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A new liquid chromatography–tandem mass spectrometry method for determination of parabens in human placental tissue samples

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ABSTRACT

Endocrine disruptors are a group of organic compounds widely used, which are ubiquitous in the environment and in biological samples. The main effect of these compounds is associated with their ability to mimic or block the action of natural hormones in living organisms, including humans. Parabens (esters of *p*-hydroxybenzoic acid) belong to this group of compounds. In this work, we propose a new liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to asses the presence of parabens most commonly used in industrial applications (methyl-, ethyl-, propyl- and butyl-paraben) in samples of human placental tissue. The method involves the extraction 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 negative mode. Deuterated bisphenol A (BPA-d_{16}) was used as surrogate. Found detection limits (LOD) ranged from 0.03 to 0.06 ng g^{-1} and quantification limits (LOQ) from 0.1 to 0.2 ng g^{-1}, while inter- and intra-day variability was under 13.8%. The method was validated using standard addition calibration and a spike recovery assay. Recovery rates for spiked samples ranged from 82% to 108%. This method was satisfactorily applied for the determination of parabens in 50 placental tissue samples collected from women who live in the province of Granada (Spain).

1. Introduction

Parabens are a group of alkyl esters of *p*-hydroxybenzoic acid widely used as antimicrobial preservatives, especially against mold and yeast, in cosmetic products and pharmaceuticals, and in food and beverage processing. Individually or in combination, parabens are used in over 13,200 formulations [1] in nearly all types of cosmetics. The antimicrobial activity of parabens increases, but water solubility decreases, as the length of the alkyl chain is increased. For this reason, methyl- and propyl-parabens are the most extensively used in cosmetics and food processing [2]. The widespread use of parabens arises from their low toxicity, broad inertness, worldwide regulatory acceptance and low cost. The estimated human exposure is 76 mg per day: with food accounting for approximately 1 mg/day; cosmetics and personal care products, 50 mg/day; and drugs, 25 mg/day [3]. However, some parabens may have estrogenic activity, whose extent depends on their structure [4,5]. *In vitro* studies suggest that parabens exhibit estrogenic activity in yeast-based assays [5–8], induce the growth of MCF-7 human breast cancer cells, and influence the expression of estrogen-dependent genes [9–12]. Similarly, *in vivo* studies suggest increased uterine weight in immature mice after exposure to butyl-isobutyl-, and benzyl-paraben [10]. Male rodents also exhibited decreased testosterone secretion and some reproductive tract alterations after exposure to butyl- and propyl-parabens [13–15], but not to methyl and ethyl paraben exposure [16]. Besides ethyl-, propyl-, and butyl-paraben evoked estrogenic responses in sexually immature rainbow trout. Estrogenic activity of parabens in animals and the presence of these compounds in human breast tissue [17] have raised some concerns about their safety.

The widespread use of parabens and their potential risk to human health have prompted interest in assessing human exposure to these compounds. Exposure may occur through inhalation, dermal contact and ingestion [3,18], and parabens metabolism may differ depending upon the exposure pathway [19,20]. Metabolism and uptake through human skin seem to be lower than in other species [20–22], and inter-individual differences among humans in dermal metabolic capacity exist [23]. Esterase enzyme can hydrolyze parabens to *p*-hydroxybenzoic acid, which can be conjugated before urinary excretion [24,25], but they can be also be excreted as intact esters [26]. Some studies demonstrate that parabens can be rapidly absorbed through the skin into the human

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body even from a single dose of body care product and esterase levels in the skin are not sufficient to hydrolyze all paraben esters to completion [27]. Moreover, *in vitro* models have shown that permeation of parabens through human skin can increase with repeated doses [18].

The estrogenic activity of free parabens and *p*-hydroxybenzoic acid has been demonstrated [28]; however, there are not yet studies that demonstrate the estrogenic activity of conjugated forms [29].

