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Determination of benzophenone-UV filters in human milk samples using ultrasound-assisted extraction and clean-up with dispersive sorbents followed by UHPLC–MS/MS analysis

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ABSTRACT

A new sample preparation method for the determination of five benzophenone UV-filters in human breast milk has been developed. The procedure involves the lyophilization of the sample, and its subsequent extraction by ultrasound sonication using acetonitrile. In order to reduce matrix effects produced by milk components that are coextracted, mainly proteins, sugars and lipids, a further clean-up step with a mixture of dispersive-SPE sorbents, C18 and PSA, was applied. Extraction parameters were optimized using experimental design, and the compounds were detected and quantified by ultrahigh performance liquid–chromatography tandem mass spectrometry (UHPLC–MS/MS) in positive ESI mode. Analytes were separated in 10 min. BP-d_{10} was used as internal standard. The limits of detection (LODs) were between 0.1 and 0.2 ng mL^{-1}, and the limits of quantification (LOQs) were between 0.3 and 0.6 ng mL^{-1} for the target analytes. The inter- and intra-day variability was $<12\%$. The method was validated using matrix-matched calibration and recovery assays with spiked samples. Recovery rates were between 90.9 and 109.5%. The method was successfully applied for the determination of these compounds in human milk samples collected from volunteers lactating mothers with no known occupational exposure to these compounds who live in the province of Granada (Spain). The analytical method developed here may be useful for the development of more in-depth studies on the prenatal exposure and biomonitoring of these commonly used UV-filters.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a group of natural and synthetic chemicals that may interfere with the normal function of the endocrine system in animals and humans [1]. EDCs can either mimic or inhibit the action of natural hormones, leading to adverse reproductive and developmental effects. In men, exposure to EDCs is associated with problems in reproductive capacity and testicular or prostate cancer [2]. In women, abnormal endocrine function may be associated with increased risk for endometriosis, reproductive and endocrine-related cancers, or impaired oocyte competence, ovarian function or menstrual cycling [3]. Although, the effects of early life exposure to EDCs remain still unclear, it has been suggested that fetal or childhood exposure may lead to abnormal sex differentiation, abnormal neurological and reproductive development, and to risk of reproductive problems or cancer later in life [4].

A wide variety of chemicals have been shown to have estrogenic activity [5], being the majority of them synthetic compounds. Many are components of personal care products, PCPs (e.g., UV-filters) [6], which comprise different groups of compounds that are currently used as additives in different common products such as cosmetic, household, food or pharmaceutical products, among others. Considerable amounts of PCPs are used in everyday human activities, so they are produced in large quantities (thousands of tons per year). Although these compounds are used in some products intended for direct ingestion, the main route of exposure to PCPs is the absorption through the skin, being further metabolized and eventually bioaccumulated and/or excreted [7–13].

Organic UV-filters are often used to protect skin against UV radiation damage. They are constituents of many daily products such as skin creams, body lotions, hair sprays, hair dyes, shampoos and sunscreen. The European Union (EU) Regulation 1223/2009 – Cosmetics Regulation – provides a robust, internationally recognized regime, which reinforces product safety. It stipulates the compounds that are allowed to be used as UV-filters in cosmetics and their maximum concentrations [14]. The family of benzophenones (BPs) is one of the most frequently used

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groups of UV-filters as they are able to absorb UV light that is harmful to the human body in the form of UVA (320 to 400 nm) and UVB (290 to 320 nm). BPs consist of 12 main compounds, called from benzophenone-1 (BP-1) to benzophenone-12 (BP-12), as well as other less known as 2-hydroxybenzophenone (2-OH-BP), 3-hydroxybenzophenone (3-OH-BP) and 4-hydroxybenzophenone (4-OH-BP) [15].

