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Rapid, sensitive and simultaneous determination of 16 endocrine-disrupting chemicals (parabens, benzophenones, bisphenols, and triclocarban) in human urine based on microextraction by packed sorbent combined with liquid chromatography tandem mass spectrometry (MEPS-LC-MS/MS)

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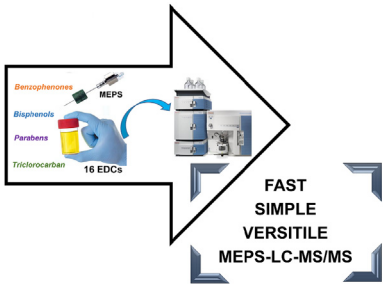
HIGHLIGHTS

- A new versatile method for the determination of 16 endocrine disruptors in urine is proposed.
- The procedure requires very low volumes of samples and organic solvents.
- The method is based on microextraction by packed sorbent and LC-MS/MS.
- The proposed method was successfully applied for the analysis of Brazilian children urine samples.

GRAPHICAL ABSTRACT



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ABSTRACT

A high demand exists in human biomonitoring studies for reliable and straightforward methods that generate data faster and simultaneously. Thus, the present study combines microextraction by packed sorbent (MEPS) and liquid chromatography coupled to mass spectrometry (LC-MS/MS) for simultaneous extraction and determination of various classes of endocrine-disrupting chemicals (EDCs), including parabens, benzophenones, bisphenols, and the antimicrobial, triclocarban in human urine samples. Optimized MEPS conditions were: i) MEPS sorbent (C18), ii) pH of sample (3), iii) volume of sample (250 μL), iv) number of draws–eject cycles (5) and (vi) desorption solvent conditions (100 μL of CH_3OH:H_2O 80:20 v/v). The calibration curves were linear over the selected ranges for all studied compound, with correlation coefficients higher than 0.99. The variation coefficient for precision was lower than 20% at lower concentrations and lower than 15% at the higher concentrations studied. The accuracy ranged from 90% to 118%. The proposed strategy affords several advantages over currently

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published approaches, including simplicity of operation and reduction of sample and solvent volumes and time for matrix clean-up. Moreover, the analytical performance of each MEPS cartridge remained stable over the analysis of at least 70 samples (RSD < 10%). Thus, the current procedure may be an interesting high-throughput alternative for large routine human biomonitoring studies. Urinary geometric mean concentrations of EDCs obtained in this study were close than those previously reported for Brazilian children.

1. Introduction

Concerns have been growing over the augmented human exposure to an extensive range of synthetic environmental chemicals (SECs). The SECs include bisphenols, parabens, benzophenones, and the antimicrobial triclocarban, among others. Human exposure to these compounds been associated with hormonal disorders, and thus they are classified as endocrine-disrupting chemicals (EDCs) (Darbre and Harvey, 2008; Howdeshell et al., 1999; Krause et al., 2012; US.EPA, 1997). Bisphenol A and its analogues are found in many everyday products mainly because of their wide use in the production of packaging. (Kang et al., 2006; Rocha et al., 2015). Parabens are used as antimicrobial preservatives in cosmetics, pharmaceuticals, and foodstuffs (Liao et al., 2013). Triclocarban has extensive use as an antimicrobial in personal care products (PCPs) (Yusa et al., 2012), whereas benzophenones are a common chemical found in PCPs for skin and hair protection against UV irradiation (Kunisue et al., 2012).

Based on a widespread use as long as their potential risk to human health, biomonitoring human exposure to these compounds has been a common practice in national exposure surveys around the world (Calafat et al., 2010, 2008; Cristina Jardim et al., 2015; Dewalque et al., 2014; Frederiksen et al., 2011; Kang et al., 2013; Karthikraj and Kannan, 2018; Larsson et al., 2014; Liao and Kannan, 2012; Ma et al., 2013; Moos et al., 2014; YE et al., 2006). Thus, a high demand exists for fast, simple, sensitive and reliable methodologies for the simultaneous determination of EDCs in clinical samples. On the other hand, simultaneous methods of sample preparation for a wide range of EDCs is challenging, due to differences in the physico-chemical properties of the compounds.

LC-MS/MS has been the chosen technique for the determination of EDCs in clinical samples (Asimakopoulos et al., 2014; Dewalque et al., 2014; Kang et al., 2013; Karthikraj and Kannan, 2018; Larsson et al., 2014; Rocha et al., 2018a, 2018b; Rocha et al., 2017; YE et al., 2006), although some groups designate gas chromatography coupled to mass spectrometry (GC-MS) as an excellent alternative technique for some of these compounds (González-Mariño et al., 2011).

