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Determination of bisphenols, parabens, and benzophenones in placenta by dispersive liquid-liquid microextraction and gas chromatography-tandem mass spectrometry

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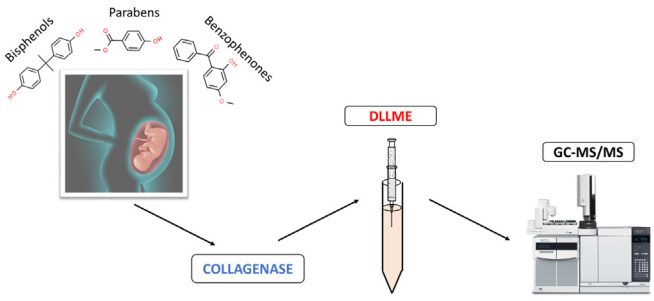
HIGHLIGHTS

- First DLLME procedure applied to placenta samples.
- Enzymatic liquefaction for enabling extraction of the target compounds by DLLME.
- Use of chemometric strategies to optimize experimental parameters.
- The analytical method provides significant operational improvements.

GRAPHICAL ABSTRACT



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ABSTRACT

Human exposure to endocrine disrupting chemicals (EDCs) is of particular concern during development. Bisphenols, parabens, and benzophenones are EDCs widely used in the manufacture of numerous goods, personal care products, and cosmetics. The aim of this study was to develop a new and practical method for determining three bisphenols, four parabens, and five benzophenones in placenta samples. It uses dispersive liquid-liquid microextraction (DLLME) in combination with gas chromatography-tandem mass spectrometry (GC-MS/MS). Several chemometric approaches were employed to optimize the experimental parameters. Limits of detection ranged from 0.04 to 0.08 ng g^{-1} and inter-day variabilities (evaluated as relative standard deviation) from 4.2% to 13.4%. The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. Recovery percentages ranged from 87.1% to 113.2%. Finally, the method was used to measure target compounds in 20 placental tissue samples from voluntary donors. This analytical procedure can provide information on the exposure of the fetus to non-persistent EDCs.

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1. Introduction

Expansion of the chemical industry over past decades has led to the ubiquitous environmental presence of anthropogenic endocrine disrupting chemicals (EDCs), whose interference with the action of hormones can increase or decrease the normal activity of the human endocrine system (Schug et al., 2016). Given the involvement of this system in all maturation and regulation processes, the high prevalence of EDCs can have a significant health impact. For instance, epidemiological studies have associated exposure to EDCs with reproductive alterations, behavioral disorders, neurodevelopment deficiencies, and an increased risk of cancer, among other diseases (Kortenkamp et al., 2011; World Health Organization, 2012; Mocarelli et al., 2011; Kumar et al., 2010). The effect of this exposure on hormonal pathways poses even greater risks during the critical period of development.

Numerous EDCs are known to pass from the mother to fetus *via* the placenta, including persistent EDCs to which she has been exposed in the past and non-persistent EDCs to which she has recently been exposed (Dassayanake et al., 2009; Chen et al., 2017). These chemicals have been detected in fetal blood circulation and tissues; therefore, the biomonitoring of their concentrations in placental tissue is crucial for evaluating their health effects in epidemiological studies (Fernández et al., 2014, 2016; Nelson et al., 2019).

Among the large number of known EDCs (The Endocrine Disruptor Exchange, 2020), the present study focuses on three groups: 1) bisphenol A (BPA) and its congeners bisphenol S (BPS) and bisphenol F (BPF); 2) benzophenones such as benzophenone 3 (BP-3) and benzophenone 1 (BP-1), among others; and 3) parabens, including methyl (MPB), ethyl (EPB), propyl (PPB), and butyl (BPB) paraben. All of these compounds are practically omnipresent in daily life. Bisphenols are the main monomers used in the manufacture of a wide range of goods based on epoxy resins and/or polycarbonate plastics, including toys, beverage/food packaging, domestic/leisure products, clothing, medical devices, and office furniture, among others (Ballesteros-Gómez et al., 2009). Benzophenones are included in sunscreens as UV-filters and in a range of cosmetics and personal care products (PCPs), and some are incorporated in plastics and food-packaging to inhibit light-induced degradation (European Commission, 2009, 2011). Parabens are extensively employed as preservatives in PCPs and in some processed foods and beverages (Andersen, 2008; Soni et al., 2005). Over the past decade, international regulations have been tightened to minimize the exposure of children and newborns to these xenobiotics. Thus, the EU prohibited the use of BPA in plastic infant feeding bottles (European Commission, 2011b, 2011c), and the European Food Safety Authority reduced its Tolerable Daily Intake (TDI) to 4 μg kgb_w day (EFSA, 2015), while Denmark outlawed the presence of parabens in PCPs for children (European Commission, 2011d) and the EU partially banned the use of long alkyl chain parabens (PPB and BPB) in these products (European Commission, 2014). In addition, the European Human Biomonitoring Initiative HBM4EU (https://www.hbm4eu.eu) was launched to coordinate and improve evidence on the actual exposure of European to prioritized chemicals (e.g., bisphenols and benzophenones) and its impact on human health.

