Journal of Chromatography B, 992 (2015) 47–55

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

New method for the determination of parabens and bisphenol A in human milk samples using ultrasound-assisted extraction and clean-up with dispersive sorbents prior to UHPLC–MS/MS analysis

R. Rodríguez-Gómez, N. Dorival-García, A. Zafra-Gómez*, F.J. Camino-Sánchez, O. Ballesteros, A. Navalón

Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, Campus of Fuentenueva, University of Granada, E-18071 Granada, Spain

ARTICLE INFO

Article history:
Received 26 December 2014
Received in revised form 8 April 2015
Accepted 10 April 2015
Available online 20 April 2015

Keywords:
Human milk
Endocrine disrupting chemicals
Ultrasound-assisted extraction
Sorbent materials
UHPLC–MS/MS

ABSTRACT

A sensitive and accurate analytical method for the determination of methyl-, ethyl-, propyl- and butylparaben and bisphenol A in human milk samples has been developed and validated. The combination of ultrasound-assisted extraction (UAE) and a simplified and rapid clean-up technique that uses sorbent materials has been successfully applied for the preparation of samples prior to ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) analysis. The analytes were extracted from freeze–dried human milk samples using acetonitrile and ultrasonic radiation (three 15-min cycles at 70% amplitude), and further cleaned-up with C18 sorbents. The most influential parameters affecting the UAE method and the clean-up steps were optimized using design of experiments. Negative electrospray ionization (ESI) in the selected reaction monitoring (SRM) mode was used for MS detection. The use of two reactions for each compound allowed simultaneous quantification and identification in one run. The analytes were separated in less than 10 min. Deuterium-labeled ethylparaben-d_5 (EPB-d_5) and deuterium-labeled bisphenol A-d_{16} (BPA-d_{16}) were used as surrogates. The limits of quantification ranged from 0.4 to 0.7 ng mL^{-1}, while inter- and intra-day variability was under 11.1% in all cases. In the absence of certified reference materials, recovery assays with spiked samples using matrix-matched calibration were used to validate the method. Recovery rates ranged from 93.8% to 112.2%. The proposed method was satisfactorily applied for the determination of four selected parabens and bisphenol A in human milk samples obtained from nursing mothers living in the province of Granada (Spain).

1. Introduction

Early life is a particularly vulnerable period of development and disturbances in the developmental process can result in persisting structural and functional changes that can manifest later in life in the form of impairment of cognitive function, chronic diseases, pubertal development or adult obesity [1,2]. Assessment of children exposure to the many environmental contaminants is of special interest.

Over the last decades, industry has grown steadily to meet the needs of today's society. This has resulted in an increase of the number of chemical compounds used to improve the standards of living. However, this dramatic industrial growth has been accompanied by an increase in human exposure to xenobiotics able to cause adverse effects on human health. This is particularly important in the case of endocrine disrupting chemicals (EDCs). EDCs are ubiquitous components of especially food and consumer products. These compounds belong to an extensive group of both synthetic and natural substances that are able to interfere with the normal hormonal and homeostatic function in wildlife and humans [3].

Synthetic chemicals such as bisphenol A (BPA) or parabens (PBs) are among the most common EDCs. BPA, widely used in the manufacture of polycarbonate plastics and epoxy resins [4], is one of the high-volume chemicals produced worldwide. On the other hand, PBs are widely used as bactericide and antimicrobial preservatives, especially against mold and yeast in cosmetic products, pharmaceuticals, food and beverages [5,6]. Individually or in combination, PBs are used in over 13,200 cosmetic formulations with a total maximum permitted concentration of 0.8% in the European Union in 2011 [7]. However, a recent review has decreased the levels of propyl- and butylparaben in cosmetic products to a combined maximum concentration of 0.19% [8]. The ability of PBs to disrupt

* Corresponding author. Tel.: +34 958 243326; fax: +34 958 243328.
E-mail address: azafra@ugr.es (A. Zafra-Gómez).

http://dx.doi.org/10.1016/j.jchromb.2015.04.022
1570-0232/

important physiological functions has been widely demonstrated [6,9–11]. In addition, the presence of non-metabolized PBs in breast cancer tissue [12] suggests their potential carcinogenic and toxic nature [11,13,14].

The main routes of human exposure to these compounds are dermal contact, ingestion or inhalation [6,15–17]. The assessment of exposure to EDCs is especially important in the case of breastfed infants, who are in the first stages of development being therefore more vulnerable and susceptible to changes in the endocrine system. Human breast milk has been proposed for the assessment of exposure to environmental chemicals [18], particularly to EDCs [19], because it is possibly the main route of exposure for babies. For this reason, the development of analytical methods for determination of EDCs at trace levels in this type of matrix becomes of great importance.

The isolation of analytes from this complex biological matrix is a critical issue and an extraction technique is usually required. Few studies have evaluated the methods for the determination of these compounds in human milk, particularly of PBs. Classical techniques such as liquid-liquid extraction (LLE) [1,20,21], solid-phase extraction (SPE) [22–25] including on-line SPE [26–28] have been used, being SPE, the most widely applied. However, several disadvantages are associated to both techniques, including the need of large sample volumes and toxic organic solvents, which make these techniques costly, time-consuming and environmentally-unfriendly. For these reasons, new extraction techniques have been developed over the last decades, including solid–liquid–liquid microextraction [29], stir-bar sorptive extraction [30], extraction with stir bar coated with molecularly imprinted polymers [31] or molecularly imprinted solid-phase microextraction [32]. In this work, the use of a simple and efficient solid-liquid extraction technique (ultrasound-assisted extraction) from freeze–dried samples is proposed. This technique is relatively simple in comparison with more recent techniques that does not require high solvent consumption, unlike classic procedures, and is highly efficient and rapid. The freeze–drying of samples as a preliminary step to extraction provides important advantages to sample treatment including smaller sample volumes and water removal to favor the partitioning of the analytes into the extraction solvent [33]. Given the complexity of biological matrices, the co-extraction of matrix components that reduces the sensitivity of the method is the major drawback of any extraction technique. A simplified clean-up step with sorbent materials is proposed in this paper in order to reduce matrix effects and improve analytical performance of the method.

In summary, the aim of the present work was to develop a sensible, selective and accurate method based on UAE followed by a clean-up step for the determination of four PBs, (methyl-, ethyl-, propyl- and butylparaben) and BPA in human milk samples. Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) was used as detection technique. The method has been satisfactorily applied for the determination of these compounds in 10 samples collected from volunteer nursing mothers.

