraction time: 40 min; n = 3.

octanol was also proven to be the most suitable SLM.

4.1.2. pH of donor and acceptor phases

The pH donor composition was studied within the range of 2–12.5, using different aqueous solutions of NaOH and HCl. The acceptor pH value was fixed at pH 12 to keep all the analytes negatively charged (Table S1) based on their pK_a value. Based on the results obtained, different phenomena were observed. The data depicted in Fig. 2 showed that the best enrichment factors were accomplished at pH 4 for all compounds. Based on their the pK_a values, PHBA, 3,4-OH-PHBA, and 3,4,5-OH-PHBA were in their neutral form below pH 4 and ionised above that pH value, meantime the rest of parabens were in their neutral form below pH 8 and ionised about that pH value. Over pH 8, a clear EME is occurring for all compounds, however, the higher EF was observed at pH 4. This could be explained based on previous reported phenomena regarding the boundary pH layer [28]. This observation could be attributed to elevated pH conditions in the donor/SLM interface (donor boundary layer). This donor boundary layer, which is located at the donor/SLM interface, has most likely a pH higher than in the bulk donor phase. The analyte molecules transfer the SLM as partially or fully deprotonated ionised species (depending on the compound pK_a value) and therefore electro-kinetic migration does also occur in the donor/SLM interface. Then, the liquid phase microextraction was electrically enhanced when the analytes reached the donor SLM/interface. On the other hand, the enrichment factor for parabens was always higher for the three metabolites (PHBA, 3,4-OH-PHBA, and 3,4,5-OH-PHBA) regardless of the pH of the donor phase. This behaviour could be associated with the lower polar character of the esters (see log P values in Table S1) compared to the acidic compounds, which confers them a higher affinity for the SLM, resulting in a better transfer rate. Hence, the analytes aqueous solution pH (≈ 4) was selected as the optimal value for the donor phase.

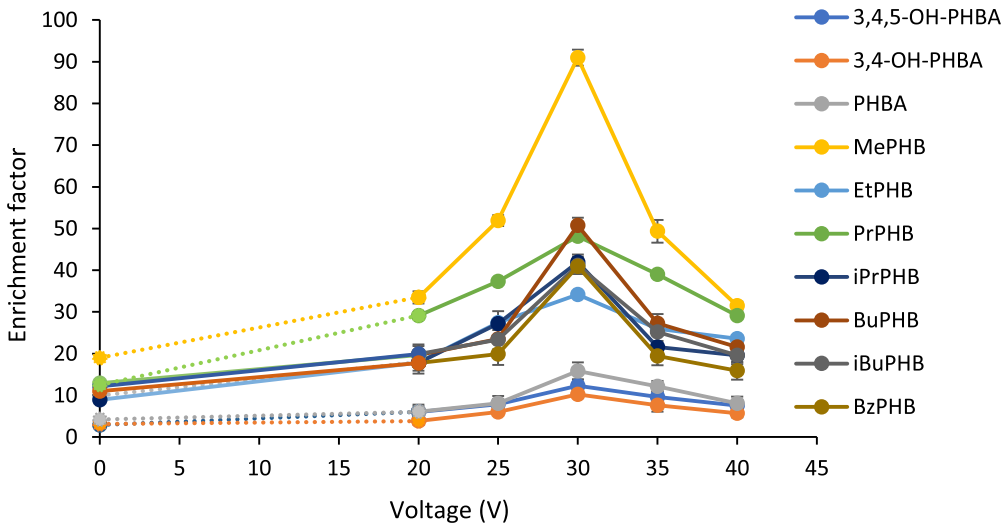

Fig. 4. Effect of applied voltage. HF-EA-LPME conditions: pH of donor solution: 4 (not adjusted); SLM:1-octanol; pH of acceptor solution: 13 (NaOH, 100 mM); Agitation speed: 400 rpm; Extraction time: 40 min; n = 3.

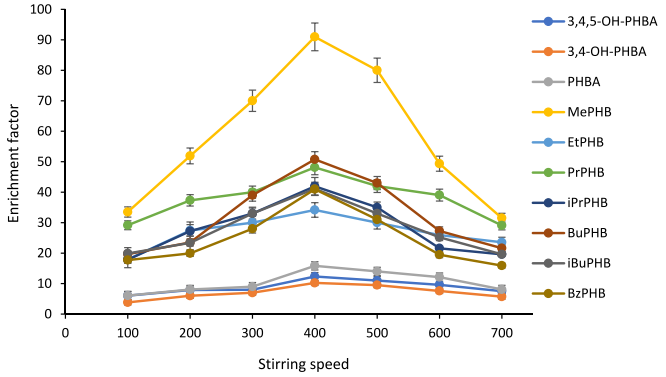

Fig. 5. Effect of stirring speed. HF-EA-LPME conditions: pH of donor solution: 4 (not adjusted); SLM:1-octanol; pH of acceptor solution: 13 (NaOH, 100 mM); Applied voltage: 30 V; Extraction time: 40 min; n = 3.