Placental tissue is a complex biological matrix that requires special treatment to detect xenobiotics, normally present at very low concentrations. Biomonitoring and large-scale epidemiological studies require analytical techniques that are not only accurate and sensitive but also inexpensive, rapid, easy to deploy and feasible in laboratories with only basic equipment. Over the past decade, several methods have been developed to measure non-persistent chemicals (e.g., bisphenols, parabens, and benzophenones) in placenta. Initial proposals were based on classic liquid-liquid extractions (LLE) and ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (Jiménez-Díaz et al., 2010, 2011; Vela-Soria et al., 2011), followed by methods that combine lyophilization with matrix solid phase dispersion (MSPD) (Vela-Soria et al., 2014, 2015) or ultrasound assisted extraction (UAE) with dispersive solid phase extraction (d-SPE) (Vela-Soria et al., 2017). These procedures all offer low limits of detection (LODs) and reliable measurements; however, they require relatively long extraction times, large volumes of solvent, and/or the use of specific devices, reducing their practicality and usefulness for large-scale biomonitoring.

Dispersive liquid-liquid microextraction (DLLME), first described by Rezaee et al. (2006), may represent a useful option for determining the concentration of EDCs in placenta samples. The very short extraction times associated to DLLME together with other advantages (e.g., reduced amount of extraction solvents and increased enrichment factors) has led to the widespread application of DLLME to determine organic compound concentrations in aqueous samples (Saraji and Boroujeni, 2014; Sajid, 2018; Sajid and Alhooshani, 2018). In solid and semi-solid matrices, however, its usefulness is limited by the need to combine it with other extraction techniques (e.g., SPE, UAE, supercritical fluid extraction (SFE) or accelerated solvent extraction (ASE)) (Sajid and Alhooshani, 2018), increasing the extraction time and solvent consumption and thereby reducing its practicality. DLLME has rarely been applied to determine EDCs in biomonitoring studies (Adoamnei et al., 2018a, 2018b) and, to the best of our knowledge, it has never been used to analyze bisphenols, parabens, or benzophenones in placenta samples.

With this background, the objective of this study was to develop a method for the determination of non-persistent chemicals (3 bisphenols, 4 parabens, and 5 benzophenones) in placental tissue by applying DLLME in combination with GC-MS/MS, overcoming the obstacle of the semi-solid state of the placental tissue by using an enzymatic liquefaction process. The proposed procedure was applied to 20 placenta samples from anonymous donors.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system (Millipore, Bedford, MA). Bisphenols (bisphenol A, (BPA), bisphenol S (BPS), bisphenol F (BPF) and labeled deuterium bisphenol A (BPA-D_{16})), parabens (methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB), butylparaben (BPB), methylparaben ring $^{13}C_6$ labeled (MPB-$^{13}C_6$), ethylparaben ring $^{13}C_6$ labeled (EPB-$^{13}C_6$), propylparaben ring $^{13}C_6$ labeled (PPB−$^{13}C_6$) and butylparaben ring $^{13}C_6$ labeled (EPB-$^{13}C_6$)), and benzophenones (benzophenone-1(BP-1), benzophenone-2 (BP-2), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8), 4-hydroxybenzophenone (4-OH-BP), and labeled deuterium benzophenone-3 (BP-3-D_5)) were purchased from Sigma-Aldrich (Madrid, Spain). The purity of these compounds was higher than 99%. Stock standard solutions of compounds (200 mg L^{-1}) were prepared in acetonitrile and stored at 4 °C in the dark. The solutions were stable for at least four months. Working standards were prepared by mixing and dilution with acetonitrile. A set of solutions from 0.005 to 1.0 mg L^{-1} were prepared for calibration and validation purposes.

Acetonitrile (HPLC-grade), ethyl acetate, and trichloromethane (TCM) were purchased from Merck (Darmstadt, Germany).

Phosphate buffer saline (PBS), sodium chloride, calcium chloride, and N,O-Bis(trimethylsilyl)trifluoro-acetamide with trimethylchlorosilane (BSTFA/1% TCMS) were purchased from Sigma-Aldrich (Madrid, Spain). Hydrated zinc acetate, polyhydrated phosphotungstic acid, and glacial acetic acid were supplied by Sigma-Aldrich, and a mixture of these substances (0.91 g, 0.55 g, and 0.60 mL, respectively) was dissolved in 10.0 mL of deionized water, yielding a fat/protein precipitation solution (FPS) that was prepared immediately before its utilization.

Collagenase type-I from *Clostridium histolyticum* was supplied by Sigma-Aldrich. The enzymatic solution was prepared immediately before its utilization by dissolving 1 mg of enzyme powder in 10 mL PBS (0.01 M, pH = 7.4) containing 5 mM Ca^{2+}.

2.2. Instruments and software

GC–MS/MS analysis was performed using an Agilent 7890 GC (Agilent Technologies, Palo Alto, CA, USA) with split-splitless inlet and 7693 ALS autosampler. The detector was an Agilent 7000D triple quadrupole mass spectrometer with inert electron-impact ion source, operated in SRM mode. Electron impact (EI) ionization was set at 70 eV. Agilent MassHunter B.03.02 software was used for instrument control and data analysis and Statgraphics Centurion XVI 16.0.07 (Manugistics Inc., Rockville, MD) for statistical analyses. A B-400 mixer (BÜCHI Labortechnik AG, Switzerland) was employed to homogenize placenta samples, and a Hei-MIX incubator 1000 (Heidolph Instruments GmbH & CO, Germany) was used for the enzymatic treatment.

