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Hollow fiber liquid-phase microextraction-gas chromatography-mass spectrometry method to analyze bisphenol A and other plasticizer metabolites☆

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ABSTRACT

Phthalates and bisphenol A are important environmental pollutants due to their toxicity for humans and animals, including actions in the endocrine system. Their metabolites in urine can be used as biomarkers to assess human exposure. This paper describes the development of a new method to determine bisphenol A and eight phthalate metabolites in urine samples using hollow fiber liquid phase microextraction (HF-LPME) and gas chromatography-mass spectrometry (GC–MS). This method showed linearity, precision, limits of detection, and quantification suitable to analyze these compounds at low concentration levels in urine. Limits of detection ranged from 0.777 to 23.3 $\mu g\,L^{-1}$, showing sensitivity for evaluating environmental exposure. Relative standard deviation (RSD) ranged from 11.7 to 19.7%. The developed method presented a good biomarker alternative for evaluating environmental exposure to bisphenol A and phthalates.

1. Introduction

Bisphenol A (BPA) and phthalates are ubiquitous pollutants used in a wide range of plastic products. Therefore, human exposure results from many environmental sources. After exposure, these compounds are quickly hydrolyzed to their respective monoesters, which can be also biotransformed into oxidative metabolites, depending on the compound. Finally, they are excreted in urine in their conjugated form, especially in the glucuronide form [1–4].

BPA, phthalates, and some of their metabolites are compounds suspected to produce endocrine system dysfunctions in humans and animals. Thus, several methods have been developed to analyze these compounds in food, air, or water samples in order to evaluate human exposure sources [5–8]. Different extraction techniques can be used to determine monoesters and BPA in different matrices. Miniaturized extraction techniques have been widely used in recent years [9]. In a study developed by Haeri, biosorption-based dispersive liquid–liquid microextraction was used to extract BPA from aqueous matrices [10]. In addition, exposure evaluation can be performed by analyzing biomarkers represented by BPA and phthalate metabolites in urine [11,12]. The biomonitoring for assessing human exposure allows for the prevention of alterations in the endocrine system and/or emergence of diseases by associating biomarkers with early effects. Hauser et al. showed a relationship between the concentration of bis-(2-ethylhexyl) phthalate metabolites in samples of damaged semen and sperm DNA [13]. Meeker and Ferguson determined a relationship between the concentration of phthalates and BPA in urine and alterations in thyroid hormones [14].

The analysis of BPA and phthalate metabolites in urine requires prior sample preparation, including conjugate metabolite hydrolysis. This hydrolysis is typically performed with specific enzymes, such as β-glucuronidase and α-glucuronidase [2,15]. The amount of enzyme required will depend on the initial amount of sample and enzyme concentrations. Frederiksen et al. used 5 μL of enzyme for 500 μL of sample [16]. Holm et al. used 250 μL for β-glucuronidase for 50 mL of urine [17]. An ideal incubation period is also required for complete hydrolysis. Frederiksen et al., Blount et al., and Kato et al. used a time of 90 min and a temperature of 37 °C for complete hydrolysis [2,16,18]. Studies conducted by Koch et al. and Holm

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et al. have shown that efficient hydrolysis can also be obtained with a temperature of 37 °C and an incubation time of 60 min [17,19]. Since each enzyme has an optimum pH level to act, hydrolysis must be controlled in the process through the use of ammonium acetate buffer solution or pH adjustment [16,17]. Hydrolysis is stopped by adding acid to the sample. Phosphoric acid and acetic acid are normally used [16]. After hydrolysis, the analytes are extracted by different techniques. Rocha et al. determined BPA and six analogs (S, F, Z, P, AF, AP) using dispersive liquid–liquid microextraction and fast liquid chromatography, coupled with mass spectrometry [20]. In recent years the use of hollow fiber liquid phase microextraction (HF-LPME) has stood out in the extraction of organic compounds due its efficiency and ease of application. HF-LPME with gas chromatography is rather unusual in the analysis of BPA and phthalates; however, HF-LPME has proven to be an efficient extraction technique. HF-LPME can be applied using different configurations [21]. This study shows the application of two-phase HF-LPME, which consist of the use of a hollow, porous, and hydrophobic fibers that are impregnated with an organic solvent, which also fills the inside of the fiber. This organic solvent is known as the acceptor phase. The fiber is placed within the sample (donor phase), followed by a stirring application that allows the analytes to migrate from the matrix to the acceptor phase. After extraction, the analytes can be determined by liquid or gas chromatography. Usually, in liquid chromatography, the analyte can be analyzed directly after the extraction of the compounds, whereas when using gas chromatography, a derivatization step is required to produce more volatile derivates. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), containing 1% trimethylchlorosilane (TMCS), was successfully applied to the derivatization of BPA and monoesters [22–24]. In accordance with Basheer et al., BSTFA proved to be a fast and efficient derivatizing agent for BPA [25].

