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A fast method for bisphenol A and six analogues (S, F, Z, P, AF, AP) determination in urine samples based on dispersive liquid-liquid microextraction and liquid chromatography-tandem mass spectrometry

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ABSTRACT

In this study, a novel method combining dispersive liquid-liquid microextraction (DLLME) and fast liquid chromatography coupled to mass spectrometry (LC-MS/MS) was developed and validated for the extraction and determination of bisphenol A (BPA) and six bisphenol analogues, namely bisphenol S (BPS), bisphenol F (BPF), bisphenol P (BPP), bisphenol Z (BPZ), bisphenol AP (BPAP) and bisphenol AF (BPAF) in human urine samples. Type and volume of extraction and disperser solvents, pH sample, ionic strength, and agitation were evaluated. The matrix-matched calibration curves of all analytes were linear with correlation coefficients higher than 0.99 in the range level of 0.5–20.0 ng mL^{-1}. The relative standard deviation (RSD), precision, at three concentrations (1.0, 8.0 and 15.0 ng mL^{-1}) was lower than 15% with accuracy ranging from 90 to 112%. The biomonitoring capability of the new method was confirmed with the analysis of 50 human urine samples randomly collected from Brazilians. BPA was detected in 92% of the analyzed samples at concentrations ranging $<$ LOQ to 10.4 ng mL^{-1}. The detection rates of BPS (10%), BPAF (4%) and BPF (2%) were much lower than 92%.

1. Introduction

Endocrine disruptors (ED) are a wide group of chemicals present in the environment that may interfere with mammals' endocrine systems by causing detrimental effects in health, growth, and reproduction [1–3]. Their ability to either imitate naturally occurring hormones or act as antagonists might leads to harmful effects on hormone biosynthesis, metabolism, distribution and mechanism of action [1,3,4]. Endocrine disruptors may be divided into two groups. One group comprises synthetic compounds such as chemicals used in agricultural threats (pesticides), pharmaceutical drugs, substances used in plastic production and plasticizers, packaging materials, medical devices, cosmetics - bisphenols (BPs), phthalates and parabens – as well as industrial solvents, building materials and paints (polychlorinated biphenyls and metals). The other group comprises natural compounds including soy phytoestrogens and extracts from plants or fungi [1–3].

Under the prospective of Gore et al. (2014), endocrine disruptors can be classified according to their occurrence in daily life contact materials. These include pesticides and other chemicals present in children and personal care products, electronics, clothing and food packaging materials [5,6]. The work we present here concerns with the determination of bisphenol A (BPA) and its analogues. These include bisphenol AF (BPAF), bisphenol AP (BPAP), bisphenol B (BPB), bisphenol F (BPF), bisphenol P (BPP), bisphenol S (BPS) and bisphenol Z (BPZ) [6,7]. Their molecular structures are shown in Fig. 1.

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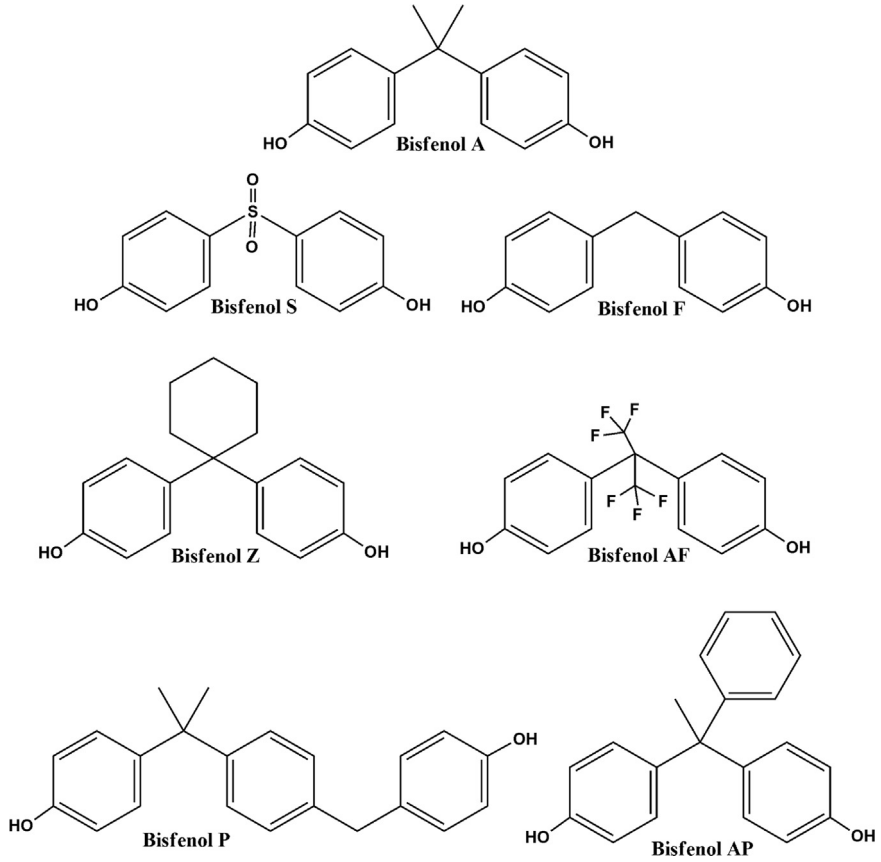

Fig. 1. Chemical structures of bisphenol A and analogues.