2. Experimental

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 from Millipore (Bedford, MA, USA). Methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB) and butylparaben (BPB) were supplied by Alfa Aesar (Massachusetts, MA, USA). BPA and deuterium-labeled bisphenol A-d_{16} (BPA-d_{16}) – used as surrogate – were supplied by Sigma–Aldrich (Madrid, Spain). Deuterium-labeled ethylparaben-d_5 (EPB-d_5) – used as surrogate – was purchased from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada).

Stock standard solutions (100 μg mL^{-1}) were prepared by weighing 10 mg of each compound into a 100 mL flask. Acetonitrile (ACN) was added up to the final volume. The solution remained stable for at least four months at 4 °C in the dark. Working standards were prepared fresh from the ACN solutions prior to the experiments. Methanol (MeOH), ethanol, ethyl acetate and ACN (HPLC-grade) – used for the preparation of standards and for the selection of the extraction solvent–were purchased from Merck (Darmstadt, Germany). LC–MS grade water, methanol, ammonia (≥25%) and formic acid (≥98%) – used for the preparation of mobile phases and pH adjustments – were purchased from Fluka (St. Louis, MO, USA). Anhydrous $MgSO_4$ was provided by Panreac (Barcelona,

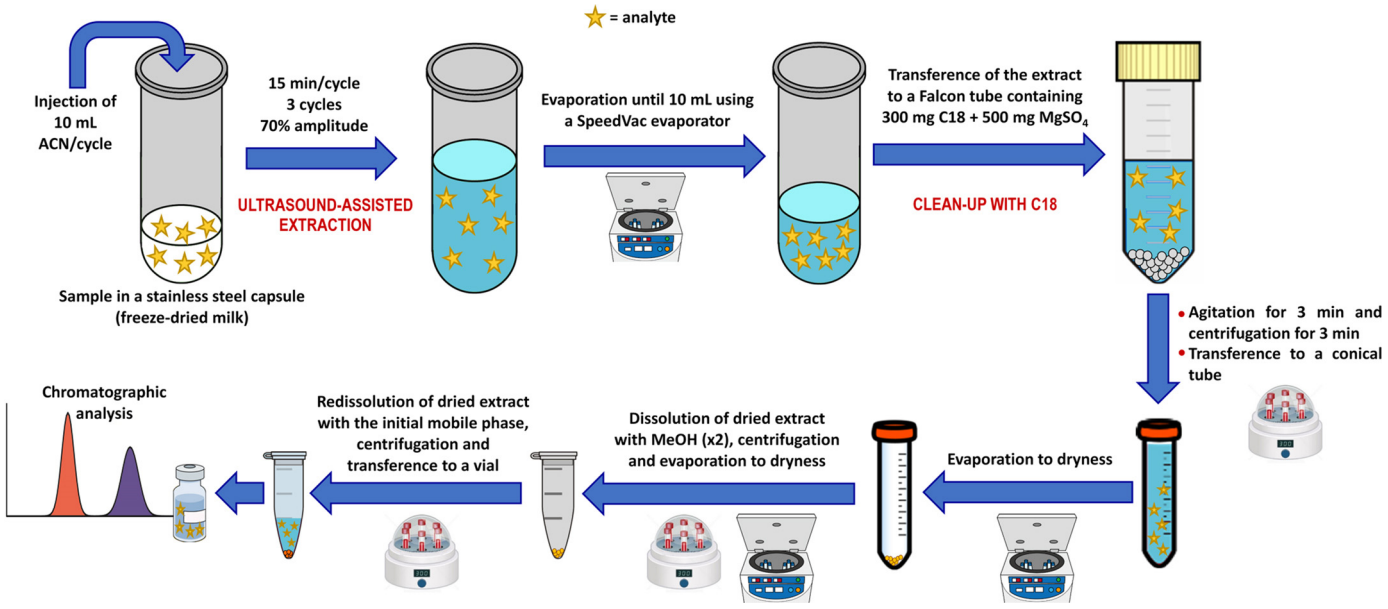

Fig. 1. Schematic illustration of the extraction procedure.

Spain). BAKERBOND® octadecyl C18 sorbent (40 μm particle size) was purchased from J.T. Baker (Deventer, The Netherlands) and primary-secondary amine sorbent (PSA) (40–60 μm particle size) was provided by Scharlab (Barcelona, Spain).

2.2. Instrumentation and software

The extraction of samples was performed with a Branson digital Sonifier® unit model S-450D (Danbury, CT, USA), operated with a standard 12.7 mm titanium disruptor horn, a flat and a temperature probe. UHPLC–MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class (Waters, Manchester, UK), consisting of an Acquity UPLC™ binary solvent manager and an Acquity UPLC™ sample manager. Separation of compounds was obtained with a UPLC™ BEH C18 column (1.7 μm, 2.1 mm × 50 mm) (Waters). A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for analyte detection. Samples were freeze–dried using a SCANVAC CoolSafe™ freeze dryer. Extracts were evaporated with a SCANVAC CoolSafe™ ScanSpeed MaxiVac centrifuge for vaccuum evaporation (Lynge, Denmark). For pH measurements, a EUTECH PCD 650 digital pH-meter with a combined glass–Ag/AgCl (KCl 3 M) electrode (EUTECH Instruments Ltd, Singapore) was used. A vortex-mixer (IKA, Staufen, Germany), a Hettich Universal 32 centrifuge (Tuttlingen, Germany) and a Spectrafuge™ 24D centrifuge from Labnet International, Inc. (New Jersey, USA) were also used. Samples agitation during the extraction procedure was carried out in an eight-position digital agitator-vibrator purchased from J.P. Selecta (Barcelona, Spain). Statgraphics Plus software version 5.1 (Statpoint Technologies Inc., Virginia, USA) was used for statistical treatment of data.

2.3. Sample collection and storage

Human milk samples were obtained from healthy nursing mothers 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. The samples were obtained under strictly controlled conditions; BPA-free plastic bags were used for sampling and mothers were advised not to apply body creams on the breast before sampling.

2.4. Basic procedure

2.4.1. Preparation of spiked samples

For the preparation of the spiked samples used for the optimization of the extraction procedure, a concentrate standard solution of the analytes was added to the sample and agitated to obtain a final concentration of 20 ng mL^{-1} for each compound. In order to allow the analytes to interact with the human milk, the spiked samples were left to stand for 24 h at 4 °C in the dark before analysis. Next, milk was divided in 10 mL glass vials and frozen at −80 °C for 12 h prior freeze–drying.

For method validation purposes (recovery assays, precision and trueness), in the absence of certified reference materials, 9.9 mL of blank human milk samples were spiked at different concentrations by adding 100 μL of the spiked standard solutions containing the analytes and surrogates. The spiked samples were treated underwent the treatment previously described. The blank samples were previously analyzed to ensure the absence of analytes or that these were below the limits of detection (LODs) of the method.