In recent years, several methods for the analysis of these compounds have been described for different matrices. Assessment in, pharmaceutical formulations [30], cosmetic products [31–33], foods [34–36], beverages [37,38], solid environmental samples [39] and virgin and recycled paper [8] have been described using different analytical techniques. However, only a few methods have focused on the determination of parabens in human samples. Table 1 shows a representative fraction of the proposed methods for the determination of these compounds in human biological fluids and tissues recently published.

The aim of this work is to develop a sensitive analytical method for the determination of four free parabens in human placental tissue samples. To our knowledge, there is no published literature on parabens determination in this matrix. The method we propose in this paper was validated and satisfactorily applied to determine free parabens in 50 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^{-1}) 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) and deuterated bisphenol A (BPA-d_{16}) was supplied by Sigma–Aldrich (Madrid, Spain).

Stock 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 four 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 and ammonia (25%) were purchased from Sigma–Aldrich (Madrid, Spain). Formic acid (98%) and sodium chloride were supplied by Panreac (Barcelona, Spain).

Before the injection into the LC system, the samples (final extracts) were filtered through 0.20 μm (4 mm diameter) non sterile regenerated cellulose filters supplied by Sartorius (Goettingen, Germany).

2.2. Instrumentation

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 online to an API 2000 (Applied Biosystems, Foster City, CA, USA) triple quadrupole mass spectrometer system that can use either APCI or electrospray ionization (ESI) interfaces. 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 previously calibrated 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 [46] was used for statistical and regression analyses (linear mode).

2.3. Sample collection and storage

Placenta samples were collected at delivery in the Maternity Unit of San Cecilio University Hospital of Granada (Spain). Each whole placenta was accurately examined and weighed, and cut in two pieces that included maternal and fetal sides as well as central and peripheral parts, was taken. Each one was fragmented, beaten and placed in a container. Then, the samples were homogenized using an ultrasonic spindle. The container was placed in a glass full of ice in order to avoid sample heating and the spindle was directly introduced in the placental tissue. Ultrasound setting consisted in pulses of 30 s followed by 30 s without sonication, until completing 5 min of effective radiation. The process was repeated and we had to add more ice into the glass. Once homogenized, samples were frozen at −86 °C and stored confidentially and anonymously until the analysis in our laboratory.

2.4. Preparation of spiked samples

Spiked samples were prepared by adding 450 μL of methanol containing the analytes at the different concentration levels every 1.5 g of placental tissue. Spiked samples were stirred and slightly heated until they recovered their 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 200 μL of methanol containing 100 ng g^{-1} of BPA-d_{16} and shaken for 10 min. Once fortified the sample was homogenized with 1.5 mL of water shaking on a vortex for 1 min. The homogenate was extracted by adding 3 mL of ethyl acetate and shaking again for 10 min and the mixture was then 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 of diameter) non sterile regenerated cellulose filter.

2.5.2. Chromatographic conditions

Chromatographic separation was performed using a Gemini C_{18} column (100 mm × 2 mm i.d., 3 μm particle size) from Phenomenex (Torrance, CA, USA). The standards and samples were separated using an isocratic mobile phase consisting of 40% of 0.1% (v/v) ammoniacal aqueous solution (solvent A) and 60% of 0.1% (v/v) ammonia in methanol (solvent B). Flow rate was 0.25 mL min^{-1}, injection volume 40 μL, the column temperature was maintained at 40 °C and total run time was 5.0 min.

2.5.3. Mass spectrometric conditions

APCI was performed in the negative 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

Table 1
Analytical methods for the determination of parabens in human samples.

Human sample	Analytical technique	Detection limit (LOD)	Reference
Blood and urine	LC–UV	2.0 ng mL^{-1}	[40]
Breast cancer tissue	GG-MS	1.05–3.75 ng g^{-1}	[41]
Milk	SPE-LC–MS/MS	0.1 ng mL^{-1}	[42]
Saliva	LC–UV	0.2–0.3 ng mL^{-1}	[43]
Serum	LC–MS/MS	0.3 ng mL^{-1}	[27]
	LC–MS/MS	0.1–0.2 ng mL^{-1}	[44]
Urine	SPE-LC–MS/MS	0.10–0.18 ng mL^{-1}	[26]
	LC–MS/MS	0.3 ng mL^{-1}	[45]

Table 2
Selected MRM transitions and optimized potentials.

