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A multiresidue method for the determination of selected endocrine disrupting chemicals in human breast milk based on a simple extraction procedure

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ABSTRACT

In recent decades, in parallel to industrial development, a large amount of new chemicals have emerged that are able to produce disorders in human endocrine system. These groups of substances, so-called endocrine disrupting chemicals (EDCs), include many families of compounds, such as parabens, benzophenone-UV filters and bisphenols. Given the demonstrated biological activity of those compounds, it is necessary to develop new analytical procedures to evaluate the exposure with the final objective of establishing, in an accurate way, relationships between EDCs concentrations and the harmful health effects observed in population. In the present work, a method based on a simplified sample treatment involving steps of precipitation, evaporation and clean-up of the extracts with C18 followed by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis for the determination of bisphenol A and its chlorinated derivatives (monochloro-, dichloro-, trichloro- and tetrachlorobisphenol A), parabens (methyl-, ethyl-, propyl- and butylparaben) and benzophenone-UV filters (benzophenone −1, −2, −3, −6, −8 and 4-hydroxybenzophenone) in human breast milk samples is proposed and validated. The limits of detections found ranged from 0.02 to 0.05 ng mL^{-1}. The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. Recovery rates ranged from 91% to 110% and the precision (evaluated as relative standard deviation) was lower than 15% for all compounds, being within the acceptable limits for the selected bioanalytical method validation guide. The method was satisfactorily applied for the determination of these compounds in human breast milk samples collected from 10 randomly selected women.

1. Introduction

The overall development occurred in the last century has led man to have available a lot of manufactured products with a wide applicability that have significantly eased the life. Nevertheless, this massive development has brought an important inconvenient to the population: the exposure to a high variety of xenobiotics that could cause negative health effects. Among these compounds, endocrine disrupting chemicals (EDCs) have become in a special concern in the last years.

EDCs cover an important range of synthetic and natural substances able to alter the normal hormone function of wildlife and humans. The endocrine and reproductive effects of those compounds are believed to be due to their ability to mimic or antagonize the effects of endogenous hormones, such as estrogens and androgens, or to disrupt synthesis and metabolism of endogenous hormones and hormone receptors [1]. Beside some naturally occurring compounds (lignans, coumestans, isoflavones, mycotoxins), numerous synthetic chemicals such as are bisphenol A (BPA) and its chlorinated derivatives, benzophenone-UV filters (BPs) and parabens (PBs) have been implicated in endocrine disruption.

Since its effects, even at very low concentrations, are more detrimental and pernicious than other EDCs, BPA has received a tremendous attention from the scientific-medical community and governments [2,3]. It is the raw material used in the manufacturing of epoxy resin and polysulfones. It is also applied as antioxidant or stabilizer. However, the most important use of BPA is the production of polycarbonate plastics for a great variety of applications such as digital media (e.g., CDs, DVDs), electrical and electronic equipment, automobiles, sports safety equipment, reusable food and drink containers, medical devices and many other products [4]. Moreover, when BPA is present in treated waters, it may react with residual chlorine originally used as disinfectant, producing chlorinated BPA derivatives depending on the pH of the medium [5]. Regarding to BPs, those compounds are used as UV filters in sunscreens to protect the skin and hair from UV irradiation as they are able to absorb UV light that is harmful to the human body in the form of

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UVA (320–400 nm) and UVB (290 to 320 nm). Finally, PBs (alkyl esters of *p*-hydroxybenzoic acid) are widely used as antimicrobial preservatives, especially against mold and yeast, in cosmetic products and pharmaceuticals, and in food and beverage processing [6].

The widespread use of BPA, PBs and BPs and their potential risk to human health have prompted interest in assessing human exposure to them. It may occur through inhalation, dermal contact or ingestion [7–10] and their metabolism may differ depending upon the exposure route [7,11]. These compounds may conjugate to β-D-glucuronide and sulfate, thus reducing their bioactivity and facilitating their urinary excretion. Although free and conjugate forms can be measured in humans, only the free forms are biologically active.

Developmental exposure to EDCs is particularly important in the first stages of life because of the increased susceptibility of the brain and other organs to estrogens during this period [12]. It has been postulated that EDCs accumulate in certain human tissues and their effects might pass to the offspring via the placenta and/or breast milk [13–17]. Breastfeeding mothers exposed to EDCs may be unknowingly exposing their children to harmful levels of these compounds. In this context, it is particularly important to develop strategies for the study of this exposure through the mother after childbirth and therefore, to develop sensitive analytical methods to monitor EDCs in human milk.

Sample preparation is a critical step in complex biological matrices analysis, such as human milk. An extraction technique is usually required to purify and isolate the target compounds. Moreover, because of the low levels of EDCs in human milk, these extraction techniques must be able to concentrate the analytes. To date, BPA and its chlorinated derivatives, PBs and BPs have been extracted from human milk using liquid–liquid extraction (LLE) [18–20], ultrasound assisted extraction (UAE) [21], off-line solid-phase extraction (SPE) [22–25] and on-line SPE [21,26–28]. In the present work, a simple and cost effective sample treatment based on a precipitation of fat and proteins followed by a clean-up using a simplification of the Quick, Easy, Cheap, Effective, Rugged & Safe (QuEChERS) methodology is proposed. QuEChERS was developed by Anastassiades et al. in 2003 for the analysis of pesticides in fruits and vegetables [29]. Since then, it has become an important and widely used technique in the analysis of multiple chemical residues, including EDCs, in a great variety of matrices. Thus, it has been used for the analysis BPA and bisphenol S in canned vegetables and fruits [30], pesticides and mycotoxins in commercial milk [31] and steroid hormones or BPA and the active metabolites of methoxychlor and vinclozolin in rat testis [32]. Recently, the efficacy of this methodology has been also proven for the extraction of organochlorine pesticides in human milk [33]. However, to our knowledge, QuEChERS has not been applied for the EDCs selected in the present work in human milk samples.

