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Determination of benzophenones in human placental tissue samples by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Benzophenones (BPs) are a family of compounds widely used to protect the skin and hair from UV irradiation. Despite human exposure to BPs through dermal application of products containing sunscreen agents and the increasing evidence that BPs are able to interfere with endocrine systems, few studies have examined the occurrence of BPs in humans. In this work, we propose a new liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to determine six BPs, namely, benzophenone-1 (BP-1), benzophenone-2 (BP-2), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8) and 4-hydroxybenzophenone (4-OH-BP) in human placental tissue samples. The method involves an extraction step of the analytes from the samples using ethyl acetate, followed by a clean-up step using centrifugation prior to their quantification by LC–MS/MS using an atmospheric pressure chemical ionization (APCI) interface in the positive mode. Benzophenone-d_{10} (BP-d_{10}) was used as surrogate. Found detection limits (LOD) ranged from 0.07 to 0.3 ng g^{-1} and quantification limits (LOQ) from 0.3 to 1.0 ng g^{-1}, while inter- and intra-day variability was under 5%. The method was validated using standard addition calibration and a recovery assay. Recovery rates for spiked samples ranged from 98 to 104%. This method was satisfactorily applied for the determination of BPs in 16 placental tissue samples collected from women who live in Granada (Spain).

1. Introduction

The increasing exposure to UV irradiation raises a growing demand for chemicals which protect the skin against sunburn, photoageing, skin cancer, and photodermatosis as well as industrial products against the damaging effects of UV radiation. Therefore, chemical UV-filters are used extensively in sunscreens, cosmetic products such as face day creams, after-shave products, makeup formulations, lipsticks, shampoos, and in plastic based packaging materials. In the European Union, 26 different organic compounds are permitted for use as sunscreen agents. The maximum content of these compounds in cosmetics is regulated by legislation [1], at a usual concentration between 0.1 and 10%.

Since most of UV-screening compounds are photostable and many are highly lipophilic [2], they are prone to bioaccumulation in humans and wildlife. Residues of these UV screens have been detected in fish and aquatic environments [3,4].

The family of benzophenones (BPs) is one of the most frequently used groups of UV-filters. BPs consists of 12 main compounds, called benzophenone-1 (BP-1) to benzophenone-12 (BP-12), as well as other less known compounds as 2-hydroxybenzophenone (2-OH-BP), 3-hydroxybenzophenone (3-OH-BP) and 4-hydroxybenzophenone (4-OH-BP). These compounds are reported to enter the human body through the food chain [5] or via skin absorption [6–9]. Despite some toxic effects of BPs, such as hepatotoxicity, they are safe for topical application in humans. However, there is increasing evidence that BPs are able to interfere with the endocrine system. The first BP to be classified as an "endocrine active chemical" (EAC) with estrogenic activity was BP-3, which stimulates the proliferation of the breast cancer cell line MCF-7 [10]. With regard to BP-2, it exerts considerable estrogenic effects on the uterus, vagina, bone and liver of ovariectomized rats [11,12]. In fish, BP-2 acts as an estrogenic disruptor causing in vitro and in vivo effects [13] and a recent work showed that BP-2 administered orally to pregnant mice induced hypospadias in the male offspring via signalling through the estrogenic receptors [14]. It has been reported that, in rats and piglets, BP-3 is metabolized to BP-1 and BP-8 [15–18]. Interestingly, there is evidence that BP-1 possesses higher estrogenic activity than BP-3 [16,19–22] and it

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Table 1
Analytical methods for the determination of benzophenones (BPs) in human samples.

Human sample	Analytical technique	Studied BPs	Detection limit (LOD)	Reference
Urine	SPE-LC–MS/MS	BP-3	0.3 ng mL^{-1}	[31]
	SPE-LC–MS/MS	BP-3	0.5 ng mL^{-1}	[32]
	LC–UV	BP-3	1.3 ng mL^{-1}	[33]
	GC–MS	OH-BP, 2-OH-BP, BP-3, BP-10	0.05–0.1 ng mL^{-1}	[34]
	GC–MS	OH-BP, 2-OH-BP, BP-3, B-P10	5–10 pg mL^{-1}	[35]
	GC–MS	BP, BP-1, BP-3, BP-10, OH-BP, 2-OH-BP, 3-OH-BP, 4-OH-BP	0.01–0.05 ng mL^{-1}	[36]
	LC–UV	BP-3	60 ng mL^{-1}	[37]
		BP-4	30 ng mL^{-1}	
	LC–MS/MS	BP-1, BP-2, B-P3, BP-8, 4-OH-BP	0.28–0.90 ng mL^{-1}	[38]
Milk	SPE-LC–MS/MS	BP-3	0.4 ng mL^{-1}	[39]
Serum	SPE-LC–MS/MS	BP-3	0.5 ng mL^{-1}	[40]
Blood	LC–UV	BP-3	3.9 ng mL^{-1}	[41]