Since most of the EDCs are found at low concentration in the biological specimens (ppt-ppb levels) and due to the complexity of clinical matrices, an initial step of sample preparation is required before chromatographic analysis to clean up the matrix and when possible also concentrate the analytes. The extraction of different classes of EDCs in urine has been routinely done through solid-phase extraction (SPE) (Dewalque et al., 2014; Kang et al., 2006; Larsson et al., 2014; YE et al., 2006) or liquid-liquid extraction (LLE) (Asimakopoulos et al., 2014; Rocha et al., 2018a, 2018b). However, these traditional sample preparation procedures have disadvantages including the reuse of the extractor phase, the use of toxic solvents and are in general time consuming.

Microextraction by packed sorbent (MEPS) is a miniaturized SPE method that was developed by AstraZeneca, Sodertalje, Sweden (Abdel-Rehim, 2004). MEPS incorporates the same sorbents as conventional SPE columns, and they are integrated into a liquid-handling syringe. However, MEPS has several advantages over traditional SPE techniques including the sorbent phase reuse and much lower volumes of sample and solvent required (Cristina Jardim et al., 2015; Fumes and Lanças, 2017; González-Mariño et al., 2011).

Despite the advantages, the use of MEPS for the simultaneous extraction of several EDCs from biological matrices is infrequent. Based on that, this study aimed to develop a new sample preparation method by using MEPS for simultaneously extracting of 16 EDCs from urine samples followed by liquid chromatography-tandem mass spectrometry determination.

2. Materials and method

2.1. Reagents and analytical standards

Urine samples were analyzed for 16 EDCs including, bisphenols, parabens, benzophenones, and triclocarban. The list with all chemical names of all compounds analyzed is shown in Table S1. Reference standards of bisphenols, parabens, benzophenones, triclocarban and the internal standards BPA-d_{16} and, parabens internal standard mix solution were obtained from Sigma-Aldrich (St. Louis, MO, USA). All stock solutions were prepared by dissolving each compound in methanol to obtain a solution at a concentration of 1 mg mL^{-1}. Mixed stock solutions containing all studied compounds (10 000 ng mL^{-1}) or all the internal standards (1000 ng mL^{-1}) were prepared in MeOH:H_2O and stored in the dark at −20 °C until use. All solvents (HPLC grade) used were purchased from JT Baker (Phillipsburg, NJ, USA). High purity deionized water (Millipore RiOs-DITM, Bedford, MA, USA) was used throughout the experiments. Details of the synthetic urine preparation are presented elsewhere (Rocha et al., 2018a, 2018b; Rocha et al., 2016; Vela-Soria et al., 2014). The β-glucuronidase enzyme from *Helix pomatia*, type H-1, partially purified powder (≥300 000 units/g solid), was purchased from Sigma-Aldrich.

2.2. Instrumental analysis, sample collection, and enzymatic hydrolysis

For the detection of the EDCs after MEPS sample preparation, the LC-MS/MS instrument conditions recommended by Rocha et al. (Rocha et al., 2018a, 2018b) was adopted.

Twenty urine samples were randomly selected from a biorepository that consists of urban resident Brazilian school children aged 6–14 years from all geographic regions of Brazil. This biorepository is part of a big study of the Brazilian Ministry of Health and our laboratory. Detailed information of the urine samples are presented elsewhere (Rocha et al., 2017, 2018a). This study was approved by the Ethics Committee of Faculdade de Ciências Farmacêuticas de Ribeirão Preto, the University of São Paulo, Brazil (CEP/FCFRP no. 459 – CAAE nº 80512717.6.0000.5403). The selected urine samples were stored in a freezer at −20 °C until chemical

analysis.

It is known that EDCs are excreted mostly as glucuronide conjugates in urine, and only a few are excreted in their free form (Frederiksen et al., 2011; Karthikraj and Kannan, 2018; YE et al., 2006). Therefore, human urine should be subjected to an initial step of hydrolysis with β-glucuronidase before determination of urinary concentrations of EDCs. 250 μL of urine sample with 20 ng of internal standards mixture was buffered with 20 μL of 1 M ammonium acetate (pH = 5) containing 50 unit of β-glucuronidase, and the sample was incubated at 37 °C for 12 h (Rocha et al., 2019).