2.3. Sample collection and storage

Human placental tissue samples were collected from 20 volunteers at delivery (San Cecilio University Hospital, Granada, Spain). All volunteers signed their informed consent to donate their placenta, and the study was approved by the institutional ethical committee of the hospital. Each placenta was examined and accurately weighed. Representativeness of the whole placenta was achieved by placing half of it (including maternal and fetal sides and central and peripheral parts) in the glass container of a mixer for homogenization. Once homogenized, 35-g aliquots were coded and stored at −86 °C until chemical analysis.

2.4. Preparation of spiked samples

A pool of two homogenized placenta samples was spiked at 10.0 ng g^{-1} for the optimization process. A placenta pool was also spiked at different concentrations between 0.1 and 20.0 ng g^{-1} for calibration and validation purposes. Spiking was done by adding 10 μL of the corresponding solution (see 2.1 section) to 0.5-g aliquots of pooled sample. Regarding mass-labeled internal standards, samples were spiked at 10.0 ng g^{-1} with 10 μL of a solution containing BPA-D_{16}, MPB-$^{13}C_6$, EPB-$^{13}C_6$, PPB-$^{13}C_6$, BPB-$^{13}C_6$, and BP-3-D_5 at 0.5 mg L^{-1}.

2.5. Sample liquefaction by enzymatic treatment

After placing 0.5 g of homogenate in a polypropylene centrifuge tube, 1 mL of collagenase solution was added, and the mixture was incubated at 37 °C for 4 h, obtaining a completely liquid sample.

2.6. Sample treatment

2.6.1. Protein and lipid denaturation

1.0 mL acetonitrile and 50 μL FPS were added to the liquefied sample, which was then vortexed for 30 s and centrifuged for 5 min at 5000 rpm; next, the supernatant was transferred to a conical glass tube and diluted with 10.0 mL of 6% NaCl aqueous solution (*w/v*), pH 2.

2.6.2. DLLME procedure

For DLLME, the prepared aqueous sample was rapidly injected with 1.2 mL TCM using a syringe. After manual shaking for 30 s and centrifugation for 10 min at 4000 rpm, the whole sedimented phase was transferred to a glass vial and the organic phase was dried under a nitrogen stream. The residue was dissolved with 100 μL of a mixture of ethyl acetate and BSTFA/1% TMCS (80:20; v/v). After undergoing derivatization for 20 min at 60 °C, the extract was ready for analysis.

2.7. Chromatography and mass spectrometry conditions

Analytes were separated in a HP–5MS-UI capillary column (30 m × 0.25 mm i. d.; 0.25 μm film thickness) from Agilent. The injection port of the GC was set at 250 °C. Samples were automatically injected in splitless-injection mode, using an Ultra Inert Liner 5190–3163 from Agilent. The injection volume was 2 μL, with a 5181–3354 10 μL Syringe supplied by Agilent. The flow of helium carrier gas (99.999% purity) was maintained at 1.2 mL min^{-1}. The initial oven temperature was set at 70 °C and held for 2.0 min, then ramped to 120 °C at 25 °C min^{-1}, held for 0.5 min, to 250 °C at 10 °C min^{-1} and, finally, to 280 °C at 120 °C min^{-1}, held for 4 min (total time of 22 min). Single reaction monitoring (SRM) mode was performed on the spectrometer, reporting two MS/MS transitions for each analyte, the first for quantification and the second for confirmation. Table 1 exhibits the mass spectrometry conditions applied. The method was divided into three segments to ensure sufficient sampling points for each chromatographic peak and adequate dwell times for a good sensitivity. The resolution was adjusted to 1.0 Da for quadrupoles 1 and 3. Temperatures of the transfer line, ion source, and quadrupoles were 280 °C, 280 °C, and 150 °C, respectively. The mass spectrometer was auto-tuned weekly.

2.8. Quality control

Background contamination was controlled by analyzing procedural blanks every 15 injections (milliQ water was used as sample for this purpose). No quantifiable concentrations of target analytes were detected. In addition, a pool of placental tissue spiked at 0.2,10 and 20 ng $g^{-1}$was analyzed in triplicate every 15 injections.

3. Results and discussion

3.1. Protein and lipid removal

After the enzymatic liquefaction process, proteins and lipids (mostly phospholipids, constituents of cellular membranes) must be discarded. The addition of acetonitrile achieved protein denaturation, but the sample was not suitable for DLLME procedure due to a gelation phenomenon. However, the addition of FPS together with acetonitrile resulted in a clear supernatant suitable for DLLME treatment. FPS has previously been used in breast milk samples for denaturation purposes (Rodríguez-Gómez et al., 2014).

The volume of FPS solution was optimized to ensure a good degree of denaturation without masking the target analytes. For this purpose, placental homogenate aliquots (spiked at 10 ng g^{-1}) were liquefied and treated with volumes of FPS ranging from 50 to 800 μL. As shown in Fig. 1, the highest extraction efficacies were obtained at 50 μL FPS, suggesting a masking of the analytes with larger FPS volumes. FPS volumes lower than 50 μL impeded a good

Table 1
Selected SRM transitions and optimized potentials.