The aim of the present work was to develop a sensitive multiresidue method based on a precipitation of fat and proteins followed by a clean-up step for the simultaneous determination of free amounts of BPA and its chlorinated derivatives (monochloro-, dichloro-, trichloro- and tetrachloro-); four PBs (methyl-, ethyl-, propyl- and butylparaben) and six BPs (benzophenone-1, 2, 3, 6, 8 and 4-hidroxybenzophenone) in human milk samples. UHPLC-ESI-MS/MS has been used as detection technique. The proposed method was satisfactorily validated and applied for the determination of the free content of the above mentioned compounds in 10 human milk samples from volunteers lactating mothers who live in the province of Granada (Spain).

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. PBs standards were supplied by Alfa Aesar (Massachusetts, MA, USA). Bisphenol A (BPA), tetrachlorobisphenol A (Cl_4-BPA), deuterium-labeled bisphenol A-d_{16} (BPA-d_{16}), benzophenone-UV filter standards (BPs) and deuterium-labeled benzophenone-d_{10} (BP-d_{10}) were supplied by Sigma-Aldrich (Madrid, Spain). Monochloro-, dichloro- and trichlorobisphenol A (Cl-BPA, Cl_2-BPA, Cl_3-BPA) were synthesized in our laboratory (purity > 99%) by direct chlorination of BPA [34]. Deuterium-labeled ethylparaben-d_5 (EPB-d_5) was purchased from Toronto Research Chemicals Inc (North York, Ontario, Canada). Stock standard solutions (100 μg mL^{-1}) were prepared by weighing 10 mg of each compound into a 100 mL flask. Then, acetonitrile up to the final volume was added. The solution remained stable for at least four months at 4 °C in the darkness. For calibration and validation purposes, two intermediate solutions, No. 1 and 2 (10 and 2.5 μg mL^{-1}) were prepared by diluting 1.0 and 0.25 mL respectively of the stock solution to 10 mL in acetonitrile (MeCN). Subsequently, two new intermediate solutions No. 3 and 4 (1.0 and 0.5 μg mL^{-1}) were prepared by diluting 1.0 and 0.5 mL respectively of solution No. 1 to a final volume of 10 mL in MeCN. Then, two new intermediate solutions No. 5 and 6 (0.1 and 0.05 μg mL^{-1}) were prepared by diluting 1 and 0.5 mL respectively of solution No. 3 to a final volume of 10 mL in MeCN. Finally, intermediate solution No. 7 (0.01 μg mL^{-1} was prepared by diluting 1 mL of solution No. 5 to a final volume of 10 mL in MeCN. Working standards for calibration and validation purposes were prepared by diluting 100 μL of the intermediate solutions No. 2 to 7 to a final volume of 10 mL in human breast milk. Working standards were prepared fresh from the MeCN solutions prior to the experiments.

Methanol (MeOH) and MeCN gradient grade were obtained from Merck (Darmstadt, Germany). LC-MS grade methanol and water, formic acid, ammonia (25%), zinc acetate dihydrate, phosphotungstic acid hydrate and primary-secondary amine (PSA), were also purchased from Sigma-Aldrich (Madrid, Spain). Octadecyl (C18) solid sorbent (40 μm) was supplied by J.T. Baker (Deventer, Netherlands). Glacial acetic acid (99%) was obtained from Panreac (Barcelona, Spain). The fat/proteins precipitation solution was prepared at time of use by dissolving 9.10 g of zinc acetate hydrated, 5.46 g of hydrated phosphotungstic acid and 5.8 mL of glacial acetic acid in 100 mL final volume of deionised water.

2.2. Instrumentation and software

UHPLC-MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class from Waters (Manchester, UK). A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for EDCs detection. An Acquity UPLC® BEH C18 (100 mm × 2.1 mm i.d., 1.7 μm particle size) from Waters (UK) was used as chromatographic column. A vacuum centrifugal evaporator was used to concentrate samples (LaboGene, Lynge, Denmark). MassLynx 4.1 software was used for instrument control, peak detection and integration. Statgraphics Plus version 5.0 (Manugistics Inc., Rockville, USA, 2000) was used for statistical and regression analyses.

2.3. Sample collection and storage

Human milk samples were obtained from healthy lactating women living in Granada, Spain. Samples were anonymized, frozen at −20 °C and stored until analysis in our laboratory. The study was performed in compliance with the *Ethical Principles for Medical Research Involving Human Subjects* issued by the World Medical Association, and all volunteers signed the informed consent form.

2.4. Preparation of fortified milk samples (calibration and validation standards)

For calibration and recovery studies, blank samples were spiked at different concentrations (from 0.1 ng mL^{-1} to 25 ng mL^{-1}) by adding 100 μL of the different spiking standard solutions (No. 2–7) to 9.9 mL of human breast milk. In order to attain equilibrium, the mixtures were vortexed for 2 min and then left to stand for 24 h at 4 °C in the dark before analysis. This allows the analytes to come into full contact with the sample. The blank samples were obtained from two different mothers and in different days. Although the exposure to these compounds through numerous pathways is very usual, the metabolism of these substances varies from one individual to another; in this sense, the fact of being exposed does not necessarily mean bioaccumulation of these compounds in every biological fluid. Thus, taking into account the results obtained in previous analyses of different tissues and biological fluids (placenta or serum of more than 50 parturient women), they were selected six mothers that had very low or even null concentrations of the analytes under study in those samples. Finally, the breast milk of each mother was analyzed for several days and the samples from two of the mothers, in different days, was found to be "free of the analytes" (under LOD of proposed method), and therefore these samples were selected as blank for further experiments.