There is increasing evidence that BPs are able to interfere with the endocrine system. In vitro studies have shown that BPs stimulate the proliferation of the breast cancer cell line MCF-7 due to their estrogenic activity and that these compounds have also antiandrogenic activity [16,17]. Carcinogenesis and reproductive organ malformations have been also reported in rodents after exposure to BP-UV filters [18,19]. During the biotransformation of BPs, they can suffer yet a "cross-transformation" to other types of BPs which often show more dangerous disrupting activities than the original forms [20]. It has been reported that BP-3 is metabolized to BP-1 and BP-8 in animals [20,21], and there is some evidence that BP-1 possesses higher estrogenic activity than BP-3 [16,21,22]. Others BPs such as 4-OH-BP are also metabolites of BP-3.

Although BPs are usually present in aquatic ecosystems and exhibit bioaccumulation in invertebrates and fish, their presence in human fluids and tissues appears to be related with consumer habits rather than with environmental exposure. It has been proved that there is a positive correlation between the use of cosmetics containing BPs and their presence in human milk [23]. In a recent study, Liao and Kannan have analyzed 231 samples of PCPs in China and the US, being positive 88% of them in BP-3 [24]. Since human milk is the main route of exposure to chemical compounds for infants, its analysis is of special interest. The early life stages are very important due to vulnerability in developmental processes and any disturbance can lead to persistent alterations in structure and function that sometimes become manifest later in life.

To date, very few studies have been focused on the development of analytical methods for the determination BPs in human milk, which are mainly focused on BP-3, since it is the most widely used and therefore the most important member of this family of compounds. Due to the complexity and composition of this biological matrix, especially in relation to its significant fat and protein content, the isolation of the target analytes becomes critical for the development of any analytical method. The selection of an adequate sample treatment and the extraction technique is very important to obtain a selective and efficient recovery of the analytes from samples, reducing matrix effects and improving the sensitivity of the method, considering the great importance of detecting trace levels of these substances, since it is proved their adverse effects even at very low concentrations. For determination of BPs in human milk samples classical techniques such as liquid–liquid extraction (LLE) [23,25] or solid phase extraction (SPE) including on-line SPE [26,27] have been used, however very few works have developed a multiresidue method [28,29].

In this work, a sensible, selective and accurate multiresidue method for determination of BP-1, BP-3, 4-OH-BP, BP-6 and BP-8 in human milk samples is proposed. This method is based on the use of ultrasound-assisted extraction, followed by a clean-up step with PSA and C18, commonly used as dispersive-SPE sorbents. UHPLC–ESI–MS/MS has been used as analysis technique. Previous lyophilization of milk samples is an important innovation, since this treatment clearly facilitates the extraction procedure, which will be completed through a further clean-up step, improving the quality parameters of the method. The method was satisfactorily validated and applied for the determination of the concentration of free forms 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. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8), 4-hydroxybenzophenone (4-OH-BP) and labelled-deuterium benzophenone (BP-d_{10}) were supplied by Sigma-Aldrich (Madrid, Spain). BP-d_{10} was used as internal standard. Stock standard solutions (100 μg mL^{-1}) were prepared in acetonitrile (MeCN). The solution remained stable for at least four months at 4 °C in the darkness. Working standards were prepared fresh from the MeCN solutions prior to the experiments. Methanol (MeOH), ethanol, ethyl acetate and MeCN (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, Spain). Bakerbond® octadecyl C18 sorbent (40 μm particle size) was purchased from J.T. Baker (Deventer, The Netherlands), and primary-secondary amine (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 replaceable 12.7 mm titanium tip and a temperature probe. 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 BPs detection. Separation of compounds was obtained with a CORTECS UPLC™ C18 column (50 mm × 2.1 mm; 1.6 μm particle size) (Waters, UK). MassLynx 4.1 software was used for instrument control, peak detection and integration. Samples were lyophilized using a SCANVAC CoolSafe™ freeze dryer and extracts were evaporated with a SCANVAC CoolSafe™ ScanSpeed MaxiVac centrifuge for vacuum 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 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 spiked milk samples

For the optimization of experimental variables, an aliquot of 9.9 mL of human milk sample was placed into a glass vial of 20 mL and 100 μL of a concentrate standard solution was added to obtain a final concentration of 20 ng mL^{-1} of all analytes. Then, the spiked samples were left to stand for 24 h at 4 °C in the dark before analysis to allow the analytes to come into contact with the whole human milk sample. Next, the samples were frozen at −80 °C for 12 h prior to lyophilization.