BPA is the most representative endocrine disruptor among the bisphenols analogues and it is one of the most used products in contact materials around the world [7–9]. BPA is an additive for the production of phenol resins, polyacrylates, polyesters, epoxy resins and polycarbonate plastics [8,10–12]. As such, human exposure to BPA occurs through several consumption products such as food and beverage packaging, adhesives, toys, water pipes, drinking containers, eyeglass lenses, sports safety equipment, medical devices, thermal receipts and electronics [10,11,13,14]. The capability of BPA to migrate from plastic bottles into drinking water is of particular concern as it might represent a direct route to human contamination [15,16]. *In vitro* and in vivo BPA studies have shown considerable damage in male and female reproductive systems [17–19], carcinogenesis [17,19], and mutagenic and genotoxicity capabilities [20]. Harmful effects in humans include neural disorders and behavioral dysfunction [21], reproductive damage [22], and high diastolic blood pressure [23].

Considering dietary and non-dietary sources of BPA exposure, European Food Safety Authority (EFSA)'s experts estimated the exposure to BPA and assessed the human health risks. In January 2015, the document about risk assessment was published in the CEF Panel's "Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuff". The EFSA's experts established a new tolerable daily intake (TDI) based on uncertainty of health effects after BPA exposure. Thus, the new safe level of BPA was considerably reduced from 50 micrograms per kilogram of body weight per day (μg/kg of bw/day) to 4 μg/kg of bw/day [24,25]. Moreover, members of the EFSA concluded that BPA concentrations that individuals of all ages are exposed, including pregnant women and elderly people, are less than the value determined for TDI (4 μg/kg of bw/day). In this sense, the exposure levels could not pose a risk to human health [24].

Regarding No Observed Adverse Effect Level (NOAEL), the FDA's BPA Joint Emerging Science Working Group has kept its value in 5 mg/kg bw/day for systemic toxicity from studies using rodents. Since 2014, this value of NOAEL was considered the most appropriate for a safety assessment of dietary and non-dietary exposures to BPA [26].

Bisphenol analogues have similar physicochemical properties to BPA which make them suitable candidates to its replacement in several industrial applications [6,17]. Unfortunately, this similarity also leads to harmful toxicological profiles. In 2005, Kitamura [27] demonstrated that BPA, BPB, and BPS were potent anti-androgens compounds. In 2015, Castro et al. [28] obtained in vivo evidence of the potential adverse effects of BPF and BPS in the developing brain of mammals and Rosenmai et al. [29] noticed that the main effect resulting from exposure to bisphenols was endocrine interference. While BPS showed low estrogenic and anti-androgenic activity, the other bisphenol analogues had toxicological behavior similar to BPA [29]. Evidence of additional harmful effects includes genotoxicity, carcinogenicity and DNA damage [29], oxidative stress [29,30] and cytotoxicity in human peripheral blood mononuclear cells (leucocytes) [30].

Human exposure to bisphenols is often monitored in urine samples [31]. After absorption into the human body, BPA is excreted in urine as a glucuronic acid conjugate [31–33]. Classical methodology includes sample pre-concentration via liquid-liquid extraction (LLE) [12], solid-phase extraction (SPE) [34,35], or solid-phase micro extraction (SPME) [36]. The work presented here focuses on dispersive liquid-liquid micro-extraction (DLLME), a sample preparation technique originally developed for the extraction and pre-concentration of organic and inorganic compounds in different matrices [37–39]. Although DLLME has been applied to the determination of BPA via high-performance liquid