2.3. Optimized microextraction by packed sorbent (MEPS) procedure

MEPS purification system used a 100 μL gastight syringe which needle contains 1 mg of sorbent (SGE Analytical Science, Melbourne, Australia). The MEPS Barrel Insert and Needle Assemblies (BINs) evaluated were two silica-based sorbents: C8 and C18. After choosing the C18 as the best sorbent material, pH of sample (3.0, 5.3 and 7.0), urine sample volume (250 μL, 500 μL and 1000 μL), number of cycles draw-eject (2, 3, 4 and 5) and desorption solvent (methanol:water 80:20 v/v, 50:50 v/v and 20:80 v/v) were evaluated. These variables were selected based upon previous reports highlighting how they might affect the MEPS extraction efficiency (Abdel-Rehim, 2011, 2004; Cristina Jardim et al., 2015; Fumes and Lanças, 2017; González-Mariño et al., 2011).

The first step of the MEPS procedure consists of sorbent condition with 4 cycles draw-eject of 100 μL of MeOH, followed by 4 cycles draw-eject of 100 μL of H_2O. Synthetic urine samples (250 μL) were spiked with bisphenols, parabens, benzophenones and triclocarban at a concentration of 20.0 ng mL^{-1} and diluted with 250 μL of ultrapure water. The urine sample loadings were carried out manually through the MEPS C18 sorbent (extraction of the analytes) and ejected, resulting in 5 cycles of 100 μL. For cleaning purpose, 100 μL of 10% methanol + 0.1% acetic acid (1 cycle) was used to remove undesired components such as inorganic salts from the sorbent. Elution was carried out with 100 μL of methanol: water (80:20 v/v) directly into the LC-MS/MS vials. In this study, the extraction was repeated multiple times using the same MEPS cartridge. Between the procedure, the sorbent was washed with 10 draw-eject cycles of 100 μL of methanol and 10 draw-eject cycles of 100 μL of water.

3. Results and discussion

3.1. Optimization of MEPS procedure

The main purpose of this study was the development of a useful analytical method that requires a small sample volume and the use of organic toxic solvents for the analysis of a wide range of chemicals. It has been possible due to the application of a single sample preparation protocol for the analysis of 16 EDCs simultaneously. For achieving the satisfied efficiency of extraction, the influences of different experimental variables that might affect MEPS extraction efficiency were evaluated and optimized, including pH of the sample, number of draw-eject extraction cycles, sample volume and desorption solvent. For this purpose, 250 μL of synthetic urine spiked with 20.0 ng mL^{-1} of studied EDCs was used to evaluate the efficiency of extraction.

The sorbent washing step and clean-up step were carried out according to previously reported MEPS studies (Abdel-Rehim, 2011; Cristina Jardim et al., 2015; González-Mariño et al., 2011) with some modifications. Briefly, we performed all the experiments using 100 μL of methanol:aqueous acetic acid 0.1% (10:90, v/v) for the purpose of washing unwanted weakly retained interferents while ensuring minimum leaking of the analytes (Cristina Jardim et al., 2015), and the clean-up step was performed by 10 draw-eject cycles of 100 μL methanol + 10 draw-eject cycles of 100 μL water for the purpose of reusing of the MEPS sorbent between the samples and avoiding the carryover phenomena often observed in MEPS procedures (Abdel-Rehim, 2011).

Considering the number of analytes in the study and for better visualization of the optimized conditions in the figures, only the main compounds of each class studied (MeP, PrP, BPA, BPS, BP1, and TCC) were chosen to be reported in the graphs in a representative manner. These selected compounds are also the most common reported in the literature in human biomonitoring studies (Asimakopoulos et al., 2014; Calafat et al., 2010, 2008; Rocha et al., 2018a, 2018b; Rocha et al., 2019; Vela-Soria et al., 2014; YE et al., 2006).

Since the sorbent selection is one of the most important factors to achieve acceptable clean-up and high extraction efficiency, two different sorbents commonly used in MEPS: C8- and C18-modified silica gel were evaluated The results were express in terms of the extraction efficiency (Fig. 1). Although the behavior of both materials is quite similar, better extraction efficiencies were obtained from C18 BIN (Fig. 1). Consequently, C18 BIN was selected as the sorbent for further experiments.

The primary extraction mechanism using MEPS is based on the partition process. Therefore, the pH of the sample matrix influence the ionization of analytes possessing a pH-dependent dissociable group (Noorashikin et al., 2014). Then, the pH was adjusted to ensure that all studied compounds were in their no ionized forms, which facilitate their partitioning into the extraction phase. Since silica is the base material of the C18 extraction phase, the selection of pH lower than 2 and higher than 8 must be avoided due to the degradation of the phase. Furthermore, at pH above 8.0, alkaline hydrolysis of some EDCs (parabens) takes place (Fumes and Lanças, 2017). Since, the almost all studied compounds are weak acids, with typical pKa values higher than 7, weak acidic conditions are expected as a good condition for reverse-phase extraction analytes from urine samples without degradation of sorbent phase. The effect of the sample pH on the retention of the analytes was investigated by extracting 5 × 100 μL (draw-eject cycles) of spiked synthetic urine (20 ng mL^{-1}) in a C18 BIN-sorbent and eluting the compounds with 100 μL of methanol: water (80:20, v/v). Three values of pH were tested (3, 5.3 and 7), and they were performed in triplicate. The best extraction efficiency was obtained without acidification.

