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A fast-multiclass method for the determination of 21 endocrine disruptors in human urine by using vortex-assisted dispersive liquid-liquid microextraction (VADLLME) and LC-MS/MS

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ABSTRACT

Simplicity, speed, and reduced cost are essential demands for routine analysis in human biomonitoring studies. Moreover, the availability of higher volumes of human specimens is becoming more restrictive due to ethical controls and to the costs associated with sample transportation and storage. Thus, analytical methods requiring much lower sample volumes associated with simultaneous detection capability (multiclass analysis) are with a very high claim. In this sense, the present approach aimed at the development of a method for preconcentration and simultaneous determination of four classes of endocrine disruptors (seven bisphenols, seven parabens, five benzophenones, and two antimicrobials) in the urine. The approach is based on vortex-assisted dispersive liquid-liquid microextraction (VADLLME) and high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). After optimization of the significant parameters of VADLLME extraction, the proposed procedure showed to be simple, fast, sensitive, requiring only 1.0 mL of urine, 400 μL of organic solvents with a total stirring time of 20 s. Moreover, a variation of inter-day and between-day runs were lower than 10.0% and 11.0%, respectively. Finally, the proposed method was successfully applied to the analysis of 50 urine samples of Brazilian pregnant women to establish reference ranges.

1. Introduction

Commonly everyday use products, including cosmetics, personal care, food, plastics utensils, detergents, toys, food cans, pharmaceuticals, may contain contaminants known as endocrine disruptors (EDCs) (Bansal et al., 2018). These chemicals, when in constant contact with the body for long periods, can modify the normal endocrinal functions, affecting the reproductive (Sifakis et al., 2017; Pollack et al., 2018; Aker et al., 2019), neurological (Przybyla et al., 2017; Preciados et al., 2016), immune systems (Bansal et al., 2018; DeWitt and Patisaul, 2018) or even muta- and carcinogenic effects (Kim and Lee, 2017; Del Pup et al., 2016; Rochefort, 2017). Some classes of EDCs are of extensive industrial production, such as bisphenols, benzophenones, antimicrobials (triclosan/triclocarban), and parabens (Chen et al., 2016; Bilal and Iqbal, 2019; Weatherly and Gosse, 2017; Ghazipura et al., 2017). Triclosan and triclocarban, which are present in most personal care products, have been associated with inhibitory effects on CYP19A1 (producer of estrogen from androgens) in humans (Li et al., 2017). Benzophenones present in sunscreens have been associated with endometriosis in women (Kunisue et al., 2012) and reduced semen quality in men (Buck Louis et al., 2015). Bisphenols with extensive use as plastic packaging are associated with numerous adverse effects on humans, including bone damage (Chin et al., 2018). Finally, parabens were associated with an increase in the number of MCF–7 mammary cancer cells, indicating a possible contribution to tumor growth (Charles and Darbre, 2013).

With the broad risks to human health, concerns about exposure to

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Table 1
Linearity and limits of quantification and detection of the method for analysis of EDCs in synthetic urine.

EDCs	Linear range (ng/mL)	Linear equation	r[a]	LOD[b] (ng/mL)	LOQ[b] (ng/mL)	RSD[c] (%)
BPA	0.5–20.0	0.7631 + 0.3439 x	0.996	0.08	0.20	5
BPS	0.5–20.0	−0.6558 + 0.5165 x	0.995	0.05	0.14	6
BPF	0.5–20.0	−0.0282 + 0.0529 x	0.997	0.05	0.15	2
BPZ	0.5–20.0	−0.1101 + 0.3019 x	0.997	0.09	0.25	6
BPP	0.5–20.0	0.2044 + 0.5601 x	0.997	0.07	0.20	7
BPAF	0.5–20.0	0.2669 + 0.1711 x	0.996	0.07	0.20	10
BPAP	0.5–20.0	5.3626 + 1.3795 x	0.994	0.06	0.18	8
OH–MeP	0.5–20.0	−0.0727 + 0.1765 x	0.998	0.02	0.05	10
OH–EtP	0.5–20.0	−0.0923 + 0.3488 x	0.998	0.01	0.03	5
MeP	0.5–20.0	0.0785 + 0.4808 x	0.998	0.05	0.15	8
EtP	0.5–20.0	−0.1066 + 0.3731 x	0.997	0.03	0.10	7
PrP	0.5–20.0	−0.0156 + 0.5855 x	0.996	0.01	0.02	8
BuP	0.5–20.0	−0.0107 + 0.5605 x	0.997	0.01	0.03	9
BzP	0.5–20.0	−0.1891 + 3.0485 x	0.998	0.02	0.08	9
BP1	0.5–20.0	−0.6347 + 2.0245 x	0.997	0.02	0.05	8
BP2	0.5–20.0	−0.7228 + 1.6842 x	0.998	0.03	0.10	10
BP3	0.5–20.0	−0.6769 + 0.2059 x	0.997	0.02	0.08	7
BP8	0.5–20.0	0.1442 + 0.5918 x	0.997	0.03	0.10	9
4–OH–BP	0.5–20.0	−0.0554 + 0.3659 x	0.997	0.10	0.40	5
TCS	1.0–20.0	0.0004 + 0.2027 x	0.996	0.20	0.50	10
TCC	1.0–20.0	3.8004 + 8.6947 x	0.997	0.02	0.05	9

[a] Correlation coefficient.
[b] *(n = 5)* for each concentration.
[c] RSD, relative standard deviation (%).

these chemicals are gaining attention worldwide, with Public Health Agencies prioritizing studies on biomonitoring of human populations (NIEHS, 2003; CDC, 2020; Bocato et al., 2019). Then, several analytical methods based on high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) are now in routine use to assess population exposure to EDCs (Sosvorova et al., 2017; Wang et al., 2019; de Oliveira et al., 2019; Cho and Song, 2018; Martín-Pozo et al., 2020; Silveira et al., 2020; van der P van der Meer et al., 2019; Rocha et al., 2018). The analyses are carried out in plasma/serum (Sosvorova et al., 2017; Wang et al., 2019), saliva (de Oliveira et al., 2019), hair (Cho and Song, 2018), nails (Martín-Pozo et al., 2020) and urine (Silveira et al., 2020; van der P van der Meer et al., 2019; Rocha et al., 2018). However, urine better represents the body burden of EDCs and has been the matrix of choice (Silveira et al., 2020; van der P van der Meer et al., 2019; Rocha et al., 2018). On the other hand, due to ethical restrictions and the costs associated with sample transportation and storage of large volumes, the availability of higher volumes of human specimens in populations' studies are becoming much more limited. Moreover, for prospective studies, the availability of samples in biobanks rarely exceeds 1.0 mL. Then, multiclass methods allow epidemiologists/toxicologists to get much more information about population exposure in less time and with the use of available volumes of samples.