2.4.2. Extraction procedure

Fig. 1 illustrates the steps of the extraction procedure. Freeze–dried samples were placed into stainless steel capsules and 10 mL of ACN were added. The capsules were vortexed for 2 min and sonicated for 15 min at 70% amplitude. Four samples could be simultaneously extracted. Three extraction cycles were required. The extracts obtained were merged and concentrated to a volume of approximately 10 mL in Falcon tubes at 40 °C using the SpeedVac concentrator. The remaining extracts were then cleaned-up with 500 mg of anhydrous $MgSO_4$ and 300 mg of C18 sorbent. The Falcon tubes were placed on the digital agitator-vibrator, stirred for 3 min at room temperature and centrifuged for 3 min at 3634 × *g*. The supernatants were decanted into conical polypropylene tubes and evaporated to dryness using the SpeedVac concentrator at 40 °C. The dried extracts were dissolved with two portions of 300 μL MeOH each one, then centrifuged using Eppendorf tubes for 20 min at 16,300 × *g*, and again evaporated to dryness. The residue was dissolved in 100 μL of the initial mobile phase, centrifuged for 30 min at 16,300 × *g* and finally injected.

2.5. UHPLC–MS/MS conditions

The chromatographic separation was performed using a binary gradient mobile phase consisting of 0.025% (v/v) aqueous ammonia solution (solvent A) and 0.025% (v/v) ammonia in MeOH (solvent B). The flow rate was 300 μL min^{-1}, the column was maintained at 40 °C and the injection volume was 10 μL. Gradient conditions were as follows: initial mobile phase, 80% (A), maintained for 2 min, then it was linearly decreased to 10% (A) within 3.0 min, and to 0% within 0.1 min and held for 1.9 min to clean the column using 100% organic mobile phase. Finally, back to 80% (A) in 0.1 min and kept for 2.9 min to equilibrate the column. Total run time was 10 min.

The mass spectrometer (MS) was operated in negative electrospray ionization mode. For increased sensitivity and selectivity, the analyses were performed in selected reaction monitoring mode (SRM) using $[M–H]^-$ as precursor ion. Instrument parameters were as follows [30]: 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 nebuliser gas pressure, 7.0 bar. Nitrogen (≥99.995%) was used as cone and desolvation gas, and argon (99.999%) as a collision gas. Dwell time for each transition was 25 ms, and inter-scan delay was set at 3 ms. In order to obtain the maximum sensitivity, the most abundant transition was used for quantification. Table 1

Table 1
SRM transitions and optimized potentials for UHPLC-MS/MS analysis of PBs, BPA, and surrogates.

	Transitions (*m*/*z*)	CV (V)	CE (eV)
BPA	227.2 → 211.9[a]	−50–50	−22–26
	227.2 → 132.9[b]		
MPB	151.1 → 91.8[a]	−38–38	−22–14
	151.1 → 135.8[b]		
EPB	165.1 → 91.9[a]	−38–38	−24–16
	165.1 → 136.0[b]		
PPB	179.1 → 91.8[a]	−42–42	−24–16
	179.1 → 136.1[b]		
BPB	193.1 → 91.4[a]	−42–42	−24–16
	193.1 → 136.1[b]		
BPA-d_{16}	241.2 → 223.0[a]	−46–46	−22–32
	241.2 → 141.9[b]		
EPB-d_5	170.1 → 92.1[a]	−38–38	−24–16
	170.1 → 136.0[b]		

[a] SRM transition used for quantification.
[b] SRM transition used for confirmation.

summarizes the transitions and potentials used for UHPLC–MS/MS analysis for the EDCs and the surrogates.

2.6. Quality assurance and quality control

Validity of the analytical results was verified by several quality assurance and quality control (QA/QC) measures. Procedural blanks were injected to monitor for background contamination. No quantifiable amount of target compounds was detected. On the other hand, in order to evaluate possible contamination and the variability of the instrument, spiked blank samples at 0, 1 and 50 $\mu g\,L^{-1}$ and a standard solution in pure solvent (50 $\mu g\,L^{-1}$) were injected in triplicate, every nine samples, for every batch of samples.

3. Results and discussion

3.1. Optimization of the extraction parameters

Although MS is a selective and sensitive detection technique, lipids and other matrix interferences can deteriorate the sensitivity of the instrumental technique by increasing the signal background. An efficient clean-up step is necessary to overcome this analytical drawback. It is necessary to count on an extraction technique to