For validation purposes (recovery assays, precision and trueness) blank samples were spiked at different concentrations (from 0.5 to 50 ng mL^{-1}) by adding 100 μL of the different spiking standard solutions to 9.9 mL of human milk, left at 4 °C for 24 h and frozen at −80 °C. The blank samples were previously analyzed in order to ensure the absence of analytes or that these were below the limits of detection (LODs) of the method.

2.5. Extraction procedure

Lyophilized human milk samples were placed into stainless steel capsules and 10 mL of MeCN were added. In order to the sample comes into contact with the solvent, these capsules were vortexed for 2 min, and then sonicated for 15 min at 70% amplitude. Three cycles were required. The obtained extracts were merged and concentrated to a volume around 10 mL using the SpeedVac concentrator at 760 × *g* and 40 °C. Then, the extracts were cleaned-up with a mixture of 500 mg anhydrous $MgSO_4$, 250 mg PSA and 300 mg C18 sorbents. The extract was stirred for 3 min at room temperature at an eight-position digital agitator–vibrator and centrifuged for 3 min at 3634 × *g*. The supernatant was transferred to a 10 mL polypropylene conical tube and evaporated to dryness in the SpeedVac concentrator at 760 × *g* and 40 °C. The residue was dissolved with two portions of 300 μL of MeOH 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, then it was filtered through a 4 mm and 0.22 μm nylon filters. The sample was ready to be injected into the chromatographic system.

2.6. Liquid chromatography–mass spectrometry analysis

The chromatographic separation of target analytes was performed using a CORTECS UPLC™ C18 (Waters) column (50 mm × 2.1 mm; 1.6 μm particle size). The compounds were separated using a gradient mobile phase consisting of 4 mM aqueous ammonium formate solution (pH = 9) as solvent A and 0.025% (v/v) ammonia in MeOH as solvent B. 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. Flow rate was 300 μL min^{-1} and the injection volume 10 μL. The column temperature was 40 °C.

For MS analysis, ESI was performed in positive ion mode. The tandem mass spectrometer was operated in the multiple reactions monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. Instrument parameters were as follows [28]: 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.

2.7. 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. Blanks were processed in the same way as the samples. Briefly, 10 mL of MeCN was introduced to stainless steel capsules and sonicated for 15 min, three cycles at 70% amplitude were applied. No quantifiable amount of target compounds was detected. On the other hand, in order to evaluate possible contaminations and the variability of the instrumental analysis, standards (spiked blank samples at 0, 10 and 25 μg L^{-1}) and a standard in pure solvent (methanol, 50 μg L^{-1}) were injected by triplicate, every 9 samples, in each analytical batch of samples.

3. Results and discussion

3.1. Sample treatment

Although MS is a selective and sensitive detection technique, human milk is a complex matrix that contains lipids, proteins, carbohydrates, minerals and vitamins that can be also co-extracted and interfere with the detection of the target analytes. The previous lyophilization of the samples, minimize the extraction of many of these matrix components, which remain precipitated during the extraction step (i.e., proteins). In this sense, ultrasound-assisted extraction and a clean-up step with sorbents were selected. Several variables were considered for the optimization of both procedures and design of experiments was applied. Each experiment was done in triplicate.

3.1.1. Selection of the extraction solvent

Since they have been widely used for the extraction of different families of EDCs from biological samples, methanol, ethanol, acetonitrile and ethyl acetate were evaluated as extraction solvents [30,31]. The following basic procedure was applied: spiked lyophilized milk samples were extracted with 10 mL of each solvent, two extraction cycles of 15 min at 70% amplitude were applied in all cases. The obtained extracts were evaporated to dryness in the SpeedVac concentrator at 760 × *g* and 40 °C. In the case of methanol and ethanol, the use of both solvents was directly discarded because their extracts contained high amounts of matrix