Classical methods reported in the literature for the determination of EDCs in urine samples employ solid-phase extraction or liquid-liquid extraction assample preparation procedures (van der Meer, 2018; Zhao et al., 2018; Heffernan et al., 2016; Yao et al., 2018; Bury et al., 2019). These techniques have several disadvantages, including very high consumption of solvents and samples as well as a very tedious and time-consuming extraction steps. On the other hand, miniaturized sample preparation techniques are very attractive alternatives to solve the limitations of conventional extraction methods. Among them, dispersive liquid-liquid microextraction (DLLME) seems to be an excellent choice for the analysis of EDCs in human urine (Rocha et al., 2018; Vela-Soria et al., 2014a,b; Jiménez-Díaz et al., 2016; Rocha et al., 2016; Cunha and Fernandes, 2010; Wang et al., 2013; Shen et al., 2017), since the extractions are much faster and easy to perform and with less cost. DLLME can instantly extract and pre-concentrate the analytes of interest by forming a cloud point using only microliters of a mixture of an extractor and a dispersant solvent (Dugheri et al., 2019; Rezaee et al., 2006). Although DLLME is a very suitable technique for sample preparation in large scale human biomonitoring studies, most of the previously proposed methods are based on the use of large sample volumes or with the limitation of application to one or just a few analytes (Rocha et al., 2018; Vela-Soria et al., 2014a,b; Jiménez-Díaz et al., 2016; Rocha et al., 2016; Cunha and Fernandes, 2010; Wang et al., 2013; Shen et al., 2017). In recent years, many variations of DLLME have been proposed to increase the pre-concentration capacity of trace analytes and to reduce the sample volumes (Campillo et al., 2017). Vortex-assisted dispersive liquid-liquid microextraction (VADLLME) is an up-and-coming technique in this sense since the aid of a vortex promotes an increase in surface tension of the mixture of extractor and dispersant solvents used in the technique, forming a light emulsion that facilitates the partition of compounds of interest between immiscible phases (Yiantzi et al., 2010; Sereshti et al., 2018).

In this sense, the present study aimed at the development of a fast and simple multiclass method for the simultaneous quantification of 21 EDCs in reduced amounts of human urine. The approach is based on VADLLMEand LC-MS/MS.After method validation, it was successfully applied to the determination of the 21 EDCs in the study in urine samples from a group of 50 Brazilian pregnant women to establish reference ranges.

2. Experimental

2.1. Chemical, reagents and standards solution

Standard stock solutions of 2,2-bis(4-hydroxyphenyl)propane (BPA), 4,4′-sulfonyldiphenol (BPS), 4,4′-(1-phenylethylidene)bisphenol (BPAP), 4,4′-(1,4-phenylenediisopropylidene)bisphenol (BPP), 4,4′-methylenediphenol (BPF), 2,2-Bis(4-hydroxyphenyl)hexafluoropropane (BPAF), 4,4′-cyclohexylidenebisphenol (BPZ), methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP) butylparaben (BuP), benzyl paraben (BzP), methyl protocatechuic acid (OH–MeP) ethyl protocatechuic acid (OH–EtP), 2-hydroxy-4-methoxybenzophenone (BP3), 2,4-dihydroxybenzophenone (BP1), 2,2′-dihydroxy-4-methoxybenzophenone (BP8), 2,2′,4,4′-tetrahydroxybenzophenone, (BP2), 4-hydroxybenzophenone (4-OH-BP), 5-chloro-2- (2,4-dichlorophenoxy) phenol triclosan (TCS), and 1-(4-chlorophenyl)-3-(3,4-dichlorophenyl) urea triclocarban (TCC) (all purchased from Sigma-Aldrich, St. Louis, MO, USA) were prepared at the concentration of 100 μg/mL in methanol:water (1:1, v/v). Calibration curves and work standard solutions at concentrations range of 0.1–2.0 μg/mL for TCS and TCC and 0.05–2.0 μg/mL for other EDCs were obtained by serial dilutions in the same solvent. The solution of mix internal standards was prepared in methanol at the concentration of 2.0 μg/mL, employing the analytical standards: (2,2-bis(4-hydroxyphenyl)propane-d_{16} (BPA-d_{16}),

Table 2
Within-day and Between-day Precision and accuracy for analysis of EDCs in synthetic urine.