SPE, solid-phase extraction; LC–MS/MS, liquid chromatography–tandem mass spectrometry; GC–MS, gas chromatography-mass spectrometry; LC–UV, liquid chromatography–ultraviolet detection; BP, benzophenone; BP-1, benzophenone-1; BP-2, benzophenone-2; BP-3, benzophenone-3; BP-4, benzophenone-4; BP-8, benzophenone-8; BP-10, benzophenone-10; OH-BP, hydroxybenzophenone; 2-OH-BP, 2-hydroxybenzophenone; 3-OH-BP, 3-hydroxybenzophenone; 4-OH-BP, 4-hydroxy benzophenones.

can also display antiandrogenic activity in vitro. Additive estrogenic effects of BP-1 and BP-3, on pS2-gene transcription in MCF-7 cells, have been reported [23]. In addition, other BPs, such as 4-OH-BP, have shown higher estrogenic activity than BP-3 [19,21].

BPs can also be conjugated with β-D-glucuronide and sulphate, reducing their bioactivity and facilitating their urinary excretion. Although free and conjugated BPs can be measured in humans, only the free forms are considered biologically active.

In recent years, some methods for the analysis of these compounds have been described in different matrices. In this sense, waters [24–28], soils and sediments [29] and fish [3,30] have been studied using different analytical techniques. However, few methods have focused on the determination of BPs in biological samples. Table 1 summarizes the proposed methods already published for the analysis of BPs in human biological fluids.

To our knowledge, there is a lack of published literature on BPs determination in placental tissue. The aim of this work is to develop a sensitive analytical method for the determination of the six free BPs with demonstrated estrogenic activity: BP-1, BP-2, BP-3, BP-6, BP-8 and 4-OH-BP in placental tissue samples. Fig. 1 shows the chemical structure of the selected compounds.

The proposed method was validated and satisfactorily applied for the determination of the above-mentioned BPs in 16 placental tissue samples collected from women who live in the province of Granada (Spain).

2. Experimental

2.1. Chemical 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). BP-1, BP-2, BP-3, BP-6, BP-8, 4-OH-BP and BP-d_{10} were supplied by Sigma-Aldrich (Madrid, Spain). Stock

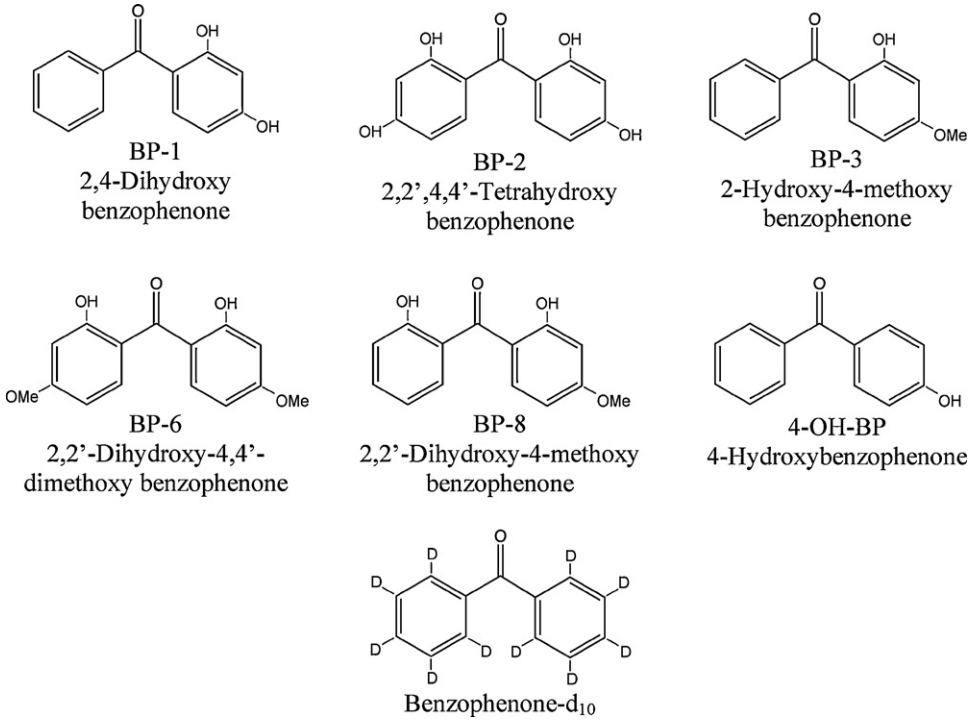

Fig. 1. Chemical structure of the studied benzophenones.