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

	Transitions	CV	CE
BP-1	214.9 → 136.8[a]	2	18
	214.9 → 105.1[b]		32
BP-3	229.0 → 150.8[a]	4	20
	229.0 → 104.9[b]		18
BP-6	275.0 → 150.9[a]	14	18
	275.0 → 94.9[b]		34
BP-8	245.0 → 120.9[a]	14	20
	245.0 → 150.9[b]		20
4-OH-BP	199.0 → 120.8[a]	36	20
	199.0 → 104.8[b]		18
BP-d_{10}	193.1 → 109.8[a]	18	16
	193.1 → 81.8[b]		30

CV: Cone voltage (V); CE: Collision energy (eV).
[a] SRM transition used for quantification.
[b] SRM transition used for confirmation.

components. The dried residues from acetonitrile and ethyl acetate extracts were redissolved with 100 μL of the initial mobile phase containing the internal standard (50 ng mL^{-1}) and directly injected into the LC system. Fig. 1 shows the comparison of the extraction efficiency between acetonitrile and ethyl acetate. Mean values of normalized areas 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 for all analysed BPs, being acetonitrile the best option for solvent, which was selected for further experiments.

3.1.2. Optimization of USE conditions

In order to optimize of the variables involved in the USE extraction procedure and to study possible interactions between them, a 15-run Box–Behnken design with three replicates of the central point was used for fitting a second-order response surface. Three factors and three levels for each one were checked: extraction time (1, 8 and 15 min), number of extraction cycles (1, 2 and 3) and volume of solvent per cycle (10, 15 and 20 mL). Amplitude was set at the maximum recommended value of 70% in all the experiments. The data were evaluated by ANOVA and the test gave determination coefficients (R^2) between 0.833 and 0.897. Since the *P*-values for the *lack-of-fit* test were $> 5\%$ 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 influential parameter, unlike extraction time and volume, which were not significant. However, a significant negative interaction between the number of cycles and volume was observed. For this reason, 10 mL was selected as optimum for the volume of solvent, which was the lowest assayed volume.

The combination of the optimized experimental values obtained for each compound allowed the determination of the best overall extraction efficiency, which was calculated with 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 extraction solvent volume set at 10 mL, is shown in Fig. 2A. Although there were not significant differences in desirability along the evaluated extraction time interval at 3 cycles, it was selected 15 min in order to assure a more complete extraction of the analytes in samples. Consequently, the optimum values corresponded to 3 extraction cycles of 15 min each one, 10 mL of extraction solvent volume and 70% of amplitude.

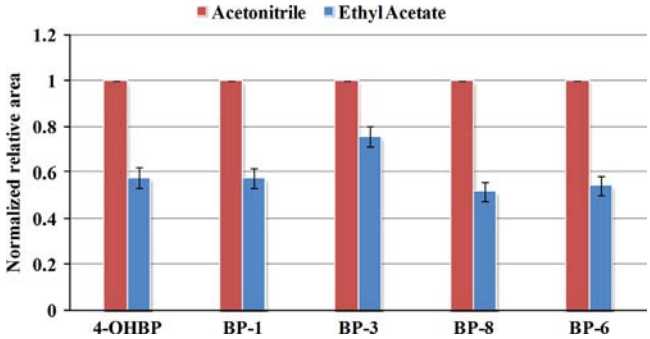

Fig. 1. Normalized relative areas for the selection between MeCN and ethyl acetate as extraction solvent. BP-1 (LSD = 1.47); BP-3 (LSD = 1.17); 4-OH-BP (LSD = 0.11); BP-6 (LSD = 0.12); BP-8 (LSD = 0.23). Significance level of 95% was selected for all cases.

3.1.3. Optimization of the clean-up procedure

A clean-up step to obtained extracts from USE was necessary, almost mandatory, especially for removal of remaining lipids from samples. It was selected a clean-up procedure using different sorbents that are frequently used in dispersive solid phase extraction. Since they are commonly used in the removal of extracted interferences in biological matrices [32–38], a mixture of two solid sorbents was evaluated: PSA and C18. C18 is specifically used for removal of co-extracted fat and other lipophilic compounds from acetonitrile extracts. 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. The composition of sorbent mixture for the clean-up step was also determined with a Box–Behnken design, being also included as a variable the amount of $MgSO_4$ that is applied during the clean-up phase as a desiccant, in order to eliminate traces of water which complicate sample evaporation and concentration.