The acceptor solution composition was investigated within the range 10–13.5. As can be seen in Fig. 3, the enrichment factor increased up to pH 13 and decreased above that pH due to electrolysis phenomena and a lower stability of the compounds at higher pH values. Most molecules are in their ionised form at pH 13, and therefore the greatest enrichments are observed for all the compounds studied. Therefore, a pH of 13 as the acceptor phase was set for the rest of the study.

4.1.3. *Applied voltage*

Since the voltage applied demonstrated a better efficiency compared to the absence of voltage, this parameter was evaluated between 20 and 40 V. Mass transfer depends on the applied voltage, which is considered the main driving force in electrokinetic extraction procedures. This finding was previously reported by Pedersen-Bjergaard et al. [31], who demonstrated that extraction efficiency should be better at high voltage. Nevertheless, by increasing the voltage, some phenomena, such as electrolysis or SLM loss, could lead to system instability and thus negatively affect the flux of analytes. As seen in Fig. 4, the enrichment factor increased gradually from 20 to 30 V and significantly decreased at voltages higher than 30 V. The latter could be attributed to the electrolysis reactions in both donor and acceptor solutions. Then, 30 V was selected as the optimum voltage.

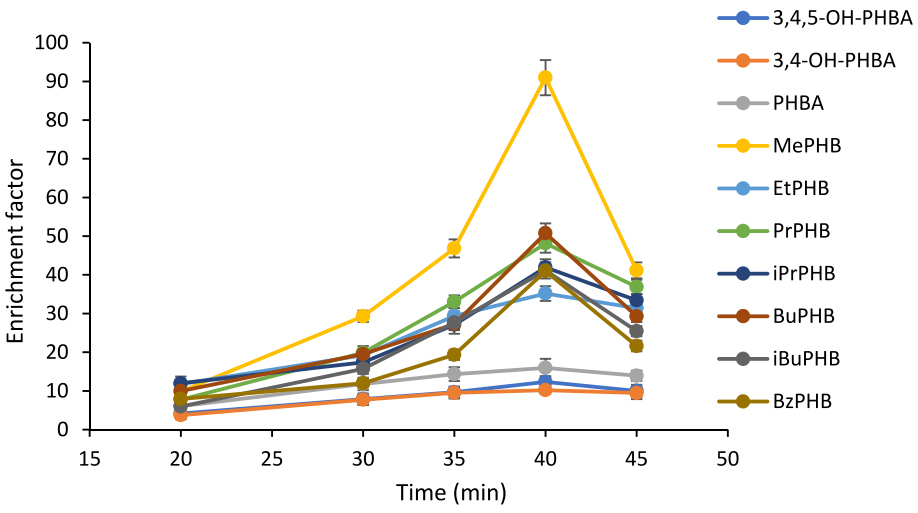

Fig. 6. Effect of extraction time. HF-EA-LPME conditions: pH of donor solution: 4 (not adjusted); SLM:1-octanol; pH of acceptor solution: 13 (NaOH, 100 mM); Agitation speed: 400 rpm; Applied voltage: 30 V; n = 3.

Table 1
Figures of merit of the optimised EA-LPME method.

Analyte	Linear range (ng mL^{-1})	(R^2)	MLOD (ng mL^{-1})		MLOQ (ng mL^{-1})		EF	Rep/IP (%RSD)		
			AF	Urine	AF	Urine		0.2	0.5	2
3,4-OH-PHBA	MLOQ-50 (AF)/100 (U)	0.9912	0.036	0.054	0.12	0.18	10	4.1/9.2	5.3/8.1	5.2/7.3
3,4,5-OH-PHBA	MLOQ-50 (AF)/100 (U)	0.9917	0.060	0.050	0.20	0.17	12	6.3/9.6	7.2/10.1	5.9/11.8
PHBA	MLOQ-50 (AF)/100 (U)	0.9915	0.030	0.024	0.10	0.080	14	7.6/8.1	6.3/6.8	5.9/9.4
MePHB	MLOQ-50 (AF)/100 (U)	0.9971	0.0067	0.0075	0.022	0.025	90	4.7/7.2	6.9/8.2	5.3/7.8
EtPHB	MLOQ-50 (AF)/100 (U)	0.9990	0.013	0.016	0.043	0.052	34	8.4/9.9	5.1/7.5	6.1/10.5
iPrPHB	MLOQ-50 (AF)/100 (U)	0.9928	0.014	0.015	0.045	0.051	41	5.3/9.4	5.5/8.6	5.0/7.9
PrPHB	MLOQ-50 (AF)/100 (U)	0.9969	0.012	0.013	0.041	0.042	48	6.1/8.7	6.4/11.3	6.7/9.4
iButPHB	MLOQ-50 (AF)/100 (U)	0.9914	0.012	0.014	0.039	0.047	41	4.3/6.5	5.2/9.9	5.5/7.7
ButPHB	MLOQ-50 (AF)/100 (U)	0.9917	0.012	0.013	0.040	0.042	50	5.9/6.5	7.1/10.2	5.2/6.9
BzPHB	MLOQ-50 (AF)/100 (U)	0.9927	0.014	0.017	0.045	0.055	40	5.1/9.5	6.3/8.0	6.1/8.3

*Data were calculated with three replicates.