standard solutions (100 mg L^{-1}) for each compound were prepared in methanol and stored at 4 °C in the dark. These solutions were stable for at least 4 months. Working standards were prepared just before use, diluted with methanol. Methanol and acetonitrile (both HPLC-grade) were purchased from Merck (Darmstadt, Germany) and analytical-grade ethyl acetate from Riedel-de-Haën (Madrid, Spain). LC–MS grade methanol, water, ammonia (25%) and formic acid (98%) were purchased from Sigma–Aldrich. Sodium chloride was supplied by Panreac (Barcelona, Spain). Before the injection into the LC system, the samples were filtered through 0.20 μm (4 mm diameter) non-sterile regenerated cellulose filters supplied by Sartorius (Goettingen, Germany).

2.2. Instrumentation and software

Analyses were performed using an Agilent 1200 series (Agilent Technologies Inc., Palo Alto, CA, USA) high-performance liquid chromatograph equipped with an binary pump, a vacuum membrane degasser, a thermostated column compartment, an automatic autosampler, an automatic injector and connected on line to an API 2000 (Applied Biosystems, Foster City, CA, USA) triple quadrupole mass spectrometer system that can use either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) interfaces being APCI the interface we used. Analyst software version 1.4.2 was used for instrument control and for data acquisition and analysis. All pH measurements were made with a Crison (Crison Instruments S.A, Barcelona, Spain) combined glass–Ag/AgCl (KCl 3 M) electrode using a Crison 2000 digital pH-meter. A Branson digital sonifier (Danbury, CT, USA), a Hettich Universal 32 centrifuge (Tuttlingen, Germany) and a vortex-mixer (Yellow line, Wilmington, NC, USA) were also used.

Statgraphics Centurion XV, version 15.1.02 software package [42] was used for statistical and regression analysis (linear mode).

2.3. Sample collection and storage

Placenta samples for the application of the method were collected during different deliveries in the Maternity Unit of San Cecilio University Hospital of Granada (Spain). Criteria for inclusion of the mothers in the present study were: (i) to live in one of the study areas, (ii) to be at least 16-year-old, (iii) not to have undergone any programme of assisted reproduction, (iv) to wish to deliver in the referenced hospital and (v) to have no communication problems. The study was approved by the Ethics Committees of the hospitals involved in the study. Pregnant women received written and oral information about the study and signed the informed consent. Recruitment took place during the first weeks of gestation or hospital admission for delivery. Extensive assessments were carried out in pregnant women and newborns. The information was gathered from a variety of sources: *ad hoc* administered questionnaires in face-to-face interviews by trained INMA – Infancia y Medio Ambiente – (Environment and Childhood Project) personnel, clinical data, physical examinations, ultrasound scans, biological samples (blood, placenta, urine, saliva, hair, nails and mother's milk).

Placentas from the participating mothers, collected at the time of delivery, without deciduas basalis and chorionic plate were examined and weighed. The placental material was taken out from the bag and was immediately coded, and frozen at −86 °C. Each sample was then divided into three portions and one of then was sent to the Laboratory of Medical Investigation, keeping them in the same conditions until processed. The laboratory was always blinded to the status of samples. Before analysis, a triangular portion was defrosted and mechanically homogenized and divided into 20 g portions. Then, each small fraction of sample was again homogenized using an ultrasonic spindle by placing the container in a glass full of ice in order to avoid sample heating and the spindle was directly introduced in the placental tissue. The ultrasound setting consisted in short pulses of 30 s on and then 30 s off, with 5 min of effective radiation. The process was repeated one more time. Once homogenized, samples were frozen at −86 °C and stored until the analysis.

2.4. Preparation of spiked samples

Spiked samples were prepared by adding 300 μL of methanol containing the analytes at three concentration levels (10, 20 and 30 ng mL^{-1}) to every gram of placental tissue. Spiked samples were stirred and slightly heated until they recovered original weight. Then, we weighted aliquots of 1.5 g of spiked placental tissue in 8 mL glass vials. The spiked samples were then ready for the experiments.

2.5. Basic procedure

2.5.1. Sample treatment

An aliquot (1.5 g) of placental tissue was placed into an 8 mL glass vial, fortified with 150 μL of methanol containing 60 ng mL^{-1} of BP-d_{10} and shaken for 10 min. Once fortified, the sample was homogenized with 1.5 mL of deionized water vortexing for 1 min. The homogenate was extracted by adding 3 mL of ethyl acetate and shaking again for 10 min and then the mixture was centrifuged for 10 min at 5000 rpm (4050 × *g*). The underlying organic layer was transferred to a clean glass vial and evaporated to dryness at room temperature under a nitrogen stream. The residue was dissolved in a mixture of 100 μL of 0.1% (v/v) ammonia in methanol and 100 μL of 0.1% (v/v) ammoniacal aqueous solution and shaken vigorously. The extract was placed in a 1.5 mL Eppendorf tube and centrifuged for 35 min at 16,500 rpm (24,960 × *g*) and finally, prior to its injection into the LC system, the extract was filtered through a 0.20 μm (4 mm diameter) non sterile regenerated cellulose filter.