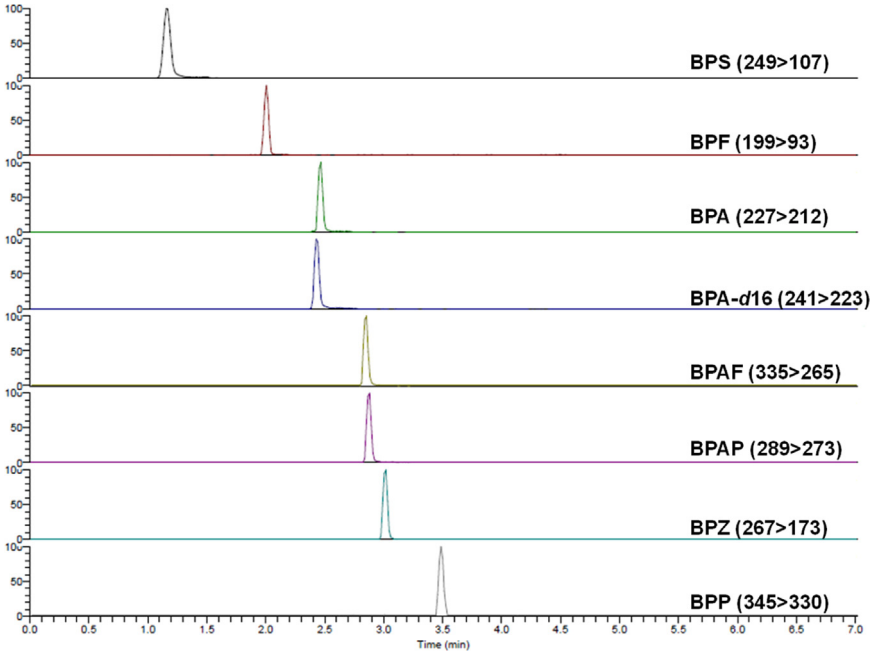

Fig. 2. LC–MS/MS chromatograms of pure standard solutions after optimized DLLME extraction in synthetic urine sample (1.0 ng mL^{-1}).

chromatography (HPLC), [38] our literature search reveals no reports on its application to the analysis of BPA analogues. Herein, DLLME is coupled to liquid chromatography-mass spectrometry (LC-MS/MS) for the simultaneous determination of BPA, BPS, BPF, BPZ, BPAP and BPAF in human urine samples. The excellent analytical figures of merit, the simplicity of the proposed DLLME procedure and the short LC-MS/MS analysis time make this approach a valid alternative for the routine monitoring of BPA and its derivatives in numerous urine samples.

2. Experimental

2.1. Chemical and reagents

Analytical standards of BPA (2,2-bis(4-hydroxyphenyl)propane), BPA-*d*16 (2-bis(4-hydroxyphenyl)propane-*d*16), BPS (4,4′-sulfonyldiphenol), BPF (4,4′-dihydroxydiphenylmethane), BPAF (4,4′-(hexafluoroisopropylidene)-diphenol), BPAP (4,4′-(1-phenylethylidene)bisphenol), BPP (4,4′-(1,4-phenylenediisopropylidene) bisphenol) and BPZ (4,4′-cyclohexylidenebisphenol) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions were prepared in methanol, stored at −20 °C and protected from light.

The following solvents (HPLC grade) were used in DLLME procedures: acetonitrile, methanol, ethanol, acetone and chloroform purchased from JT Baker (Phillipsburg, NJ, USA); dichloromethane and carbon tetrachloride obtained from Sigma (St. Louis, MO, USA); and 1,2-dichloroethane, acquired from Fluka (Buchs, Switzerland). High purity de-ionized water (resistivity 18.2 MΩ cm) used throughout the experiment was obtained using a Milli-Q water purification system (Millipore RiOs-DITM, Bedford, MA, USA).

The reagents (analytical grade) for the preparation of synthetic urine were obtained from Sigma-Aldrich (St. Louis, MO, USA), and they are: 3.8 g of potassium chloride, 8.5 g of sodium chloride, 24.5 g of urea, 1.03 g of citric acid, 0.34 g of ascorbic acid, 1.18 g of potassium phosphate, 1.4 g of creatinine, 0.64 g of sodium hydroxide, 0.47 g of sodium bicarbonate, and 0.28 mL of sulfuric acid were added in 500 mL of mili-Q water and stirred for 60 min. The synthetic urine was ultrasonicated for 30 min and stored at −4 °C until further use. The β-glucuronidase enzyme from Helix pomatia, type HP-2, aqueous solution (197,000 units mL^{-1}) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Instrumentation

Chromatographic analysis was performed with a Thermo Scientific LC system equipped with a pump (Accela 600 pump) and an autosampler coupled with Thermo Scientific TSQ Quantum Access Max with an electrospray triple quadrupole mass spectrometer. The Xcalibur software version 2.0 (Thermo Fisher Scientific) was used to control the instruments and to process data. The fast chromatographic separation was carried out on a Supelco Ascentis Express C18 column (75 mmx2.1 mm i.d. and 2.7 μm particle size, Sigma-Aldrich, St. Louis, MO, USA) and methanol:water, with 0.1% of ammonium hydroxide (*v*/*v*) as mobile phase (the use of ammonium hydroxide in the mobile phase enabled bisphenols analysis with a simple DLLME preparation without matrix effects). Gradient elution was adopted, starting with 30% MeOH and reaching 95% MeOH in 3 min, maintaining this percentage for 2 min (3–5 min); and finally to 30% MeOH and held for 2 min prior to the next injection (total run time of 7 min). The column temperature was kept at 50 °C. The Mobile phase flow-rate was set 500 μL min^{-1} and the injection volume was 10 μL. The use of a C18 (Fused-Core™) column provided a high efficient chromatographic separation with a rapid analysis time, less than 7 min, working at low backpressures (Fig. 2).