Moreover, it was observed that at pH 3 the EDCs are poorly

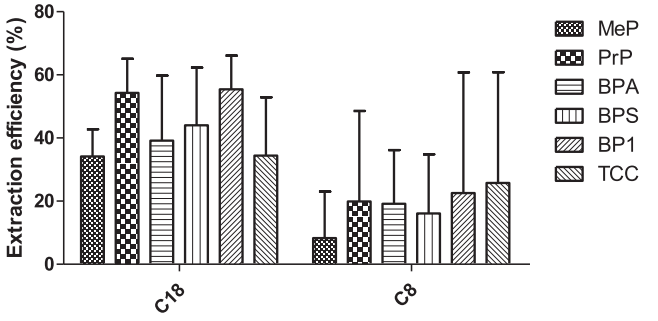

Fig. 1. Influence of MEPS sorbent for extraction of EDCs from urine by MEPS. Conditions: urine volume, 500 μL; pH urine sample, 5.3 (no pH correction); number of draw-eject extraction cycles (100 μL), 5; elution solvent, 100 μL of methanol:water (80:20 v/v). These assays were performed in triplicate (n = 3). MeP: methylparaben, PrP: propylparaben, BPA: bisphenol A, BPS: bisphenol S, BP1: benzophenone 1, TCC: triclocarban.

extracted utilizing a MEPS procedure, while at higher pH values, e.g., 5.3 and 7 the extraction presented similar results (Fig. S1). The reason for this behavior is not clear when considering only their pKa values. This effect may be partially attributed to their protonation at low values of pH values. It is import to note that pH lower than 7 is enough to ensure the neutrality of the selected EDCs (González-Mariño et al., 2011; Rocha et al., 2018a, 2018b). As the common pH of human urine is between 5.5 and 7.0. So, sample pH adjustment was not necessary.

Further experiments evaluated the influence of sample volume on analyte extraction (250 μL, 500 μL, and 1000 μL). The samples were diluted with water and the volume used was equal to the sample volume used (depending on the test). As much as the dilution volume has not been studied, we follow a MEPS tutorial (Abdel-Rehim, 2011) which says that biological samples need dilution because it contains a large number of impurities that can clog MEPS. To this end, the synthetic urine was spiked with EDCs (20 ng mL^{-1}), and 5 draw-eject cycles of 100 μL were performed. As observed in Fig. S2, the higher the sample volume, the lower is the extraction efficiency. In this specific case, the sample volume is closely related to the number of draw-eject cycles. If there is a large sample volume, it is necessary to increase the number of draw-eject cycles to maximize analyte contact with the sorbent material. Although this procedure increases the extraction efficiency, it also increases the time for sample preparation. Thus, 250 μL of the sample volume in addition to the 250-μL initial water volume was selected. This dilution reduces urine viscosity and extends the reuse of the C18 (Abdel-Rehim, 2011; Cristina Jardim et al., 2015).

The extraction efficiency increases with the number of loading cycles until the sorption equilibrium is established (Abdel-Rehim, 2011). In the present study, 3, 4, 5 and 6 extraction cycles were investigated. The results showed that the extraction efficiency increased linearly from 3 draw-eject cycles (100 μL) to 6 cycles (Fig. 2). Although extraction efficiency increases with more loading cycles, the time for sample preparation also increases and a high number of draw-eject cycles results in much more time for perfom the procedure. Therefore, as a compromise between the efficiency of extraction and time of analysis, 5 cycles (100 μL) of extraction were selected for further the studies combined with 500 μL of sample volume (250 μL synthetic urine + 250 μL water).

C18 sorbent is based on hydrophobic interactions. For this reason, nonpolar solvents are the most suitable to interrupt the interactions between the analytes and the sorbent (González-Mariño et al., 2011). For this reason, it was evaluated three different proportions of the mixture methanol: water (80:20, 50:50 and 20:80, v/v). Moreover, using the mobile-phase composition as a desorption solvent prevented unnecessary steps of sample drying and resuspension with the desorption solvent (Abdel-Rehim, 2011; Fumes and Lanças, 2017). Better extraction efficiency was achieved by using MeOH:H_2O (80:20, v/v), and the lower the methanol concentration, the lower is the extraction efficiency (Fig. 3). It can be observed that the analytes have a higher affinity for the methanol, which can be explained by the hydrophobic characteristics of the EDCs in the study. Concentrations over 80% of methanol were not evaluated.