2.5.2. Chromatographic conditions

Chromatographic separation of compounds was performed using a Gemini C_{18} column (100 mm × 2 mm i.d., 3 μm particle size) from Phenomenex (Torrance, CA, USA). Standards and samples were separated using a gradient mobile phase consisting of 0.1% (v/v) ammoniacal aqueous solution (solvent A) and 0.1% (v/v) ammonia in methanol (solvent B). Gradient conditions were: 0.0–3.5 min, 60% B; 3.5–4.0 min, 60–100% B; 4.0–6.5 min, 100% B and back to 60% in 0.5 min. Flow rate was 0.25 mL min^{-1}, injection volume 40 μL, the column temperature was maintained at 40 °C. Total run time was 8.0 min and the post-delay time for reconditioning the column with 60% B was 3.5 min.

2.5.3. Mass spectrometric conditions

APCI was performed in the positive ion mode. The tandem mass spectrometer was operated in the multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (10 mg L^{-1}). The ion source temperature was maintained at 350 °C. The IonSpray voltage was set at 3 kV. Nitrogen was used as both the curtain gas at 30 psi and ion source gas 1 and 2 at 50 and 30 psi respectively; collision gas was helium at 10 psi. Other adjustments like entrance potential (EP), declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were optimized for each analyte. The dwell time of each compound was set at 200 ms (Table 2).

Table 2
Selected MRM transitions and optimized potentials.

Compound	Transitions	Dwell time (ms)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
BP-1	215.0 → 137.1[a]	200	8	280	8	27	18
	215.0 → 105.1[b]						
BP-2	247.0 → 137.1[a]	200	8	380	10	23	18
	247.0 → 108.9[a]						
BP-3	229.0 → 150.8[a]	200	21	280	7	30	23
	229.0 → 105.0[b]						
BP-6	275.0 → 150.9[a]	200	7	375	4	17	19
	275.0 → 95.0[b]						
BP-8	245.0 → 120.9[a]	200	9	37	7	25	18
	245.0 → 150.9[b]						
4-OH-BP	198.9 → 120.9[a]	200	13	200	5	21	18
	198.9 → 105.0[b]						
BP-d_{10}	193.0 → 110.0[a]	200	12	280	9	24	15
	193.0 → 82.1[b]						

DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.
[a] MRM transition used for quantification.
[b] MRM transition used for confirmation.

3. Results and discussion

3.1. Liquid chromatographic separation

We tested both a Gemini C_{18} liquid chromatography column (100 mm × 2 mm i.d., 3 μm particle size) from Phenomenex (Torrance, CA, USA) and an Acquity UPLC column (100 mm × 2.1 mm i.d., 1.7 μm particle size) from Waters (Mildford, MA, USA). Although both columns offered similar resolution for all the analytes investigated, Acquity UPLC column generated pressures close to the maximum allowed by the chromatographic system. Consequently, the Gemini C_{18} column was the one we selected for our study.

Our aim was to obtain high sensitivity and selectivity in a short time. We started using water and methanol as mobile phase. First, the pH of mobile phase was studied and formic acid and ammonia were tested as additives. The best separation, peak shapes and ionization of the compounds were obtained with a mixture of 0.1% (v/v) ammoniacal aqueous solution as solvent A and 0.1% (v/v) ammonia in methanol as solvent B. Second, we analyzed the effect of substituting methanol for acetonitrile but no improvements were observed in peak shapes or resolution, so we selected the mobile phase previously mentioned. A linear gradient, as described in the previous section, was used.

Lastly, we increased the injection volume in order to enhance the analytical signal and consequently the LOD of the method. A range from 5 to 40 μL (maximum allowed by the chromatographic system) was analyzed and we observed no extra broadening of the peaks even at maximum value. Accordingly, 40 μL was chosen as injection volume.

3.2. Mass spectrometry analysis

ESI and APCI interfaces in positive and negative modes were evaluated for all the compounds analyzed. APCI in positive mode was selected because of its higher sensitivity for all the compounds. Fig. 2 shows a representative chromatogram of a standard mixture of the studied compounds in a spiked placental tissue sample (10 ng g^{-1} of each BP and 6 ng g^{-1} of surrogate) in MRM-APCI positive mode.