4.1.4. Stirring speed

Stirring in the donor phase improved the transportation of analytes while concurrently reducing the boundary layer's size at the donor/SLM interface. As seen in Fig. 5, the stirring speed was study from 0 to 1000 rpm, observing an increase in the enrichment up to 400 rpm. Above 400 rpm, a decrease in the enrichment was observed possibly due to the loss of the organic solvent in the supported liquid membrane. Then, a stirring rate of 400 rpm was selected.

4.1.5. Extraction time

The mass transfer in EA-LPME is also a time-dependent process and the extraction yield increases with time until the system reaches the steady state, where mass transfer stops and no further gain in recovery is observed [32]. The extraction time was investigated within the range of 20–45 min. As can be seen in Fig. 6, the highest enrichment was reached at 40 min for all compounds. A clear increase was shown up to 40 min till the system reached the steady state. In contrast, the lower EF obtained above 40 min might be related to changes in the pH of the acceptor solution caused by electrolysis reactions after longer extraction times [30]. Then, extractions were carried out for 40 min for the rest of the study.

Finally, the optimal extraction conditions were established as follows: 10 mL (pH 4) of donor aqueous donor solution (containing 1 mg·L^{-1} of all target analytes, 1-octanol as SLM, and 50 μL (pH 13, 100

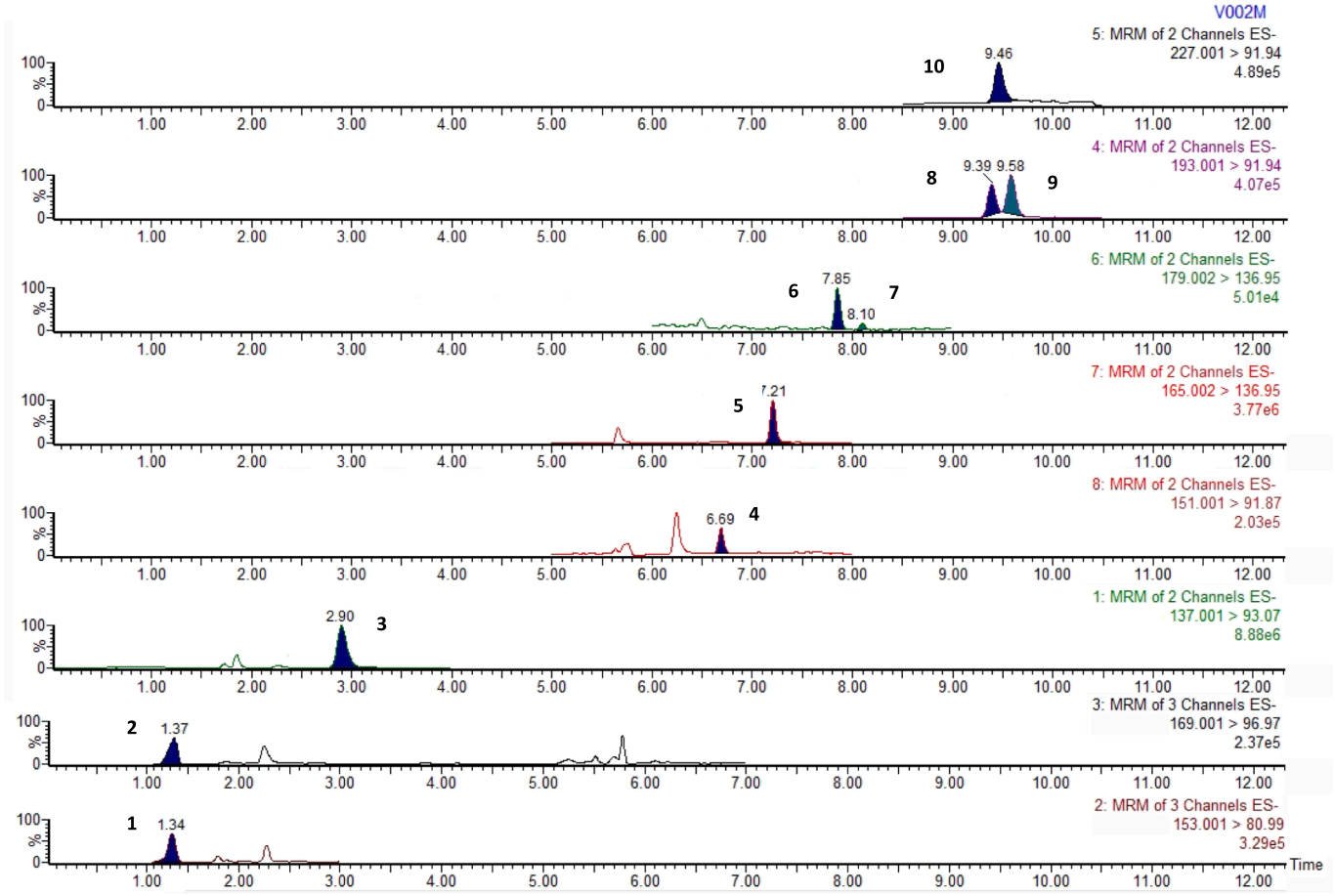

Fig. 7. Chromatogram of a spiked urine sample at 1 μg L^{-1} for all compounds. 1: 3,4,5-OH-PHBA, 2: 3,4-OH-PHBA, 3: 4-OH-PHBA, 4: MePHB, 5: EtPHB. 6: PrPHB, 7: iPrPHB, 8: iBuPHB, 9: ButPHB, 10: BzPHB.

mM NaOH) as acceptor solution. The extractions were carried out at 30 V for 40 min at 400 rpm.

4.2. Validation of the EA-LPME procedure

To evaluate the applicability of the optimised method, the figures of merit of the methodology were validated based on the EURACHEM (European Analytical Chemistry) guide [33]. Linearity, sensitivity, precision, and accuracy were evaluated as quality parameters. Validation data are summarised in Table 1. Linearity was evaluated by a ten-point external calibration curve in the concentration range of 0.001–10 ng mL^{-1}. The calibration curve was plotted by least-squares linear regression analysis of standard mixtures at different concentrations submitted in triplicate to the proposed HF-EA-LPME procedure. According to the data, calibration curves were linear in the range of 0.1–5 ng mL^{-1}, with coefficients of determination (R^2) between 0.9912 and 0.9990. Sensitivity was investigated in terms of limits of detection (LOD) and quantitation (LOQ). LODs and LOQs were calculated based on a signal-to-noise (S/N) ratio of 3 and 10, respectively. Table 1 shows the method limits of detection (MLOD) and quantitation (MLOQ) in AF and urine. As it can be observed, the values showcase exceptional sensitivity, with MLODs hovering around a hundredth of a ng mL^{-1} for most of the analysed compounds, except for the metabolites, for which higher values were obtained. This