In this study a novel HF-LPME method was developed for analysis of mono-methyl phthalate (MMP), mono-isobutyl phthalate (MiBP), mono-butyl phthalate (MBP), mono-cyclohexyl phthalate (MCHP), mono-(ethylhexyl) phthalate (MEHP), mono-isononyl phthalate (MiNP), mono-octyl phthalate (MOP), mono-benzyl phthalate (MBzP), and bisphenol A (BPA) in women's urine by means of GC–MS. This study was submitted to and approved by the Research Ethics Committee of Universidade Federal de Minas Gerais (UFMG).

2. Materials and methods

2.1. Reagents and samples

MBP and MEHP standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). MBzP was purchased from Cambridge Isotope Laboratories (Andover, USA). The MiNP, MOP, MMP, MCHP, and MiBP standards were purchased from AccuStandard (New Haven, USA). A stock solution of 1000.0 mg L^{-1} was prepared in acetonitrile HPLC grade (JT Baker, Xalostoc, Edo Mex, Mexico) for each compound. BSTFA, containing 1% of TMCS, was purchased from Sigma-Aldrich (St. Louis, MO, USA). To develop the method, the standard solution of BPA and phthalate metabolites were prepared in pooled urine from adult volunteers among UFMG students. Application of the HF-LPME method to real samples was carried out through the analysis of urine samples from volunteer women patients at the UFMG Clinical Hospital. The samples were frozen at −18 °C until analysis by GC–MS.

2.2. HF-LPME method

The first step of this study was to choose the best solvent for the extraction and concentration of BPA and phthalate metabolites through the HF-LPME method. Two solvents were evaluated as acceptor phases for the extraction of the compounds: octanol and ethyl decanoate. To this end, a hollow fiber of 10 cm was dipped in octanol for 10 s. The solvent excess was then removed by introducing the fiber into a vial containing water, following by sonication for 5 s. The fiber was filled with 35.0 μL of octanol and was subsequently introduced into a vial containing 16.0 mL of aqueous solution in the concentration of 1.0 mg L^{-1} for each studied compound. The vial was shaken for 60 min at room temperature under magnetic stirring. The same procedure was repeated using ethyl decanoate as a solvent.

A 2^3 factorial design with a center point was used to optimize extraction and derivatization parameters using an aqueous solution of the compounds at a concentration of 500.0 μg L^{-1}. For factorial design, a minimum and maximum point was defined for each variable. The center point was defined as the average value of the minimum and maximum points. The variables evaluated for the extraction of the compounds were: time (40, 55, and 70 min); stirring (100, 400, and 700 rpm); and adding salt, NaCl (0, 5, and 10%), to the salt in out effect. In this extraction study, the center point was 55 min, 400 rpm, and 5% of salt. The following variables were evaluated to determine the derivatization: time (5, 15, and 25 min); temperature (25.0 °C, 36.5 °C, and 50.0 °C); and the amount of derivatizing agent (25.0, 30.0, and 35.0 μL). In the derivatization study, the center point was 15 min, 36.5 °C, and 30.0 μL of derivatizing agent.

Analysis of BPA and plasticizer metabolites requires a sample pre-treatment, considering that these compounds are eliminated in urine linked to endogenous substrates (glucuronic acid). To carry out the hydrolysis of conjugate metabolites, 16.0 mL of sample was transferred to a 22 mL vial, 200.0 μL of acetate buffer (2.0 mol L^{-1}) was added, and the pH was adjusted to 6.0-6.5 using an ammonium hydroxide solution (50% V/V) or phosphoric acid (6.0 mol L^{-1}). After pH adjustment, 80.0 μL of the enzyme ß−glucuronidase (*E. coli*) was added to the vial for enzymatic deconjugation at 37 °C for 90 min [16]. Finally, the sample pH was adjusted to 2 using phosphoric acid.