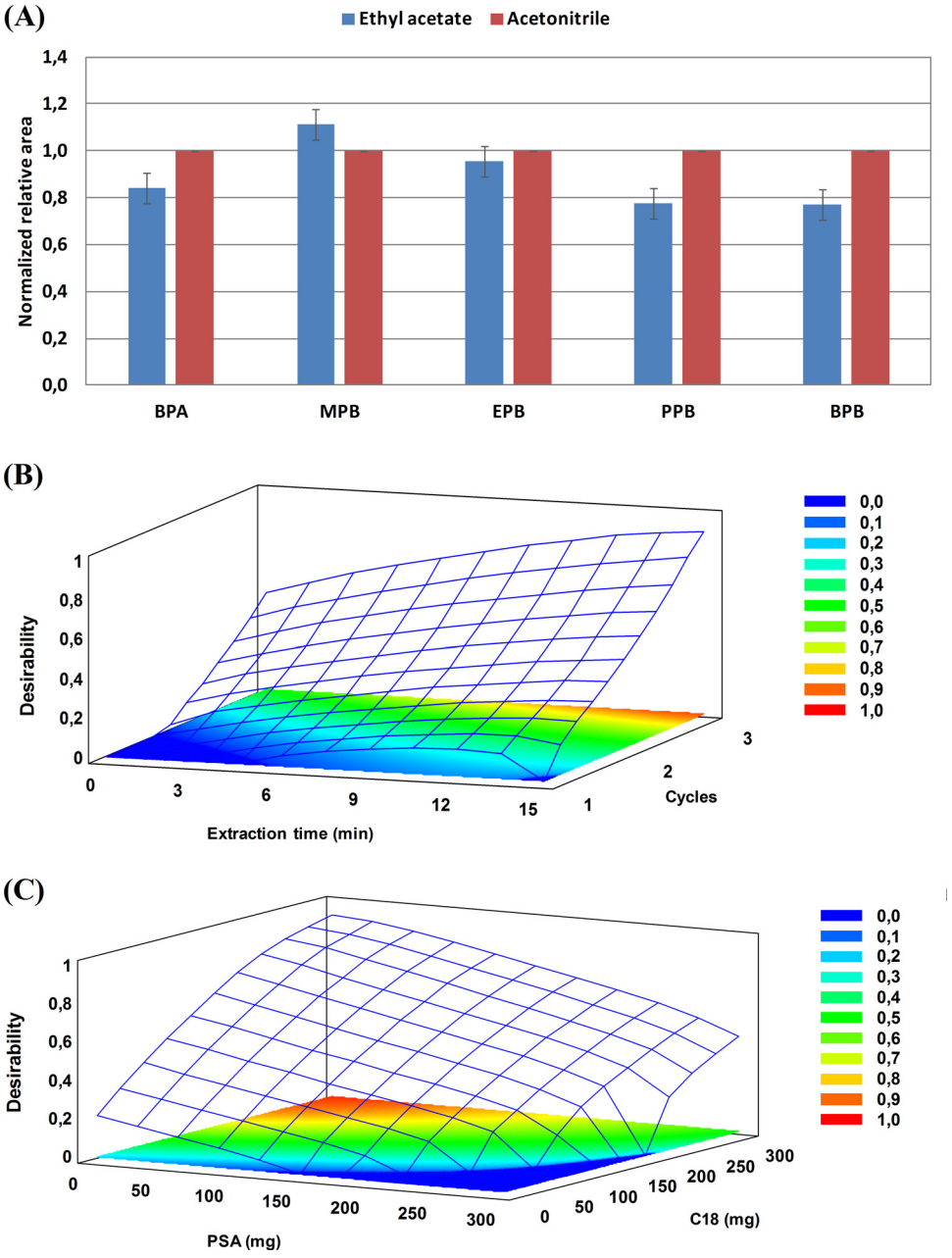

Fig. 2. (A) Normalized relative areas for the selection between ACN and ethyl acetate as extraction solvent. BPA (LSD = 0.08); MPB (LSD = 0.10); EPB (LSD = 0.16); PPB (LSD = 0.15); BPB (LSD = 0.39). Significance level of 95% was selected for all cases. Representation of the global desirability function versus (B) Extraction time and number of cycles for ultrasonic extraction, and (C) Amount of PSA and C18 sorbents for the clean-up step for the determination of targets EDCs.

disrupt the matrix and efficiently extract the analytes. In this sense, the variables affecting the UAE method and the clean-up steps were optimized using design of experiments. Each assay was done in triplicate.

3.1.1. Selection of the extraction solvent

The extraction solvent is a critical variable for developing an efficient UAE method. Four organic solvents (methanol, ethanol, acetonitrile and ethyl acetate) widely used for the extraction of different families of EDCs from biological and environmental samples were evaluated [5,34,35]. The following basic procedure was applied: spiked freeze–dried milk samples were extracted using UAE with 10 mL of the evaluated solvent during 15 min at 70% amplitude. Two extraction cycles were applied in all cases. The obtained extracts were evaporated to dryness using the SpeedVac concentrator at 40 °C. The dried residues were dissolved with the initial mobile phase and injected into the LC system.

MeOH and ethanol extracts were characterized by the highest extraction of matrix components, making difficult the handling of the extracts and were not analyzed further. The results of the comparison of the extraction efficiency of ACN and ethyl acetate are shown in Fig. 2(A). Mean values of relative area for each target analyte with each solvent were compared using the least significant difference (LSD) multiple range test with a 95.0% confidence level. The results showed statistically significant differences only for PPB and BPB, being acetonitrile the optimal solvent. In the case of MPB, EPB and BPA, no significant differences were found between both solvents. ACN was selected as optimal solvent.

3.1.2. Optimization of UAE conditions

In order to optimize the UAE parameters and determine possible interactions between them, a 15-run Box–Behnken design including three replicates at the center point was used for fitting a second-order response surface, with three factors and three levels for each one: extraction time (1, 8 and 15 min), number of extraction cycles (1, 2 and 3) and extraction solvent volume per cycle (10, 15 and 20 mL). The maximum amplitude was 70% in order to not reduce the lifespan of the ultrasound probe. This amplitude was used in all assays because some studies indicate that this value provides the highest extraction efficiency for EDCs [36]. The matrix applied in the Box–Behnken design is shown as supplementary material.

The data were analyzed using ANOVA, which provided determination coefficients (R^2) between 0.813 and 0.930. Since the P values for the *lack-of-fit* test were >0.05 in all cases, the model appears to be satisfactory with the 95% of confidence level. It was observed that the number of extraction cycles was the most important parameter, followed by the extraction time, which was not significant only for BPA. The observed tendency for all the analytes was that both parameters had always a positive effect. These results proved that although the long exposure to solvent allows the matrix to swell, thus improving the penetration of solvent into the sample interstices and the contact of the solvent with the analytes. There is an important advantage in increasing the number of cycles instead of increasing the extraction time. The introduction of fresh solvent maintains a favorable solvent/sample equilibrium hence, improving partitioning into the liquid phase and increasing recoveries [35]. Finally, the volume of extraction solvent was not an influential variable in any case. It was set at 10 mL, the lowest volume assayed.

The combination of the optimized experimental values obtained for each compound for the evaluated variables allowed the determination of the best overall extraction efficiency, which was calculated using the desirability function. Responses for each compound in the experiments of the Box–Behnken design were first normalized between 0 and 1, and the global desirability function was defined as their geometric mean. The plot of this function versus the number of cycles and the extraction time, with the volume of the extraction solvent at 10 mL, is shown in Fig. 2(B). The optimal values corresponded to three extraction cycles of 15 min, 10 mL of extraction solvent and 70% of amplitude.

3.1.3. Optimization of the clean-up procedure

When an extraction procedure is applied to a biological sample, many interfering substances are also co-extracted. Therefore, it was of interest to perform a clean-up step to remove matrix components, especially the lipid content of the extract. A clean-up procedure with sorbents that are frequently used in dispersive solid-phase extraction was used.

For the development of the clean-up procedure, a combination of two solid sorbents was evaluated: PSA and C18, commonly used for the removal of extracted interferences in biological matrices. In addition, the combination of these two types of sorbents was used because the extracted interferences in milk, such as fatty acids, sugars, triglycerides, phospholipids and cholesterol have higher affinity for this combination than for the other types of sorbents [37–41]. C18 is specifically used for removal of co-extracted fat and other lipophilic compounds from ACN extracts. In addition, it is

Table 3
Recovery assay, precision and trueness of PBs and BPA determination in human milk.