high sensitivity was not only attributed to the possibility of preconcentration provided by hollow fiber-based set-ups but also to the analytical instrumentation employed (UPLC-MS/MS). Precision was studied according to repeatability (inter-day precision) and intermediate precision (intraday precision). Aqueous standard solutions at three concentration levels (0.2, 0.5 and 2 ng mL^{-1}) were submitted in triplicate to the entire EA-LPME procedure on one single day (repeatability) and one single day per week over one month (intermediate precision). Repeatability (Rep) and intermediate precision (IP), both expressed as relative standard deviation percentages (% RSD), ranged from 4 to 9 % and 7–12 %, respectively. The EF calculated as described in section 3.3 ranged from 10 to 90, being the higher values for MePHB, ButPHB and PrPHB and the lower for the metabolites. In addition, the EFs were also calculated from the slopes of the external and extracted calibration curves, showing very similar values, whose deviation in no case exceeded the precision of the method.

In order to check the applicability of the proposed EA-LPME method to biological samples, the accuracy was evaluated by means of recovery assays on maternal urine and amniotic fluid samples. Because of the saline composition of urine and amniotic fluid, dilution was necessary to minimise electrolysis effects and ensure a stable EA-LPME performance. Maternal urine and amniotic fluid were diluted at a ratio of 1:20 (v/v) and 1:10 (v/v), respectively, which also helps to avoid or reduce possible matrix effects. First, selected maternal urine and amniotic fluid samples were analysed to check whether some of the target analytes might be present. Once the absence or presence of analytes was confirmed, these samples were employed for the assay, subtracting the corresponding signal in case any of the compounds were present. MePHB was present in both samples (0.18 and 0.76 ng mL^{-1} in urine and AF respectively) and 3,4,5-OH-PHB (0.27 ng mL^{-1}) and PHBA (0.12 ng mL^{-1}) were quantified in AF and urine, respectively. The samples were spiked at three concentration levels, microfiltered (0.22 μm), and diluted with ultrapure water for final concentration levels of 0.5 and 2 ng mL^{-1} prior to EA-LPME. Fortification levels were selected considering the optimum linearity of the method. The pH of diluted samples was adjusted to 4 before extraction and finally submitted to the optimised EA-LPME procedure. The relative recovery percentages (RR%) at each concentration level are summarised in Table S3. According to the obtained results, recoveries over 87 % were obtained, and no significant differences were found between the two spiked concentration levels. Therefore, it can be stated that the proposed EA-LPME procedure was suitable for the selective extraction and preconcentration of target analytes in maternal urine and amniotic fluid samples. Figure 7 shows a representative chromatogram of a spiked sample at 1 ng mL^{-1}.

Fig. 8. BAGI index pictogram for proposed analytical method.