Parameters	MPB	EPB	PPB	BPB	BPA-d_{16}
Transitions	151.1 → 91.9[a]	165.1 → 91.9[a]	179.1 → 92.0[a]	192.9 → 91.9[a]	241.2 → 142.0[a]
	151.1 → 136.1[b]	165.1 → 137.0[b]	179.1 → 135.9[b]	192.9 → 136.0[b]	241.2 → 223.0[b]
Dwell time (ms)	200	200	200	200	200
DP (V)	−40	−28	−40	−42	−43
FP (V)	−350	−360	−370	−390	−160
EP (V)	−8	−7	−8	−10	−11
CE (V)	−25	−30	−33	−30	−32
CXP (V)	−18	−15	−13	−15	−20

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 for confirmation.

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. Dwell time for each compound was set at 200 ms. Optimized parameters for each compound are listed together with the mass transitions in Table 2.

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 liquid chromatography 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 tested, Acquity UPLC column generated pressures close to the maximum allowed by the chromatographic system. Consequently, the Gemini C_{18} column was the selected one 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. Secondly, we analyzed the effect of substituting methanol for acetonitrile but no improvements were observed neither in peak shapes nor resolution, so we selected methanol. We tested several isocratic percentages and selected 60% of 0.1% (v/v) ammonia in methanol (solvent B) and 40% of 0.1% (v/v) ammoniacal aqueous solution (solvent A) as the most appropriate.

Lastly, we performed a study in order to enhance the analytical signal, and consequently the detection limits of the method, evaluating the possibility of increasing the injection volume. A range from 5 to 40 μL (maximum allowed by the chromatographic system) was analyzed observing no extra broadening of the peaks even at maximum value. Accordingly, 40 μL was chosen as injection volume.

3.2. Mass spectrometric analysis

The MS/MS detection method was optimized by direct individual infusion of each compound in order to optimize the response of the precursor ion. ESI and APCI interfaces in positive and negative modes were evaluated for all the compounds. APCI negative mode was selected because of its higher sensitivity for all the compounds. $[M-H]^-$ 151.1, 165.1, 179.1 and 192.9 m/z were the selected precursor ions for MPB, EPB, PPB and BPB, respectively. The precursor ion selected for BPA-d_{16}, used as surrogate, was 241.2 m/z. Fig. 1 shows a representative chromatogram of a standard mixture of the target compounds in a spiked placental tissue sample (6.0 ng g^{-1} of each paraben and 100 ng g^{-1} of surrogate) in MRM-APCI negative mode.

Two product ions (two reactions) were monitored: one for quantification and the other for confirmation. For quantification, the most abundant transition was selected to obtain the maximum sensitivity. The parameters optimized for the precursor ions were: declustering potential (DP), focusing potential (FP) and entrance potential (EP); for product ions the optimized parameters were: collision energy (CE) and collision cell exit potential (CXP). Regarding sensitivity, the most influential parameters were DP and CE.

3.3. Extraction procedure

We tested an ultrasonic probe using a Branson digital sonifier and a vortex-mixer as extraction techniques. We placed 1.5 g of placental tissue in an 8 mL glass vial, fortified with the surrogate and homogenized with 1.5 mL of water and vortexed for 1 min. Then, 3 mL of ethyl acetate were added in order to carry out both the extractions: vortexing for 10 min or applying ultrasonic radiation for 10 min at 70% amplitude. Recoveries obtained under these conditions were similar for both procedures (from 81 to 102% for vortex extraction and from 75 to 105% for ultrasonic