Box–Behnken matrix also consisted of 15 experiments, including three central points. The variables studied (at 3 levels), were C18 (0, 150 and 300 mg), PSA (0, 150 and 300 mg) and $MgSO_4$ amounts (0, 250, 500 mg). Fig. 2B shows the plot of the desirability function versus the amounts of PSA and C18 sorbents, with the amount of $MgSO_4$ set at 500 mg, corresponding to the optimum value according to the Box–Behnken design. The three variables resulted influential, $MgSO_4$ and C18 amounts showed a positive effect for all analytes, while PSA amount had a positive influence for BP-3, BP-6 and BP-8, but negative for 4-OH-BP and BP-1. Moreover, significant positive interactions among all variables were observed: PSA and C18, as well as $MgSO_4$ with each one of the sorbents. According to the observed positive influence of the variables, the optimum values were the maximum for C18 and $MgSO_4$, but slightly lower for PSA, due to the negative effect showed for some compounds. Consequently, a mixture of 250 mg of PSA, 300 mg of C18 and 500 mg of $MgSO_4$ was selected as optimum for the clean-up step.

3.2. Analytical performance

A calibration curve for each compound, at six concentration levels (six fold) was built, in the range from the limit of quantification (LOQ) to 50 ng mL^{-1}. The curves were constructed using analyte/surrogate peak area ratio versus concentration of analyte. Calibration graphs were made using SRM mode. Internal standard (BP-d_{10},) was added at a concentration of 50 ng mL^{-1}. Each calibration level was made in triplicate and analyzed twice. Table 2 shows the statistical and the analytical parameters obtained for each compound.

The presence/absence of matrix effect was evaluated with two calibration curves for each compound, one in the initial mobile phase and the other in blank human milk. A Student's *t*-test was applied in order to compare the calibration curves, showing high statistical differences among slope values for the calibration curves in all cases and consequently, a significant matrix effect was observed. A possible explanation for this not correction of the matrix effects by the internal standard, could be that the chemical structure and, consequently, the physical and chemical properties of the analyzed compounds are relatively variable. Therefore, although the compounds studied have a similar basic structure, and the use of this internal standard is accepted in scientific literature, they differ due to the presence of different substituents in the molecule. Then, it was decided to work with matrix-matched calibration in all cases.

3.3. Method validation

Validation in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity, was performed according to the US