4.3. Evaluation of method practicality

White Analytical Chemistry (WAC), a concept developed in 2021 by Nowak et al. serves as an extension and complement to green analytical chemistry, combining the ecological, analytical and practical perspectives of an analytical method. A more recent complement regarding the practicality of the method has been proposed by Manousi et al. in 2023 [34].

Using a simple metric tool named blue applicability grade index (BAGI), which provides the called BAGI index, a score is obtained that determine the applicability of the method. Actually, two different types of correlated results are obtained, a score and a pictogram. The overall result of the assessment is a pictogram in the shape of an asteroid with the number in its centre. The scores range from 25 to 100, the higher the score, the more practical the method is. A score of 60 or more is considered to demonstrate the applicability of the method. The pictogram shows where the strengths and weaknesses of the method lie.

The proposed method was evaluated using BAGI 0.9.2 free software [35]. The software tool takes into account the attributes: type of analysis; number of analytes that are simultaneously determined; analytical technique and required analytical instrumentation; number of samples that can be simultaneously treated; sample preparation; number of samples that can be analysed per hour; type of reagents and materials used in the analytical method; requirement for preconcentration, automation degree and amount of sample. The pictogram obtained is shown in Fig. 8, as it can be seen, the BAGI score of 60 that was assigned to the method demonstrates its applicability. The method demonstrates to have the highest strong points in preconcentration, and the quantitative and confirmatory results provided by the method, while the weak points are mainly in the degree of automation, the number of samples that can be processed simultaneously and the use of a sophisticated instrumental technique.

4.4. Application to real samples

To evaluate the applicability of the optimised method, seven maternal urine and amniotic fluid samples obtained at delivery from pregnant women who gave informed consent, were submitted to the EA-

Table 2
Determination of parabens in biological samples reported recently in literature.

Parabens Determined	Matrix	Analytical features	Sample treatment	Analytical technique	Sample volume	Conc. range in samples (ng mL^{-1})	Extraction time (min)	Ref.