For the HF-LPME extraction method, 35.0 μL of octanol was introduced in a hollow fiber of 10 cm that had been previously soaked in octanol for 10 s. The hollow fiber was then placed in a vial containing 16.0 mL of sample under magnetic stirring for 70 min at room temperature. Subsequently, the fiber was removed from the sample vial and the octanol containing the analytes was transferred through a syringe into an insert enclosed in a 2.0 mL vial. An aliquot of 13.0 μL of octanol was transferred to another vial of 2.0 mL containing 25.0 μL of BSTFA and 5.00 μL of acetonitrile. The vial was shaken for 30 s and then left to rest for 5 min. After derivatization, the sample was analyzed by GC–MS.

2.3. Merit parameters studied

A urine pool spiked with BPA and phthalate metabolites was used to determine the merit parameters. It was prepared a standard solution, from stock solutions of 1000.0 mg L^{-1}, at concentrations of 16.0 mg L^{-1} for MiBP and MOP, and 8.00 mg L^{-1} for MMP and MBP, and standard solutions of 1.00 mg L^{-1} and 4.00 mg L^{-1} for MCHP, MEHP, MiNP, MBzP, and BPA. The calibration curves were constructed at different concentration ranges, using independent triplicates for each point. Aliquots of the solution containing MiBP, MOP, MMP, and MBP were added to different flasks containing the urine pool. The volumes used were 40.00 μL, 120.0 μL, 360.0 μL, 540.0 μL, 1000 μL, and 2000 μL. Aliquots of the solution containing MCHP, MEHP, MiNP, MBzP, and BPA were added to different flasks containing the urine pool. The volumes used here were: 80.00 μL of the 1.00 mg L^{-1} and 40.00 μL, 80.00 μL, 160.0 μL, 240.0 μL, 320.0 μL, 400.0 μL, and 800.0 μL^{-1} of the 4.00 mg L^{-1}. The

final volume in the flasks was 16.0 mL. The limit of detection (LOD) and limit of quantification (LOQ) were determined according the equations presented in the Eurachem 2014 Guide using urine pool samples (blanks) [26]. Within-day and between-day precision were carried out through analysis of urine samples spiked with standard solutions of 120.0 $\mu g\,L^{-1}$ for MiBP and MOP, 60.00 $\mu g\,L^{-1}$ for MMP and MBP, and 20.00 $\mu g\,L^{-1}$ for MCHP, MEHP, MiNP, BPA, and MBzP.

2.4. GC–MS system

The analyses were performed on a gas chromatograph system (Agilent – 7890C) coupled to a mass spectrometer equipped with a quadrupole mass analyzer (Agilent – 5975C). The chromatographic analysis was performed via injection in splitless mode for 2 min at 250 °C using a 5% phenyl, 95% dimethylpolysiloxane column—DB-5MS Agilent (60 m × 250 μm × 0.25 μm) with a helium flow of 1.5 mL min^{-1}. The oven temperature ramp started at 120 °C and was then increased to 220 °C at a rate of 5 °C min^{-1}, where it was maintained for 1 min. The temperature was then increased to 270 °C at a rate of 3 °C min^{-1}, further increased to 320 °C at a rate of 20 °C min^{-1}, and finally maintained for 5 min. The total run time was 45.2 min. The analysis was conducted in a selected ion monitoring (SIM) mode with electron ionization (EI) at 70 eV. The monitored mass/charge ratios (m/z) are presented in Table 1.

2.5. Statistical analysis

The areas obtained in the comparison of the solvents, octanol, and ethyl decanoate, were compared, using the Student's t-test. The software "Statistica" v.8 (Hewlett–Packard, Palo Alto, CA, USA) was used to elaborate and evaluate the 2^3 factorial design's results of the optimization of extraction and derivatization conditions. The statistical tests to evaluate the calibration curve were selected according to the tests suggested by Souza and Junqueira [27]. The Ryan-Joiner Test, Durbin-Watson Test, Brown-Forsythe Test, and ANOVA Test were applied using excel software (Microsoft, Redmond, USA).

3. Results and discussion

The chromatographic method presents adequate selectivity with a short analysis time. Fig. 1 shows a chromatogram of urine sample spiked with derivatized BPA and phthalate metabolites.

HF-LPME parameters were optimized to obtain best extraction and derivatization conditions. Two acceptor phases were tested, the results of which are shown on Fig. 2. Octanol, when used to coat and fill the hollow fiber, showed a higher extraction efficiency for all compounds; therefore, it was chosen as the extraction solvent.