For each compound two product ions (two reactions) were monitored: one for quantification and the other for confirmation. Optimized parameters for each compound are listed together with the precursor ions and the mass transitions in Table 2.

3.3. Extraction procedure

We tested an ultrasonic probe and a vortex-mixer as extraction techniques. Recoveries were similar for both procedures (from 91 to 109% for vortex extraction and from 84 to 103% for ultrasound extraction), so we chose the simplest procedure, vortex extraction.

We tested different organic solvents during the extraction: ethyl acetate, methanol, ethanol and acetonitrile. Ethyl acetate appeared to be the most effective solvent to extract the analytes. 50:50 (v/v) mixtures of ethyl acetate and the above mentioned solvents were also tested but not improvements were found.

The effect of different pH values on extraction yield was analyzed. Different amounts of formic acid or ammonia (0.01, 0.05, 0.1,

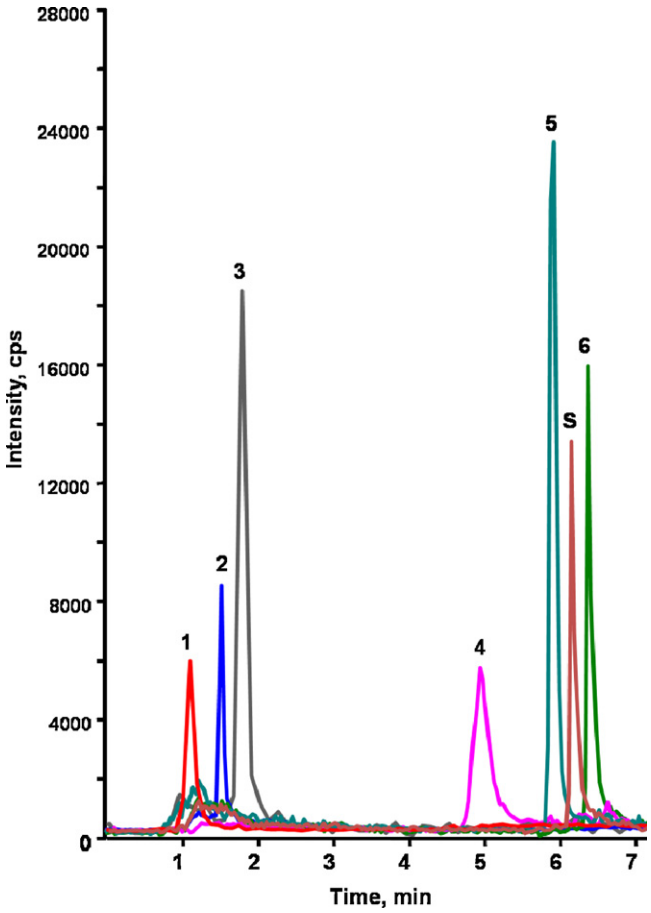

Fig. 2. MRM mode chromatogram of a standard mixture of the target compounds in a spiked placental tissue sample (10.0 ng g^{-1} of each compound). Peak identification: (S) Surrogate; (1) BP-2; (2) BP-1; (3) 4-OH-BP; (4) BP-8; (5) BP-6; (6) BP-3.

0.5, 1 and 10%; v/v) were added to the sample prior to extraction. We observed that recoveries did not improve with the lower percentages and decreased drastically with percentages above 0.1% (v/v). Finally, we determined the possibility of using a saturated aqueous solution of sodium chloride to generate a salting-out effect, but negligible differences were found compared to using deionized water. So placenta samples were homogenized with deionized water.

3.4. Analytical performance

For calibration purposes, six concentration levels (1.0, 2.0, 5.0, 10.0, 15.0 and 20.0 ng g^{-1}) were prepared and calibration curve was built. The calibration standards were prepared adding 450 μL of methanol containing the analytes to 1.5 g increments of placental tissue and each level of concentration was made in triplicate. The samples were stirred and slightly heated until they recovered their original weight and the extraction procedure previously explained was applied.

Calibration curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. Calibration graphs were made using MRM mode. BP-d_{10} (6 ng g^{-1}) was used as surrogate.

In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound: one in solvent (initial mobile phase) and the other in the matrix. A Student's *t*-test was applied in order to compare the calibration curves. First, we had to compare the variances estimated as $S^2_{y/x}$ by means of an Snedecor's *F*-test. The Student's *t*-test showed statistical differences among slope values for the calibration curves in all cases and, consequently, the use of matrix-matched calibration was necessary. Table 3 shows the analytical parameters obtained.

3.5. Method validation

Validation in terms of linearity, precision, accuracy, sensitivity, and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [43].