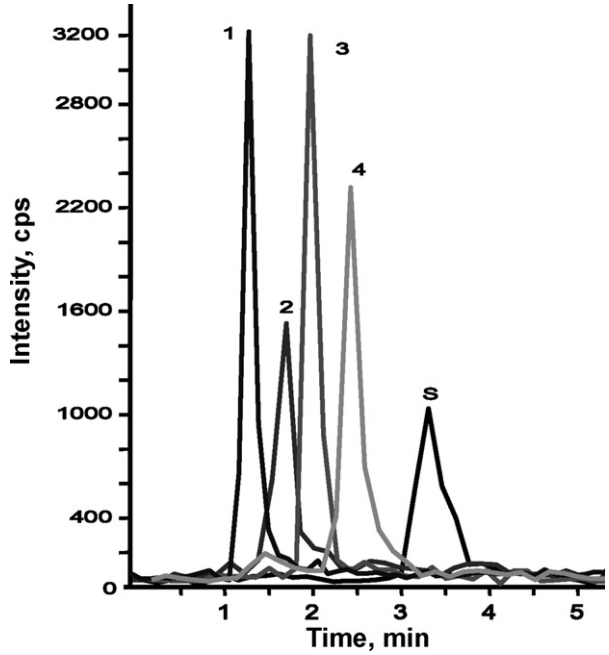

Fig. 1. MRM mode chromatogram of a standard mixture of the target compounds in a spiked placental tissue sample (6.0 ng g^{-1} of each paraben and 100 ng g^{-1} of surrogate). Peak identification: (S) surrogate (BPA-d_{16}); (1) methylparaben; (2) ethylparaben; (3) propylparaben; and (4) butylparaben.

extraction), so we chose the simplest procedure, i.e., 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 also analyzed. Different amounts of formic acid or ammonia (0.01, 0.05, 0.1, 0.5, 1 and 10%; v/v) were added to the sample prior to extraction. We observed that the recoveries did not improve with the lower percentages and decreased drastically with percentages above 0.1%. 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 deionised water. Finally, placenta samples were homogenized with deionised water.

3.4. Analytical performance

For calibration purposes, seven concentration levels (0.20, 0.50, 0.75, 1.0, 2.5, 5.0 and 10.0 ng g^{-1}) were prepared and calibration curve was built. Calibration standards were prepared by adding 450 μL of methanol containing the analytes every 1.5 g of placental tissue. Each level of concentration was made in triplicate. For each concentration we weighted 6.0 g of placental tissue and added 1.8 mL of methanol containing the appropriate concentration of analytes. The samples were stirred and slightly heated to remove the added methanol until they recovered original weight. Then, we weighted 1.5 g of placental tissue in triplicate in 8 mL glass vials in order to perform the extraction procedure.

Calibration curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. Calibration graphs were made using MRM mode. Because we cannot afford to purchase deuterated parabens, BPA-d_{16} (at 100 ng g^{-1}) was used as surrogate. In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each selected compound: one in solvent (initial mobile phase) and the other in the matrix. The *t*-Student test was applied to compare the calibration curves. First, we compare the variances estimated as $S^2_{y/x}$ by means of an *F*-Snedecor test. The *t*-Student test showed statistical differences among slope values 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, selectivity, precision, accuracy and sensitivity was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [47].

3.5.1. Linearity

A concentration range from the minimal quantified amount up to two higher orders of magnitude (0.1–10 ng g^{-1}) was selected. Linearity of the calibration graphs was tested using the correlation coefficients (R^2) and the *P* values of the *lack-of-fit* test (P_{lof} %) [48]. R^2 values ranged from 99.78 to 99.94% and P_{lof} values were higher than 5% in all cases. These facts indicate a good linearity within the stated ranges. As well, in our application, no levels higher than 10.0 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 samples. No interferences from endogenous substances were observed at the retention time of the analytes. Methylparaben, ethylparaben, propylparaben and butylparaben eluated at 1.30 min, 1.85 min, 2.20 min and 2.75 min, respectively. Surrogate appears at 3.70 min. These findings suggest that the spectrometric conditions ensured high selectivity of the LC–MS/MS method.