Compound	Transitions	CE (eV)	t_R (min)	Compound	Transitions	CE (eV)	t_R (min)
BPA	356.6 → 199.1[a]	20	17.5	**PPB-$^{13}C_6$**	257.8 → 201.1[a]	15	11.9
	371.6 → 199.1[b]	30			257.8 → 216.1[b]	10	
BPS	378.6 → 165.2[a]	20	20.4	**BPB**	209.7 → 151.1[a]	5	13.0
	378.6 → 181.0[b]	30			209.7 → 195.0[b]	15	
BPF	343.6 → 179.0[a]	20	16.9	**BPB-$^{13}C_6$**	215.8 → 157.1[a]	5	13.0
	343.6 → 163.0[b]	30			215.8 → 201.1[b]	15	
BPA-D_{16}	367.7 → 197.2[a]	20	17.5	**BP-1**	342.6 → 271.0[a]	20	16.9
	385.7 → 197.2[b]	30			342.6 → 105.1[b]	30	
MPB	223.7 → 177.0[a]	5	10.0	**BP-3**	284.7 → 242.1[a]	20	16.4
	223.7 → 209.1[b]	30			284.7 → 212.2[b]	30	
MPB-$^{13}C_6$	229.7 → 183.1[a]	5	10.0	**BP-6**	402.7 → 360.0[a]	20	19.0
	229.7 → 215.1[b]	30			402.7 → 73.0[b]	30	
EPB	237.7 → 195.0[a]	5	10.8	**BP-8**	372.6 → 73.0[a]	30	17.9
	237.7 → 223.1[b]	30			298.7 → 73.0[b]	20	
EPB-$^{13}C_6$	243.7 → 199.1[a]	5	10.8	**4-OH-BP**	269.7 → 193.1[a]	10	15.8
	243.7 → 229.1[b]	30			269.7 → 251.1[b]	10	
PPB	251.7 → 195.1[a]	5	11.9	**BP-3-D_5**	290.0 → 247.2[a]	20	16.4
	251.7 → 210.2[b]	10			290.0 → 217.1[b]	30	

[a] SRM transition used for quantification.
[b] SRM transition for confirmation; CE, Collision energy; t_R, Retention time.

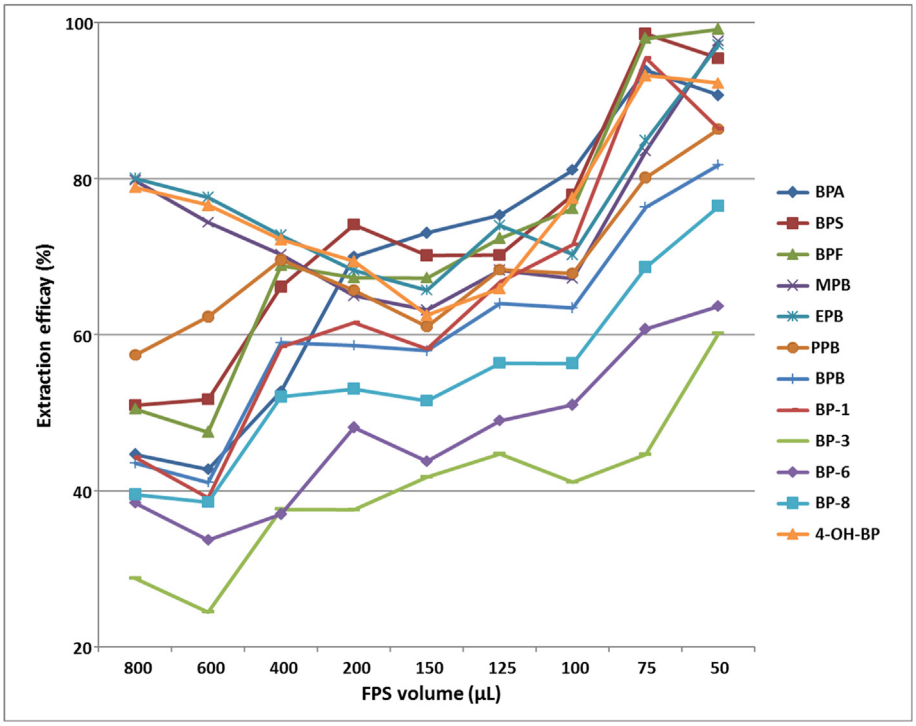

Fig. 1. Extraction efficacies obtained at different FPS volumes (mean of three determinations).

performance in the subsequent DLLME. Hence, 50 µL was selected as the optimal FPS volume.

3.2. Optimization of DLLME conditions

In a first set of experiments, the influence of four variables on the chromatographic peak area was studied for each selected analyte. For this purpose, a two-level 2^4 factorial experimental design (six replicates of the central point) was used to evaluate the effects of TCM volume (A), pH of aqueous solution (B), percentage of NaCl in aqueous solution (C) and extraction time (D) (experimental range and domain of this design are exhibited in Table S1). As depicted Fig. 2, TCM volume had a highly significant positive effect, clearly above the experimental error, while the pH and percentage NaCl had a lower influence, mainly manifested by the interaction between these factors (the effect of extraction time was negligible).