	Spiked (ng mL^{-1})	[a] Found ± CI (ng mL^{-1})	RSD (%)	Recovery (%)
BPA	1	1.02 ± 0.03	5.8	102.7
	10	9.9 ± 0.2	3.1	99.6
	50	49.6 ± 0.5	1.3	100.2
MPB	1	0.93 ± 0.04	6.5	94.0
	10	9.94 ± 0.3	5.0	100.0
	50	49.6 ± 1.2	3.2	100.3
EPB	1	0.93 ± 0.03	5.1	93.8
	10	10.6 ± 0.5	7.3	107.0
	50	50.0 ± 2.0	4.5	101.1
PPB	1	1.09 ± 0.07	9.3	109.6
	10	10.1 ± 0.8	11.1	101.7
	50	49.8 ± 1.2	3.7	100.7
BPB	1	1.12 ± 0.03	3.3	112.2
	10	9.68 ± 0.5	6.8	97.4
	50	49.7 ± 0.8	2.4	100.5

[a] Mean of 18 determinations; CI: confidence interval (P = 95% and 17 freedom degrees); RSD: relative standard deviation.

Table 2
Analytical and statistical parameters for UHPLC-MS/MS analysis of PBs and BPA in human milk.

	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	1.278×10^{-2}	1.010×10^{-4}	99.8	47.4	0.2	0.5	0.5–50
MPB	1.870×10^{-2}	1.340×10^{-4}	99.8	17.1	0.2	0.5	0.5–50
EPB	1.952×10^{-2}	1.723×10^{-4}	99.6	17.5	0.1	0.5	0.5–50
PPB	1.590×10^{-2}	1.322×10^{-4}	99.7	54.0	0.1	0.4	0.4–50
BPB	8.057×10^{-3}	5.509×10^{-5}	99.8	15.0	0.2	0.7	0.7–50

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

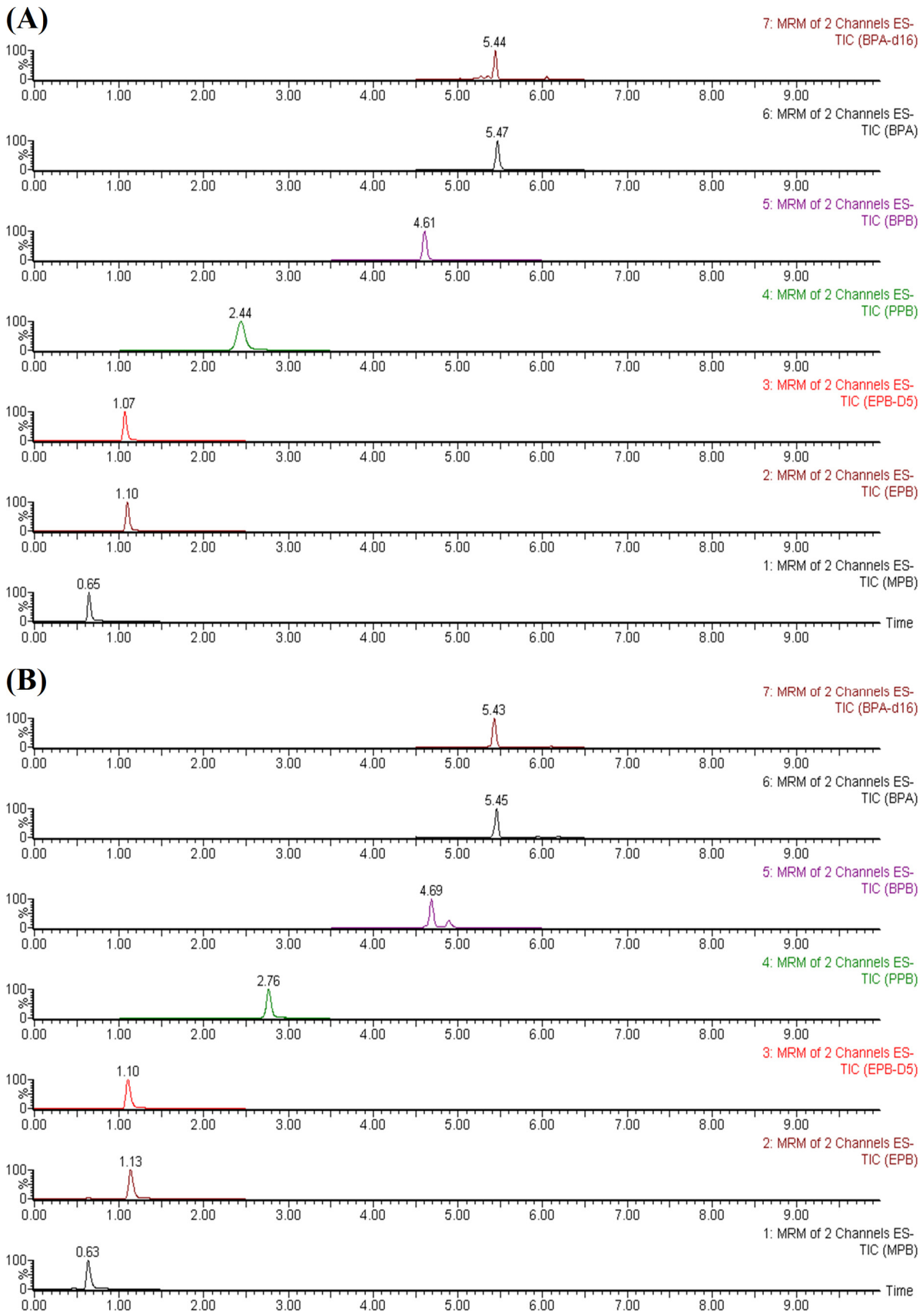

Fig. 3. UHPLC–MS/MS chromatograms of: (A) a spiked blank; (B) a positive human breast milk sample with the target analytes, containing the corresponding surrogates (BPA-d_{16} and EPB-d_5).

worth noting, that although bulk fats represented by triacylglycerols (TAGs) are not soluble in ACN, some TAGs and other lipophilic compounds move to the organic layer during a partitioning step in the form of micro-micelles and can cause clogging of the analytical column. To eliminate these non-polar co-extracts, the use of C18 sorbent can also be an efficient alternative [35,42]. On the other hand, PSA could be also a good alternative for clean-up, since its bidentate structure is responsible for its high chelating effect. As a result of its secondary and primary amino groups, the retention of free fatty acids and other polar matrix compounds is very strong. Therefore, matrix components can be more efficiently removed from the extracts resulting in cleaner chromatograms. The composition of sorbent mixture for the clean-up step was determined with a Box–Behnken design. The mass of the desiccant ($MgSO_4$) was also included as a variable. The Box–Behnken design matrix also involved 15 experiments, including three central points. The variables, evaluated at three levels, were C18 mass (0, 150 and 300 mg), PSA mass (0, 150 and 300 mg) and $MgSO_4$ mass (0, 250, 500 mg). The matrix design, i.e. the extraction conditions for each run, is shown as supplementary material.