It is well known that the desorption procedure should be carried out with a small volume of solvent due to the small amount of sorbent used in MEPS. Using larger solvent volumes leads to analyte dilution and decreased analytical signal (Abdel-Rehim, 2011; Cristina Jardim et al., 2015; Fumes and Lanças, 2017; González-Mariño et al., 2011). Moreover, since that the depth of the injection syringe limits volume aspirated into the insert and the nature of the LC autosampler operation, the accomplishment of the desorption procedure was carried out using 100 μL of MeOH:H_2O (80:20, v/v).

Based on our results, the optimized procedure was as follows: C18 BIN, 250 μL of urine sample diluted with 250 μL of water, five draw–eject cycles (5 × 100 μL) and elution with 100 μL of MeOH and H_2O solution (80:20, v/v).

3.2. Method performance

Figures of merit for the proposed method were studied using synthetic urine due to the difficulty of finding real human urine without any of the EDCs studied in this work. The synthetic urine mimics the human urine composition without the presence of EDCs (Rocha et al., 2018a, 2018b; Rocha et al., 2019, 2016; Vela-Soria et al., 2014) allowing method development. The MEPS-HPLC–MS/MS method performance was evaluated using synthetic urine samples spiked with the internal standard (20.0 ng mL^{-1}) and EDCs standard solutions.

Calibration curves using MEPS-LC-MS/MS were obtained by least-squares linear regression analysis of the ratio between the analytes and the internal standard peak areas using seven concentration levels in triplicate. For this purpose, synthetic urine was spiked with known EDCs concentrations, treated with the proposed MEPs procedure and then analyzed. The correlation coefficients (R) ranging from 0.990 to 0.997 are obtained for all the analytes indicating that the developed method had good linearity (Table 1).

Estimated LODs and LOQs of the proposed method (calculated for a signal-to-noise ratio of 3 and 10, respectively) ranged from 0.005 to 0.01 ng mL^{-1} and 0.02–0.33 ng mL^{-1} in synthetic urine, respectively. The proposed method LOQs and LODs obtained in this study (Table 1) were similar to those previously reported (Table 2), and it should be a feasible method for quantification of EDCs in

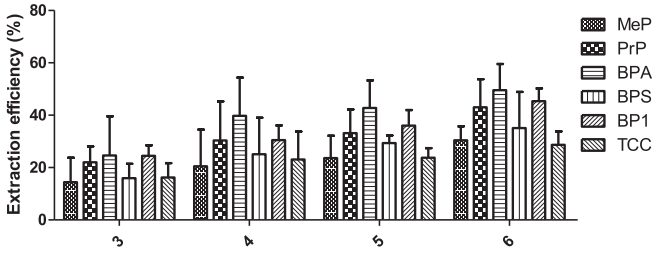

Fig. 2. Influence of number of draw-eject extraction cycles (100 μL) for extraction of EDCs from urine by MEPS. Conditions: MEPS sorbent, C18; urine volume, 250 μL + 250 μL of water; pH urine sample, 5.3 (no pH correction); elution solvent, 100 μL of methanol:water (80:20 v/v). These assays were performed in triplicate (n = 3). MeP: methylparaben, PrP: propylparaben, BPA: bisphenol A, BPS: bisphenol S, BP1: benzophenone 1, TCC: triclocarban.

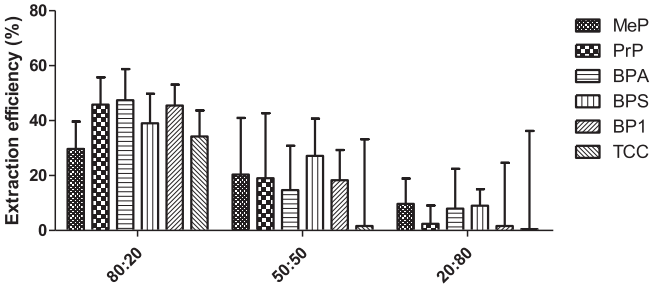

Fig. 3. Influence of methanol:water (100 μL) as elution solvent for extraction of EDCs from urine by MEPS. Conditions: MEPS sorbent, C18; urine volume, 250 μL + 250 μL of water; pH urine sample, 5.3 (no pH correction); number of draw-eject extraction cycles (100 μL), 5. These assays were performed in triplicate (n = 3). MeP: methylparaben, PrP: propylparaben, BPA: bisphenol A, BPS: bisphenol S, BP1: benzophenone 1, TCC: triclocarban.

Table 1
Analytical performance of the proposed method for determination of endocrine-disrupting chemicals in urine.