EDCs	Nominal concentration (ng/mL)	Wihtin-day*			Between-day*,[a]		
		Obtained concentration (ng/mL)	Accuracy (%)	Precision, RSD[b] (%)	Obtained concentration (ng/mL)	Accuracy (%)	Precision, RSD[b] (%)
BPA	1.0/12.5/20.0	0.8/10.5/17.4	80/84/87	1.0/7.1/4.8	1.0/11.0/19.4	100/88/95	8.1/7.2/2.8
BPS	1.0/12.5/20.0	0.9/12.4/20.6	90/99/103	7.4/6.0/3.2	0.9/12.0/20.9	97/98/100	5.0/1.5/4.3
BPF	1.0/12.5/20.0	0.9/12.1/20.9	90/97/104	0.7/4.9/5.0	0.8/11.5/20.9	99/88/102	7.0/3.5/0.5
BPZ	1.0/12.5/20.0	0.9/12.3/21.3	92/98/106	8.6/7.4/4.8	0.9/13.7/18.9	99/104/89	5.0/4.5/4.8
BPP	1.0/12.5/20.0	0.9/12.2/18.7	91/96/94	8.0/3.8/7.0	1.0/12.4/19.1	100/98/96	3.0/1.5/4.0
BPAF	1.0/12.5/20.0	1.0/12.2/21.0	100/95/102	2.0/2.5/4.3	1.0/13.7/21.1	102/104/102	2.9/5.7/4.3
BPAP	1.0/12.5/20.0	0.8/11.3/19.0	81/80/85	2.0/2.3/4.7	0.8/11.5/18.6	82/92/93	11.0/2.3/1.5
OH–MeP	1.0/12.5/20.0	0.9/13.2/21.2	93/102/104	1.6/5.1/6.1	0.9/12.9/21.0	98/99/108	6.0/5.4/3.3
OH–EtP	1.0/12.5/20.0	1.1/12.3/21.1	102/99/104	10.0/4.3/4.7	1.1/11.2/21.2	104/88/111	5.0/4.3/4.7
MeP	1.0/12.5/20.0	1.0/12.3/20.3	100/98/100	3.0/5.6/2.1	0.9/12.2/18.6	99/97/94	3.2/5.0/5.0
EtP	1.0/12.5/20.0	1.1/12.0/21.1	101/96/102	4.3/1.5/6.0	0.9/13.5/21.3	97/109/104	5.0/6.8/1.3
PrP	1.0/12.5/20.0	0.9/12.0/21.4	94/98/101	8.4/0.6/4.6	1.1/13.7/19.1	102/102/97	8.0/7.4/4.4
BuP	1.0/12.5/20.0	1.1/12.8/21.3	102/100/103	5.0/1.1/2.7	0.8/12.2/20.7	94/98/101	5.0/3.0/2.7
BzP	1.0/12.5/20.0	0.8/11.6/18.5	98/109/105	1.0/6.1/2.6	0.7/13.4/20.2	93/108/100	11.0/6.0/0.7
BP1	1.0/12.5/20.0	0.8/10.4/17.5	74/75/78	5.6/7.2/6.3	0.8/11.2/17.9	73/76/78	8.0/9.8/0.4
BP2	1.0/12.5/20.0	0.9/13.7/18.9	98/111/93	6.9/8.2/5.2	1.1/13.7/18.8	101/105/94	4.5/7.0/6.0
BP3	1.0/12.5/20.0	1.1/12.8/21.0	100/105/102	8.0/5.0/5.0	0.9/12.3/21.0	98/100/110	5.0/2.0/5.0
BP8	1.0/12.5/20.0	0.9/12.0/21.6	96/95/104	7.5/0.7/5.2	1.2/11.9/21.9	107/93/113	10.0/7.0/4.7
4-OH-BP	1.0/12.5/20.0	1.1/12.6/18.8	100/100/88	2.3/3.0/5.1	1.0/13.6/21.3	99/112/109	2.9/6.4/4.3
TCS	1.0/12.5/20.0	0.6/11.9/19.1	95/95/96	5.0/2.8/7.0	0.9/12.2/21.1	93/93/98	6.0/3.0/5.0
TCC	1.0/12.5/20.0	0.8/9.5/16.0	75/76/77	2.0/5.0/7.0	0.9/10.1/16.6	74/73/79	10.0/4.2/1.5

*($n = 5$) for each concentration.
[a] Based on three consecutive days.
[b] RSD, relative standard deviation.

benzophenone-d_{10} (BP-d_{10}), paraben internal standards mix solution [containing methyl-4-hydroxybenzoate-$^{13}C_6$ (MeP$^{13}C_6$), ethyl-4-hydroxybenzoate-$^{13}C_6$ (EtP$^{13}C_6$) propyl-4-hydroxybenzoate-$^{13}C_6$ (PrP$^{13}C_6$) and butyl-4-hydroxybenzoate-$^{13}C_6$ (BuP$^{13}C_6$)] (all purchased from Sigma-Aldrich, St. Louis, MO, USA) and $^{13}C_{12}$-2′,4,4′-trichloro-hydroxy diphenyl)ether (TCS$^{13}C_{12}$) (purchased from Cambridge Isotope Laboratories, Andover, MA, USA). All these solutions were stored at −20 °C in polypropylene tubes and protected from direct light.

All solvents (methanol, dichloromethane, dichloroethane, trichloromethane, 2-propanol, acetone, and acetonitrile) were of HPLC grade and were obtained from J. T. Baker (Phillipsburg, NJ, USA). Synthetic urine was employing in the VADLLME optimization and validation procedure. Therefore, the following reagents were used (analytical grade): potassium chloride, sodium chloride, urea, citric acid, ascorbic acid, potassium phosphate, creatinine, sodium hydroxide, sodium bicarbonate, and sulfuric acid. For the enzymatic hydrolyze of glycuronate conjugates, the enzyme β-glucuronidase *Helix pomatia*, type HP–2, aqueous solution (≥100,000 units/mL) and ammonium acetate (analytical grade) were used. All these reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). High purity water (resistivity 18.2 MΩ cm) used in all experimental steps was obtained from a Milli-Q water purification system (Millipore RiOs-DITM, Bedford, MA, USA).

2.2. Instrumentation, analytical conditions, and sample analysis

The determination of EDCs was carried out with a Thermo Scientific LC-MS/MS system equipped with a quaternary pump (Accela 600 pump model) and an automatic sampler coupled with a triple quadrupole mass spectrometer analyzer type (model TSQ Quantum Access Max) with source ionization by electrospray (Thermo Fisher Scientific, USA). The chromatographic separation was performed with a Brownlee Aq C18 column (100 mm × 4.6 mm i. d., 5 μm particle size, PerkinElmer, Waltham, MA, USA) and carried out by gradient elution. The mobile phase was defined as follows: mobile phase A had water as the solvent, and mobile phase B consisted of methanol. A gradient program was used with a total run time of 12 min: 0.0–5.50 min 35–98% B; 5.51–9.80 min was maintained with 98% B; 9.81–10.50 min a gradient up to 35% B; 10.51–12.00 min was kept at 35% B again. The column was kept at room temperature (23 °C) and the autosampler was kept at 15 °C. The mobile phase flow was set at 500 μL/min, and the injection volume used was 10 μL. The MS/MS parameters were individually optimized for each compound by direct infusion on the mass spectrometer using a standard solution in methanol: water (1:1, v/v) at a concentration of 1.0 μg/mL. The instrument parameters were capillary tension at ±4000 V and capillary and vaporizer temperature of 222 °C and 250 °C, respectively. Nitrogen was used as a coating gas and to aid in flows of 20 and 15 arbitrary units, respectively. Argon was used as a collision-induced dissociation gas (CID) at a pressure of 1.9 mTorr. Xcalibur version 2.0 software (Thermo Fisher Scientific) was used to control instruments and data processes. Negative mode selective reaction monitoring (SRM) was employed for major EDCs, and the positive mode was employed only for BP-d_{10} internal standard. The transition channels optimized, collision energy (CE), tube lens, and retention times obtained for each EDC are described in Table S1. The precursor ions and their corresponding product ions with a dwell time of 0.4 s. Fig. S1 shows the resulting LC-MS/MS chromatograms for all analytes.