Next, a Doehlert-type quadratic response surface design was used to select optimal values for TCM volume, pH, and percentage NaCl, setting the extraction time at 30 s. This design allows the simultaneous optimization of three variables, studying seven levels for the first (TCM volume), five levels for the second (percentage NaCl) and three levels for the third (pH of aqueous solution), as shown in Table S2. Optimal values were obtained by using the desirability function, a chemometric procedure to obtain the best compromise values of experimental factors for multiple simultaneous responses. A desirability value of 1 indicates an ideal

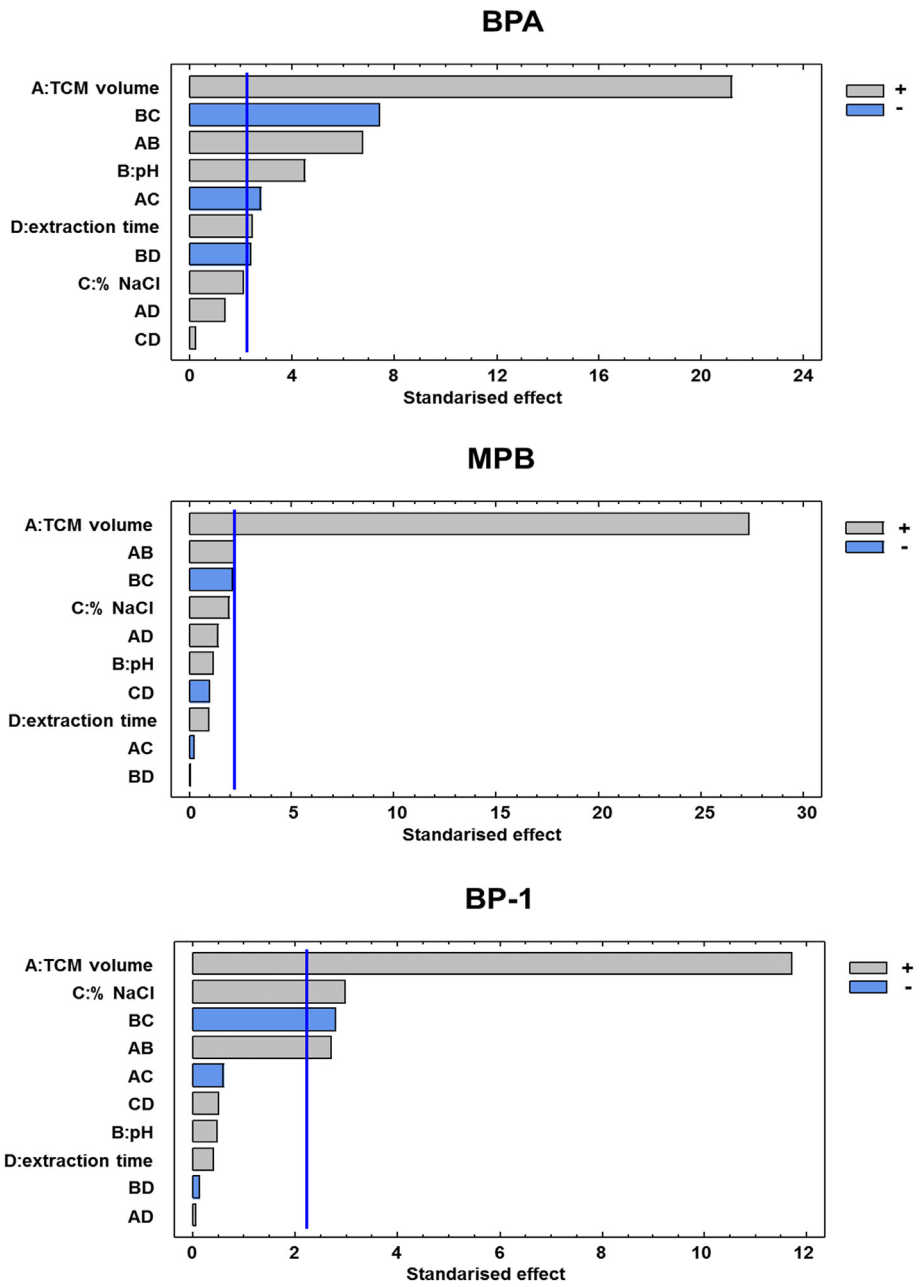

Fig. 2. Examples of standardized effects of DLLME conditions on peak areas. Blue line represents the standardized effect associated to the experimental error. The two-letter combinations represent the interactions between assayed factors. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

solution, in which all individual responses are optimized (a more detailed explanation can be read in Candioti et al., 2014). In the present case, a maximum desirability value of 0.78 was obtained for 1200 μL TCM, a pH of 2, and 6% NaCl. Fig. 3 depicts the response surface associated with the desirability function obtained.

3.3. Analytical performance and method validation

The linearity, sensitivity, accuracy (trueness and precision), and selectivity of the method was tested according to US Food and Drugs Administration (FDA) guidelines for Bioanalytical Method Validation (Guidance for Industry, 2001).

A calibration curve was constructed for each analyte with ten concentration levels (four replicates) from 0.1 to 20 ng g^{-1}, utilizing

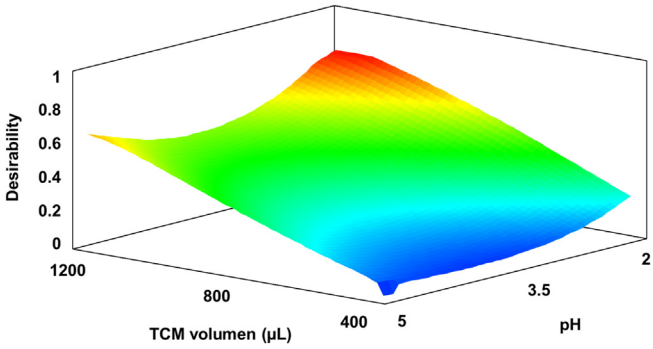

Fig. 3. Estimated response surface for the desirability function.

the analyte/mass-labeled surrogate peak area ratio against the analyte concentration. Fig. S1 depicts a chromatogram from placenta spiked at 10 ng g^{-1}.