EDC	Linear range (ng mL^{-1})	Linear equation	R	Spiked (ng mL^{-1})	Within-day[a]		Between-day[c]		LOD (ng mL^{-1})	LOQ (ng mL^{-1})
					Precision[b] (CV%)	Accuracy[d]	Precision[b] (CV%)	Accuracy		
MeP	1.0–20.0	0.090x-0.046	0.994	1.0	7	96	8	98	0.10	0.33
				10.0	9	101	4	102		
				20.0	3	101	6	104		
EtP	1.0–20.0	0.052x-0.038	0.994	1.0	13	115	15	116	0.03	0.10
				10.0	6	104	7	109		
				20.0	3	98	3	97		
PrP	0.5–20.0	0.084x-0.042	0.995	0.5	9	114	8	116	0.01	0.03
				10.0	4	101	5	98		
				20.0	5	101	3	98		
BzP	1.0–20.0	0.158x-0.070	0.995	1.0	12	116	10	116	0.01	0.03
				10.0	6	104	5	109		
				20.0	1	101	2	97		
BuP	0.5–20.0	0.095x-0.031	0.997	0.5	11	98	13	106	0.01	0.03
				10.0	6	103	8	94		
				20.0	4	101	6	98		
OH–MeP	2.5–20.0	0.095x-0.110	0.992	2.5	12	98	13	103	0.04	0.13
				10.0	10	99	10	96		
				20.0	5	99	4	102		
OH–EtP	1.0–20.0	0.081x-0.058	0.995	1.0	10	114	12	115	0.03	0.10
				10.0	6	103	8	104		
				20.0	2	99	2	98		
BPA	2.5–20.0	0.008x+0.026	0.991	2.5	15	108	15	110	0.02	0.07
				10.0	1	104	5	105		
				20.0	7	106	9	108		
BPS	0.5–20.0	0.068x-0.028	0.993	0.5	14	104	13	105	0.01	0.03
				10.0	4	101	5	98		
				20.0	6	101	7	97		
BPAP	2.5–20.0	0.054x-0.101	0.990	2.5	15	91	16	92	0.01	0.03
				10.0	6	99	9	104		
				20.0	6	107	5	110		
BPAF	2.5–20.0	0.319x-0.410	0.995	2.5	4	110	6	112	0.005	0.02
				10.0	6	99	5	96		
				20.0	1	99	5	97		
BP1	1.0–20.0	0.093x-0.055	0.993	1.0	15	113	10	110	0.005	0.02
				10.0	4	105	5	105		
				20.0	4	99	6	99		
BP2	0.5–20.0	0.143x-0.050	0.993	0.5	14	18	16	118	0.01	0.03
				10.0	3	102	1	104		
				20.0	7	101	10	109		
BP8	1.0–20.0	0.035x-0.033	0.996	1.0	5	112	9	110	0.03	0.10
				10.0	7	99	5	97		
				20.0	3	102	6	106		
4OH-BP	0.5–20.0	0.064x-0.016	0.993	0.5	13	90	15	92	0.01	0.03
				10.0	10	102	12	106		
				20.0	7	97	9	103		
TCC	2.5–20.0	0.113x-0.218	0.991	2.5	10	116	8	114	0.01	0.03
				10.0	7	101	9	102		
				20.0	6	103	4	109		

[a] Number of replicates: 3.
[b] Coefficient of variation.
[c] Based on three different days (See text for additional details).
[d] Recovery.

Table 2
Comparison of the proposed method using microextraction by packed sorbent with other previously published extraction methods for the determination of endocrine-disrupting chemicals in urine samples.

Sample procedure and analysis	Compounds	LOD (ng mL^{-1})	Sample Volume (μL)	Reference
SPE-LC-MS/MS	Parabens	0.10–0.18	100	YE et al. (2006)
LLE–LC–MS/MS	Bisphenol A diglycidyl ethers, parabens, benzophenones, triclosan, and triclocarban	NR	500	Asimakopoulos et al. (2014)
DLLME-LC-MS/MS	Bisphenols	0.005–0.2	5000	Rocha et al. (2016)
AALLME-LC-MS/MS	Parabens, bisphenols, benzophenones, triclosan and triclocarban	0.01–0.30	5000	(Rocha et al., 2018a, 2018b)
SPE-UPLC-MS/MS	Phthalates, parabens and benzophenone 3	0.09–0.37	3000	Dewalque et al. (2014)
MEPS-UPLC-MS/MS	Parabens	0.5[a]	200	Cristina Jardim et al. (2015)
MEPS-LC-MS/MS	Parabens, bisphenols, benzophenones and triclocarban	0.005–0.10	250	This study

NR- Not reported. The authors reported LOQ values only.
[a] Reported as lower limit of quantification.

human urine samples.