Table 3
Analytical and statistical parameters.

Parameter[a]	MPB	EPB	PPB	BPB
n	21	21	21	21
a	0.371	0.322	0.313	0.05
s_a	0.356	0.169	0.085	0.133
b (g ng^{-1})	2.937	1.590	1.356	1.949
s_b	0.063	0.030	0.015	0.023
R^2 (%)	99.78	99.83	99.94	99.93
$s_{y/x}$	0.995	0.473	0.237	0.370
LOD (ng g^{-1})	0.03	0.06	0.06	0.06
LOQ (ng g^{-1})	0.1	0.2	0.2	0.2
LDR (ng g^{-1})	0.1–10.0	0.2–10.0	0.2–10.0	0.2–10.0

[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.

Compound	Spiked ($ng\,g^{-1}$)	Observed[a] ± SD (%, RSD)	Recovery (%)	$t_{calculated}$	P_{value} (%)	n
Intra-day						
	1.0	1.0 ± 0.1 (10.0)	99	0.76	48.2	6
MPB	2.5	2.4 ± 0.2 (8.3)	96	1.22	27.7	6
	7.5	7.7 ± 0.5 (6.5)	103	1.68	15.4	6
	1.0	1.1 ± 0.1 (9.4)	106	0.95	38.6	6
EPB	2.5	2.6 ± 0.2 (7.7)	104	0.99	36.8	6
	7.5	7.3 ± 0.7 (9.6)	97	1.14	30.6	6
	1.0	0.9 ± 0.1 (10.6)	94	0.87	42.4	6
PPB	2.5	2.5 ± 0.3 (12.0)	100	1.35	23.5	6
	7.5	7.6 ± 0.6 (7.8)	101	0.97	37.7	6
	1.0	1.0 ± 0.1 (10.2)	98	0.98	37.2	6
BPB	2.5	2.6 ± 0.2 (7.7)	104	0.89	41.4	6
	7.5	7.4 ± 0.3 (4.1)	99	1.45	20.7	6
Compound	Spiked ($ng\,g^{-1}$)	Observed[a] ± SD (%, RSD)	Recovery (%)	$t_{calculated}$	P_{value} (%)	n
Inter-day						
	1.0	1.0 ± 0.1 (11.2)	98	1.35	19.5	18
MPB	2.5	2.6 ± 0.3 (10.6)	105	1.74	10.0	18
	7.5	7.9 ± 0.5 (6.0)	108	0.80	43.5	18
	1.0	1.1 ± 0.1 (9.5)	105	2.00	6.2	18
EPB	2.5	2.4 ± 0.2 (8.7)	96	0.66	51.8	18
	7.5	6.9 ± 0.9 (13.8)	82	1.29	21.4	18
	1.0	1.0 ± 0.1 (9.6)	104	0.69	50.0	18
PPB	2.5	2.4 ± 0.1 (5.4)	96	0.98	34.1	18
	7.5	7.0 ± 0.6 (8.9)	92	0.35	73.1	18
	1.0	0.9 ± 0.1 (12.2)	90	0.77	45.2	18
BPB	2.5	2.4 ± 0.2 (8.5)	94	1.82	8.6	18
	7.5	7.7 ± 0.4 (5.7)	102	1.35	19.5	18

SD, standard deviation; RSD, relative standard deviation; n, number of determinations; P_{value}, probability of obtaining a test statistic at least as extreme as the one that was actually observed, assuming that the null hypothesis is true; $t_{calculated}$: calculated value of the statistic t-Student.
[a] Mean value ($ng\,g^{-1}$).

3.5.3. Precision and accuracy

The precision and accuracy data for the analytical procedures are shown in Table 4. Intra-day and inter-day precision (as relative standard deviation, RSD) of the method were lower than 14%, and were within the acceptable limits proposed by the guidelines for bioanalytical method validation (≤20%). To evaluate the precision of the assay, laboratory reproducibility and repeatability were estimated at three different concentrations for each compound (1.0, 2.5 and 7.5 $ng\,g^{-1}$). 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. The repeatability and within-laboratory reproducibility, expressed as RSD, are summarized in Table 4. Precision and accuracy data indicated that the methodology to extract the selected compounds from placental tissue is highly reproducible and robust.