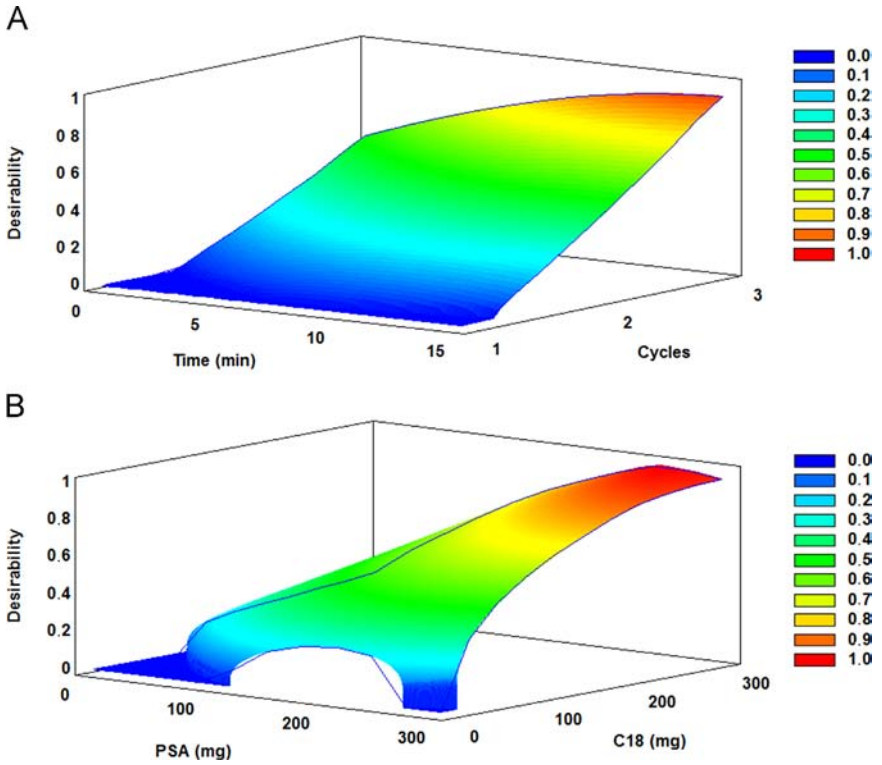

Fig. 2. Representation of the global desirability functions. (A) Extraction time and number of cycles for ultrasonic extraction, and (B) amount of PSA and C18 sorbents for the clean-up step for the determination of BPs.

Table 2
Analytical and statistical parameters.

	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})
BP-1	1.334×10^{-1}	1.191×10^{-3}	99.6	12.1	0.1	0.5	0.5–50
BP-3	4.402×10^{-2}	4.910×10^{-4}	99.5	6.5	0.2	0.6	0.6–50
BP-6	6.021×10^{-2}	5.417×10^{-4}	99.7	89.4	0.1	0.5	0.5–50
BP-8	4.992×10^{-2}	3.363×10^{-4}	99.8	91.5	0.1	0.4	0.4–50
4-OH-BP	5.898×10^{-1}	5.650×10^{-3}	99.6	7.3	0.1	0.3	0.3–25

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

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 50 ng mL^{-1} was selected for BP-1, BP-3, BP-6 and BP-8, and to 25 ng mL^{-1} for 4-OH-BP. The determination coefficient (R^2) and the *lack-of-fit* test (P_{lof}) were evaluated. The values obtained for R^2 ranged from 99.5 to 99.8% and P_{lof} values were $>5\%$ in all cases [40]. This indicates a good linearity within the stated ranges (LOQ-50 ng mL^{-1}).

3.3.2. Sensitivity

The limits of detection (LODs) and quantification (LOQs) were calculated by taking into consideration the minimum concentration of analyte that the method can detect and with a signal-to-noise ratio of 3 for LODs and 10 for LOQs, using the quantification transition. Found LODs ranged from 0.3 for 0.6 ng mL^{-1}. The results are also summarized in Table 2.

3.3.3. 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} for BP-1, BP-3, BP-6 and BP-8; and 1, 10 and 25 ng mL^{-1} for 4-OH-BP. Precision, expressed as relative standard deviation (%, RSD) was determined from triplicate spiked samples during the same day and in

Table 3
Recovery assay, precision and trueness of target compounds in human milk.

	Spiked (ng mL^{-1})	[a]Found ± CI (ng mL^{-1})	RSD (%)	Recovery (%)
BP-1	1.0	1.09 ± 0.05	8.4	108.7
	10	9.21 ± 0.2	4.0	92.1
	50	50.6 ± 0.9	3.5	101.1
BP-3	1.0	1.02 ± 0.03	5.1	102.1
	10	9.6 ± 0.2	5.0	95.9
	50	50.7 ± 0.9	3.4	101.3
BP-6	1.0	1.12 ± 0.05	9.4	109.5
	10	9.7 ± 0.4	7.6	97.1
	50	50.0 ± 0.9	3.8	100.1
BP-8	1.0	1.12 ± 0.07	12.3	108.6
	10	9.9 ± 0.1	2.0	98.6
	50	50.1 ± 0.9	3.5	100.3
4-OH-BP	1.0	1.05 ± 0.03	5.0	104.9
	10	9.1 ± 0.2	3.3	90.9
	25	25.3 ± 0.3	2.6	101.2