The matrix effect (ME) was studied by comparing the slopes of two calibration curves for each compound, one in milliQ water (W) and the other in placenta (P). The percentage ME was calculated as follows:

ME (%) = [1 - (calibration slope of in P/calibration slope in W)] x 100.

ME values for BPA, MPB, EPB, PPB, BPB, and BP-3 were negligible, ranging from −5.7 to 4.9%. However, ME values above 30% were obtained for BPS, BPF, BP-1, BP-6, BP-8, and 4-OH-BP. It was therefore necessary to conduct a matrix-matched calibration using a pool of two blank placentas, which were selected by screening several tissue donations not included as analyzed samples in the present study. Fig. S2 depicts a chromatogram from this pool.

3.3.1. Accuracy (precision and trueness)

A recovery study was carried out to assess the accuracy of the method, using a spiked blank placenta pool at four concentrations (0.2, 1.0, 10, and 20 ng g^{-1}) on six consecutive days. The precision and trueness of the procedure were ratified, obtaining values below 15% for the relative standard deviation (RSD) and percentage difference in recovery values. Table 2 displays the results obtained in this study.

3.3.2. Limits of detection and quantification

The limit of detection (LOD) was defined as the lowest concentration at which signals were three-fold higher than the background noise. The limit of quantification (LOQ) was determined as the lowest concentration at which trueness and precision were within ±20%. For the proposed method, the LOQ was 0.2 ng g^{-1} for all analytes, and LOD values ranged from 0.04 to 0.08 ng g^{-1}, as reported in Table 3.

3.3.3. Linearity

A range of concentrations from the LOQ to 20 ng g^{-1} was established as the linear dynamic range (LDR) (Table 3). The determination coefficients (% R^2) ranged from 99.1% to 99.7% and the p-values of the lack-of-fit test (% P_{lof}) were >5% in all cases, confirming linearity within the stated range.

3.3.4. Selectivity

The selectivity of the method was evaluated by analyzing chromatograms of the procedure blank and the corresponding pooled blank sample. No interference from endogenous substances was observed at the analyte retention times.

3.4. Method application

The proposed method was used to determine the target EDCs in 20 placenta samples. BPA, MPB, EPB, PPB, and BP-3 were detected in at least one of the samples, whereas BPB, BPS, BPF, BP-1, BP-6, BP-8, and 4-OH-BP were not detected in any sample. Table 4 summarizes these results. A chromatogram from sample M15 is shown in Fig. S3.

In general, discrepancies between the present findings and published results can be attributed to differences in exposure patterns among populations. BPA was detected by Jiménez-Díaz et al. in 20% of 50 samples studied at concentrations ranging from 5.7 to 22.2 ng g^{-1} (Jiménez-Díaz et al., 2010), and maximum levels of 53.1 ng g^{-1} and 273.9 ng g^{-1} (dried tissue) were described by Lee et al. (2018) and Troisi et al. (2014). In the present study, BPA was detected in all 20 samples but at much lower concentrations (maximum of 0.28 ng g^{-1}).

In the case of parabens, the global profile of detection frequencies and ranges appears more similar between the present study and previous observations. Thus, an earlier study by our research found that MPB was highly prevalent, with detection frequencies close to 100% at concentrations ranging from 0.2 to 16.8 ng g^{-1}, followed by PPB (0.2–3.4 ng g^{-1}) and EPB (0.2–2.2 ng g^{-1}) and with virtually no detection of BPB (Jiménez-Díaz et al., 2011; Vela-Soria et al., 2014, 2015, 2017). Nevertheless, Valle-Sistac et al. (2016) detected not only MPB (maximum level: 11.8 ng g^{-1}) but also BPB (maximum level: 0.9 ng g^{-1}) and other congeners such as benzyl paraben in 100% of samples from 12

Table 2
Recovery assay, precision, and trueness of the method.

	Spiked (ng g^{-1})	Found[a] (ng g^{-1})	Recovery (%)	RSD (%)		Spiked (ng g^{-1})	Found[a] (ng g^{-1})	Recovery (%)	RSD (%)
BPA	0.2	0.19	94.7	12.1	BPB	0.2	0.17	87.1	11.3
	1.0	1.10	110.3	8.9		1.0	1.13	112.7	4.6
	10	9.47	94.7	9.2		10	10.0	100.1	5.6
	20	22.4	112.2	11.1		20	20.1	100.8	4.9
BPS	0.2	0.18	89.1	9.9	BP-1	0.2	0.19	95.6	10.7
	1.0	0.91	91.2	6.7		1.0	1.01	100.9	10.4
	10	9.72	97.2	12.6		10	10.2	102.3	8.5
	20	21.5	107.6	13.1		20	20.4	101.9	8.4
BPF	0.2	0.18	88.9	10.0	BP-3	0.2	0.21	106.6	11.2
	1.0	1.03	102.7	10.1		1.0	1.05	105.2	11.3
	10	0.924	92.4	9.0		10	10.2	101.8	4.7
	20	17.8	88.8	12.7		20	21.2	106.3	8.8
MPB	0.2	0.20	100.4	11.1	BP-6	0.2	0.23	113.2	13.4
	1.0	0.99	98.9	13.3		1.0	1.11	111.1	10.9
	10	9.85	98.5	5.3		10	11.2	112.1	10.4
	20	17.9	89.6	4.2		20	20.8	103.9	12.6
EPB	0.2	0.22	110.5	9.7	BP-8	0.2	0.18	90.6	12.3
	1.0	1.09	108.9	11.6		1.0	1.03	102.8	8.3
	10	9.13	91.3	7.1		10	10.7	106.9	8.2
	20	18.7	93.4	7.4		20	20.0	100.1	7.9
PPB	0.2	0.19	95.5	8.4	4-OH-BP	0.2	0.21	104.4	11.9
	1.0	1.08	108.1	11.3		1.0	1.10	110.4	10.3
	10	10.5	105.5	7.9		10	10.8	108.0	12.0
	20	20.4	101.8	9.1		20	21.4	107.3	13.2

[a] Mean of 18 determinations.