2.5. Sample treatment

An aliquot of 9.9 mL of human breast milk sample was placed into a 45 mL centrifuge tube and 100 μL of a 0.5 μg mL^{-1} acetonitrile solution of the surrogates (BPA-d_{16}, BP-d_{10} and EPB-d_5) was added. The final concentration of surrogates in sample was 5 ng mL^{-1}. The sample was vigorously shaken in a vortex-mixer for 1 min and then, 7.5 mL of MeCN and 7.5 mL of the fat/proteins precipitation solution were added. The mixture was stirred on a vortex-mixer for 1 min and centrifuged for 10 min at 4050 × *g*. The underlying liquid layer was filtered through a 0.22 μm nylon filter and transferred to a Falcon tube for evaporation to dryness in a vacuum centrifugal evaporator at 760 × *g* and 60 °C.

After evaporation was complete, a clean-up step was carried out. For this purpose, the dry residue obtained was dissolved in 7.5 mL of MeCN and 150 mg of C18 were added as clean-up sorbent. $MgSO_4$ (about 0.1 g) was also added to the mixture in order to remove any traces of moisture. The mixture was stirred for 3 min at room temperature and after centrifugation at 4050 × *g*, the supernatant was transferred to a 8 mL glass vial and evaporated to dryness in the vacuum centrifugal evaporator at 760 × *g* and 60 °C. The residue was dissolved with 300 μL of MeOH (twice) and transferred to a 1.5 mL Eppendorf tube for evaporation to dryness at room temperature. Finally, the residue was dissolved in 100 μL of initial mobile phase and after stirring for 60 s in vortex, it was filtered through a 4 mm and 0.22 μm nylon filter. The sample was ready to be injected into the chromatographic system.

2.6. Liquid chromatography-mass spectrometry analysis

The chromatographic separation of targets analytes was performed using an Acquity UPLC® BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size). The compounds were separated using a gradient mobile phase consisting of 0.1% (v/v) aqueous ammonium formate solution (pH=9) as solvent A and 0.1% (v/v) ammonia in MeOH as solvent B. Gradient conditions were as follows: 0.0–4.0 min, 40% B; 4.0–6.0 min, 40–90% B; 6.0–6.1 min, 90–100% B; 6.1–7.5 min, 100% B and back to 40% in 0.5 min. Then, 5 min for conditioning of column were added. Total run time was 13.0 min. Flow rate was 300 μL min^{-1} and the injection volume was 10 μL. The column temperature was maintained at 40 °C.

ESI was performed in both negative and positive ion modes. The tandem mass spectrometer was operated in the multiple reactions monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each analyte by continuous infusion of concentrate standard solutions (1 μg mL^{-1}). The ion source temperature was maintained at 150 °C. Other instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1} and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell times were set at 25 ms. Collision energies (CE) and cone voltages (CV) were optimized for each analyte. Optimized parameters for each compound are listed together with the mass transitions in Table 1.

3. Results and discussion

3.1. Sample treatment

3.1.1. Protein and fat precipitation

The sample treatment techniques for isolation of EDCs from biological samples published in the scientific literature are usually laborious and time-consuming and particularly, in the case of human breast milk, there is an important lack of information. After a carefully study of possible sample treatments for cow milk-related products a simple precipitation procedure with a solution containing zinc and tungsten salts in an acidic media was selected. This solution has been currently used for milk treatment in the analysis of lactulose as described in the method by the International Dairy Federation [35]. It was decided to evaluate a similar procedure to remove fat and proteins from human milk by precipitation. The results were excellent in terms of sensitivity and sample handling and on the one hand a white solid fraction corresponding to protein, fat and other precipitated salts was obtained, and it was also separated a clear solution containing the analytes that after filtering was completely transparent.

Since heavy metals are present in the precipitation solution, in order to minimize the amounts of reagents and to decrease final residues on the analysis, the ratio of precipitation solution/acetonitrile and human milk sample was optimized for maximum

Table 1
Transitions and optimized potentials for UHPLC–MS/MS analysis.

	Transitions	CV	CE		Transitions	CV	CE
BPA	227.2→211.9[a]	−50	−22	BP-1	214.9→136.8[a]	2	18
	227.2→132.9[b]	−50	−26		214.9→105.1[b]	2	32
Cl-BPA	261.1→182.0[a]	−56	−30	BP-2	245.1→134.8[a]	−40	−16
	261.1→210.0[b]	−56	−22		245.1→108.9[b]	−40	−22
Cl_2-BPA	295.0→215.9[a]	−74	−30	BP-3	229.0→150.8[a]	4	20
	295.0→243.9[b]	−74	−24		229.0→104.9[b]	4	18
Cl_3-BPA	329.0→249.8[a]	−2	−32	BP-6	275.0→150.9[a]	14	18
	329.0→277.9[b]	−2	−24		275.0→94.9[b]	14	34
Cl_4-BPA	365.0→313.9[a]	−50	−28	BP-8	245.0→120.9[a]	14	20
	365.0→285.9[b]	−50	−32		245.0→150.9[b]	14	20
MPB	151.1→91.8[a]	−38	−22	4-OH-BP	199.0→120.8[a]	36	20
	151.1→135.8[b]	−38	−14		199.0→104.8[b]	36	18
EPB	165.1→91.9[a]	−38	−24	BPA-d_{16}	241.2→223.0[a]	−46	−22
	165.1→136.6[b]	−38	−16		241.2→141.9[b]	−46	−32
PPB	179.1→91.8[a]	−42	−24	EPB-d_5	170.1→92.1[a]	−38	−24
	179.1→136.1[b]	−42	−16		170.1→136.0[b]	−38	−16
BPB	193.1→91.4[a]	−42	−24	BP-d_{10}	193.1→109.8[a]	18	16
	193.1→136.1[b]	−42	−16		193.1→81.8[b]	18	30

CV: Cone voltage (V); CE: Collision energy (eV).

[a] SRM transition used for quantification.

[b] SRM transition used for confirmation.

recovery. For 10 mL of milk sample, volumes ranging from 2.5 to 10 mL of precipitation solution and MeCN were assayed; being the optimum value obtained 7.5 mL for both them.