2.3. Sample analysis

The following mass spectrometer conditions were used: capillary and cone voltage kept at −4000 V and −30 V, respectively and vaporizer temperature of 395 °C. Nitrogen was used as a sheath gas, ion sweep gas and auxiliary gas at flow rates of 40, 10 and 55 arbitrary units, respectively, and ion transfer tube temperature was set at 220 °C for tandem mass spectrometry, the deprotonated molecule $[M-H]^-$ was used as precursor ion. Argon was used as a collision-induced-dissociation (CID) gas at 1.5 mTorr. The Xcalibur software version 2.0 (Thermo Fisher Scientific) was

used to control the instruments and to process data. To optimize the source working conditions and to carry out the tandem mass spectrometry experiments, a 1 μg mL^{-1} stock standard methanol: water solution of each compound was infused at a flow-rate of 30 μL min^{-1} using direct infusion via a syringe pump integrated into the TSQ instrument to mix the standard solution with the mobile phase (470 μL min^{-1}, methanol:water). The selective reaction monitoring (SRM) in negative mode was used, and two SRM transitions were used, one for quantification(^{Q}) and one for confirmation(^{C}) and both for each compound. The followings channels were used: 227 > 212^{Q} and 227 > 133^{C} for BPA (collision energy (CE) 17 and 23); 249 > 108^{Q} and 249 > 156^{C} for BPS (CE 26 and 23); 241 > 223^{Q} and 241 > 142^{C} for BPA-d_{16} (CE 19 and 21); 199 > 93^{Q} and 199 > 105^{C} for BPF (CE 19 and 18); 345 > 330^{Q} and 345 > 315^{C} for BPP (CE 26 and 29); 267 > 173^{Q} and 267 > 197^{C} for BPZ (CE 25 and 28); 289 > 273^{Q} and 289 > 208^{C} for BPAP (CE 20) and 335 > 265^{Q} and 335 > 197^{C} for BPAF (CE 21 and 30). Argon was used as collision gas. Fig. 2 shows the resulting LC-MS/MS chromatogram recorded from pure standard solutions of BPA and the studied analogues.

A six-concentration-level calibration curve was built for each studied BP within the 0.5–20 ng mL^{-1} concentration range in synthetic urine (matrix-matching calibration). Deuterated internal standard (BPA-*d*16) was employed to compensate for possible matrix effects and analyte losses. The chemical composition of synthetic urine was formulated according to Vela-Soria et al. [2], which closely mimics the composition of uncontaminated human urine samples.

2.4. Sample collection and enzymatic treatment

A total of 50 urine samples from human adult (20 women and 30 men) were collected from healthy volunteers in 50 mL polypropylene tubes. All volunteers consented to participate in this study. All urine specimens were kept at −80 °C until analysis. Previous to using, all propylene tubes were screened for the possible presence of BPs. This was accomplished via ultra-sonication with methanol for 24 h at room temperature followed by LC-MS/MS of the methanol extract.

BPs are excreted in urine as glucuronide conjugates [31]. Since only minor amounts of BPs are excreted in their free forms, concentration levels were determined after urine hydrolysis with β-glucuronidase. The amount of enzyme used in the hydrolysis step was 400 units mL^{-1} of urine sample. To analyze the total concentration of the BPs, the urine samples were thawed and mixed. Then, 5 mL of urine was transferred into 15 mL polypropylene tube with conical bottom and spiked with 0.050 mL of BPA-*d*16 internal standard solution (1000 ng mL^{-1}) and the sample was spiked with 0.100 mL of 1.0 mol L^{-1} ammonium acetate buffer containing 2000 units of β-glucuronidase (pH 5.0; 0.77 g of ammonium acetate dissolved in 8.4 mL of Milli-Q water and 0.6 mL of acetic acid with 1.02 mL of β-glucuronidase commercial solution (197,000 units mL^{-1}), daily prepared). After mixing, the samples were incubated (overnight) at a temperature of 37 °C [31]. Deconjugation efficiency using these conditions was approximately 100% in all cases.

2.5. Optimized dispersive liquid–liquid microextraction (DLLME) procedure

DLLME was performed in 15 mL polypropylene tubes. After enzymatic hydrolysis, 5 mL of urine sample was diluted to 10 mL with 10% NaCl aqueous solution and mixed. pH adjustment was not necessary. After that, a mixture of 750 μL of acetone (disperser solvent) and 500 μL of 1,2-dichloroethane (extraction solvent) was rapidly injected into the sample tube with a 2.50 mL syringe-gastight (Hamilton, Reno, NV, USA). Each tube was closed, gently shaken by hand for 10 s, and further centrifuged at 4000 rpm for 20 min at 20 °C (2500 *g*). All sediment phase was collected and transferred to an Eppendorf tube using a 1.0 mL micropipette. The organic phase was evaporated and the dried residue was dissolved with 100 μL of mixture of methanol:water (0.1% ammonium hydroxide), vortexed for 30 s and injected into the LC-MS/MS.

3. Results and discussion

3.1. Optimization of DLLME conditions

The initial conditions of study of experimental parameters for the optimization of the DLLME procedure was based on a previous report for the extraction of BPA and its chlorinated derivatives, bisphenol S, parabens, and benzophenones from human urine samples [2,40]. The conditions evaluated include type of extraction solvent and volume, type of disperser solvent and volume, pH sample, ionic strength and sample agitation time. All optimization studies were carried out in triplicates. To determine the best experimental parameters with best extraction efficiencies, the absolute recovery of each condition was evaluated. Urine samples spiked with BPs at concentration of 5 ng mL^{-1} were submitted to the DLLME procedures. The areas obtained for these urine samples were compared with the areas achieved by direct injection of pure stock solutions containing the same amount of each analyte dissolved in mobile phase. The recovery was expressed as a percentage of the extracted amount.