3.5.4. Sensitivity

A fundamental aspect that needs to be examined in the validation of any analytical method is its LOD and LOQ in order to determine if an analyte is present in the sample. The DL is the minimum amount of analyte that could be detected 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 [49]. 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. [50]. It can be assumed that the chromatographic peak shape is a Gaussian-type one, 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 in 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 of the chromatograms' baseline taking a width $t_R \pm 0.5W_{b0}$, 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 that better adjusted to a statistical evaluation are implemented in Table 3.

3.5.5. Recovery

Due to the absence of certified materials, we performed a recovery assay in order to validate the method. We had to use two placenta samples because all of the samples contained at least one of the four parabens tested. Each blank spiked placenta was previously analyzed in order to ensure that it did not contain the compounds of interest or that they were below the LOD of the method. Accuracy 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 in the standard calibration curve within the linear dynamic range and compared to the amount of analytes previously added to the samples. A recovery test (t-Student test) was used. The results are shown in Table 4. Because all calculated P-values calculated were >0.05 (5%) in all cases, the null

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

Sample	Concentration ($ng\,g^{-1}$)[a]			
	Methylparaben	Ethylparaben	Propylparaben	Butylparaben
M01	8.2	D	0.6	ND
M02	0.6	ND	0.2	ND
M03	3.9	0.8	0.3	0.4
M04	0.3	0.6	0.3	D
M05	1.9	ND	0.8	ND
M06	0.8	ND	0.3	ND
M07	0.6	ND	0.9	ND
M08	10.0	0.3	0.7	ND
M09	4.5	ND	0.2	ND
M10	2.7	ND	0.7	ND
M11	5.9	D	0.6	ND
M12	0.4	ND	ND	ND
M13	1.6	2.2	ND	ND
M14	8.3	D	1.2	ND
M15	1.0	0.2	1.1	ND
M16	3.8	0.4	0.6	ND
M17	D	ND	0.3	ND
M18	4.0	ND	0.3	ND
M19	1.1	ND	1.7	ND
M20	1.7	0.4	0.8	D
M21	5.2	5.3	0.7	ND
M22	ND	0.7	2.2	0.6
M23	9.7	0.3	0.5	ND
M24	ND	0.3	0.4	ND
M25	0.4	D	ND	ND
M26	0.4	ND	ND	ND
M27	1.0	D	ND	ND
M28	0.2	0.4	0.5	D
M29	3.3	D	0.3	ND
M30	0.8	ND	0.2	ND
M31	3.2	1.2	1.7	0.5
M32	2.2	D	0.2	ND
M33	0.9	0.3	0.6	ND
M34	0.8	0.2	0.2	ND
M35	3.6	0.6	0.4	ND
M36	2.1	D	0.3	ND
M37	4.2	1.2	1.1	D
M38	1.5	ND	0.8	ND
M39	0.4	D	0.5	ND
M40	0.9	ND	0.3	ND
M41	0.3	D	0.4	ND
M42	2.1	D	0.4	ND
M43	3.9	0.3	0.2	0.2
M44	0.7	ND	0.3	ND
M45	9.4	0.2	0.5	ND
M46	0.7	ND	0.5	ND
M47	0.3	ND	0.2	ND
M48	1.9	D	0.3	ND
M49	1.2	0.5	0.9	ND
M50	0.2	D	0.4	ND

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

hypothesis appears to be valid, i.e., recoveries are close to 100%. The results indicate the high extraction efficiency of the procedure.

3.6. Method application

We used the proposed method to determine free methylparaben, ethylparaben, propylparaben and butylparaben in 50 placental tissue samples obtained from women living in the province of Granada (Spain). All the samples were analyzed in triplicate. The results obtained as mean of three determinations are shown in Table 5. Fig. 2 shows an example of chromatograms corresponding to two of the analyzed samples.