3.1.2. Optimization of the clean-up sorbent

After the precipitation of proteins and fat, the extract was completely evaporated to dryness in a vacuum centrifugal evaporator

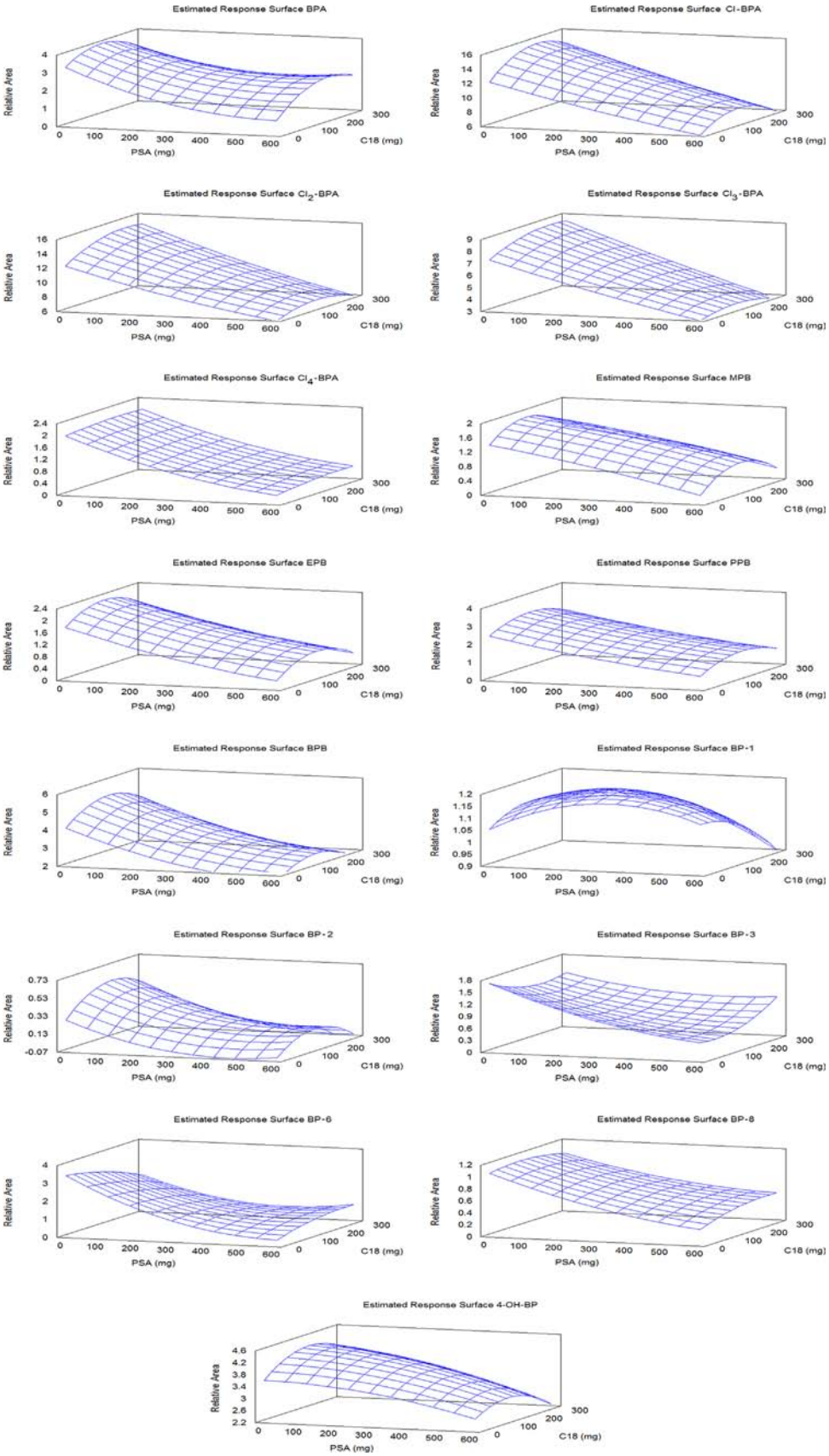

Fig. 1. Optimization of the amount of clean-up sorbent. Response surfaces obtained for the factorial experimental design.

at 760 × *g* and 60 °C. A final extract with an oily aspect was obtained. In consequence, a clean-up step was necessary in order to minimize matrix effects and to avoid instrumental problems in the UHPLC system. Based on our previous experience with this type of analytes in other biological and environmental matrices, and in the principles of extraction technique QuEChERs, acetonitrile was chosen as solvent to dissolve the extract. In order to minimize the solvent amounts, the volume of acetonitrile was studied in the range from 1 to 10 mL and the minimal amount of solvent necessary to dissolve adequately the oily extract was 7.5 mL.

Then, a mixture of PSA and C18 as clean-up sorbents was studied. Since the optimal amount of both sorbents in the mixture could be related each other, a simple factorial 3^2 response surface design (three central points) was performed in order to optimize the mass of both sorbents. The design allows the simultaneous optimization of two variables at three levels [36,37]. The pairs selected for the assay and the optimum values for each variable are shown as supplementary material (Table S01). Fig. 1 shows the response surfaces obtained for each compound. The following model was determined for each response: $y=b_0+b_1\chi_1+b_2\chi_2+b_{12}X_1\chi_2+b_{11}\chi_1^2+b_{22}\chi_2^2$ [38] where, y is the measured response (relative area) for each compound, b_0 the intercept, b_i the regression coefficients and X_i the values of variables (X_1=mass of PSA; X_2=mass of C18).

As the figure evidence, the presence of PSA does not affect the response and therefore, its presence in the cleaning up process is not necessary. In the case of C18 sorbent, some differences in the optimal values obtained were observed depending on the compound. The combination of the optimized experimental values obtained for each compound for the two variables allowed the determination of the best overall extraction efficiency, which was calculated with the desirability function. The plot of this function versus sorbent amounts is shown in Fig. 2.

Responses for each compound in the different experiments of the design were first normalized between 0 and 1, and then the global desirability function, was defined as their geometric mean. As Fig. 2 shows, the optimal quantities were 0 mg for PSA and approximately 150 mg for C18, and these values were then used for further experiments.