3.1.1. Selection of extraction and disperser solvents

The most important step in the optimization of DLLME is the selection of the extraction solvent. In addition to a high extraction capability for the compounds of interest, the selected solvent should have both low aqueous solubility and higher density than water [41]. Low water solubility provides a distinct, well defined two-phase system with minimum analyte loss. An extraction solvent denser than water facilitates the decantation of the aqueous phase formed at the top the two-phase system [42]. Under this prospective, the following solvents were evaluated: chloroform, dichloromethane, 1,2-dichloroethane and carbon tetrachloride. Since the best extraction efficiencies were obtained with 1,2-dichloroethane (see Table 1), all further studies were carried out with this solvent.

In order to best promote the dispersion of extraction solvent droplets in the sample, the disperser solvent should be miscible in both the extraction solvent and the aqueous phase [43]. Acetone, methanol, ethanol and acetonitrile were then selected for these studies. As shown in Table 2, the best extraction efficiencies were

Table 1
Efficiency of extraction solvents for the DLLME of BPs in urine samples.

Analytes	Extraction efficiency (%, mean ± standard deviation)			
	Chloroform	Dichloromethane	1,2-dichloroethane	Carbon tetrachloride
BPF	40.0 ± 7.9	49.5 ± 2.7	55.2 ± 1.9	12.2 ± 4.8
BPA	35.0 ± 5.5	51.4 ± 4.8	57.2 ± 6.7	28.2 ± 2.6
BPS	60.7 ± 7.2	70.8 ± 9.4	73.7 ± 1.9	1.6 ± 1.1
BPZ	18.6 ± 1.6	31.4 ± 2.1	38.7 ± 0.4	20.9 ± 3.0
BPAP	28.9 ± 1.3	45.1 ± 1.4	56.5 ± 4.0	32.1 ± 3.0
BPAF	37.7 ± 2.4	54.0 ± 2.0	63.4 ± 5.8	37.2 ± 2.1
BPP	49.4 ± 3.6	71.0 ± 3.0	75.1 ± 5.8	51.5 ± 5.2

Extraction conditions: urine volume, 5 mL; NaCl aqueous solution (10%, w/v), 5 mL; extraction solvent volume, 750 μL; disperser solvent (acetone) volume, 500 μL; agitation for 10 s.

Table 2
Efficiency of disperser solvents for the DLLME extraction of BPs from urine.

Analytes	Extraction efficiency (%, mean ± standard deviation)			
	Acetone	Methanol	Ethanol	Acetonitrile
BPF	59.2 ± 7.9	6.2 ± 1.4	7.8 ± 0.6	34.6 ± 7.7
BPA	55.2 ± 2.7	14.2 ± 2.9	13.7 ± 1.1	37.5 ± 2.4
BPS	70.7 ± 7.2	1.0 ± 1.0	3.4 ± 0.1	30.5 ± 0.6
BPZ	32.7 ± 1.6	6.2 ± 1.1	7.3 ± 0.7	15.1 ± 2.2
BPAP	59.5 ± 1.3	13.7 ± 2.2	15.0 ± 2.4	27.7 ± 2.9
BPAF	66.4 ± 2.4	16.9 ± 1.1	13.3 ± 1.9	32.6 ± 4.3
BPP	78.1 ± 3.6	24.9 ± 3.3	26.7 ± 2.0	38.0 ± 2.4

Extraction conditions: urine volume, 5 mL; NaCl aqueous solution (10%, w/v), 5 mL; extraction solvent (1,2-dichloroethane) volume, 750 μL; disperser solvent volume, 500 μL; agitation for 10 s.

obtained with acetone in all cases. All further studies were then conducted with acetone as the disperser solvent.

3.1.2. Effects of extraction and disperser volumes, sample pH, ionic strength (salt addition) and extraction time

The ratio extraction/disperser solvent volumes were carefully evaluated for best DLLME efficiency. In order to obtain the best analyte pre-concentration factor - i.e., ratio between analyte concentration in the sedimented phase and initial analyte concentration in the sample - the ideal extraction solvent should provide complete (100%) analyte extraction with minimum volume [44]. The volume of extraction solvent (1,2-dichloroethane) was then investigated within the 250–1000 μL range. In these studies, the volume of disperser solvent was kept constant at 500 μL. The obtained results are shown in Fig. 3A. Comparison of the individual recoveries reveals a similar trend for all studied BPs. The majority of the best recoveries were obtained with 500 μL of 1,2-dichloroethane. The clear improvement observed from 250 μL to 500 μL is probably due the insufficiency of extraction solvent at relatively lower volumes. The efficiency drop beyond 500 μL could