Pareto charts obtained in a 2^3 factorial design were used to evaluate the significant parameters in the extraction process. The minimum time of 40 min was defined, considering that the variable can influence the extraction; hence, the time should not be too short. The maximum time of 70 min was defined considering the viability of a routine analysis. The analysis of the effects using Pareto charts showed that the variable extraction time and agitation were significant at a 95% confidence level for all of the studied compounds. Higher time and agitation furnished the best conditions to extract the compounds. Therefore, a time of 70 min and stirring at 700 rpm were used to extract the compounds. The addition of salt proved to be significant, with a 95% confidence interval for all of the studied compounds except for BPA and MCHP, and enhanced the extraction of MMP, MiBP, and MBP. By contrast, the addition of salt decreased the extraction efficiency of MEHP, MiNP, MOP, and MBzP. It was further observed that the addition of salt significantly affected the extraction efficiency of compounds at low concentrations. Therefore, salt was removed from the extraction method.

Pareto charts were used to evaluate the significant parameters of derivatization process. Results obtained for each compound showed that none of the studied parameters were significant at a 95% confidence level within the evaluated experimental domain. Therefore, derivatization was performed at 25 °C for 5 min. Other studies also reported that some compounds react in seconds with BSTFA. Thus, a short derivatization time is required in these cases [25].

The pH of the donor phase is one of the parameters that affects the extraction efficiency [28,29]. The pH study showed that compound extractions were inefficient, and analytes went undetected when using a basic pH. Instead, acid pH showed that analytes presented a high affinity for the acceptor phase. Therefore, the pH of the sample (donor phase) was maintained at 2 in HF-LPME method. At pH 2, the analytes are all protonated and are therefore not found in an ionized form. The non-ionized form decreases the solubility of the analytes in water and favors migration through the membrane to the acceptor phase [28,30].

The calibration curves were constructed in different ranges for each metabolite. The results concerning linear range, regression equation, LOD, LOQ, and precision are presented in Table 2. The parameters of merit for the developed method were evaluated according to EURACHEM guidelines [26].

The linearity of the curves were evaluated using statistical tests involving the normality of residues (Ryan–Joiner Test), the independence of residues (Durbin–Watson Test), the homoscedasticity of residues (Brown–Forsythe Test), significance regression, and deviation from linearity based on an analysis of variance (ANOVA) [27]. The statistical analysis showed that the residues were homoscedastic and independent, and followed a normal distribution for all compounds.

The precision evaluation used a urine pool sample spiked with BPA and phthalate metabolites in a concentration of 120.0 $\mu g\,L^{-1}$ for MiBP and MOP, 60.00 $\mu g\,L^{-1}$ for MMP and MBP, and 20.00 $\mu g\,L^{-1}$ for MCHP, MEHP, MiNP, BPA, and MBzP, in turn resulting in coefficients of variation of less than 20%. The obtained values were similar to those found in other studies using other extraction techniques. In findings from Dewalque et al., phthalate metabolites were identified in urine samples that showed coefficients of variation ranging from 18% to 20% for MBP, MiBP, MBzP, and MEHP, using solid phase extraction (SPE) as the extraction technique [31]. Frederiksen et al. obtained coefficients of variation varying between 3.9% and 21.7% when analyzing MBP, MiNP, MOP, MiBP, MBzP, and MEHP in urine samples, also using SPE to extract the compounds [16]. In this study the analysis of the compounds was conducted using GC–MS, and due to polarities of the analytes, a derivatization step was required. The use of HF-LPME simplifies the derivatization step, since in techniques like SPME the derivatizing agents, such as BSTFA, can cause damage to some fibers' coatings [32,33]. A study conducted by Basheer and Lee showed that HF-LPME is a good alternative to headspace solid phase microextraction (HS-SPME) and liquid–liquid extraction (LLE) to extract compounds, such as BPA and alkylphenols, which need to be derivatized to obtain a good response in the analyses using GC–MS [34]. Moreover, when compared to other techniques, HF-LPME shows the advantage of being a relatively simple and inexpensive technique that uses a very small amount of organic solvent.

3.1. Application of the method to real samples

Environmental analysis of phthalates or studies of human contamination by plasticizers are used to evaluate health risks. However, to evaluate the real and full exposure, it is necessary to know the true amount of these compounds in the body. In the present study, this evaluation was performed by analyzing BPA and phthalate metabolites in urine samples of women between 18 and

Table 1
Merit parameters of LPME-GC/MS method for metabolites studied (n = 10).