3.2. Analytical performance

A calibration curve for each compound, with six concentration levels (six fold) was built. The curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. Calibration graphs were made using SRM mode. Surrogates (BPA-d_{16}, EPB-d_5 and BP-d_{10}) were added at a concentration of 5 ng mL^{-1}.

In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound, one in the initial mobile phase and the other in blank human milk. The Student's *t*-test was applied in order to compare the calibration curves. First, the variances estimated as $S^2_{y/x}$ were compared by means of a Snedecor's *F*-test. The Student's *t*-test showed statistical differences among slope values for the calibration curves of some of the target analytes and consequently, a significant matrix effect was observed in those cases. A possible explanation for this not correction of the matrix effects by the surrogates employed, could be that the chemical structure and, consequently, the physical and chemical properties of the analyzed compounds are relatively variable within the same family

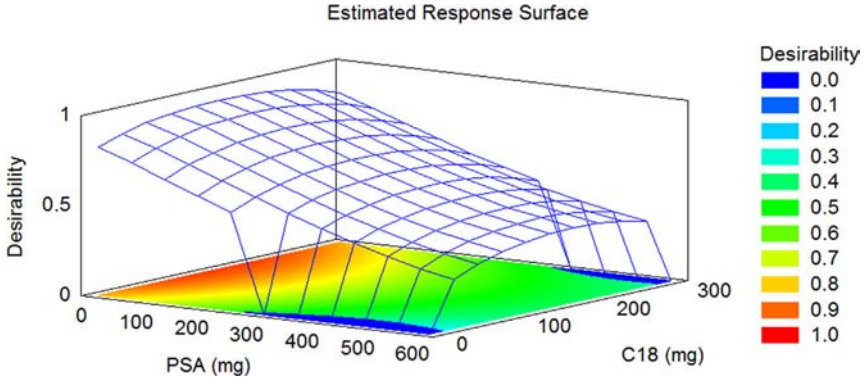

Fig. 2. Representation of the global desirability function vs PSA and C18 amounts. Results were evaluated using a 95% confidence interval.

Table 2
Analytical and statistical parameters.

Compound	b (mL ng^{-1})	s_b (mL ng^{-1})	R^2 (%)	P_{Lof} (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
BPA	0.190	1.457×10^{-3}	99.8	64.7	0.05	0.15	LOQ–25.0
Cl-BPA	1.160	6.426×10^{-3}	99.9	30.0	0.04	0.12	LOQ–25.0
Cl_2-BPA	0.870	5.961×10^{-3}	99.8	40.2	0.04	0.12	LOQ–25.0
Cl_3-BPA	0.472	6.334×10^{-3}	99.4	29.6	0.04	0.14	LOQ–25.0
Cl_4-BPA	0.198	1.015×10^{-3}	99.9	31.0	0.04	0.13	LOQ–25.0
MPB	0.468	2.553×10^{-3}	99.9	13.6	0.03	0.09	LOQ–25.0
EPB	0.631	3.906×10^{-3}	99.9	5.1	0.03	0.09	LOQ–25.0
PPB	0.846	4.381×10^{-3}	99.9	92.6	0.03	0.09	LOQ–25.0
BPB	0.961	7.095×10^{-3}	99.8	32.0	0.03	0.10	LOQ–25.0
BP-1	0.220	1.361×10^{-3}	99.9	36.9	0.03	0.09	LOQ–25.0
BP-2	0.408	2.672×10^{-3}	99.8	27.8	0.03	0.09	LOQ–25.0
BP-3	0.109	7.398×10^{-4}	99.9	7.8	0.03	0.09	LOQ–25.0
BP-6	0.319	3.805×10^{-3}	99.5	48.1	0.03	0.10	LOQ–25.0
BP-8	0.131	7.339×10^{-4}	99.9	40.0	0.02	0.08	LOQ–25.0
4-OH-BP	0.862	6.414×10^{-3}	99.8	99.2	0.03	0.09	LOQ–25.0

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

of compounds, especially in the case of BPs. Therefore, although the compounds selected have a similar basic structure compared to the analytes of the same family, and the use of these compounds as internal standards or as surrogates is accepted in scientific literature, they differ slightly due to the presence of different substituents in the molecule. Since it is impossible to have the corresponding isotopically labeled standard for each one of the studied analytes (many of them do not exist in the market), it was decided to work with matrix-matched calibration in all cases. Table 2 shows the statistical and the analytical parameters obtained for each compound.

3.3. Method validation

Validation in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [39].

3.3.1. Linearity

A concentration range from the minimal quantified amount, limit of quantification (LOQ) (see Table 2) to 25 ng mL^{-1} was selected. Linearity of the calibration graphs was assessed with the determination coefficients (% R^2) and the *P*-values (% P_{lof}) of the *lack-of-fit* test [40]. The values obtained for R^2 ranged from 99.4% for Cl_3-BPA to 99.9% for Cl-BPA, Cl_4-BPA, MPB, EPB, PPB, BP-1, BP-3 and BP-8, and P_{lof} values were higher than 5% in all cases. This indicates a good linearity within the stated ranges.

3.3.2. Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) are two fundamental parameters that need to be calculated in the validation of any analytical method in order to determine if an analyte is present in the sample. In the present work, these parameters were established as the minimum concentration of analyte that the method can detect and with a signal-to-noise ratio of 3 for LOD and 10 for LOQ, using the quantification transition. The values obtained for the LOQ ranged from 0.08 ng mL^{-1} for BP-8 to 0.15 ng mL^{-1} for BPA. The results are also summarized in Table 2.

3.3.3. Accuracy (precision and trueness)

Due to the absence of certified materials and in order to evaluate the trueness and the precision of the method, a recovery study with spiked human breast milk samples, at three concentrations levels for each compound (0.5, 5.0 and 25 ng g^{-1}), was performed on six different days. The precision was expressed as relative standard deviation (RSD) and the trueness was evaluated with the percentage of recovery. The precision and the trueness of the proposed analytical method are shown in Table 3.