Table 3
Analytical and statistical parameters.

	b (g ng^{-1})	s_b (g ng^{-1})	R^2 (%)	LOD (ng g^{-1})	LOQ (ng g^{-1})	LDR (ng g^{-1})
BPA	$1.1 \cdot 10^{-1}$	$2.8 \cdot 10^{-3}$	99.2	0.06	0.2	0.2–20
BPS	$4.1 \cdot 10^{-2}$	$8.9 \cdot 10^{-4}$	99.2	0.08	0.2	0.2–20
BPF	$6.5 \cdot 10^{-2}$	$1.4 \cdot 10^{-3}$	99.3	0.04	0.2	0.2–20
MPB	$1.2 \cdot 10^{-1}$	$2.2 \cdot 10^{-3}$	99.3	0.05	0.2	0.2–20
EPB	$1.2 \cdot 10^{-1}$	$1.8 \cdot 10^{-3}$	99.4	0.04	0.2	0.2–20
PPB	$9.2 \cdot 10^{-2}$	$8.4 \cdot 10^{-4}$	99.7	0.04	0.2	0.2–20
BPB	$8.4 \cdot 10^{-2}$	$9.0 \cdot 10^{-4}$	99.6	0.04	0.2	0.2–20
BP-1	$1.5 \cdot 10^{-2}$	$2.2 \cdot 10^{-4}$	99.6	0.08	0.2	0.2–20
BP-3	$9.8 \cdot 10^{-2}$	$8.7 \cdot 10^{-4}$	99.7	0.05	0.2	0.2–20
BP-6	$8.9 \cdot 10^{-3}$	$9.0 \cdot 10^{-5}$	99.5	0.06	0.2	0.2–20
BP-8	$8.4 \cdot 10^{-3}$	$8.9 \cdot 10^{-5}$	99.5	0.05	0.2	0.2–20
4-OH-BP	$5.1 \cdot 10^{-2}$	$1.2 \cdot 10^{-3}$	99.1	0.07	0.2	0.2–20

b, slope; s_b, slope standard deviation; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range.

Table 4
Application of the proposed method to placenta samples.

Sample	Found concentration, ng g^{-1} (RSD %)[a]				
	BPA	MPB	EPB	PPB	BP-3
M01	D	0.43 (2.4)	D	D	D
M02	D	0.30 (9.1)	ND	D	D
M03	D	0.47 (12.0)	D	ND	ND
M04	D	0.80 (8.4)	D	D	ND
M05	D	9.12 (8.1)	ND	2.60 (10.1)	ND
M06	0.22 (9.7)	0.72 (5.2)	1.11 (10.3)	ND	D
M07	D	7.38 (11.8)	5.37 (6.8)	0.50 (6.8)	D
M08	D	7.63 (4.7)	0.29 (4.60)	1.46 (8.7)	D
M09	D	D	D	D	0.20 (13.4)
M10	D	0.68 (9.1)	ND	D	ND
M11	0.20 (9.4)	5.70 (4.3)	ND	0.97 (4.9)	ND
M12	D	0.95 (2.9)	ND	0.36 (10.2)	ND
M13	D	0.30 (11.7)	ND	ND	D
M14	D	2.10 (9.7)	1.25 (11.4)	ND	ND
M15	D	16.38 (12.5)	ND	4.02 (13.2)	ND
M16	0.20 (14.0)	0.20 (7.3)	D	0.23 (7.9)	ND
M17	D	0.51 (12.1)	ND	0.45 (7.6)	ND
M18	0.28 (9.7)	2.64 (7.9)	0.34 (8.8)	0.84 (12.8)	ND
M19	0.24 (13.2)	3.25 (11.8)	0.35 (3.7)	0.50 (11.0)	D
M20	D	7.81 (10.9)	D	2.25 (13.1)	ND
Det. (n, (%))[b]	20 (100)	20 (100)	12 (60)	16 (80)	8 (40)
Median	0.22	0.95	0.35	0.84	–
C.range[c]	D-0.28	D-16.38	ND-5.37	ND-4.02	ND-0.20

[a] Mean of 3 determinations; RSD: relative standard deviation; ND, not detected (<LOD); D, detected (>LOD and <LOQ).
[b] Detected.
[c] Concentration range.

donors, also reporting that EPB and PPB were observed in more than 75% of them, with concentration ranging from 0.09 to 0.62 ng g^{-1} and from 0.09 to 1.28 ng g^{-1}, respectively.