MetPHB,EtPHB,PrPBH, iPrPHB, BuPHB, iBuPHB, BzPHB, PHBA, 3,4-OH-PHBA, 3,4,5-OH-PHBA	Maternal urine, AF	MLOD:0.0067–0.060 ng mL^{-1} MLOQ: 0.022–0.20 ng mL^{-1} R (%): 87.0–98.0	EA-HF-LPME	UPLC-ESI-MS/MS	0.5 mL urine 1 mL AF (both diluted to 10 mL)	MetPHB: <LOQ-1.3 EtPHB: <LOQ-60 PrPBH: <LOQ-0.31 iPrPHB: N.D. BuPHB: <LOQ-0.13 iBuPHB. N.D. BzPHB:<LOQ-0.33 PHBA: <LOQ-1.9 3,4-OH-PHBA: <LOQ-0.28 3,4,5-OH-PHBA: <LOQ-3.4	40 min	This study
MetPHB,EtPHB,PrPBH, iPrPBH, BuPHB, BzPHB	Urine, serum, AF	LOD: 0.04–0.08 ng mL^{-1} R (%): n.d.	SPE	ID-UPLC-MS/MS	n.d	MetPHB:<0.38–52.5 EtPHB: N.D.-2.25 PrPBH: N.D.-10.3 iPrPBH: <0.07 BuPHB:<0.05 BzPHB:<0.05	>30 min	[5]
MetPHB,EtPHB,PrPBH, BuPHB,HepPHB, BzPH B, PHBA, 3,4-OH-PHBA, OH-MePBH OH-EtPHB	Urine, fetal serum, AF	LOQ: 0.05–0.20 ng mL^{-1} R (%): 84–102	LLE	LC-MS/MS	0.5 serum 2 mL urine and AF	MetPHB:<0.01–26 EtPHB:<0.01–6.5 PrPBH:<0.01–9.9 BuPHB:<0.01–1.9 HepPHB:<0.01–1.6 BzPHB:<0.01 PHBA:<0.2–1620 3,4-OH-PHB:<0.02–1370 OH-MePBH:<0.005–48 OH-EtPHB:<0.01–20	>60 min	[6]
MetPHB, EtPHB, PrPBH, BuPHB	AF	LOD: 0.044–0.080 ng mL^{-1} LOQ: 0.1452–0.2640 ng mL^{-1} R (%): 95.6–98.7	DLLME	CG-MS	7–9 mL	MetPHB: ND-5.75 EtPHB: ND-1.64 PrPBH: ND-1.29 BuPHB: N.D-35.40	24 h + 15 min aprox.	[7]
MetPHB, EtPHB, PrPBH, iPrPHB, BuPHB, iBuPHB	Urine, blood, and breast milk	LOD: 0.0002–0.0018 ng mL^{-1} LOQ: 0.0006–0.006 ng mL^{-1} R (%): 86–104	Cont-SPE-DER	CG-MS	5 mL	MetPHB: 0.65–3.2 EtPHB: 0.93–14 PrPBH: D iPrPBH:0.9- D BuPHB: 0.95–9 iBuPHB: 0.1–0.28	>30 min	[11]
MetPHB,EtPHB,PrPHB, BuPHB, BzPHB	Urine	LOD:3 pg (BzPHB 1 pg) R (%): 80.6–95.6	SPE	LC-ESI-MS/MS	4 mL	MetPHB: 0.0–31 EtPHB: 0.2–25.8 PrPHB: 0.1–23.1 BuPHB: 0.01–020 BzPHB: 0–0.0003	n.d	[12]
MetPHB, EtPHB, PrPBH, BuPHB	Placenta	MLOD:0.04–0.05 ng mL^{-1} MLOQ: 0.2 ng mL^{-1} R (%): 87.1–112.7	DLLE	CG-MS	0.5 g	MetPHB: D-16.38 EtPHB: ND-5.37 PrPBH: D-4.02 BuPHB: N.D.	>30 min	[15]
EtPHB, PrPHB	Urine	MLOD:10 ng mL^{-1} MLOQ:30 ng mL^{-1} R (%): 98.4–100.2	μF-LPME	HPLC-DAD	n.d.	spiked	15 min	[18]
MetPHB,EtPHB, PrPBH, BuPHB, HepPHB, BzPHB, PHBA	Urine, serum	LOD: 0.003–0.033 ng mL^{-1} LOQ: 0.010–0.100 ng mL^{-1} R (%): 61–125	SPE/USAE	LC-MS/MS	0.5 mL	MetPHB:0.16–18.86 EtPHB: <LOD-1.14 PrPBH: N.D.-10.37 BuPHB: N.D. HepPHB: N.D. BzPHB: N.D. PHBA: 28.2–735	>30 min	[36]
MetPHB, PrPBH, BuPHB, BzPHB	Umbilical cord blood	MLOD:0.01–0.41 ng mL^{-1} MLOQ: 0.04–1.38 ng mL^{-1} R (%): 72.25–121.6	LLE	LC-MS/MS	0.5 mL	1 sample n.d	>30 min	[38]
MetPHB, EtPHB, PrPBH, BuPHB,	Urine	LOD: 0.0008–0.05 ng mL^{-1} LOQ: 0.003–0.18 ng mL^{-1} R (%): 96–109	SPE-MS	UHPLC-MS/MS	15 mL	MetPHB: <LOD-20 EtPHB: <LOD PrPBH: <LOD-4.10 BuPHB: <LOD-12	>30 min	[39]
MetPHB, EtPHB PrPBH, BuPHB, HepPHB, BzPHB	Urine	LOD: 0.0005–0.0050 ng mL^{-1}	LLE	LC-MS/MS	0.2 mL	n.a	>30 min	[40]