Trueness was evaluated by determining the recovery of known amounts of the tested compounds in spiked human breast milk samples. Samples were analyzed using the proposed method and the concentration of each compound was compared with the amount of analyte previously added to the samples. In all cases, the recoveries were close to 100%. Precision (expressed as relative standard deviation, RSD) was lower than 15% for all compounds. Therefore, it was within the acceptable limits for bioanalytical method validation, which are considered $\leq$ 15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. The data, also shown in Table 3, demonstrated that the proposed method is reproducible. Therefore, precision and trueness data indicate that the method is highly accurate.

3.3.4. Selectivity

The specificity of the method was demonstrated by analyzing the chromatograms of a human milk sample spiked with the analytes and the corresponding blank. No interferences from endogenous substances were observed at the retention time of the compounds. These findings suggest that the spectrometric conditions ensured high selectivity of the UHPLC-MS/MS method. Fig. 3 shows a chromatogram of a spiked milk sample.

3.4. Application of the proposed method

The validated method was applied to the determination of the selected EDCs in 10 samples of human breast milk. The results obtained as mean of six determinations are summarized in Table 4. Fig. 4 shows the chromatograms obtained for a natural sample (M01).

Table 3
Recovery assay, precision and trueness of the method.

	Spiked (ng mL^{-1})	Found[a] (ng mL^{-1})	RSD (%)	Recovery (%)		Spiked (ng mL^{-1})	Found[a] (ng mL^{-1})	RSD (%)	Recovery (%)
BPA	0.50	0.55	4.6	109.8	BPB	0.50	0.47	7.5	96.0
	5.00	4.77	8.0	95.5		5.00	4.72	4.1	94.4
	25.0	25.0	3.5	100.2		25.0	24.9	3.6	99.9
Cl-BPA	0.50	0.51	5.4	102.6	BP-1	0.50	0.54	7.7	108.2
	5.00	5.19	4.6	103.8		5.00	5.09	6.1	101.8
	25.0	25.1	1.5	100.4		25.0	25.1	1.9	100.3
Cl_2-BPA	0.50	0.54	6.9	108.0	BP-2	0.50	0.55	3.6	109.3
	5.00	5.17	5.3	103.5		5.00	4.98	5.0	99.5
	25.0	25.1	2.9	100.5		25.0	25.2	2.3	100.6
Cl_3-BPA	0.50	0.47	4.2	93.1	BP-3	0.50	0.49	3.1	99.0
	5.00	4.93	14.6	98.6		5.00	4.86	5.0	97.2
	25.0	25.2	5.2	100.8		25.0	24.9	2.0	99.7
Cl_4-BPA	0.50	0.55	3.2	110.0	BP-6	0.50	0.55	7.1	109.8
	5.00	4.89	3.0	97.8		5.00	5.13	5.4	102.7
	25.0	25.1	2.0	100.3		25.0	25.1	5.3	100.6
MPB	0.50	0.46	4.6	93.0		0.50	0.53	7.1	106.6
	5.00	4.76	2.0	95.1	BP-8	5.00	4.82	5.4	96.4
	25.0	25.0	2.5	100.0		25.0	25.1	1.9	100.2
EPB	0.50	0.46	12.4	91.6		0.50	0.53	5.8	106.0
	5.00	4.73	4.4	94.6	4-OH-BP	5.00	5.02	6.2	100.4
	25.0	24.9	2.4	99.8		25.0	25.0	2.4	100.1
PPB	0.50	0.45	6.3	91.0					
	5.00	4.98	3.0	99.6					
	25.0	24.9	2.0	99.9					

[a] Mean of 18 determinations; RSD: relative standard deviation.