The accuracy and precision of the method for the determination of EDCs in urine were determined by spiking synthetic urine samples at three different concentration levels (lower calibration point, 10.0, 20.0 ng mL^{-1}) and the internal standard at 20.0 ng mL^{-1} concentration. The inter-day (three replicate measurements per day were done in three consecutive days) and intra-day (three replicate measurements were performed on the same day) accuracy and precision. The accuracy was determined by recovery experiments, and the precision was calculated by the coefficient of variation (CV). Values outside the range of ±15% of the nominal value were not accepted, except for the lower calibration point, for which values outside the range of ±20% of the nominal value were not allowed. The accuracy and precision of the proposed method are given in Table 1. The method showed within-day accuracy ranging from 90 to 118%, and the precision was 1–15%. Accuracy and precision between-day ranged from 92 to 118% and from 1 to 16%, respectively.

Matrix effects (MEs) were found for all target chemicals analyzed, probably due to the presence of matrix components that co-elute during the evaporation on the electrospray housing showing ionization suppression or signal enhancement. The use of internal standards may compensate these effects, maintaining the fidelity of the method. Furthermore, all experiments were carried out using a matrix quite similar to human urine instead of solvents, which better approximates real samples, even for the matrix effect. So, matrix effects were investigated using synthetic urine and a pool of 6 urine samples (randomly selected) spiked at low and high analyte concentrations (5.0 ng mL^{-1} and 15.0 ng mL^{-1}), respectively. This determination was conducted in triplicate, and matrix effects were calculated by the ratio between the peak area of the analytes in the presence of matrix (measured by analyzing spiked blank matrix after extraction of the analyte) and the peak area of the analytes in the absence of matrix (pure solution of the analyte in 100 μL of methanol: water (80:20 v/v)). The IS normalized matrix factor (MF) was calculated by dividing the MF of the analyte by the MF of the IS. The CV of the IS-normalized MF calculated should not be greater than 15%. The calculated CVs of the IS-normalized MF (matrix effects) range from 4% for BPA to 15% for BP1 and TCC at a concentration of 5.0 ng mL^{-1} and from 3% for BPAP to 18% for OH–MeP at a concentration of 15.0 ng mL^{-1}. Only OH–MeP had a CV of the IS-normalized MF over the desired limit of 15%. The results are shown in Table S2. Due to the presence of matrix effects, the use of matrix-matched calibration was used.

Carry-over was checked by performing the MEPS procedure on a blank synthetic urine sample and injecting it promptly after analysis of the synthetic urine sample spiked with analytes at the concentration that corresponded to the upper calibration point (20.0 ng mL^{-1}). Carry-over was investigated by running a blank after running the highest calibrator and should not be greater than 20% of the analyte signal from the lower calibration point chromatogram and not greater than 5% of the internal standard signal (IS). Carry-over in the non-spiked urine samples that were evaluated immediately after sampling (20.0 ng mL^{-1}) presented an analytical signal from bisphenol AF (BPAF); however, the signal was not higher than 20% of the analyte signal at lower calibration point, and not greater than 5% of the IS signal.

3.3. Comparison of MEPS-HPLC-MS/MS with other methods for EDCs determination in different matrices

A comparison between our proposed method with previous methods is summarized in Table 2. Among solid phase extractions procedures used for EDCs determination in urine (Almeida and Nogueira, 2014; Chen et al., 2012; Cristina Jardim et al., 2015; Dewalque et al., 2014; Dias et al., 2015; Fumes and Lanças, 2017; González-Mariño et al., 2011; YE et al., 2006), MEPS is faster because it does not need an equilibration step, reducing sample preparation time. MEPS needs a fast sorbent conditioning equilibration instead SPE that needs some minutes to an hour to achieve equilibrium and the extraction of analytes. Among studies that used MEPS as microextraction procedure, we know briefly that the authors, taking in account the sampling and cleaning steps with different volumes, they spent nearly 14 cycles (Cristina Jardim et al., 2015), 32 cycles (FUMES & LANÇAS, 2017), 40 cycles and 15 min (González-Mariño et al., 2011). In this study, we spent 25 cycles resulting around 6 min to prepare each sample. Although the authors of previous works have not reported the exact time they spend in their extraction procedures, we might do a humble time comparison between studies because the times, in these cases, is relative to the cycles number and also it is relative to aspirated volume. The ones who spent more cycles also spent more time to do the procedure. Unlike for exemple, we can remember authors that used SBSE method to extract EDCs. They reported in their procedure times varying between 1.5 and 3 h (Dias et al., 2015; Almeida and Nogueira, 2014).