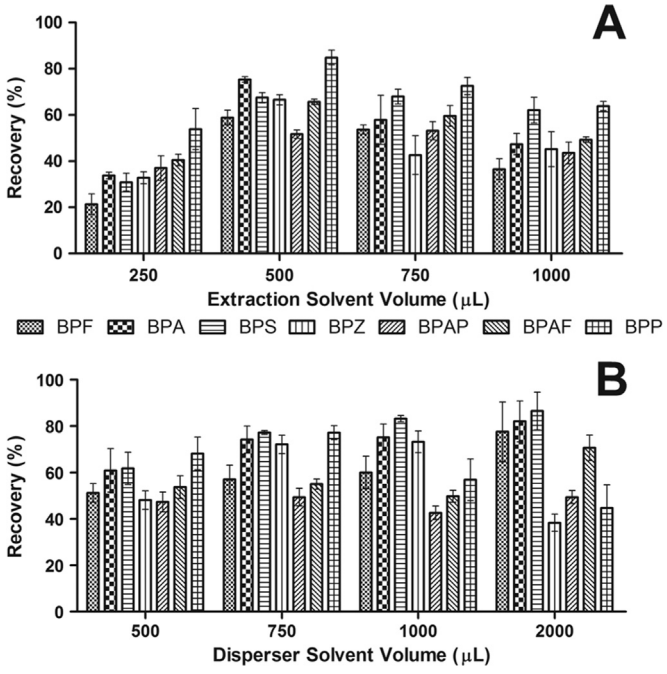

Fig. 3. Influence of extraction (1,2-dichloroethane) and disperser (acetone) solvent volume for extraction of BPs from urine by DLLME. (A) Extraction solvent volume range; (B) Disperser solvent volume range. Extraction conditions: urine volume, 5 mL; NaCl aqueous solution (10%, w/v), 5 mL; extraction solvent, 1,2-dichloroethane; disperser solvent, acetone; agitation for 10 s.

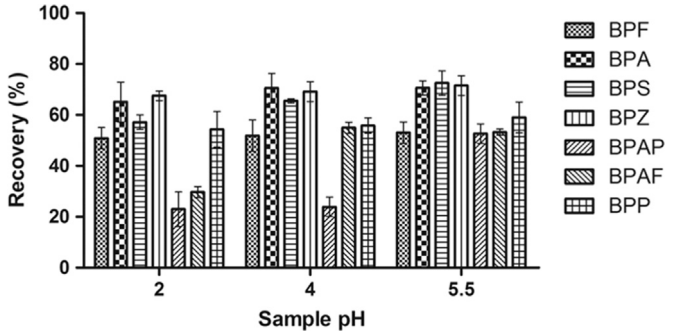

Fig. 4. Influence of pH sample. Extraction conditions: urine volume, 5 mL; NaCl aqueous solution (10%, w/v), 5 mL; extraction solvent (1,2-dichloroethane), 500 μL; disperser solvent (acetone),750 μL; agitation for 10 s.

be attributed to inefficient cloud point formation due to the use of 500 μL of disperser solvent. Fig. 3B shows the effect of acetone (disperser volume) on the extraction efficiency of BPs. These experiments were carried out the 500–2000 μL volume disperser range using 500 μL of 1,2-dichloroethane. Since the best acetone volume depended on the investigated BP, a compromise was made for all future studies to use a volume (750 μL) that provided satisfactory recoveries for all studied BPs.

The pH was adjusted to ensure that all BPs were in their neutral forms, which facilitate their partitioning into the extracted phase [45]. BPs are weak acids with typical pK_a values around 10. In order to avoid deprotonation, the urine was made acid by the addition of 0.1 mol L^{-1} HCl. The efficiency of extraction from urine samples without the addition of acid (natural pH close to 5.5) was then compared to those from urine samples acidified to pH 4.0 and pH 2.0. The best recoveries were obtained without acidification. Apparently, a pH 5.5 is enough to ensure the neutrality of the studied BPs (see Fig. 4).

The addition of salt to the urine can improve the efficiency of extraction due to the salting-out effect [41]. BPs recoveries from natural urine samples (diluted with 5 mL of water) were then compared to those previously mixed with 5 mL of NaCl aqueous solutions. NaCl concentrations were adjusted to obtain final salt concentrations equal to 5% and 10% (w/v). Since the addition of NaCl favored the extraction of all studied BPs, and no significant gain was observed with 10% NaCl, all further studies were conducted in the presence of 5% NaCl (see Fig. 5).