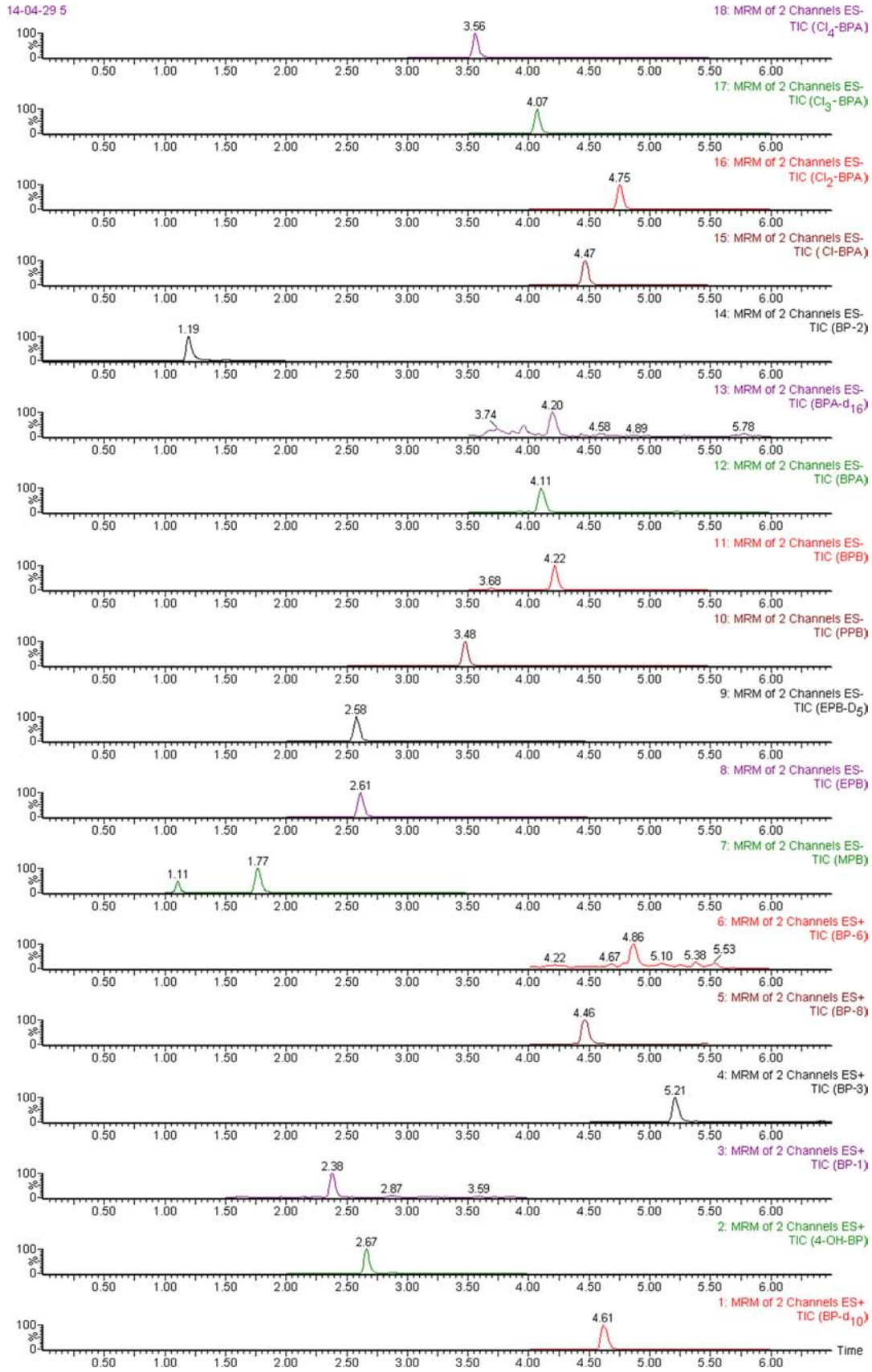

Fig. 3. Chromatograms of a standard mixture in human milk (5 ng mL^{-1} of each studied analyte).

As it is shown in Table 4, ten of the fifteen analyzed compounds were detected and quantified in almost one of the samples. Regarding BPA, the compound was quantified in six of the ten samples in a concentration range from 0.6 to 13.8 ng mL^{-1} (mean: 4.6 ng mL^{-1}). However, in the case of chlorinated derivatives, only Cl_2-BPA was detected in two of the samples at concentration levels

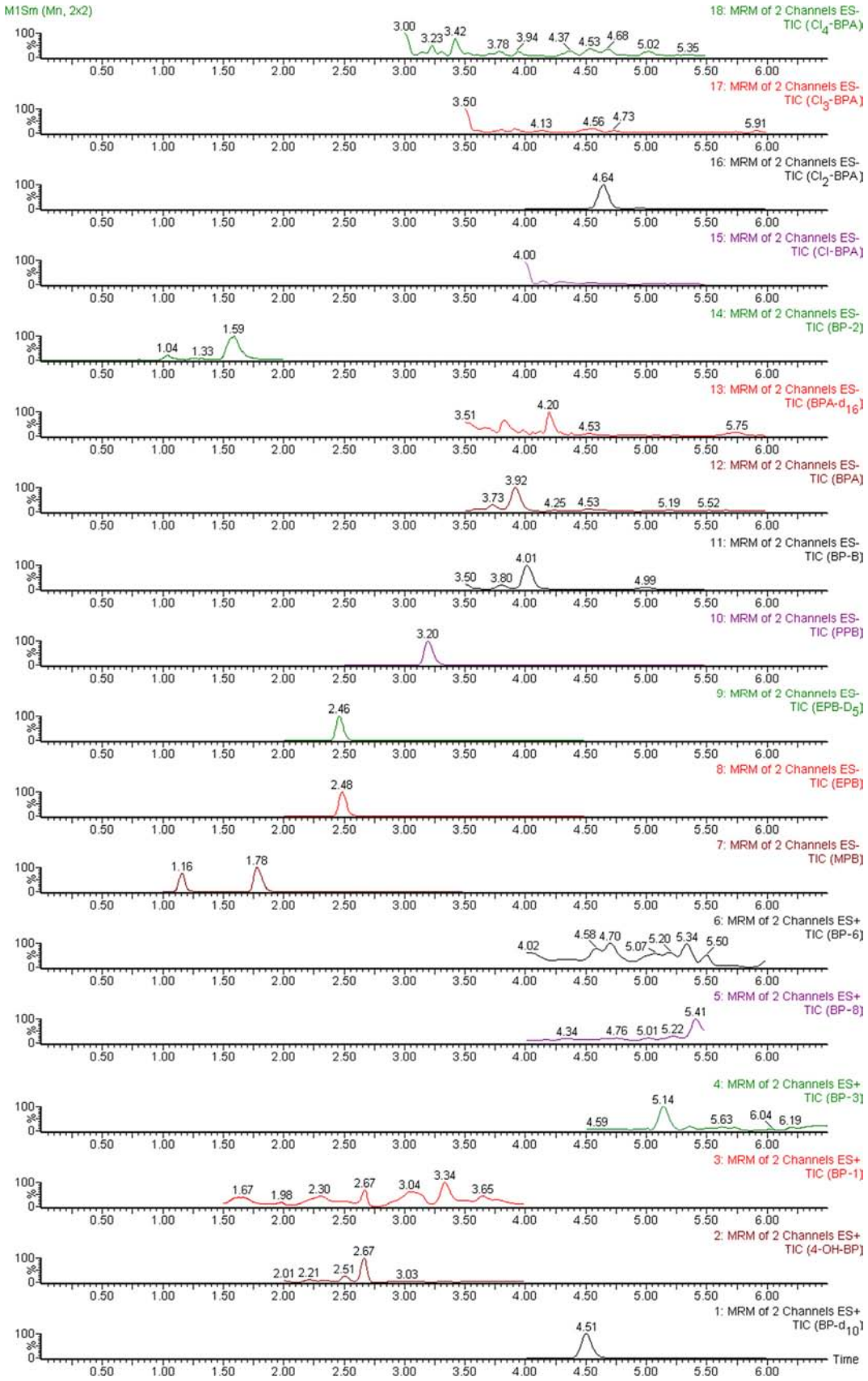

Fig. 4. Chromatograms of a real human milk sample (M01).

lower than 0.5 ng mL^{-1}. PBs were detected in almost samples, being MPB and PPB clearly the predominant. MPB was detected and quantified in 9 of the analyzed samples, in a concentration range from 0.4 to 3.5 ng mL^{-1} (mean: 1.7 ng mL^{-1}). PPB was also detected in nine samples in concentrations ranging from 0.1 to 7.5 ng mL^{-1} (mean: 2.3 ng mL^{-1}). EPB was quantified in nine of

Table 4
Application to human breast milk samples.