In addition, our washing step almost eliminates carry-over and MEPS could be reused over 70 times with the reproductive and minimum loss of analyte signal (RSD < 10%). It should be mentioned that a conventional column of SPE can only be used once (Abdel-Rehim, 2011; Cristina Jardim et al., 2015). Moreover, the proposed MEPS procedure is straightforward, since it requires only a sample dilution with water. When compared with extraction procedures based on LLE principles (Asimakopoulos et al., 2014; Rocha et al., 2018a, 2018b; Rocha et al., 2016; Shen et al., 2017; Tarazona et al., 2013; Vela-Soria et al., 2014), the proposed MEPS procedure presents some advantages, since it does not require resuspension and drying steps. In addition, MEPS allows the matrix to be washed and, therefore, the decrease of impurities that could go to the chromatographic system and cause damage. Simultaneous determination of several EDCs is possible with much lower solvent and sample volumes, but with similar or better LODs than previously reported methods in the literature. Furthermore, it does not use extremely toxic liquids, such as chlorinated solvents.

3.4. Analysis of endocrine-disrupting chemicals in the urine of Brazilian children

To evaluate the applicability of the proposed method, 20 human urine samples were extracted using the MEPS method and analyzed

Table 3
Concentrations (ng/mL) of endocrine-disrupting chemicals (EDCs) in the urine of Brazilian children.

EDC	Detection rate %	Geometric Mean	Minimum	Maximum
MeP	100	42.5	3.21	982
EtP	80	0.50	< LOQ	28.6
PrP	90	4.22	< LOQ	92.4
BzP	0	–	–	–
BuP	40	0.28	< LOQ	6.75
OH–MeP	100	2.65	0.38	31.5
OH–EtP	90	0.48	< LOQ	4.20
BPA	90	1.40	< LOQ	14.3
BPS	10	0.62	< LOQ	1.63
BPAP	0	–	–	–
BPAF	0	–	–	–
BP1	90	4.23	< LOQ	57.6
BP2	0	–	–	–
BP8	0	–	–	–
4OH-BP	0	–	–	–
TCC	80	0.05	< LOQ	0.85

by LC-MS/MS. Table 3 summarizes the results of 16 potential EDCs (ng mL^{-1}). Among parabens, MeP was detected in 100% of the evaluated urine samples, and it was quantified as the highest concentration (geometric mean = 42.5 ng mL^{-1}) showing that this paraben is the most abundant among six parabens tested. PrP also was detected in almost all the samples (90%), and this could be explained due to MeP and PrP are the most widely used (Jackson, 1992) in several materials like pharmaceutical, cosmetics and even food (Liao et al., 2013). Except for MeP, other parabens were detected in most cases at low concentration (geometric mean = 0.28–4.22 ng mL^{-1}). BzP was not found in any evaluated urinary samples. BPA was detected in 90% of the analyzed urine samples while BPS was detected only in 10% of the urine samples (0.62 ng mL^{-1}). This result can be explained by the gradually BPA replace by other bisphenols analogues mainly BPS due to BPA possible endocrine disrupting effects. Nevertheless, BPA is still the most common form of bisphenol present in biomonitoring studies (Liao et al., 2012). Other bisphenol analogues tested (BPAP and BPAF) were not found in any samples. BP1 was present in 90% of the samples tested while other benzophenones were not detected. The antimicrobial TCC was found in 80% of the analyzed samples (geometric mean = 0.05 ng mL^{-1}). The results present in Table 3 showed different rates of detection, ranging from low to higher concentrations. This wide range of levels could be related to lifestyle as exposure of EDCs and individual factors as metabolism and kinetics variations. Therefore, urinary levels of several EDCs may be correlated with massive consumption of PCPs by Brazilian (Rocha et al., 2018a, 2018b, 2019, 2017). The baseline values for the urinary concentration of the EDCs in Brazilian children was previously established (Rocha et al., 2018a). The urinary geometric mean concentrations of EDCs found in this study were similar to those previously published in the literature for the same group of samples (Rocha et al, 2018a, 2018b).

4. Conclusions

To the authors' best knowledge, this is the first method based on MEPS and LC-MS/MS for the simultaneously extract and analysis of 16 endocrine-disrupting chemicals in urine samples. The MEPS-HPLC–MS/MS method is rapid, simples, sensitive and accurate. It requires very low sample volumes (250 μL) and organic solvents. Moreover, the MEPS procedure offers several other advantages, including matrix clean-up and multiple reuses of packed syringe cartridges. These advantages make the proposed method a desirable green alternative extraction procedure for human biomonitoring studies.

Conflicts of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

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