Fig. 2(C) shows the plot of the desirability function versus the mass of PSA and C18 sorbents, with the mass of $MgSO_4$ set at 500 mg, corresponding to the optimum value according to the Box–Behnken design. Parameters corresponding to both sorbents, particularly their quadratic terms, resulted statistically significant for all compounds. Despite the indicated advantages in the removal of matrix interferences, the use of PSA showed an important negative effect. This sorbent is a weak anion exchanger, and therefore could be not suitable due to the acidic nature of analytes. It was also observed a significant positive interaction between C18 sorbent and $MgSO_4$. This could be explained by the fact that $MgSO_4$ eliminates traces of water that could emulsify with C18. This fact hurts the clean-up step and makes difficult the separation of the cleaned extract. According to the results of the Box–Behnken design, a mixture of 300 mg of C18 and 500 mg of $MgSO_4$ was selected as the compromise mixture used in the clean-up step.

3.2. Method validation

A seven-point matrix–matched calibration curve was obtained for each studied compound in the range from the limit of quantification (LOQ) to 50 $\mu g\,mL^{-1}$. Calibration curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. EPB-d_5 and BPA-d_{16} (50 $\mu g\,mL^{-1}$) were selected as surrogates for PBs and BPA, respectively. Each calibration level was made in triplicate and analyzed twice. Table 2 shows the statistical and the analytical parameters obtained for each compound.

The analytical method was validated in terms of linearity, selectivity, sensitivity and accuracy (trueness and precision), according to the protocols described in the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [43].

Linearity. The determination coefficient (R^2) and the *lack-of-fit* test (P_{lof}) were evaluated. The values obtained for R^2 ranged from 99.6 to 99.8% and P_{lof} values were >5% in all cases [44], and good linearity was observed within the concentration range (LOQ–50 $ng\,mL^{-1}$).

Sensitivity. The LODs and LOQs were calculated by taking into consideration the minimum concentration of analyte that the method can detect and a signal-to-noise ratio (S/N) of three for LODs and ten for LOQs, using the quantification transition. Found LODs ranged from 0.1 to 0.2 $ng\,mL^{-1}$ and found LOQs ranged from 0.4 to 0.7 $ng\,mL^{-1}$. These results are also summarized in Table 2.

Accuracy (precision and trueness). The precision of the method in terms of intra- and inter-day variability was evaluated using spiked human milk samples at three concentration levels (1, 10 and 50 $ng\,mL^{-1}$). Precision, expressed as relative standard deviation (%, RSD) was determined from triplicate spiked samples during the same day and in six different days. The values obtained are summarized in Table 3. *RSD* values fell between 1.3% and 11.1%. Therefore, all compounds were within the accepted established limits for 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 indicate that the methods are reproducible.

For method validation, trueness was assessed from recovery assays. The recovery of the tested compounds in milk samples was evaluated by comparing the known concentration in spiked samples with the concentration of each compound determined using the method proposed. As shown in Table 3, the recoveries were close to 100% (93.8–112.2%) in all cases.

Precision and trueness data indicate that the method is accurate and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Selectivity was assessed by UHPLC–MS/MS analysis of blanks. A blank sample and a spiked blank sample containing a known concentration of the analytes were extracted and their chromatograms were compared. No interferences from endogenous substances were observed at the retention time of the analytes. These findings suggest that the spectrometric conditions ensure the high selectivity of the method. Fig. 3(A) shows the SRM chromatograms obtained from a spiked milk sample.

3.3. Application of the method

The proposed method was applied to determine free concentrations of selected PBs and BPA in ten anonymous human milk samples collected from women with no known occupational exposure to these compounds. The results obtained as a mean of six determinations are summarized in Table 4.

BPA was found in eight samples but quantified in five of them at concentrations ranging from 0.6 to 2.1 $ng\,mL^{-1}$. These concentration levels of free BPA are similar to those reported by previous studies in Caucasian women, one conducted in France (0.80–3.29 $ng\,mL^{-1}$) [45] and two in the United States (0.22–10.8 $ng\,mL^{-1}$) [24,27]. These studies suggest a significant association between BPA concentration and race. Caucasian women had significantly higher levels of free BPA in their milk than non-Caucasian women (mean value 0.52 $ng\,mL^{-1}$) [24]. Concentrations levels of PBs were similar in most of the samples (1–2 $ng\,mL^{-1}$), except sample No. 1 that showed concentrations slightly higher than the others, and sample No. 3 contained about 10 times higher concentrations of PBs than the rest. Only one sample did not contain any of the analyzed compounds. Except for sample No. 3, the rest of the results are in agreement with a previous work

Table 4
Concentrations of BPA and PBs determined in human milk samples.

Sample	Concentration ($ng\,mL^{-1}$)[a,b]				
	BPA	MPB	EPB	PPB	BPB
1	1.31 (0.04)	2.80 (0.09)	2.00 (0.06)	4.0 (0.4)	5.4 (0.4)
2	ND	1.82 (0.06)	1.62 (0.06)	1.3 (0.2)	D
3	D	16.3 (0.5)	18.1 (1.2)	12.6 (1.5)	12.1 (1.0)
4	D	4.65 (0.25)	D	1.98 (0.07)	1.19 (0.09)
5	0.60 (0.04)	1.74 (0.09)	0.97 (0.03)	1.39 (0.06)	D
6	1.76 (0.07)	1.26 (0.07)	D	1.02 (0.08)	D
7	2.10 (0.09)	1.99 (0.07)	1.15 (0.02)	1.13 (0.09)	1.06 (0.05)
8	D	1.81 (0.06)	1.13 (0.03)	D	1.17 (0.03)
9	D	D	D	ND	D
10	ND	ND	ND	ND	ND

ND: not detected (<LOD); D: detected (>LOD and <LOQ).
[a] Mean of 6 determinations.
[b] Standard deviations are into parentheses.

(0.32–3.04 ng mL^{-1}) [27]. In general, these results confirm that these substances are ubiquitous, which could be due to the daily and massive use of personal care products, especially by women. Fig. 3(B) shows the SRM chromatograms obtained from one of the positive analyzed samples.