2.3. Synthetic urine preparation

Since there is no human urine exempt from the chemical compounds studied, it was necessary to prepare synthetic urine (SU) to optimize the VADLLME extraction and to perform the analytical validation. Synthetic urine preparation was carried out according to previous publications (Silveira et al., 2020; Rocha et al., 2018; Vela-Soria et al., 2014a,b; Rocha et al., 2016). To prepare 1.0 L of synthetic urine, the following reagents were employed: 7.6 g of potassium chloride, 17.0 g of sodium chloride, 49.0 g of urea, 2.06 g of citric acid, 0.68 g of ascorbic acid, 2.36 g of potassium phosphate, 2.8 g of creatinine, 1.28 g of sodium hydroxide, 0.94 g of sodium bicarbonate, and 560 μL of sulfuric acid. Reagents were mixed and dilluted in 1000 mL of mili-Q water. The synthetic urine was ultrasonicated for 30 min and stored at −4 °C until

Table 3
Matrix Effect of EDCs in synthetic urine.

EDCs	Nominal concentration (ng/mL)	Matrix Effect* (%)	RSD[a] (%)
BPA	2.5/12.5/20.0	−19.5/−19.1/−19.3	6.2/13.0/11.6
BPS	2.5/12.5/20.0	−14.0/−19.0/−15.0	1.0/1.8/1.2
BPF	2.5/12.5/20.0	−11.9/−9.6/−7.0	0.4/3.6/4.6
BPZ	2.5/12.5/20.0	−17.9/−13.8/−19.7	9.8/4.6/9.1
BPP	2.5/12.5/20.0	−10.9/−16.4/−15.4	8.6/14.7/12.0
BPAF	2.5/12.5/20.0	−13.9/−10.8/−16.5	5.6/5.6/2.2
BPAP	2.5/12.5/20.0	−24.1/−27.9/−23.1	11.7/8.1/6.7
OH–MeP	2.5/12.5/20.0	−6.8/−6.2/−6.2	2.6/2.5/2.3
OH–EtP	2.5/12.5/20.0	−1.7/−1.3/−1.3	2.5/2.4/4.0
MeP	2.5/12.5/20.0	−4.0/−4.5/−4.2	6.0/9.2/8.3
EtP	2.5/12.5/20.0	−8.1/−8.0/−7.9	4.9/5.4/5.9
PrP	2.5/12.5/20.0	−9.3/−9.0/−9.9	0.8/1.2/0.2
BuP	2.5/12.5/20.0	−8.4/−7.3/−7.9	0.9/3.8/1.1
BzP	2.5/12.5/20.0	−16.8/−10.3/−16.0	4.6/5.5/2.4
BP1	2.5/12.5/20.0	−17.3/−15.3/−17.2	7.6/5.2/4.5
BP2	2.5/12.5/20.0	−8.3/−7.7/−8.8	3.6/12.0/1.3
BP3	2.5/12.5/20.0	−1.8/−0.9/−1.4	2.9/2.5/1.4
BP8	2.5/12.5/20.0	−7.7/−8.1/−8.0	1.2/2.3/1.5
4-OH-BP	2.5/12.5/20.0	−6.7/−6.7/−7.4	0.4/2.7/1.9
TCS	2.5/12.5/20.0	−8.7/−9.2/−9.3	0.8/0.9/0.1
TCC	2.5/12.5/20.0	−9.1/−9.7/−8.2	2.2/2.4/2.9
Internal Standards			
$MeP^{13}C_6$	20.0	−2.0	13.1
$EtP^{13}C_6$	20.0	−3.4	5.4
$PrP^{13}C_6$	20.0	−4.1	2.5
$BuP^{13}C_6$	20.0	−2.5	3.9
BPA-d_{16}	20.0	−2.0	7.2
BP-d_{10}	20.0	−2.8	6.0
$TCS^{13}C_{12}$	20.0	−4.7	4.9

($n = 3$) for each concentration.
[a] RSD, relative standard deviation.

further use. The concentration of the analytes in study were below the LOD in the SU.

2.4. Sample collection and enzymatic hydrolysis

A total of 50 urine samples from pregnant women were randomly selected from a Brazilian Biomonitoring Study. The study was approved by the ethics committee of Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (CNAE: 96788518.9.1001.5403). All urine samples were kept at −80 °C until analysis.

The total concentration of EDCs present in the samples was determined after hydrolysis with an β-glucuronidase enzyme solution (Silveira et al., 2020; Rocha et al., 2016, 2018). First, the urine samples were thawed and homogenized. Then 1.0 mL of urine was transferred to a 15.0 mL conical-bottom polypropylene tube and 100 μL of enzyme solution (a 1.0 mol/L ammonium acetate solution containing 2000 units/mL of β-glucuronidase) was added to the sample. The enzyme solution was prepared daily, as previously described (Rocha et al., 2016). After that, the samples were incubated for 24 h at a temperature of 37 °C. Following this procedure, the urines were treated with a mix solution of the internal standard at a final concentration of 20.0 ng/mL and subjected to extraction by VADLLME for further analysis.

2.5. VADLLME procedure

The VADLLME procedure was carried out by using a 4.0 mL screw-capped polypropylene tube. For the real samples, after performing the enzymatic hydrolysis procedure and adding the internal standards (as described in the previous item), a dilution with 1.0 mL of aqueous solution 5% (w/v) NaCl was performed. After that, a solvent mixture of 150:250 μL dichloromethane/2-propanol (extractor/dispersant) was rapidly injected into the sample with the aid of a 1000 μL glass syringe (purchased from Hamilton, needle dimensions: 40 mm × 1.6 mm id). Due to the dispersion of the mixture of solvents droplets in the urine samples, a cloudy solution (cloud point) was formed, and the analytes were rapidly extracted. To increase analyte recovery and cloud point formation, after the solvent mixture injection step, the samples were submitted at vortex agitation for 20 s. After this step, the mixture was further centrifuged at 4000 rpm (2500×*g*) for 8.0 min at 20 °C. Then, 100 μL of the sedimented phase was collected with the aid of a 250 μL microsyringe, transferred to 1.0 mL Eppendorf® and brought to dryness in a concentrator plus speed vacuum (Eppendorf, Hamburg, Germany). Subsequently, the residue was solubilized in 100 μL of the mobile phase and injected into the LC-MS/MS system. For the analytical validation procedure, the same extraction procedure was performed in synthetic urine (except the enzyme hydrolysis step). Fig. S2 shows the schematic VADLLME procedure.

2.6. Method validation

Analytical figures of merit as linearity, LOD, LOQ, precision, and accuracy had been validated as next as possible according to the Food and Drug Administration bioanalytical method recommendations (FDA, 2018). Additionally, the matrix effect was evaluated as described previously (Asimakopoulos et al., 2014a, 2014b; Matuszewski et al., 2003; Panuwet et al., 2016). Isotopically labeled internal standards were used to achieve better reproducibility in the analyzes.