(*continued on next page*)

Table 2 (*continued*)

Parabens Determined	Matrix	Analytical features	Sample treatment	Analytical technique	Sample volume	Conc. range in samples (ng mL^{-1})	Extraction time (min)	Ref.
		LOQ: 0.0002–0.010 ng mL^{-1} R (%): 76.73–103.51						
MetPHB, EtPHB, PrPBH, BuPHB, BzPHB, OH-MePBH OH-EtPHB	Urine	LOD: 0.01–0.05 ng mL^{-1} LOQ: 0.03–0.15 ng mL^{-1} R (%): n.d	VADLLME	LC-MS/MS	1 mL	MetPHB: 0.24–145 EtPHB:<LOQ-2.3 PrPBH:<LOQ-7.2 BuPHB:<LOQ-1.0 BzPHB:<LOQ-1.9 OH-MePBH:<LOQ-11.2 OH-EtPHB:<LOQ-0.8	>8 min	[41]
MetPHB, EtPHB, PrPBH, BuPHB, BzPHB, OH-MePBH OH-EtPHB	Urine	LOD: 0.02–0.30 ng mL^{-1} LOQ: 0.05–1.0 ng mL^{-1} R (%): n.d.	AALLME	LC-MS/MS	5 mL	MetPHB: 2.29–1118 EtPHB:<LOQ-33.6 PrPBH:<LOQ-78.4 BuPHB:<LOQ-11.3 BzPHB:<LOQ-0.13 OH-MePBH: 0.18–28.5 OH-EtPHB:<LOQ-7.8	>30 min	[42]
MetPHB, EtPHB, PrPBH, BuPHB	Urine	LOD: 0.06 ng mL^{-1} LOQ: 0.20 ng mL^{-1} R (%): 85–104	Dilute and shoot	LC-APCI-MS/MS	0.5 mL	MetPHB: 0.2–2952 EtPHB: N.D.-25 PrPBH: N.D.-4.7 BuPHB N.D.-0.5	>90 min	[43]
MetPHB,EtPHB,PrPBH, BuPHB	Placental tissue	LOD:0.1–0.3 ng g^{-1} LOQ:0.2–0.7 ng g^{-1} R (%): 90–122	SLE	UHPLC MS/MS	1.5 g	MetPHB: 0.5–7.1* EtPHB: 0.5–4.5* PrPHB: 0.5–9.1* BuPHB: N.D	>30 min	[44]
MetPHB, EtPHB, PrPBH, iPrPBH, BuPHB, iBuPHB, BzPHB	Urine, whoole blood, plasma	LOD: 30 ng mL^{-1} LOQ: 100 ng mL^{-1} R (%): n.d	FPSE	HPLC-PDA	180 μL blood, 450 μL plasma 0.9 mL urine	MetPHB:N.D. EtPHB: 680 PrPBH: N.D. iPrPBH: N.D. BuPHB: N.D. iBuPHB: N.D. BzPHB: N.D.	n.d.	[45]
MetPHB, PrPBH, BuPHB, BzPHB, OH-MePBH OH-EtPHB	Urine	MLOQ: 0.5–2.5- ng mL^{-1} R (%): 96–115	MEPS	LC-MS/MS	250 μL	MetPHB: 3.21–982 EtPHB:<LOQ-28.6 PrPBH:<LOQ-28.6 BuPHB:<LOQ-6.75 BzPHB: N.D. OH-MePBH:0.38–31.5 OH-EtPHB:<LOQ-4.20	n.d.	[46]
MetPHB, EtPHB, PrPBH, BuPHB	Urine	LOD: 0.1 ng mL^{-1} LOQ: 0.3 ng mL^{-1} R (%): 86–100.9	SUPLE	ID-UPLC-MS/MS	0.1 mL	MetPHB: 78.1–959.4 EtPHB: 0.3–1200.9 PrPBH:17.4–343.7 BuPHB:0.7–13.7	n.d	[47]
MetPHB,EtPHB,PrPBH, iPrPBH, BuPHB, iBuPHB	Urine	LOD:70–120 ng mL^{-1} LOQ:240–380 ng mL^{-1} R (%): 87.3–113.6	μLLE	UHPLC MS/MS	4 mL	MetPHB: 1.6-D EtPHB: 0.5-D PrPBH:0.8–0.5 iPrPBH: 1.8-D BuPHB: n.a iBuPHB: n.a.	>20 min	[48]
MetPHB, EtPHB, PrPBH, BuPHB, BzPHB, 3,4-OH-MePBH 3,4-OH-EtPHB	Urine	LOD: 0.01–0.30 ng mL^{-1} LOQ: 0.03–1.0 ng mL^{-1} R (%): 94–115 %	LDS-AALLME	LC-MS/MS	2 mL	MetPHB: 2–6312 EtPHB: 0.10–56.0 PrPBH:1.05–74.0 BuPHB: <LOQ-2.15 BzPHB: N.D OH-MePBH: <LOQ-547 OH-EtPHB: <LOQ-5.24	>20 min	[49]

LPME procedure, and analysed by UPLC-ESI-MS/MS. The obtained results are summarised in Table S4. According to the data, target analytes were detected and/or quantified in the analysed samples, which confirms the direct exposure of these women to these compounds. Among them, MePHB and EtPHB and the three metabolites (3,4-OH-PHBA, 3,4,5-OH-PHBA, and PHBA) were the most often detected in both types of samples. The predominant parent compound was EtPHB, which was quantified in ten out of fourteen samples, with a maximum concentration of 60 ng mL^{-1} in MU2 and 4.3 ng mL^{-1} in AF1. On the other hand, the predominant metabolite was PHBA, which was quantified in eleven out of fourteen samples. Nevertheless, the metabolite with the highest concentration was 3,4,5-OH PHBA, with 3.4 ng mL^{-1} in AF3. Regarding the other measured parabens, PrPHB, ButPHB, and BzPHB were only quantified at low levels in AF6 and AF7, with concentrations of 0.091 ng mL^{-1} in AF6 for BzPHB and 0.33 ng mL^{-1} in AF7 for BzPHB also. None of the isomeric forms, iPrPHB and iButPHB, were found in any of the evaluated samples. The latter is in accordance with respect to the usage of parabens as preservatives since the ones with long alkyl chains are less

commonly used. For this reason, the abundance of short alkyl chain parabens (MePHB and EtPHB) in MU and AF samples analysed in this study is higher than that of long alkyl chain parabens (PrPHB, ButPHB, and BzPHB) [5,36]. In general, the average concentrations of each found compound decrease in the following order in MU: EtPHB > MePHB > 3,4,5-OH-PHBA > PHBA > 3,4-OH-PHBA, and in AF: EtPHB > 3,4,5-OH-PHBA > MePHB > PrPHB > PHBA > BzPHB > 3,4-OH-PHBA > ButPHB. Compared to other previous studies in maternal urine and amniotic fluid [35], the results obtained in our analysis were similar in terms of frequency of appearance in samples, as MePHB and EtPHB presented the highest frequencies and concentrations, but the levels quantified in our samples were lower in urine samples. Our data showed ranges 0.3–1.5 ng mL^{-1} for MePHB and 0.08–60 ng mL^{-1} in urine, while Karzi et al. [37] obtained ranges 5.3–3501.3 ng mL^{-1} for MePHB and 0.8–81.7 ng mL^{-1} for EtPHB. Amniotic fluid however, showed very similar results.