The assisted DLLME was employed in order to increase the efficiency of extraction. The mechanical shaking use external force to increase the contact between extractor and donor phases [46]. This work compared extraction efficiency after the cloud point formation and also using manual hand-shake for 10 and 20 s. The results that were obtained show that there was a significant

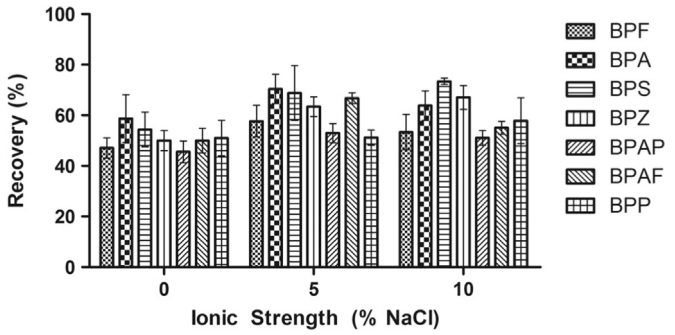

Fig. 5. Influence of ionic strength. Extraction conditions: urine volume, 5 mL; NaCl aqueous solution (10%, w/v), 5 mL; extraction solvent (1,2-dichloroethane), 500 μL; disperser solvent (acetone),750 μL; agitation for 10 s.

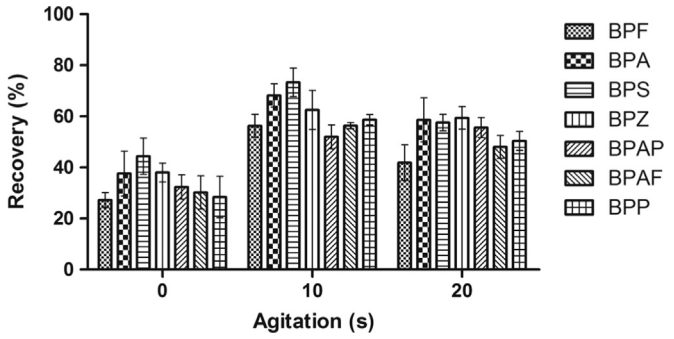

Fig. 6. Influence of agitation time. Extraction conditions: urine volume, 5 mL; NaCl aqueous solution (10%, w/v), 5 mL; extraction solvent (1,2-dichloroethane), 500 μL; disperser solvent (acetone),750 μL; agitation for 10 s.

improvement with agitation, moreover, 10 s proved to be the ideal time (Fig. 6).

3.2. Analytical figures of merit

The analytical figures of merit were evaluated with synthetic urine samples due absence of blank urine samples without BPs. The chemical composition of synthetic urine was formulated according to Vela-Soria et al. [2], which closely mimics the composition of uncontaminated human urine samples.

The present method was validated according to the EMA guidelines [47]. A six-concentration-level calibration curve was built for each studied BP within the 0.5–20 ng mL^{-1} concentration range. Deuterated internal standard (BPA-*d*16) was employed in all cases to compensate for possible matrix effects and analyte losses. Each signal intensity plotted in a calibration graph corresponds to the average ratio of the analyte-to-internal standard peak areas obtained from three independent chromatographic runs. The obtained results are shown in Table 3. The linearity of the calibration plots provided R^2 values close to unity (>0.99) and the ANOVA lack-of-fit tests. Relative standard deviations (RSD) at medium concentrations lower than 15% indicate satisfactory reproducibility of measurements within the linear dynamic ranges of the calibration curves.

The lowest linear concentrations correspond to the limits of quantitation (LOQ), which were calculated according to the formula $LOQ=10S_B/m$; where S_B is the standard deviation of the average blank signal estimated from one-fifth of the peak-to-peak noise ($N_{p\text{-}p}/5$) and m is the slope of the calibration curve. The limits of detection (LOD) were calculated with the equation $LOD=3S_B/m$. The Np-p was measured at the base peak of each eluted analyte over a sufficient time range of the chromatogram. The LOQs and LODs obtained in this study are comparable to those previously reported in the literature.

The precision and accuracy of method were assessed by the within-run (five spiked synthetic urine samples for each concentration on the same day) and between-run (five spiked synthetic urine samples for each concentration for three consecutive days). Table 4 provides information on the accuracy and precision of the proposed method. The precision results were expressed as the relative standard deviation (RSD%) and accuracy results were expressed in terms of percentage accuracy according to the EMA guidelines. Accuracy studies were conducted with synthetic urine samples previously spiked with known concentrations of BPA and the studied analogues. Each compound was investigated at three concentration levels, namely 1.0, 8.0 and 15.0 ng mL^{-1}. Blank measurements were made with non-spiked urine samples, which showed no evidence of the presence of the studied compounds. The accuracy values were within 15% of the nominal concentrations. The relative standard deviations were lower than 15%. These values of method for within- and between-run met the FDA guidelines (RSD% and relative error values below 15%).

3.3. Determination of bisphenols in Brazilian urine samples

The proposed analytical method was applied for the determination of urinary levels of bisphenol A and analogues in urine samples of 50 volunteers from both genders. Table 5 shows found values (arithmetic mean (AM) and geometric mean (GM) along with a comparison and detection rate of BPA in human urine from different countries. Due to the predominant use, BPA is the most commonly form of bisphenol evaluated in biomonitoring studies worldwide. However, since in the last years the concern to the toxic effects of BPA is growing, BPA is gradually being replaced by other analogues, mainly BPS [53].