The linearity of the method was performed in fivefold replicate at 7 concentration levels in the range of 0.5–20.0 ng/mL for all EDCs except to TCS and TCC that the range was 1.0–20.0 ng/mL. This concentration range for the analytes was chosen according to results previously obtained and reported in the literature (Silveira et al., 2020; Rocha et al., 2016, 2018). Since the residual of the analytical curve showed a heteroscedasticity behavior, their results were weighted by $1/y^2$ (Almeida et al., 2002). Calibration curves using VADLLME-LC-MS/MS were obtained by weighted analysis of the ratio between the analytes and the internal standard peak areas using seven concentration levels in triplicate. For this, the analytical curves were obtained of the VADLLME procedure employing aliquots of 1.0 mL synthetic urine spiked with 20 μL calibration curve solutions of EDCs and 20 μL the mix of the internal standards (IS) (whose final concentration of 20.0 ng/mL for each IS). The LOQ and LOD were estimated as the concentration at a signal-to-noise ratio of 10 and 3, respectively, with relative standard deviation (RSD%) lower than 20%.

The precision and an accuracy assay were evaluated by spiking the synthetic urine at three concentrations for each EDCs (1.0, 12.5, and 20.0 ng/mL) in fivefold replicate ($n = 5$) on the same day (within-day) and in three consecutive days (between-day). The results of the precision and accuracy of the method were expressed as relative standard deviation (RSD, %) and percentage of recovery (%), respectively.

Nonspecific selectivity (matrix effect) in the ionization of EDCs by MS was evaluated ($n = 3$) employing three concentrations (2.5, 12.5, and 20.0 ng/mL). In this study, synthetic urine blanks were subjected to extraction by VADLLME, and after that, the decanted phase was dried, and the residue fortified with 20 μL EDCs standard solution and 20 μL internal standard mix ("post-extraction matrix spiked"). These samples were injected into LC-MS/MS, and their areas were monitored. Simultaneously, pure standard solutions of EDCs and IS at the same concentrations and amount were analyzed (Asimakopoulos et al., 2014b; FDA, 2018). The results were expressed as a percentage of matrix effect and as relative standard deviation (RSD, %).

For additional validation purposes, 10 ordinary urine samples were

Table 4
Comparison between DLLME methods in human urine in the literature.

Methods	EDCs analyzed and number of classes of EDCs	Sample volume (mL)	Final volume of solvents	Time dispensed on sample preparation	Running time (min)	LOD (ng/mL)	Total time (min)	Reference
DLLME and CG–MS/MS	MeP, EtP, IsoPrP, PrP, IsoBuP, BuP, BP1, BP2, BP3, BP6, BP8, 4–OH–BP, BPA and, BPS (14 analytes, 3 classes)	5.0	1250 μL (750 μL of acetone 500 μL of trichloromethane)	10 s of extraction + 20 min of centrifugation	26	0.04–0.20	46.1	Vela-Soria et al. (2014a)
AALLME (Air-assisted liquid-liquid Microextraction) and LC–MS/MS	MeP, EtP, PrP, BuP, OH–MeP, OH–EtP, BzP, BPA, BPS, BPF, BPZ, BPAP, BPP, BPAF, TCC, TCS, BP1, BP3, BP8, and, 4–OH–BP (21 analytes, 4 classes)	5.0	750 μL of dichloroethane	30 s of extraction + 20 min of centrifugation	10	0.01–0.30	30.3	Rocha et al. (2018)
DLLME and UHPLC–MS/MS	MeP, EtP, PrP, BuP, BP1, BP2, BP3, BP6, BP8, 4–OH–BP and, BPA (11 analytes, 3 classes)	5.0	1250 μL (750 μL of acetone and 500 μL of trichloromethane)	10 s of extraction + 25 min of centrifugation	10	0.20	35.1	Jiménez-Díaz et al. (2016)
DLLME and UHPLC–MS/MS	MeP, EtP, BuP, BP1, BP2, BP3, BP6, BP8, 4–OH–BP, BPA, monochloro–, dichloro–, trichloro– and tetrachloro–BPA, BPS (15 analytes, 3 classes)	5.0	1250 μL (750 μL of acetone and 500 μL of trichloromethane)	10 s of extraction + 20 min of centrifugation	10	0.03–0.20	30.1	Vela-Soria et al. (2014b)
LLE and LC–ESI–MS	BP, BP1, BP2, BP8, 4-OH-BP, BADGE, BADGE·H2O, BADGE·2H2O BADGE·HCl·H2O, BADGE·HCl, MeP, EtP, PrP, BuP, BzP, HeptP and OH–EtP, TCS, TCC (19 analytes, 4 classes)	0.5	3000 μL of ethyl acetate	60 min extraction + 10 min of centrifugation (samples were extracted 3 times)	30	Not provided	110	Asimakopoulos et al. (2014b)
LLE and LC–ESI–MS/MS	MeP, EtP, PrP, BuP, BzP, HeP, *p*-HB (7 analytes, only one class)	0.5	3000 μL of ethyl acetate	60 min extraction + 5 min of centrifugation (samples were extracted 3 times)	17.5	Not provided	217.5	Wang et al. (2013)
LLE and LC–ESI–MS/MS	BPA, BP1, BP2, BP3, BP8, 4-OH–BP, MeP, EtP, PrP, BuP, BzP, TCS, 8–OHdG (13 analytes, 4 classes)	2	3000 μL of ethyl acetate	10 min extraction + 10 min of centrifugation (samples were extracted 2 times)	15	0.01–0.23	95	Chen et al. (2019)
SPE–LC–ESI–MS/MS	MeP, EtP, PrP, BuP and BzP BPA, TCS, 8-OHdG (8 analytes, 3 classes)	4			43	61.0		Ren et al. (2016)
SPE–UPLC–ESI–MS/MS	MeP, EtP, PrP, BuP; BP3, MEP, MiBP, MnBP, 5–OH–MEHP, 5–oxo-MEHP, MBzP, MEHP (12 analytes, 3 classes)	3			20	0.09–0.37		Dewalque et al. (2014)
SPE–APCI–LC–MS	BPA, BP3, MeP, EtP, PrP, BuP, TCC, t–OP, OPP, 2,4-DCP, 2,5–DCP, 2,4,5–TCP, 2,4,6–TCP (14 analytes, 7 classes)	1			20	0.1–1.0		Gavin et al., 2014
Continuation of Table 4								
VADLLME and LC–MS/MS	MeP, EtP, PrP, BuP, OH–MeP, OH–EtP, BzP, BPA, BPS, BPF, BPZ, BPAP, BPP, BPAF, TCC, TCS, BP1, BP3, BP8, and, 4–OH–BP (21 analytes, 4 classes)	1.0	400 μL (150 μL of dichloromethane and 250 μL of 2–propanol)	20 s of extraction + 8 min of centrifugation	12	0.01–0.20	20.2	Present method