Methylparaben was detected in 96% ($n = 48/50$) of the analyzed samples and quantified in 94% ($n = 47/50$) in concentrations ranging from 0.2 to 10.0 $ng\,g^{-1}$ (mean: 2.6 $ng\,g^{-1}$; median: 1.6 $ng\,g^{-1}$). Ethylparaben was detected in 66% ($n = 33/50$) of the analyzed samples and quantified in 40% ($n = 20/50$) in concentrations ranging from 0.2 to 5.3 $ng\,g^{-1}$ (mean: 0.8 $ng\,g^{-1}$; median: 0.4 $ng\,g^{-1}$). Propylparaben was detected in 90% ($n = 45/50$) of the analyzed samples and quantified in 90% ($n = 45/50$) in concentrations ranging from 0.2 to 2.2 $ng\,g^{-1}$ (mean: 0.6 $ng\,g^{-1}$; median: 0.5 $ng\,g^{-1}$). Butylparaben was detected in 16% ($n = 8/50$) of the analyzed samples and quantified in 8% ($n = 4/50$) in concentrations ranging from 0.2 to 0.6 $ng\,g^{-1}$ (mean: 0.4 $ng\,g^{-1}$; median: 0.5 $ng\,g^{-1}$).

The presence of methyl-, ethyl-, propyl- and/or butyl-paraben depends on the exposure to these compounds as well as on their different metabolisms. All the analyzed human placenta samples had at least one of the selected parabens.

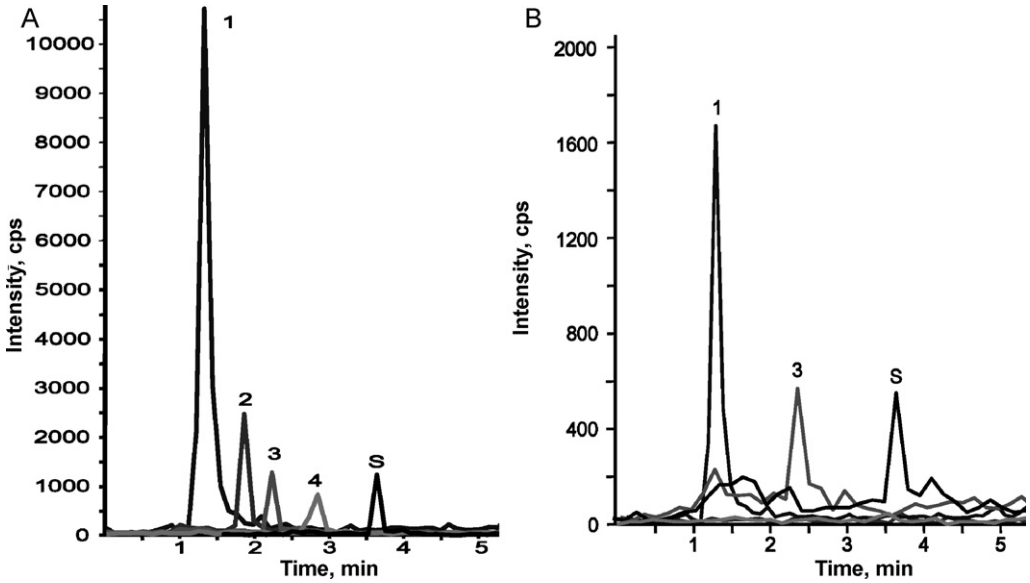

Fig. 2. MRM mode chromatogram of placental tissue samples of two women. (A) Contaminated sample with (1) methylparaben, (2) ethylparaben, (3) propylparaben and (4) butylparaben. (B) Contaminated sample with (1) methylparaben and (3) propylparaben.

4. Conclusions

Determination and quantification of free parabens 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 living in the province of Granada (Spain).

Studies on human exposure to parabens 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 here allows determining the levels of free parabens and might be used to perform studies on human populations.

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