4. Conclusions

A relatively simple, efficient and robust extraction technique that uses ultrasounds and a clean-up step that uses C18 sorbent material has been proposed as a convenient alternative for sample preparation for determining some selected EDCs (PBs and BPA) at trace levels in freeze–dried human milk samples by UHPLC–MS/MS. Due to the complexity of biological matrices, the obtained extracts shown significant matrix effects. The optimization, using experimental designs, of the UAE process and the clean-up step, together with the selective and sensitive quantification by UHPLC–MS/MS, allowed the validation of the analytical performance of the method. Excellent analytical parameters in relation to sensitivity, selectivity, accuracy and precision were obtained. Low LOQs (between 0.4 and 0.7 ng mL^{-1}), high recoveries and precision were achieved. The proposed sample preparation procedure was considered optimal because of its high extraction yield and easy operation, especially when compared to the SPE technique, traditionally used for human milk analysis. In addition, UAE saves time and requires lower volumes of solvents than SPE, reducing costs and residues.

This method is useful for the determination of PBs and BPA levels in human milk samples and it could be used in biomonitoring studies, since human milk may serve as indicator of both maternal and prenatal exposure to many different environmental chemicals, particularly to EDCs. Human milk is a major route of exposure for breastfed infants to exogenous contaminants. The development of analytical methods for the determination of these substances will allow the development of further studies on the incidence and onset of diseases and other adverse effects related to exposure to EDCs.

Acknowledgments

This study was supported by the Regional Government of Andalusia (Project of Excellence No. P09-CTS-4470). The authors are grateful to the Regional Government of Andalusia for the fellowship granted to R. Rodríguez-Gómez.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.04.022

References

[1] M. Schlumpf, K. Kypke, M. Wittassek, J. Angerer, H. Mascher, D. Mascher, C. Vökt, M. Birchler, W. Lichsteinsteiger, Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlor pesticides, PBDEs, and PCBs in human milk: correlation of UV filters with use of cosmetics, Chemosphere 81 (2010) 117–1183.
[2] I. Pan, J.L. Daniels, A.H. Herring, W.J. Rogan, A.M. Siega-Riz, B.D. Goldman, A. Sjödin, Lactational exposure to polychlorinated biphenyls, dichlorodiphenyltrichloro-ethane, and dichlorodiphenyldichloroethylene and infant growth: an analysis of the pregnancy, infection and nutrition babies study, Paediatr. Perinat. Epidemiol. 24 (2010) 262–271.
[3] C. Sonnenschein, A.M. Soto, An updated review of environmental estrogen and androgen mimics and antagonists, J. Steroid. Biochem. 65 (1998) 143–150.
[4] Bisphenol A Website, Document available on line at: http://www.bisphenol-a.org (visited 07.04.15).
[5] I. Jiménez-Díaz, F. Vela-Soria, A. Zafra-Gómez, A. Navalón, O. Ballesteros, N. Navea, M.F. Fernández, N. Olea, J.L. Vílchez, A new liquid chromatography-tandem mass spectrometry method for the determination of parabens in human placental tissue samples, Talanta 84 (2011) 702–709.
[6] M.G. Soni, I.G. Carabin, G.A. Burdock, Safety assessment of esters of p-hydroxybenzoic acid (parabens), Food Chem. Toxicol. 43 (2005) 985–1015.
[7] EU Cosmetics Directive 76/768/EEC. Available at: http://ec.europa.eu/consumers/archive/sectors/cosmetics/documents/directive/index_en.htm (visited 07.04.15).
[8] European Commission, Scientific Committee on Consumer Safety (2011) Opinion on Parabens. SCCS/1348/10, 1-36. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_041.pdf (visited 07.04.15).
[9] J.A. van Meeuwen, O. van Son, A.H. Piersma, P.C. de Jong, M. van den Berg, Aromatase inhibiting and combined estrogenic effects of parabens and estrogenic effects of other additives in cosmetics, Toxicol. Appl. Pharm. 230 (2008) 372–382.
[10] J.R. Byford, L.E. Shaw, M.G. Drew, G.S. Pope, M.J. Sauer, P.D. Darbre, Oestrogenic activity of parabens in MCF7 human breast cancer cells, J. Steroid Biochem. 80 (2002) 49–60.
[11] J. Boberg, C. Taxvig, S. Christiansen, U. Hass, Possible endocrine disrupting effects of parabens and their metabolites, Reprod. Toxicol. 30 (2010) 301–312.
[12] P.D. Darbre, A. Aljarrah, W.R. Miller, N.G. Coldham, M.J. Sauer, G.S. Pope, Concentrations of parabens in human breast tumours, J. Appl. Toxicol. 24 (2004) 5–13.
[13] P.D. Darbre, P.W. Harvey, Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks, J. Appl. Toxicol. 28 (2008) 561–578.
[14] R. Golden, J. Gandy, G. Vollmer, A review of the endocrine activity of parabens and implications for potential risks to human health, Crit. Rev. Toxicol. 35 (2005) 435–458.
[15] C.G.J. Hayden, S.E. Cross, C. Anderson, N.A. Saunders, M.S. Roberts, Sunscreen penetration of human skin and related keratinocyte toxicity after topical application, Skin Pharmacol. Phys. 18 (2005) 170–174.
[16] S. El Hussein, P. Muret, M. Berard, S. Makki, P. Humbert, Assessment of principal parabens used in cosmetics after their passage through human epidermis-dermis layers (ex-vivo study), Exp. Dermatol. 16 (2007) 830–836.
[17] L.N. Vandenberg, R. Hauser, M. Marcus, N. Olea, W.V. Welshons, Human exposure to bisphenol A (BPA), Reprod. Toxicol. 24 (2007) 139–177.
[18] C.M. Berlin, J.S. LaKind, S.E. Fenton, R.Y. Wang, M.N. Bates, R.L. Brent, M. Condon, B.L. Crase, M.L. Dourson, A.S. Ettinger, B. Foos, P. Furst, G.P. Giacoia, D.A. Goldstein, S.G. Haynes, K.D. Hench, S. Kacew, G. Koren, R.A. Lawrence, A. Mason, M.A. McDiarmid, G. Moy, L.L. Needham, I.M. Paul, L.C. Pugh, Z. Qian, L. Salamone, S.G. Selevan, B. Sonawane, A.J. Tarzian, M. Rose Tully, K. Uhl, Conclusions and recommendations of the expert panel: technical workshop on human milk surveillance and biomonitoring for environmental Chemicals in the United States, J. Toxicol. Environ. Health. Part A 68 (2005) 1825–1831.
[19] M. Stefanidou, C. Maravelias, C. Spiliopoulou, Human exposure to endocrine disruptors and breast milk, Endocr. Metab. Immune Disord Drug Targets 9 (2009) 269–276.
[20] Y. Sun, M. Irie, N. Kishikawa, M. Wada, N. Kuroda, K. Nakashima, Determination of bisphenol A in human breast milk by HPLC with column-switching and fluorescence detection, Biomed. Chromatogr. 18 (2004) 501–507.
[21] B. Yi, C. Kim, M. Yang, Biological monitoring of bisphenol A with HPLC/FLD and LC/MS/MS assays, J. Chromatogr. B 878 (2010) 2606–2610.
[22] H. Otaka, A. Yasuhara, M. Morita, Determination of bisphenol A and 4-nonylphenol in human milk using alkaline digestion and cleanup by solid-phase extraction, Anal. Sci. 19 (2003) 1663–1666.
[23] R. Kuruto-Niwa, Y. Tateoka, Y. Usuki, R. Nozawa, Measurement of bisphenol A concentrations in human colostrum, Chemosphere 66 (2007) 1160–1164.
[24] S.M. Zimmers, E.P. Browne, P.W. O'Keefe, D.L. Anderton, L. Kramer, D.A. Reckhow, K.F. Arcaro, Determination of free Bisphenol A (BPA) concentrations in breast milk of U.S. women using a sensitive LC/MS/MS method, Chemosphere 104 (2014) 237–243.
[25] L.P. Melo, M.E.C. Queiroz, A molecularly imprinted polymer for microdisc solid-phase extraction of parabens from human milk samples, Anal. Methods 5 (2013) 3538–3545.
[26] X. Ye, Z. Kuklenyik, L.L. Needham, A.M. Calafat, Measuring environmental phenols and chlorinated organic chemicals in breast milk using automated on-line column-switching–high performance liquid chromatography–isotope dilution tandem mass spectrometry, J. Chromatogr. B 831 (2006) 110–115.
[27] X. Ye, A.M. Bishop, L.L. Needham, A.M. Calafat, Automated on-line column-switching HPLC-MS/MS method with peak focusing for measuring parabens, triclosan and other environmental phenols in human milk, Anal. Chim. Acta 622 (2008) 150–156.
[28] K. Mendonca, R. Hauser, A.M. Calafat, T.E. Arbuckle, S.M. Duty, Bisphenol A concentrations in maternal breast milk and infant urine, Int. Arch. Occup. Environ. Health 87 (2014) 13–20.
[29] R. Rodríguez-Gómez, M. Roldán-Pijuán, R. Lucena, S. Cárdenas, A. Zafra-Gómez, O. Ballesteros, A. Navalón, M. Valcárcel, Stir-membrane solid–liquid–liquid microextraction for the determination of parabens in human breast milk samples by ultra high performance liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1354 (2014) 26–33.
[30] R. Rodríguez-Gómez, A. Zafra-Gómez, F.J. Camino-Sánchez, O. Ballesteros, A. Navalón, Gas chromatography and ultra high performance liquid chromatography tandem mass spectrometry methods for the determination of selected endocrine disrupting chemicals in human breast milk after stir-bar sorptive extraction, J. Chromatogr. A 1349 (2014) 69–79.
[31] W. Zhan, F. Wei, G. Xu, Z. Cai, S. Du, X. Zhou, F. Li, Q. Hu, Highly selective stir bar coated with dummy molecularly imprinted polymers for trace analysis of bisphenol A in milk, J. Sep. Sci. 35 (2012) 1036–1043.