BADGE: bisphenol A diglycidyl ether; BADGE·H2O: bisphenol A (2,3-dihydroxypropyl) glycidyl ether; BADGE·2H2O: bisphenol A bis (2,3-dihydroxypropyl) ether; BADGE·HCl·H2O: bisphenol A (3-chloro-2-hydroxypropyl) (2,3-dihydroxypropyl) ether; BADGE·HCl: bisphenol A (3-chloro-2-hydroxypropyl) glycidyl ether; HeptP: heptyl parabéns; OH–EtP: ethyl-protocatechuate; MEP: Monoethyl phthalate; MiBP: Mono-iso-butyl phthalate; MnBP: Mono-n-butyl phthalate; 5-OH-MEHP: Mono-2-ethyl-5-hydroxyhexylphthalate; 5-oxo-MEHP: Mono-2-ethyl-5-oxohexylphthalate; MBzP: Monobenzyl phthalate; MEHP: Mono-2-ethylhexylphthalate; t-OP: 4-tert-octylphenol; OPP: ortho-phenylphenol; 2,4-DCP: 2,4-dichlorophenol; 2,5-DCP: 2,5-dichlorophenol; 2,4,5-TCP: 2,4,5-trichloropheno; 2,4,6-TCP: 2,4,6-trichlorophenol.
[a]The "total time" was considered as the time dispensed to analyze 1 sample.

analyzed by the proposed procedure and by using a previously published method (Rocha et al., 2018) (see Table S2).

3. Results and discussion

3.1. Chromatographic separation

The chromatographic separation described in this work was initially based on the method of Rocha et al. (2018), with some modifications.

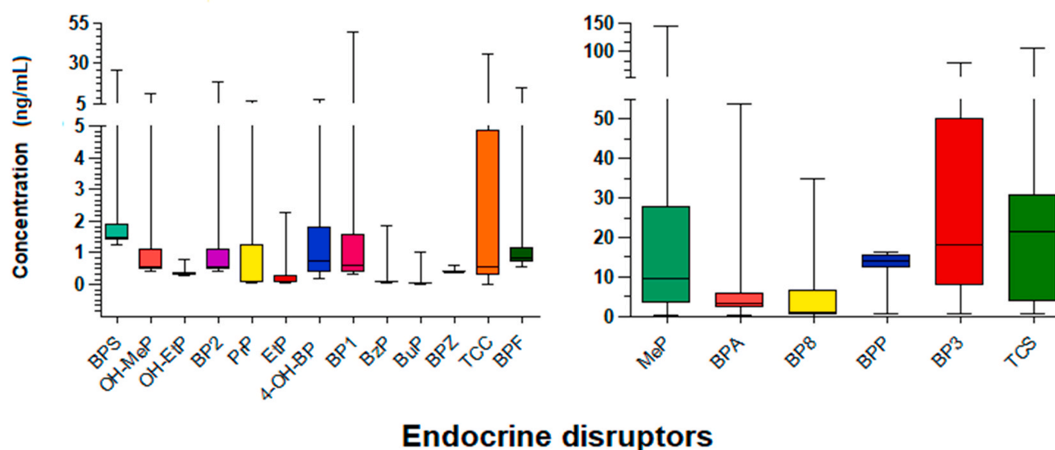

Fig. 1. Urinary concentrations of EDCs (ng mL^{-1}) in Brazilian pregnant women. The horizontal lines represent 10th, 50th and 90th percentiles and the boxes represent 25th and75th percentiles.

Table 5
Concentrations of endocrine-disrupting chemicals (EDCs) in the urine o Brazilian pregnant urine samples.

EDCs	Detection Rate (%)	Geometric Mean (ng/mL)	Aritmetic Mean (ng/mL)	Minimum (ng/mL)	Maximun (ng/mL)
BPA	98	3.0	6.2	< LOQ	53.8
BPS	17	1.8	2.5	< LOQ	11.2
BPF	64	1.0	1.5	< LOQ	15.2
BPZ	50	0.4	0.40	< LOQ	0.6
BPP	18	12.3	13.1	< LOQ	16.3
BPAF	0	–	–	–	–
BPAP	0	–	–	–	–
OH–MeP	96	0.8	1.1	< LOQ	11.2
OH–EtP	83	0.4	0.4	< LOQ	0.8
MeP	100	8.9	23.2	0.24	145.0
EtP	86	0.1	0.3	< LOQ	2.3
PrP	94	0.2	0.9	< LOQ	7.2
BuP	62	0.1	0.1	< LOQ	1.0
BzP	7	0.1	0.1	< LOQ	1.9
BP1	98	0.9	2.5	< LOQ	49.6
BP2	66	0.8	1.7	< LOQ	18.8
BP3	88	15.9	27.2	< LOQ	80.6
BP8	3	0.8	4.3	< LOQ	34.7
4–OH–BP	74	0.8	1.5	< LOQ	7.7
TCS	38	11.7	23.5	< LOQ	106.5
TCC	78	1.0	4.7	< LOQ	35.8

Retention times are similar only for the isotopically labeled internal standards and their respective EDCs (see Fig. S1).

3.2. VADLLME optimization

The main parameters that influence VADLLME procedure were optimized: types of dispersant and extractor solvents, dispersant and extractor solvent volumes, ionic strength and vortex assisted stirring time (Yiantzi et al., 2010; Sereshti et al., 2018). All steps were performed in three replicates (n = 3) by the addition of 20 μL of a mixture of each analyte studied in 1.0 mL of synthetic urine whose the final concentration for each analyte were of 20.0 ng/mL. Additionally, each tube was added to 1.0 mL of deionized water. The final pH was close to 6.0 (which is the physiological urine pH), totaling a final volume of 2.0 mL in the extraction tube. The results obtained were expressed as graphs of the areas of the analytes obtained regarding the parameter evaluated.