In the present study, BPA was detected in 92% of the analyzed urine samples (AM and GM concentrations of BPA were 2.8 and 1.9 ng mL^{-1}) and the range of concentrations is very close to previous reports from other geographic regions (see Table 5). Other analogues were detected in much lower detection rates: BPS (10% of samples), BPAF (4% of samples) and BPF (2% of samples).It can be pointed out that BPS has also being detected in other studies with different populations, but in higher detection rates: Japan (100% of samples), USA (97% of samples), China (82% of samples), India (76% of samples), Korea (42% of samples) [53] and Australia (10% of samples) [53, 54].

4. Conclusion

The increasing awareness and public concern with hazard exposure to bisphenol and its analogues calls for methods capable to handle numerous urine samples in short analysis time. The DLLME-LC-MS/MS method proposed here is capable to determine

Table 3
Analytical Figures of Merit for the DLLME-LC-MS/MS analysis of bisphenol A and its analogues.

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Linear equation[a]	R^2 [b]	ANOVA lack-of-fit	
					F-value[c]	*p*-value
BPA	0.1	0.4	0.1669x+0.3321	0.9957	0.23	0.919
BPS	0.01	0.04	1.0087x+0.3550	0.9976	1.80	0.194
BPF	0.2	0.5	0.0420x+0.0016	0.9983	0.39	0.812
BPP	0.1	0.4	0.0238x+0.0084	0.9985	0.12	0.220
BPZ	0.01	0.03	0.1268x+0.0268	0.9958	0.17	0.952
BPAP	0.04	0.1	0.4947x+0.1919	0.9958	2.58	0.091
BPAF	0.005	0.02	6,4200x+0.5308	0.9942	0.12	0.974

[a] Three replicates for each compound concentration; [b]Coefficient of determination; [c]$F_{tabled}=3.11$

BPA, BPS, BPF, BPP, BPZ, BPAP and BPAF in approximately 7 min per sample run (it means 23 samples per working day (8 h) or 115 samples per week. Moreover, samples can be easily left overnight for the enzymatic treatment and analyzed in the day after. In comparison to previously reported methodologies, sample preconcentration via DLLME reduces considerably the time of analysis (since less steps of sample preparation are required), the usage of organic solvents at no expenses of analytical performance. Accuracies close to 100% were obtained in all cases at the ng mL^{-1} concentration level. To the extent of our literature search, this is the first report on the urine analysis for the simultaneous determination of BPA and its analogues using DLLME. Moreover, our study is the first reporting concentrations of BPA in urine samples of Brazilians. The obtained results show a similar trend to those reported for BPA in urine samples from European countries.

Table 5
The mean concentrations and detection rates of urinary bisphenol A (ng mL^{-1}).

Country	AM[a]	GM[b]	Detection rate (%)	Reference
Brazil	2.8	1.9	92	This study
China	3.8	1.1	94	[48]
Vietnam	3.3	1.4	94	[48]
India	2.0	1.6	94	[48]
Kuwait	4.1	1.2	94	[48]
Japan	2.0	0.8	94	[48]
Korea	3.5	2.0	94	[48]
USA	–	0.7	97	[49]
USA	–	0.7	95	[50]
USA	–	2.6	93	[51]
6 European member states	–	1.8	91	[52]

[a] Arithmetic mean;
[b] Geometric mean.

Table 4
Accuracy and precision of the DLLME-LC-MS/MS method for BPA and its analogues.

Bisphenols	Spiked (ng mL^{-1})	Within-run[a]			Between-run[c]		
		Found (ng mL^{-1})	Accuracy (%)	Precision[b] (RSD%)	Found (ng mL^{-1})	Accuracy (%)	Precision[b] (RSD%)
BPA	1.0	1.0	101	8.6	1.1	105	10.1
	8.0	8.1	101	2.5	8.2	103	8.2
	15.0	15.3	102	3.3	15.2	101	5.1
BPS	1.0	1.0	101	5.3	1.0	104	7.2
	8.0	8.1	101	6.8	8.1	102	5.8
	15.0	15.2	102	2.2	15.2	101	4.1
BPF	1.0	1.0	101	6.3	1.1	107	7.4
	8.0	8.2	102	6.4	8.2	103	8.7
	15.0	15.3	102	5.7	15.2	102	6.8
BPP	1.0	1.0	97	6.3	1.0	102	9.1
	8.0	7.8	97	4.6	7.9	99	6.4
	15.0	15.6	104	4.6	14.9	99	5.9
BPZ	1.0	1.1	105	1.9	1.1	112	5.2
	8.0	8.0	100	14.8	8.2	103	14.6
	15.0	15.4	103	7.2	15.4	103	9.6
BPAP	1.0	0.9	90	4.0	1.0	97	6.7
	8.0	8.2	102	5.8	7.8	97	8.9
	15.0	15.5	104	4.3	15.7	105	4.3
BPAF	1.0	1.0	99	11.3	1.0	104	13.6
	8.0	7.6	95	10.4	8.5	106	11.7
	15.0	15.2	101	7.0	15.4	103	9.8

[a] Number of replicates = 5;
[b] Relative standard deviation;
[c] based on three different days.(see text for additional details).

Conflict of Interest

The authors have declared no conflict of interest.

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