3.5.1. Linearity

A concentration range from the minimal quantified amount (0.3 ng g^{-1} for BP-1, BP-2, BP-3 and 4-OH-BP and 1.0 ng g^{-1} for BP-6 and BP-8) to 20.0 ng g^{-1} was selected. Linearity of the calibration graphs was tested using the determination coefficients (R^2%) and the *P*-values of the *lack-of-fit* test (P_{lof}%) [44]. The values obtained for R^2 ranged from 99.0 for BP-6 to 99.5% for BP-1, and P_{lof} values were higher than 5% in all cases. These facts indicate a good linearity within the stated ranges. In our application, no levels higher than 20.0 ng g^{-1} were detected.

3.5.2. Selectivity

The specificity of the method was determined by comparing the chromatograms of blank with the corresponding spiked placental tissue. No interferences from endogenous substances were observed at the retention time of the analytes eluated at 1.10 min, 1.50 min, 1.65 min, 5.10 min, 5.9 min, and 6.45 min for BP-2, BP-1, 4-OH-BP, BP-8, BP-6, and BP-3, respectively. Surrogate, BP-d_{10}, appears at 6.2 min. These finds suggest that the spectrometric conditions ensured high selectivity of the LC–MS/MS method.

3.5.3. Accuracy: precision and trueness

Due to the absence of certified materials, we performed a recovery assay in order to validate the method in terms of trueness. We used a blank spiked placenta previously analyzed, in order to ensure that it did not contain the compounds of interest or they were below the LOD of the method. Trueness was evaluated by determining the recovery of known amounts of the tested compounds in placental tissue samples. Samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation from the standard calibration curve within the linear dynamic range and compared with the amount of analytes previously added to the samples. As is shown in Table 4 the recoveries are close to 100% (98–104%). The results also indicate the high extraction efficiency of the procedure.

To evaluate the precision of the global method the intra- and inter-day precisions (as relative standard deviation, RSD) were assessed at three concentration levels for each compound (2.5, 5.0 and 10.0 ng g^{-1}) to assure precise quantifications. Placental tissue samples were spiked, extracted and analyzed in duplicate. The procedure was repeated three times on the same day to evaluate intra-day variability and was repeated on three consecutive days to determine inter-day variability. Samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation in the standard calibration curve within the linear dynamic range and compared to the amount of analytes previously added to the samples. The repeatability and within-laboratory reproducibility, expressed as RSD, are also summarized in Table 4. RSD values fell between 4.1% and 2.0% and were within the acceptable limits to meet the guidelines for bioanalytical method validation which are considered $\leq$15% of the actual value except at LOQ, where it should not deviate by more than 20%. Precision and trueness data indicated that the methodology to extract the compounds from placental tissue is highly accurate, reproducible and robust and/or the low influence of the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry.

3.5.4. Sensitivity

Two fundamental aspects, which need to be examined in the validation of any analytical method, are the LOD and LOQ in order

Table 3
Analytical and statistical parameters.

Parameter[a]	BP-1	BP-2	BP-3	BP-6	BP-8	4-OH-BP
n	18	18	18	18	18	18
a	1.1×10^{-2}	9.0×10^{-3}	1.3×10^{-2}	4.5×10^{-2}	1.7×10^{-2}	1.4×10^{-2}
s_a	1.3×10^{-3}	1.2×10^{-3}	3.1×10^{-3}	4.1×10^{-3}	3.3×10^{-3}	3.6×10^{-3}
b (ng g^{-1})	6.3×10^{-2}	4.6×10^{-2}	1.3×10^{-1}	1.9×10^{-1}	9.4×10^{-2}	1.4×10^{-1}
s_b	2.0×10^{-3}	1.0×10^{-3}	3.0×10^{-3}	1.3×10^{-3}	2.0×10^{-3}	3.0×10^{-3}
R^2 (%)	99.5	99.2	99.2	99.0	99.3	99.2
$s_{y/x}$	3.6×10^{-2}	3.1×10^{-2}	8.2×10^{-2}	1.1×10^{-1}	4.5×10^{-2}	8.8×10^{-2}
LOD (ng g^{-1})	0.07	0.07	0.07	0.3	0.3	0.07
LOQ (ng g^{-1})	0.25	0.25	0.25	1.0	1.0	0.25
LDR (ng g^{-1})	0.25–20.00	0.25–20.00	0.25–20.00	1.0–20.0	1.0–20.0	0.25–20.00

[a] *n*, points of calibration; *a*, intercept; s_a, intercept standard deviation; *b*, slope; s_b, slope standard deviation; R^2, determination coefficient; $s_{y/x}$, regression standard deviation; LOD, detection limit; LOQ, quantification limit; LDR, linear dynamic range.