3.2.1. Dispersant and extractor solvent selection

The first optimized parameter was the type of dispersant solvent. This solvent should be soluble in the extractor solvent and the sample, allowing dispersion of the organic phase into the aqueous phase (forming the cloudy solution known as the "cloud point") and thus facilitating the mass transfer of the analytes to the organic phase (Rezaee et al., 2006; Campillo et al., 2017; Yiantzi et al., 2010; Sereshti et al., 2018). The type of dispersant solvent was optimized using trichloromethane as the extractor solvent (100 μL), and the solvents evaluated were: methanol, acetonitrile, acetone, and 2-propanol (300 μL). As can be seen in Fig. S3A, the best dispersant solvent for the extraction of most EDCs into synthetic urine was 2-propanol. Similarly, now fixing 2-propanol as a dispersant solvent (volume of 300 μL), the type of extractor solvent was evaluated. In conventional DLLME, the extractor solvent used were traditionally the chlorinated solvents and they have a higher density value than water, so that it is possible to keep the extracted analytes in the sedimented phase and easily collect them after centrifugation (Rezaee et al., 2006; Li et al., 2015; Saraji and Boroujeni, 2014). This solvent should also be able to extract the analytes of interest and have low water solubility (Rezaee et al., 2006). The following extractor solvents were evaluated: dichloroethane, dichloromethane, and trichloromethane, employing a volume of 100 μL each. As can be seen in Fig. S3B, dichloromethane was the best extractor solvent for the extraction of the analytes.

3.2.2. Dispersant and extractor solvent volume

After the determination of the solvent mixture (dispersant/extractor), their volumes were evaluated, starting with the volume of the dispersant solvent optimization. For this, 100 μL dichloromethane was used, and the volume of 2-propanol was varied in the following range: 150 μL, 250 μL, 300 μL, and 400 μL. The best result was obtained using 250 μL 2-propanol (Fig. S4A). Increasing the volume of the dispersant solvent leads to an improvement in the dispersion of analytes in the organic phase, which facilitates cloud point formation, and thus extraction. However, this process continues to a specific volume. From then on, an increase in the volume of the dispersant solvent leads to a loss in the solubility of the analytes in the extractor phase and thus decreasing their extraction (Rezaee et al., 2006; Li et al., 2015). This behavior was observed for all EDCs except to triclosan and triclocarban that showed no significant differences with the dispersant solvent variation. After fixing the dispersant solvent volume to 250 μL, the extractor solvent volume was optimized by varying the dichloromethane volume from 75 to 250 μL (Fig. S4B). In DLLME, the volume of the extractor solvent should be enough to extract the analyte and, whenever possible, the smallest volume should be chosen, thus obtaining a high enrichment factor with less use of organic solvents (Psillakis, 2019). Except for bisphenols that did not show a significant change between 100 and 150 μL extractor solvent volume, the best volume for most EDCs was 150 μL

of dichloromethane (Fig. S4B). Therefore, 150 μL was the volume chosen for further studies.

3.2.3. Ionic strength influence

The salting-out effect was also evaluated. This effect is caused by the addition of an inert soluble salt in aqueous samples, which leads to a solvation process of water molecules, leaving the organic compounds freer in the medium and, therefore, more available to reach the extraction phase (Martins et al., 2012). In DLLME, this effect can improve analyte recovery and the detectability of the method. The effect of sodium chloride addition on the extraction of EDCs in synthetic urine was studied by preparing samples with concentrations of 0–20% (w/v) of this salt. As can be seen in Fig. S4C addition of salt increased the analyte recoveries for most ECDs studied, except for bisphenols where the presence of a higher amount of salt (higher than 5%) caused a decrease in their extraction (Fig. S4C). The appropriate salt concentration to continue the next experiments was defined as 5% (w/v).

3.2.4. DLLME assisted by vortex agitation

Conventional DLLME considers that the mass transfer of analytes from the aqueous phase (matrix) to the organic phase (extractor solvent) occurs in cloud point formation by rapidly injecting the mixture of organic solvents with the aid of a syringe (Rezaee et al., 2006; Martins et al., 2012). However, this process sometimes do not promote very fine droplets of the organic solvents in the sample. In this case, the effect of a vortex stirrer on immiscible liquids at high speeds results in the organic phase rupturing into finer droplets. This procedure increases the surface area of extractor solvent and promote the extraction of the analytes to equilibrium faster due to the shorter diffusion distance (Yiantzi et al., 2010; Saraji and Boroujeni, 2014) and so, affect analytes recoveries. Thus, the vortex agitation time was evaluated from 0 to 90 s, using a speed of 1200 rpm (Fig. S4D). Lower speeds were not shown due to the low dispersibility of the droplets into the matrix. It was also observed that higher speeds than 1200 rpm caused foaming in human urine, which impaired the formation of solvent droplets. As can be seen in Fig. S4D, the mass transfer equilibrium was obtained within 20 s of agitation for most EDCs. Only the bisphenol class continued to increase in recovery over time. However, to reduce the time spent in the sample preparation procedure, 20 s was chosen as the optimum vortex agitation extraction time.

The final conditions established for VADLLME in the analysis of 21 EDCs in synthetic urine were: 2-propanol as dispersant solvent (250 μL) and dichloromethane extractor solvent (150 μL) employing 1.0 mL synthetic urine plus 1.0 mL 5% (w/v) NaCl solution. Vortex agitation for 20 s at 1200 rpm. After, the extraction tubes were submitted at centrifugation for 8.0 min at 2500×*g* at a temperature of 20 °C. Finally, the recoveries were also achieved. As can be seen in Table 2, the recoveries (expressed as accuracy) for all EDCs range is 73–111%, and the standard deviation was below 15%.

3.3. Method validation

Analytical figures of merit were obtained using VADLLME-LC-MS/MS in synthetic urine. The following parameters were obtained: linearity, detection limit (LOD), and limit of quantification (LOQ), within- and between-day precision and accuracy and matrix effect.

The proposed method presented linearity over the concentration range of 0.5–20.0 ng/mLfor all EDCs except for the TCS and TCC that was 1.0–20.0 ng/mL, with correlation coefficients (r) higher than 0.994. For all cases, the accuracy and relative standard deviation for each point were lower than 15%, respectively (Table 1). The results indicated good reproducibility between the measurements for all EDCs concentrations in the calibration curve. Based on that, the validation method was realized in synthetic urine. In the present method, the limit of detection (LOD) is defined as the signal-to-noise (S/N) ratio of 3 and the (LOQ) by a signal-to-noise (S/N) ratio of 10. The obtained results for LOD and LOQ are also shown in Table 1. LOQs range from 0.02 to 0.50 ng/mL, and the LODs ranged from 0.01 to 0.20 ng/mL. The LOQs and LODs achieved in this method are similar to those previously reported in other studies that employed (U)HPLC-MS/MS and DLLME (Rocha et al., 2016, 2018; Jiménez-Díaz et al., 2016; Vela-Soria et al., 2014b) and inother previous studies that employed conventional extraction techniques such as LLE (Chen et al., 2019; Asimakopoulos et al., 2014b) and SPE (Ren et al., 2016; Dewalque et al., 2014).