There have been marked differences in the detection of benzophenones. Only BP-3 was detected in the present investigation, whereas BP-1 and 4-OH-BP have also been observed in quantifiable concentrations by other studies at maximum concentrations of 9.8 ng g^{-1} and 1.8 ng g^{-1} respectively in some cases (Vela-Soria et al., 2011, 2014, 2017). In contrast, Valle-Sistac et al. (2016) reported a more discreet profile in placenta samples, with detected but not quantified values for BP-1, BP-3 and 4-OH-BP. The heterogeneity of detection frequencies and concentrations reported in the literature suggest disparate patterns of exposure and metabolism.

3.4. Comparison with previous methods

Table 5 summarizes the main features of available procedures and the present proposal to facilitate detailed comparisons. As can be observed, all other approaches also provide reliable determinations with good LODs but have certain practical drawbacks that are resolved by the present procedure.

In comparison to the proposed DLLME protocol, classic LLE procedures require more time for the extraction step and for the more exhaustive post-extraction process, reducing their practical usefulness (Jiménez-Díaz et al., 2010, 2011; Vela-Soria et al., 2011). For their part, MSPD procedures are known to avoid interferences and co-extracted substances (Vela-Soria et al., 2014, 2015). However, they also require more time to perform, because they involve previous lyophilization of the tissue, manual blending of the sample with solid sorbents, and assembly of the cartridges; in addition, the extraction step consumes a high volume of solvents. In comparison to the present proposal, the UAE procedure (Vela-Soria et al., 2017) also needs more time and consumes more solvent, as well as requiring an ultrasound probe and a d-SPE clean-up step. The DLLME procedure therefore appears to be a superior method for processing large numbers of placenta samples.

4. Conclusions

Non-persistent EDCs were successfully determined in placenta samples from 20 randomly selected women using DLLME combined with GC–MS/MS. An enzymatic liquefaction step allowed the application of DLLME in semi-solid placental tissue. In other words, “the sample has been adapted to the extraction technique”, in reverse to the usual way of proceeding in analytical chemistry (i.e. selecting the technique according to the sample). Experimental parameters were optimized using chemometric approaches, and the procedure was validated. The proposed method has operational advantages that may make it the approach of choice for large-scale biomonitoring studies on the exposure of embryos and fetuses to prevalent EDCs.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

Authorship contributions

M.F. Fernandez: Conceptualization, Resources, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. V. Mustieles: Conceptualization, Formal analysis, Investigation, Writing – original draft. B. Suárez: Validation, Formal analysis. I. Reina-Pérez: Validation, Formal analysis. A. Olivas: Validation,

Table 5
Comparison of this DLLME procedure with previous methods for determining target analytes in placenta samples.

EDCs	Sample pre-treatment	Extraction technique[a]	Sample amount	Extraction global time/solvent consumption	Post-extraction process prior to chromatographic injection	Instrumental technique[b]	LOD[c] (ng g^{-1})	Ref.
BPA and chlorinated derivatives	Mechanical homogenization	LLE	1.5 g	10 min/3 mL	Drying and redissolution Centrifuging, 35 min Filtering	LC-MS/MS	0.2 −0.6	Jiménez-Díaz et al. (2010)
BP-1, BP-2, BP-3, BP-6, BP-8, 4-OH-BP	Mechanical homogenization.	LLE	1.5 g	10 min/3 mL	Drying and redissolution Centrifuging, 35 min Filtering	LC-MS/MS	0.07 −0.3	Vela-Soria et al. (2011)
MPB, EPB, PPB, BPB	Mechanical homogenization.	LLE	1.5 g	10 min/3 mL	Drying and redissolution Centrifuging, 35 min Filtering	LC-MS/MS	0.03 −0.06	Jiménez-Díaz et al. (2011)
MPB, EPB, PPB, BPB, BP-1, BP-3, BP-6, BP-8, 4-OH-BP	Mechanical homogenization. Lyophilization	MSPD (C18 sorbent)	0.25 g (lyophilized tissue)	−/20 mL	Drying and redissolution	LC-MS/MS	0.1	Vela-Soria et al. (2014)
MPB, EPB, PPB, BPB, BPA and chlorinated derivatives, BP-3	Mechanical homogenization. Lyophilization	MSPD (silica and PSA sorbents)	0.25 g (lyophilized tissue)	−/12.5 mL	Drying and redissolution	LC-MS/MS	0.1	Vela-Soria et al. (2015)
MPB, EPB, PPB, BPB, BP-1, BP-3, BP-6, BP-8, 4-OH-BP and other UV-Filters.	Mechanical homogenization. Lyophilization	UAE	2 g	10 min/3 mL	d-SPE (C18 and PSA sorbents) Centrifuging, 5 min Drying and redissolution	LC-MS/MS	0.05 −0.1	Vela-Soria et al. (2017)
BPA, BPS, BPF, MPB, EPB, PPB, BPB, BP-1, BP-3, BP-6, BP-8, 4-OH-BP.	Mechanical homogenization. Enzymatic treatment with collagenase.	DLLME	0.5 g	30 s/2.2 mL	Drying and derivatization, 20 min	GC-MS/MS	0.04 −0.08	This study

[a] LLE: liquid-liquid extraction; SPE: solid phase extraction. MSPD: matrix solid phase dispersion. PSA: poly secondary amine. DLLME: dispersive liquid-liquid micro-extraction. UAE: ultrasound assisted extraction.
[b] LC: liquid chromatography; MS: mass spectrometry; GC: gas chromatography.
[c] LOD: limit of detection.

Formal analysis. F. Vela-Soria: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, writing-review and 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

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