	Found amount (ng mL^{-1})[a] (RSD, %)									
	MPB	EPB	PPB	BPB	BPA	Cl_2-BPA	BP-1	BP-2	BP-3	4-OH-BP
M01	3.5 (4.3)	3.4 (2.9)	7.5 (5.8)	0.3 (5.8)	0.8 (4.8)	0.2 (6.2)	ND	0.4 (8.1)	8.1 (7.0)	2.3 (4.3)
M02	0.8 (2.5)	0.3 (2.2)	1.3 (3.2)	0.4 (8.2)	ND	ND	D	ND	0.9 (4.4)	0.4 (2.7)
M03	2.9 (5.4)	0.2 (2.5)	0.8 (3.6)	0.2 (3.9)	ND	ND	D	ND	7.1 (5.9)	4.1 (5.2)
M04	0.9 (3.0)	1.5 (3.0)	6.5 (7.3)	1.3 (3.3)	0.7 (10.9)	ND	D	ND	17.4 (2.1)	3.4 (3.8)
M05	1.1 (6.1)	0.2 (2.2)	0.3 (5.1)	D	0.6 (6.5)	0.4 (4.2)	D	0.5 (8.5)	6.8 (3.6)	5.8 (4.4)
M06	0.4 (8.1)	0.2 (3.5)	0.1 (6.8)	0.2 (4.3)	ND	ND	0.2 (4.8)	D	7.4 (4.1)	2.7 (3.5)
M07	3.3 (2.3)	0.8 (2.2)	0.8 (2.3)	0.3 (1.2)	2.3 (4.4)	ND	D	ND	2.4 (2.2)	1.3 (4.6)
M08	0.6 (3.4)	0.8 (4.9)	0.6 (4.2)	0.5 (4.9)	9.3 (3.1)	ND	0.5 (3.9)	ND	D	D
M09	1.4 (1.8)	0.3 (4.3)	2.4 (2.3)	1.3 (1.3)	13.8 (1.1)	ND	1.3 (5.1)	2.1 (5.4)	11.8 (0.8)	3.3 (2.2)
M10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Mean of 6 determinations; ND, not detected (< LOD); D: detected (> LOD and < LOQ); RSD: Relative standard deviation.

the samples but at lower concentrations than MPB and PPB (mean: 0.9 ng mL^{-1}). Finally, BPB was quantified in 8 samples and detected in one more of the analyzed samples (mean: 0.6 ng mL^{-1}). On the other hand, only four of the six BPs were detected and/or quantified in at least one sample. BP-3 and 4-OH-BP were the most frequently detected compounds being detected in nine of the ten samples and quantified in eight of them in a concentration ranges from 0.9 ng g^{-1} to 17.4 ng mL^{-1} (mean: 7.7 ng mL^{-1}) and from 0.4 to 5.8 ng mL^{-1} (mean: 2.9 ng mL^{-1}) respectively. BP-1 was detected in eight of the samples but quantified in only three of them (mean: 0.7 ng mL^{-1}) while BP-2 was detected in four samples and quantified in three of them (mean: 1.0 ng mL^{-1}). BP-6 and BP-8 were not detected in any of the analyzed samples.

Not many papers on the determination of these compounds in breast milk been published in the literature. When the results obtained in the present study are compared with those found by other authors, it can be concluded that the data are consistent but slightly different. Some authors [18,19,21–23,27] have found BPA in 100% of analyzed samples (n=23,100,3 and 4 respectively), while other authors such as Zimmers et al. [24], Ye et al. [26] and in our case, have found BPA in 60% of the analyzed samples (n=21, 20 and 10, respectively). Regarding PBs, Schlumpf et al. [20] established the presence of MPB, EPB and PPB in 26%, 15% and 11% of samples (n=54) respectively, while Ye et al. [27] determined PPB at MPB in 50% and 25% of the samples (n=4). In the present work, four PBs (MPB, EPB, PPB and BPB) were detected in most of the samples (10 samples, 90% of positives). Finally, in relation to BPs, Schlumpf [20] analyze only BP-2 and BP-3 in 54 samples and he only found BP-3 in 13% of them. Ye et al. analyzed BP-3 and BP-4 in 20 breast milk samples, with 25% [27] and 15% [26] of positive samples, compared to 90% of positive samples found in our study. Furthermore, we have found trace amounts of BP-1, BP-2 and 4-OH-BP in 80%, 40% and 90% of the samples, respectively.

4. Conclusions

The identification and quantification of free concentrations of BPA and chlorinated derivatives, four PBs and six BPs in human breast milk samples was successfully performed using a simple precipitation procedure followed by a clean-up with C18 and UHPLC-MS/MS analysis. The isolation of analytes from samples was accurately optimized and the procedure was validated. The methods were satisfactorily applied for the determination of target compounds in human milk samples from 10 randomly selected women. The analytical method can be applied can be very useful for the design of further studies for the determination of human exposure to EDCs. To our knowledge and to date, the proposed method presents the lower detection limits published in the scientific literature for the multiresidue determination of these fifteen compounds. This is a potent analytical tool that can be used in further studies for the determination of human exposure to those EDCs through human milk in the first stages of the life. As evidenced in this study and in studies by other authors [18–28], EDCs transfer from mother to child is not limited to the pregnancy period across the placenta, but it extends to the period of lactation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.07.047.

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