The precision is the parameter that evaluates how closely the results obtained from independent samples are, and accuracy demonstrates the agreement of the obtained result and the real value. For within-day precision and accuracy, three concentrations were evaluated (1.0, 12.5, and 20.0 ng/mL), and the results obtained were expressed in Table 2. The same 3 concentrations were evaluated for determination of between-day precision and accuracy for 3 consecutive days, and the results are shown in Table 2. The results obtained for accuracy and precision agree with the validation guide for bioanalytical methods (FDA, 2018), with the RSD remaining below 15%.

The matrix effect on analytes ionization was also estimated, and the results were summarized in Table 3. The matrix effect was found for all proposed EDCs with a range of 0.9 at 27.9% with BzP (16.8%), BP1 (17.2%) and bisphenols class presenting the higher values (16.5–27.9%) while the other EDCs presented no significant matrix effect (<10.0%). The negative values in Table 3 showed that occurred a little ionization suppression, a matrix effect type that is prevalent when complex matrices are evaluated (such biological fluids, foods, for example), mainly because of presence of its endogenous compounds that may compete with the analytes in the ionization source and reduce the mass intensity values in the instrument of analysis (Panuwet et al., 2016). It is essential to highlight that only one of the DLLME methods for multiclass EDCs found in the literature (Table 3) mention matrix effects and did not report the suppression values obtained. In other studies that employed conventional extraction techniques (LLE and SPE) the evaluation of matrix effect is also uncommon, but Asimakopoulous et al. (2014b) reported signal suppression, whereas Chen et al. (2019) and Gavin et al. (2014) reported non-significant matrix effects. Although the proposed method had shown an ionization suppression, the use of isotopically labeled internal standards ensured a good reproducibility (Matuszewski et al., 2003; Panuwet et al., 2016) between analyzes, and the method is then reliable and acceptable for routine human biomonitoring studies.

For additional validation purposes, 10 ordinary urine samples were analyzed by the proposed procedure and by using a previously published method (Rocha et al., 2018). Obtained results were in good agreement between the methods with no statistical differences (*t*-test, 95%, Table S2).

3.4. Comparison of analytical figures of merit between the proposed method and previous reports

Analytical figures of merit obtained with the proposed procedure and previously published multiclass methods for EDCs determination in urine samples is summarized in Table 4 (Rocha et al., 2018; Vela-Soria et al., 2014a,b; Jiménez-Díaz et al., 2016; FDA, 2018; Wang et al., 2013; Shen et al., 2017; Dewalque et al., 2014; Gavin et al., 2014; Chen et al., 2019; Ren et al., 2016; Asimakopoulos et al., 2014b). As can be seen, the present method, in general, provides the following advantages: i) requires lower sample volumes; (ii)-lower consumption of organic solvents which generates a smaller volume of residues; (iii)-similar or lower LODs; (iv)- reduced total time of analysis (approximately 20 min per sample); (v)- multiclass analysis with simultaneous determination of 21 EDCs. The lower sample volumes required may add additional advantages since the method can be applied to the analysis of samples stored in biobanks (prospective studies). Moreover, it reduces the costs for transportation and storageof samples (−80 °C freezers) of large-scale human biomonitoring studies (hundreds to thousands of samples).

3.5. Analysis of EDCs in brazilian pregnant urine samples for establishing reference ranges

After validation, the proposed method was employed for the analysis of 50 urine samples collected from Brazilian pregnant with 20–35 years old. The urinary dispersion in the concentrations of each EDCs in Brazilian pregnant humans issummarized through the boxplot graphic in Fig. 1. The geometric and arithmetic means, minimum and maximum concentration values, as well as the percentage of the samples in which they were found, are shown in Table 5. In the bisphenol class, BPA, BPF, and BPZ were found in more than 50% of the samples. The BPA was the one with the highest prevalence, detected in 98% of the samples, and with a very high variation in the concentrations into the pregnant population (range of 0.08–53.75 ng/mLand the geometric mean of 2.96 ng/mL). BPF and BPZ were detected in 64 and 50% of the samples, respectively, with geometric means of 1.01 (BPF) and 1.09 ng/mL (BPZ). These results also demonstrated that BPA is still the most widely used bisphenol by the industry, but the BPA analogs are already appearing on a large scale of products. An interesting result from the bisphenol class was found for bisphenol P, which, although it appeared in only 18% of the evaluated samples, the geometric mean proved to be the highest at a value of 12.25 ng/mL. For the parabens class, all were detected, with the highlights for MeP, EtP, PrP, and the metabolites OH–MeP and OH–EtP, which appeared in almost all samples evaluated. MeP is the most used in the cosmetic and pharmaceutical industry and was detected in 100% of the samples, with a very significant variation with a concentration range of 0.24–145.0 ng/mL. Among benzophenones, BP1 and BP3 were widely found, BP2 was also found in 66% of the samples, and the geometric means for BP1, BP2, and BP3 were 0.85, 0.81, and 15.95 ng/mL, respectively. Finally, TCS and TCC antimicrobials, extensively used in personal care products, were also detected, with TCC being the most expressive (78% of samples). Some EDCs found in higher concentrations for pregnant women compared to Brazilian children (Rocha et al., 2018) and adult men (Vela-Soria et al., 2014b) may be related to the fact that women use cosmetics on a larger scale, demonstrating that the female population may be more exposed to EDCs.

4. Conclusion

The multiclass proposed method (21 EDCs in urine) is simpler and faster when compared with other previously DLLME methods reported for endocrine disruptors determination in urine. Moreover, it requires only 1.0 mL of sample. These advantages result in a suitable attractive alternative methodology for large scale human biomonitoring studies (reduction of costs of sample storage, transportation) orin prospective studies of a small volume of samples stored and available in biobanks.

Author statement file

Mariana Zuccherato Bocato: Conceptualization, Data curation, Formal analysis, Investigation; Methodology, Roles/Writing - original draft . Cibele Aparecida Cesila: Data curation, Formal analysis, Investigation, Beatriz Favero Lataro: Data curation, Formal analysis, Investigation, Anderson Rodrigo Moraes de Oliveira: Resources, Writing - review & editing. Andres Dobal Campíglia: Resources, Writing - review & editing. Fernando Barbosa Jr: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.109883.

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