Table 4
Recovery assay, precision and accuracy of target compounds in placental tissue.

	Spiked (ng g^{-1})	Observed[a] (ng g^{-1})	SD (ng g^{-1})	Recovery (%)	RSD (%)	*n*
Intra-day						
BP-1	2.5	2.6	0.1	104	3.8	6
	5.0	4.9	0.2	98	4.1	6
	10.0	10.0	0.2	100	2.0	6
BP-2	2.5	2.5	0.1	100	4.0	6
	5.0	4.9	0.2	98	4.1	6
	10.0	10.2	0.2	102	2.0	6
BP-3	2.5	2.5	0.1	100	4.0	6
	5.0	5.0	0.2	100	4.0	6
	10.0	9.9	0.4	99	4.0	6
4-OH-BP	2.5	2.5	0.1	100	4.0	6
	5.0	5.1	0.2	102	3.9	6
	10.0	9.9	0.4	99	4.0	6
BP-6	2.5	2.5	0.1	100	4.0	6
	5.0	5.0	0.2	100	4.0	6
	10.0	9.9	0.4	99	4.0	6
BP-8	2.5	2.6	0.1	104	3.8	6
	5.0	5.1	0.2	102	3.9	6
	10.0	9.9	0.4	99	4.0	6
Inter-day						
BP-1	2.5	2.6	0.1	104	3.8	18
	5.0	4.9	0.2	98	4.1	18
	10.0	9.9	0.2	99	2.0	18
BP-2	2.5	2.6	0.1	104	3.8	18
	5.0	4.9	0.2	98	4.1	18
	10.0	10.2	0.2	102	2.0	18
BP-3	2.5	2.5	0.1	100	4.0	18
	5.0	5.0	0.2	100	4.0	18
	10.0	9.9	0.4	99	4.0	18
4-OH-BP	2.5	2.5	0.1	100	4.0	18
	5.0	5.1	0.2	102	3.9	18
	10.0	10.0	0.4	100	4.0	18
BP-6	2.5	2.5	0.1	100	4.0	18
	5.0	5.0	0.2	100	4.0	18
	10.0	9.9	0.4	99	4.0	18
BP-8	2.5	2.6	0.1	104	3.8	18
	5.0	5.1	0.2	102	3.9	18
	10.0	9.8	0.4	98	4.1	18

[a] Mean value; SD, standard deviation; RSD, relative standard deviation; *n*, number of determinations.

to determine if an analyte is present in the sample. The LOD is the minimum amount of analyte detectable in the sample while the LOQ is the minimum amount that could be quantified. They are based on the theory of hypothesis testing and the probabilities of false positives and false negatives [45]. In this work, these parameters were calculated from the blank standard deviation. In order to estimate the chromatographic blanks, we applied the methodology proposed by González-Casado et al. [46]. It can be assumed that the chromatographic peak shape is a Gaussian-type one, then the estimation of base width (W_b) for 99.73% of the peak-area is $W_b = 6\sigma = 2.548\, W_{0.5\,h}$, where $W_{0.5\,h}$ is the half-width of the peak. Extrapolation of the graph of $W_{0.5\,h}$ at different concentrations of analyte can give us a statistically significant idea of the width of the base for "zero concentration". The blank signal for each analyte can be determined by integration over the baseline of the chromatograms taking a width $t_R \pm 0.5\, W_{b0}$ where t_R is the retention time of the analyte and W_{b0} has been evaluated as explained above. It relies on studying the blank standard deviation in a time interval corresponding to the peak width at its base, extrapolated to zero concentration. LOD and LOQ better adjusted to statistical evaluation are implemented in Table 3.

3.6. Method application

We used the proposed method to determine BP-1, BP-2, BP-3, BP-6, BP-8 and 4-OH-BP in 16 placental tissue samples from women who live in the province of Granada (Spain). The samples were analyzed in triplicate. The results obtained are shown in Table 5. Fig. 3 shows the chromatograms obtained for two analyzed placental tissue samples.

BP-1 was detected and quantified in fourteen of the sixteen samples, in concentrations ranging from 0.5 to 9.8 ng g^{-1}; BP-2 in seven samples in concentrations from 1.2 to 8.9 ng g^{-1}; 4-OH-BP in eleven samples in concentrations from 0.6 to 1.8 ng g^{-1}; BP-6 was detected

Table 5
Free benzophenones observed concentration in analyzed placental tissue samples.