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

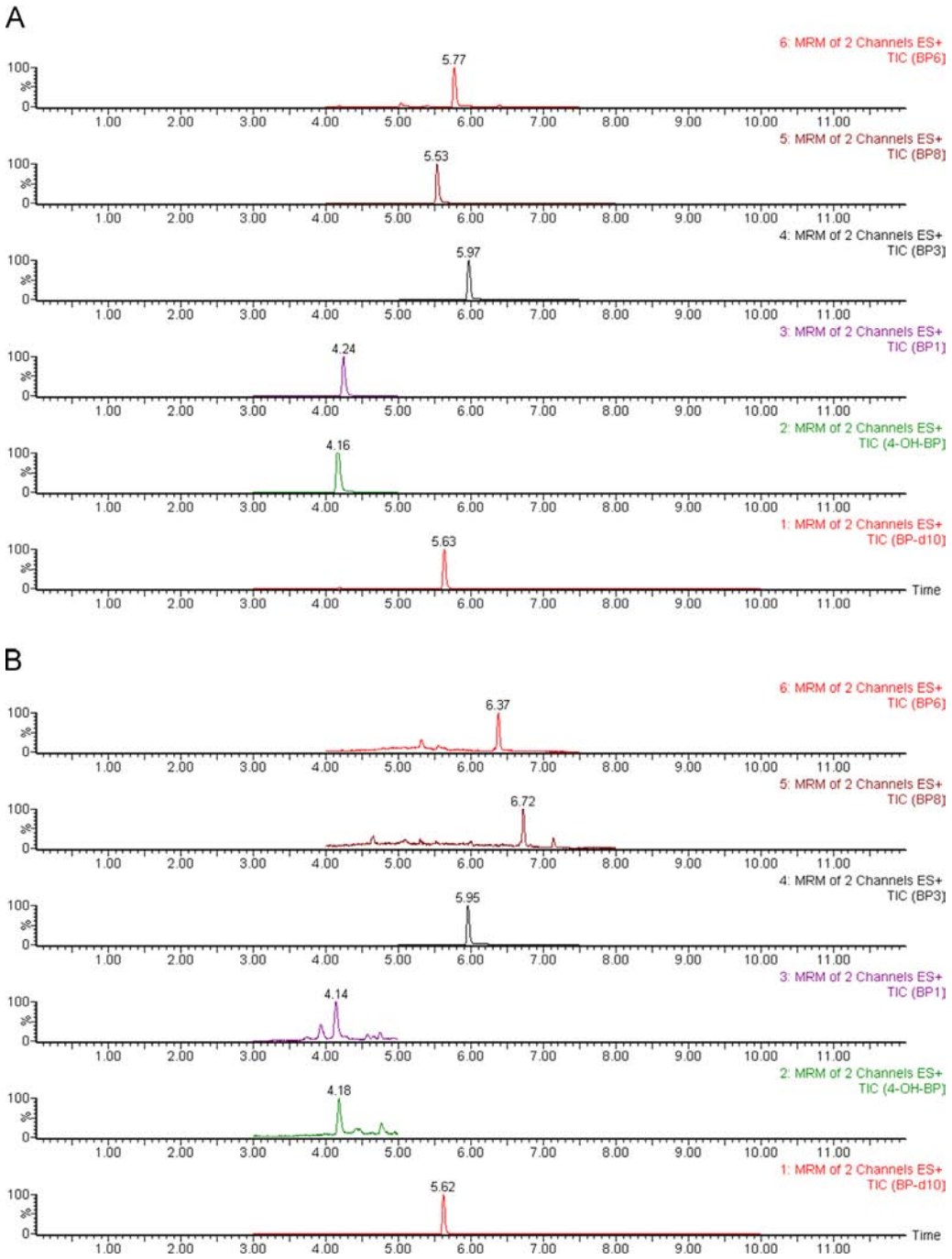

Fig. 3. UHPLC–MS/MS chromatograms of: (A) a spiked blank sample; (B) a positive human milk sample with the target analytes, containing the corresponding internal standard (BP-d_{10}).

6 different days. The values obtained are summarized in Table 3. RSD values fell between 2.0 and 12.3% being in 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 indicate that the method is reproducible.