Compound	Linear Range μg L⁻	Equation	R^2	LOD μg L^{-1}	LOQ μg L^{-1}	RSD (%)	
						Intra essay	Inter essay
MMP	20–1000	y = 7900.5x + 12069	0.9953	11.5	19.2	12.0	15.9
MiBP	40–1000	y = 58117 x – 16899	0.9886	23.3	38.9	16.0	18.6
MBP	20–500	y = 66698x + 370655	0.9961	9.84	16.4	12.8	17.9
MCHP	5–100	y = 84831 x – 260516	0.9926	1.34	2.23	12.2	18.0
MEHP	10–200	y = 98403 x – 404866	0.9867	3.03	5.05	12.6	19.5
MiNP	5–200	y = 34695 x – 51604	0.9920	1.83	3.04	15.4	19.7
MOP	40–1000	y = 79339 x – 722901	0.9904	0.777	1.29	15.7	18.6
MBzP	20–100	y = 66773 x – 372719	0.9747	1.75	2.91	11.7	19.3
BPA	5–100	y = 327231x + 521643	0.9826	1.82	3.04	13.9	17.1

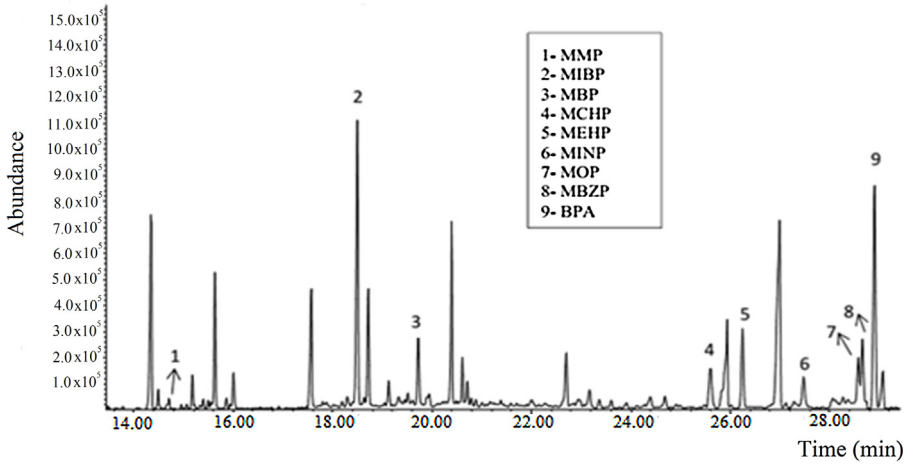

Fig. 1. Chromatographic profiles of derivatized standard solution BPA and plasticizer metabolites. HF-LPME extraction using GC-qMS analysis in SIM mode. Chromatographic conditions in text.

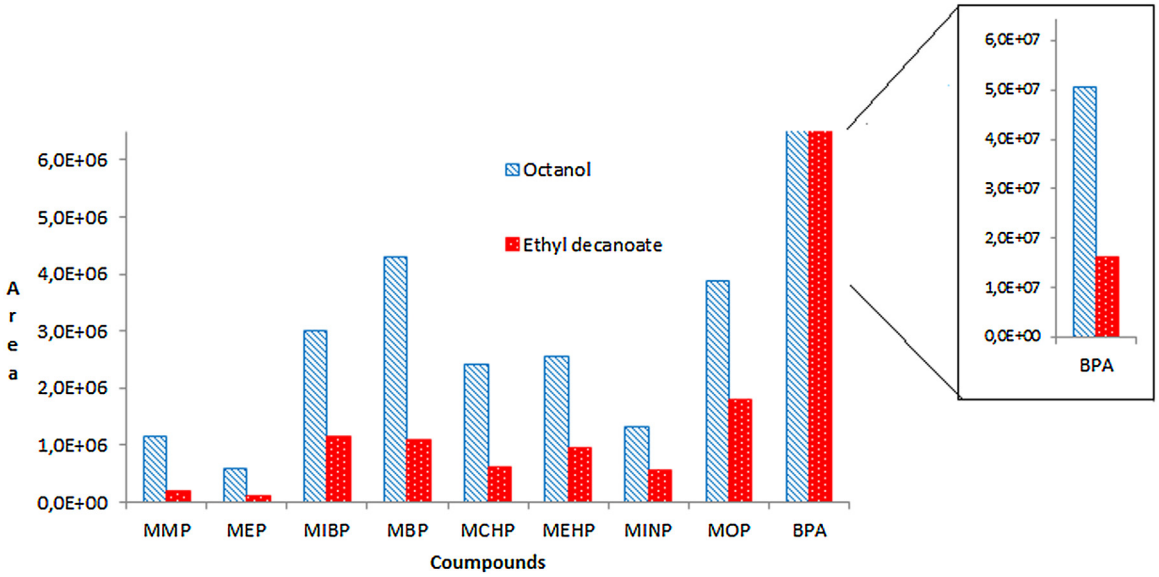

Fig. 2. Comparison of the solvents used to extract BPA and plasticizer metabolites in urine samples.