As can be seen, the exposure of women to parabens is very widespread, mainly due to the ubiquitous presence of this group of emerging pollutants in daily consumer products. In addition, the higher frequency of detection of parabens in AF samples than in MU supports the idea of a potential transplacental passage occurrence.

4.5. Comparison with previous methods

We have conducted a comprehensive comparison of our proposed method with some methods previously reported in the literature for similar samples in recent years. Detailed information is listed in Table 2. Overall, compared to other literatures, our method allows for the determination of 7 parabens and 3 of their main hydroxy metabolites. Notably, only the method introduced by Song et al. (2020) [6] allows the determination of such a substantial number of parabens and their metabolites. However, it should be noted that their procedure involves longer sample treatment times than ours, resulting in higher LOQs. As can be seen in the table, the described procedures are predominantly tailored for urine samples. Only six studies [5–7,15,38,44] were applied to samples associated with placental transfer, such as placenta, AF (amniotic fluid), umbilical cord, or fetal serum. These approaches generally exhibit LOQs higher than those achieved by our proposed method. Two noteworthy procedures, proposed by Pellicer et al. (2022) [39] for the determination of four parabens among other endocrine disruptors, requiring a sample volume of 15 mL, and Ao et al. (2021) [40], which boasts lower LOQs and involves a smaller sample volume but only allows the determination of 6 parabens and was not applied to samples, demonstrate slightly superior LOQs. The method introduced by Azzouz et al. (2016) [11] exhibits lower LOQs; however, it employs derivatization and CG-MS, entailing a complex sample treatment.

Additionally, the method proposed by Bocato et al. [41] shows comparable LOQs to our proposed method, with a shorter extraction time and a required sample volume of 1 mL. Nonetheless, it was solely applied to urine and a smaller number of compounds.

5. Conclusions

In this study, a hollow fiber electro-assisted LPME (HF-EA-LPME) combined with UPLC-ESI-MS/MS analysis for the simultaneous preconcentration and sensitive determination of seven parabens (MePHB, EtPHB, PrPHB, iPrPHB, ButPHB, iButPHB, and BZPHB) and three of their main hydroxy metabolites (3,4-OH-PHBA, 3,4,5-OH-PHBA, and PHBA) is presented. The usage of daily consumer products during pregnancy that may contain parabens either in their composition or in their packaging has led to the need for monitoring surveys. The proposed method is straightforward and offers substantial enrichment factors, reaching up to 90 for MePHB and ranges from 10 to 50 for the remaining compounds. This, combined with the of Mass Spectrometry (MS) detection, enables a high sensitivity, with LODs ranging from 0.0067 to 0.060 ng·mL^{-1} and 0.0075 to 0.054 ng·mL^{-1} for amniotic liquid and urine, respectively. The methodology employed demonstrated its adequacy for the analysis of paired maternal urine and amniotic fluid samples, offering excellent clean-up and selectivity. Notably, there were no matrix components that interfered with the signals of the analytes. Additionally, to evaluate the practicality of the method the BAGI metric tool software has been applied, providing remarkable strengths in preconcentration, quantitation and confirmatory results. As far as we know, this is the first time this metric tool has been applied to assess the practicality of an analytical microextraction technique based on the use of supported liquid membranes. On the other hand, we have conducted a comprehensive comparison of our proposed method with some other methods previously reported in the literature for similar samples in recent years. The results of the comparison indicate that our method is simpler and allows for the determination of a significantly larger number of parabens and their metabolites involving two different types of matrices, with LOQs lower than those achieved by other proposed methods. In all seven pairs of samples, we successfully detected and quantified all target parabens and/or hydroxylated metabolites, with MePHB and EtPHB emerging as the predominant parabens in both sample types. Additionally, the presence of the non-specific hydroxy metabolites suggests a direct exposure of mothers to at least one of these compounds during pregnancy. Of particular significance is the observation that PrPHB, ButPHB, and BzPHB were exclusively quantified in amniotic fluid. This finding, coupled with the increased frequency of paraben detection in amniotic fluid samples compared to maternal urine (MU) samples, lends support to the hypothesis of a potential transplacental transfer from mothers to their unborn babies.

CRediT authorship contribution statement

Eduardo Leo-Martos: Formal analysis, Investigation, Validation. **Noemí Aranda-Merino:** Visualization, Writing – original draft. **Rocío Sanchez-Ruiz:** Formal analysis, Investigation, Validation. **Isabel María Moreno:** Conceptualization, Funding acquisition. **Rut Fernández-Torres:** Methodology, Supervision, Writing – original draft. **María Ramos-Payán:** Funding acquisition, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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