Meanwhile, the trueness was evaluated by a recovery assay. The recovery of known amounts of tested compounds in milk samples was evaluated by comparing the spiked amount with the concentration of each compound determined by using the proposed method. In all cases, the recoveries were close to 100% in all cases (between 90.9 and 109.5%).

3.3.4. *Selectivity*

The specificity of the method was demonstrated by analyzing the chromatograms of the blank and the corresponding spiked human milk sample. No interferences from endogenous substances were observed at the retention time of the analytes. These findings suggest that the spectrometric conditions ensured high selectivity of the UHPLC–MS/MS method. Fig. 3A shows the SRM chromatograms obtained from the spiked blank sample (sample 5).

3.4. *Application of the method*

The proposed method was applied to the determination of the content of the free forms of the selected BPs in human milk

Table 4
Application to human breast milk samples.

Sample	[a,b]Found amount (ng mL^{-1})				
	BP-1	BP-3	BP-6	BP-8	4-OH-BP
01	D	5.8 (0.5)	D	ND	0.31 (0.02)
02	D	D	ND	D	ND
03	D	5.9 (0.4)	D	0.73 (0.02)	D
04	D	4.7 (0.2)	D	ND	ND
05	D	6.7 (0.2)	D	D	0.48 (0.05)
06	0.61(0.03)	8.5 (0.8)	ND	ND	1.92 (0.04)
07	D	4.7 (0.3)	ND	ND	ND
08	D	15.7 (0.5)	ND	D	3.9 (0.3)
09	ND	ND	ND	ND	ND
10	D	4.5 (0.1)	ND	ND	D

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

samples collected from 10 anonymous women with no known occupational exposure to these compounds. The results obtained as a mean of six determinations are summarized in Table 4.

Although the number of analyzed samples is not very high, it is possible to formulate some interesting conclusions. The results demonstrate the widespread occurrence of BP-3 in human samples. It was detected in nine of the analyzed samples, and quantified in eight of them at concentrations ranging from 4.5 to 15.7 ng mL^{-1} (mean: 7.1 ng mL^{-1}). The presence of 4-OH-BP was also important, since it was detected in the 60% of analysed samples, and quantified in the 40% of them, but in lower concentrations than BP-3 (0.31 to 1.92 ng mL^{-1}, mean: 1.6 ng mL^{-1}). It is important to remark that both compounds are the most used in commercial applications. BP-1 was detected in 9 of the samples but quantified in only one of them. This compound is described as one of the most important metabolites of BP-3. Finally, BP-6 and BP-8 were the least detected. Both were detected in four samples, but BP-8 was quantified in one of them. Fig. 3B shows the SRM chromatograms obtained from a human milk sample (sample 5).

4. Conclusions

There are very few analytical works published in the scientific literature for the determination of the selected compounds in human milk samples. Most of them have been focused on the determination of BP-3 using classical extraction techniques for compound isolation such as SPE (off- and on-line) or LLE. Other papers analyze BP-2, BP-3 and BP-4 and very few publications have been developed for multiresidue analysis.

In this context, the application of ultrasound-assisted extraction followed by a clean-up step using C18 and PSA sorbents is proposed for the identification and quantification of free forms of five BPs in human milk samples previously lyophilized. UHPLC–MS/MS is proposed as analytical technique. The isolation of analytes from samples was properly optimized by means of two Box–Behnken type experimental designs. The proposed sample preparation procedure was considered a good choice, because of its higher extraction yields and easy operation, especially when it is compared with the SPE technique that is traditionally used for human milk analysis, so that this alternative saves time and requires lower volumes of solvents than SPE, reducing costs and waste.

The procedure was accurately validated, being useful for the determination of trace levels of BPs in human milk, since limits of quantification ranged between 0.3 and 0.6 ng mL^{-1}, therefore it may be used to perform biomonitoring studies, since this matrix is a valuable biological fluid that may serve as a biomarker of both maternal and prenatal exposure to many different environmental chemicals, particularly to EDCs.

The method was satisfactorily applied for the determination of target compounds in human milk samples from 10 randomly selected women.

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