45 years of age. The urine samples were collected from volunteer patients at the UFMG Clinical Hospital. The diuretic variation of individuals was considered by measuring creatinine through the colorimetric method. A total of 29 urine samples were analyzed, but the analytes were not detected in all samples. Table 1 shows the mean values and the range of concentrations found in this study.

The concentration of plasticizer metabolites in urine has been determined in other studies using different methods for extraction and analysis. In a study carried out by Hartmann et al., plasticizer metabolites were analyzed in urine samples of Austrian individuals of different ages. The concentrations found ranged from not detectable to 78 μg g^{-1} of creatinine for MBP, from not detectable to 5.0×10^3 μg g^{-1} of creatinine for MiBP, from not detectable to

Table 2
Creatinine-adjusted concentrations of compounds studied in urine samples.

Compound	Retention time	Monitored *m/z*	Number of samples with detectable concentrations	Mean concentration $\mu g\,g^{-1}$ of creatinine	Concentration range $\mu g\,g^{-1}$ of creatinine
MMP	14.7	89, 163, 237	7	81.0	15.5–256
MiBP	18.6	223, 221, 41	18	185	49.1–447
MBP	19.7	223, 221, 41	10	81.4	41.9–192
MCHP	25.6	221, 223, 239	9	11.3	2.74–41.3
MEHP	26.2	223, 221, 239	14	30.3	6.01–79.7
MiNP	27.4	221, 223, 239	14	51.5	8.36–249
MBzP	28.6	91, 179, 163	2	23.8	28.5–19.0
MOP	28.5	223, 221, 163	1	670	670
BPA	28.9	357, 372, 207	3	9.52	4.53–15.9

40 $\mu g\,g^{-1}$ of creatinine for MBzP, from not detectable to 6.1 $\mu g\,g^{-1}$ of creatinine for MEHP, from not detectable to 1.3 $\mu g\,g^{-1}$ of creatinine for MOP, and from not detectable to 45 $\mu g\,g^{-1}$ of creatinine for MiNP [35]. In another study by Kuo et al., plasticizer concentrations were determined in urine samples of pregnant women, showing concentrations ranging from not detectable to 92 $\mu g\,g^{-1}$ of creatinine for MMP, from 8.8 to 6.6 $\times 10^2$ $\mu g\,g^{-1}$ of creatinine for MBP, from 4.7 to 4.9 $\times 10^2$ $\mu g\,g^{-1}$ of creatinine for MiBP, from 3.1 to 3.0 $\times 10^2$ $\mu g\,g^{-1}$ of creatinine for MEHP, and from 0.23 to 57 $\mu g\,g^{-1}$ of creatinine for MBzP [36]. The results of current study, as well as findings from Hartmann et al. and Kuo et al., presented a large concentration variability of the plasticizer in urine samples [35,36]. High concentrations of plasticizer metabolites have been associated with infertility and other disorders. A study by Tranfo et al. showed that the concentration of MBP and MBzP in infertile urine samples and a control group were significantly different [37]. Many studies still need to be conducted to evaluate plasticizer metabolite concentrations and diseases related to the endocrine system. However, these studies must use analytical methods that allow one to quantify the different plasticizer metabolites in different concentration ranges. The method developed in the current study is a good alternative for this type of analysis.

4. Conclusions

HF-LPME proved to be an efficient method to extract BPA and phthalate metabolites from urine samples by means of GC–MS, and thus represents an alternative for biomarker analyses. This technique is relatively simple, considering factors such as time, efficiency, and cost, and has the added advantage of using small amounts of organic solvent. The derivatization step is necessary when analyzing BPA and phthalate metabolites by means of gas chromatography. HF-LPME simplifies the derivatization step when compared to other sample preparation techniques, such as SPME. The method proposed in this study proved to be suitable for the quantification of BPA and phthalate metabolites in urine. This procedure was validated, and presented linearity, accuracy, and limits of detection and quantification within the range of interest to assess environmental exposure by biomonitoring.

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