[32] F. Tan, H. Zhao, X. Li, X. Quan, J. Chen, X. Xiang, X. Zhang, Preparation and evaluation of molecularly imprinted solid-phase microextraction fibers for selective extraction of bisphenol A in complex samples, J. Chromatogr. A 1216 (2009) 5647–5654.
[33] J.L. Capelo, A.M. Mota, Ultrasonication for analytical chemistry, Curr. Anal. Chem. 1 (2005) 193–201.
[34] I. Jiménez-Díaz, A. Zafra-Gómez, O. Ballesteros, N. Navea, A. Navalón, M.F. Fernández, N. Olea, J.L. Vílchez, Determination of bisphenol A and its chlorinated derivatives in placental tissue samples by liquid chromatography-tandem mass spectrometry, J. Chromatogr. B 878 (2010) 3363–3369.
[35] E.M. Golet, A. Strehler, A.C. Alder, W. Giger, Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction, Anal. Chem. 74 (2002) 5455–5462.
[36] N. Dorival-García, A. Zafra-Gómez, A. Navalón, J.L. Vílchez, Analysis of bisphenol A and its chlorinated derivatives in sewage sludge samples. Comparison of the efficiency of three extraction techniques, J. Chromatogr. A 1253 (2012) 1–10.
[37] A.R. Fontana, A. Camargo, L.D. Martínez, J.C. Altamirano, Dispersive solid-phase extraction as a simplified clean-up technique for biological sample extracts. Determination of polybrominated diphenyl ethers by gas chromatography–tandem mass spectrometry, J. Chromatogr. A 1218 (2011) 2490–2496.
[38] D. Lankova, O. Lacina, J. Pulkrabova, J. Hajslova, The determination of perfluoroalkyl substances, brominated flame retardants and their metabolites in human breast milk and infant formula, Talanta 117 (2013) 318–325.
[39] S.J. Lehotay, K. Mastovská, S.J. Yun, Evaluation of two fast and easy methods for pesticide residue analysis in fatty food matrixes, J. AOAC Int. 88 (2005) 630–638.
[40] B. Kinsella, S.J. Lehotay, K. Mastovská, A.R. Lightfield, A. Furey, M. Danaher, New method for the analysis of flukicide and other anthelmintic residues in bovine milk and liver using liquid chromatography–tandem mass spectrometry, Anal. Chim. Acta 637 (2009) 196–207.
[41] E.M. Malone, C.T. Elliott, D.G. Kennedy, L. Regan, Rapid confirmatory method for the determination of sixteen synthetic growth promoters and bisphenol A in bovine milk using dispersive solid-phase extraction and liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 878 (2010) 1077–1084.
[42] O. Lacina, P. Hradkova, J. Pulkrabova, J. Hajslova, Simple high throughput ultra-high performance liquid chromatography/tandem mass spectrometry trace analysis of perfluorinated alkylated substances in food of animal origin: milk and fish, J. Chromatogr. A 1218 (2011) 4312–4321.
[43] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) Guidance for Industry, Bioanalytical Method Validation, 2001.
[44] Analytical Methods Committee, Is my calibration linear? Analyst 119 (1994) 2363–2366.
[45] A. Cariot, A. Dupuis, M. Albouy-Llaty, B. Legube, S. Rabouan, V. Migeot, Reliable quantification of bisphenol A and its chlorinated derivatives in human breast milk using UPLC–MS/MS method, Talanta 100 (2012) 175–182.