Sample	Concentration[a] (ng g^{-1})					
	BP-1	BP-2	BP-3	BP-6	BP-8	4-OH-BP
S01	3.5	7.1	ND	ND	ND	ND
S02	3.0	8.9	ND	ND	ND	1.1
S03	ND	ND	ND	ND	ND	0.7
S04	1.5	ND	ND	ND	ND	0.9
S05	3.2	5.3	ND	ND	ND	0.9
S06	2.0	1.2	ND	ND	ND	ND
S07	4.0	3.5	ND	ND	ND	1.0
S08	9.8	2.6	ND	ND	ND	ND
S09	8.3	ND	ND	ND	ND	ND
S10	2.6	ND	ND	D	ND	1.8
S11	1.3	ND	ND	ND	ND	ND
S12	ND	ND	ND	ND	ND	0.7
S13	0.5	ND	ND	ND	ND	0.9
S14	1.9	ND	ND	ND	ND	1.2
S15	3.3	3.6	ND	ND	ND	0.6
S16	2.3	ND	ND	ND	ND	0.7

D, detected (between LOD and LOQ); ND, not detected (<LOD).
[a] Mean value of 3 determinations.

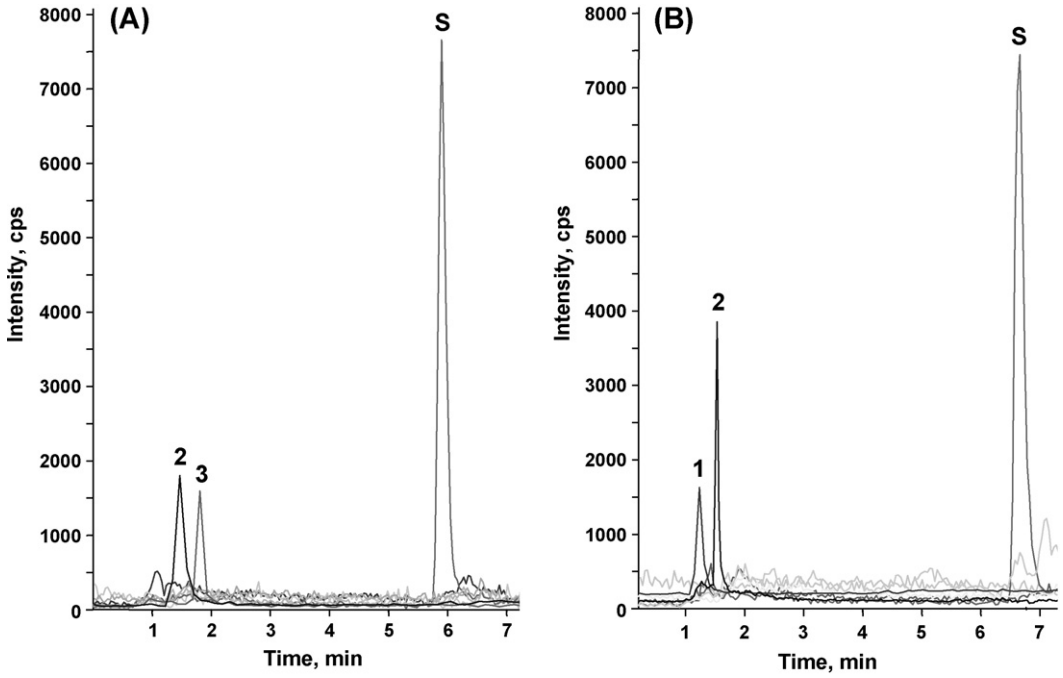

Fig. 3. MRM mode chromatograms of two placental tissue samples: (A) contaminated with BP-1 (2) and 4-OH-BP (3); (B) contaminated with BP-1 (2) and BP-2 (1).

in one sample but the quantification was not possible (concentration lower than LOQ), and BP-3 and BP-8 were not detected. The presence/absence of these compounds depends on the exposure to these compounds as well as their different metabolism.

4. Conclusions

Determination and quantification of BPs using LC–MS/MS in placental tissue samples was successfully performed on a Gemini C_{18} column using 0.1% (v/v) ammonia in methanol and 0.1% (v/v) ammoniacal aqueous solution as mobile phase. The analytical performance of this method was validated and the method has been successfully used for determination of these compounds in samples collected from women who live in the province of Granada (Spain).

Studies on exposure during pregnancy and childhood have recommended the use of biomarkers to quantify individual exposure to EDCs. The INMA Project is a network of birth cohorts in Spain that aim to study the role of environmental pollutants during pregnancy and early childhood. The studied population includes pregnant women of general population resident in seven Spanish study areas (Ribera d'Ebre, Menorca, Granada, Valencia, Sabadell, Asturias and Gipuzkoa) and their children. Studies on human exposure to BPs are needed to address the question of whether maternal exposure to these compounds can lead to adverse health effects in the offspring. The method we propose allows the determination of free BPs levels and it might be used to conduct exposure studies on human populations.

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