Journal of Chromatography B, 878 (2010) 3363–3369

Contents lists available at ScienceDirect

Journal of Chromatography B

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

# Determination of Bisphenol A and its chlorinated derivatives in placental tissue samples by liquid chromatography–tandem mass spectrometry

I. Jiménez-Díaz[a,b], A. Zafra-Gómez[a], O. Ballesteros[a,*], N. Navea[b], A. Navalón[a], M.F. Fernández[b], N. Olea[b], J.L. Vílchez[a]

[a] Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, University of Granada, Campus of Fuentenueva, E-18071 Granada, Spain
[b] Laboratory of Medical Investigations, San Cecilio University Hospital, University of Granada, CIBER Epidemiology and Public Health (CIBERESP), E-18071 Granada, Spain

ARTICLE INFO

*Article history:*
Received 7 July 2010
Accepted 24 October 2010
Available online 30 October 2010

*Keywords:*
Bisphenol A
Bisphenol A chlorinated derivatives
Liquid chromatography–tandem mass spectrometry (LC–MS/MS)
Placental tissue analysis

ABSTRACT

The group of compounds commonly called endocrine disruptors covers a wide range of synthetic and natural substances able to alter the normal hormone function of wildlife and humans, consequently causing adverse health effects. Bisphenol A (BPA) and its chlorinated derivatives are some of these compounds. In this work, we propose a new liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to determine these compounds in human placental tissue samples. The method involves an extraction phase of the extracts from the samples using ethyl acetate, followed by a clean-up phase by 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 internal standard. Found detection limits (DL) ranged from 0.2 to 0.6 ng $g^{-1}$ and quantification limits (QL) from 0.5 to 2.0 ng $g^{-1}$ for Bisphenol A and its chlorinated derivatives, while inter- and intra-day variability was under 8.1%. The method was validated using standard addition calibration and a spike recovery assay. Recovery rates for spiked samples ranged from 97% to 105%. This method was satisfactorily applied to the determination of BPA and its chlorinated derivatives in 49 placental tissue samples collected from women who live in the province of Granada (Spain).



## 1. Introduction

It is well known that about 100 of industrial chemicals have estrogenic activity in addition to their desired chemical properties. Most of these compounds, commonly called endocrine disrupting chemicals (EDCs), are organic compounds widely used, which are ubiquitous in the environment and in biological samples [1–3]. The main effect of these compounds is associated with their capability to mimic or block the action of natural hormones in living organisms, including humans. Estrogen mimics have been perhaps the most studied of all types of EDCs and the ones to capture much of the public's attention as a public health issue. Many researchers hypothesize that exposure to these endocrine disruptors during critical periods of development – *in utero* or early postnatal life – could cause morphologic and functional alterations in wildlife and humans affecting growth, reproduction and development [4–6]. It has also been postulated that EDCs accumulate in certain human tissues and their effects might pass to the offspring via the placenta and/or breast milk [7].

One of the representative compounds of the EDCs group is BPA. BPA is a compound with high reactivity and is the raw material used in a large amount of manufactured products, such as polycarbonate plastics, epoxy resins used to line metal cans, and in many plastic consumer products including toys, water pipes, drinking containers, eyeglass lenses, sports safety equipment, dental monomers, medical equipment and tubing and consumer electronics [8]. BPA is one of the highest volume chemicals produced worldwide, with an estimated production of 3.9 million tons in 2006 [10] and over 100 tons released into the atmosphere from its annual production [9]. When BPA is present in treated waters, it may react with residual chlorine originally used as a disinfectant, producing chlorinated BPA derivatives depending on the pH of the medium [11]. Polychlorinated BPA has been recently identified and biodegradation test using activated sludge revealed that it is not easily biodegraded [12], in this sense, chlorinated BPA derivatives are more cytotoxic than BPA [13] and all of them show estrogenic activity [14].

Estrogenic activity of BPA was first reported in 1993 [15]. BPA affinity for estrogen receptors is 10,000 to 100,000 fold weaker than that of estradiol, so it has been considered a very weak environmental estrogen. Recent *in vitro* studies have shown that BPA effects are mediated by both genomic and epigenomic estrogen-response mechanisms, with the disruption of the cell function occurring at

* Corresponding author. Tel.: +34 958 243326; fax: +34 958 243328.
*E-mail address:* oballest@ugr.es (O. Ballesteros).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.10.021

doses as low as 1 pM (0.23 ng $L^{-1}$) [16]. Some reports also indicate the potential of BPA to disrupt thyroid hormone action [17], to cause proliferation of human prostate cancer cells [18] and to block testosterone synthesis [19] at very low doses. This extensive new literature concerning low-dose effects of BPA has given rise to controversy about the BPA limit values set by regulatory agencies for consumer health protection and a new risk assessment has been strongly recommended [16]. Currently, the Tolerable Daily Intake set by the European Union (EU) Commission [20] and the reference dose established by the US Environmental Protection Agency (EPA) [21] is 0.05 mg BPA $kg^{-1}$ body weight/day. This value derived from the accepted overall Non-Observed-Adverse-Effect Level of 5 mg $kg^{-1}$. Similarly, a specific migration limit for BPA from food contact plastic materials of 600 ng $g^{-1}$ was set by the EU Commission in 2004 [22].

Although air, dust or water (including skin contact during bathing and swimming) are possible sources of exposure to BPA, the most important source is through the diet, BPA in food and beverages accounts for the majority of daily human exposure [23–25]. After ingestion, the majority of BPA is quickly bound to glucuronic acid to produce BPA-glucuronide [24]. Glucuronidation makes BPA more soluble in water and, therefore, easier to eliminate in the urine. To a lesser extent, unconjugated BPA (commonly referred to as "free" BPA) is converted to other metabolites, primarily BPA sulphate, as well easier to eliminate in the urine. Although free BPA and its metabolites can all be measured in humans, only free BPA is considered to be biologically active.

The aim of this work is to develop a sensitive LC–MS/MS method for the simultaneous determination of free BPA and its chlorinated derivatives in placental tissue samples. In the last years, LC–MS/MS has been quite used for the determination of BPA and related compounds in different environmental matrices [26,27], food samples as milk or infant formulas [28,29] and biological samples as urine or serum [30–32]. However, to our knowledge, there is not much published literature on BPA determination in placental tissue samples. The most relevant work is the one proposed by Schönfelder et al. [33] that involves the extraction of analyte from the samples using ethyl acetate, followed by a derivatization step prior to the gas chromatography–mass spectrometry (GC–MS) analysis. Our proposed method shows some advantages compared to the Schönfelder method. First, we propose the determination of not only BPA but also of its chlorinated derivatives; on the other hand, we leave out the derivatization phase and finally, we introduce MS/MS detection to confirm unequivocally the presence of the compounds in the analyzed samples. The method was validated and satisfactorily applied to the determination of BPA and its chlorinated derivatives in 49 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 was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Bisphenol A (BPA), tetrachlorobisphenol A ($Cl_4$-BPA) and Bisphenol A-$d_{16}$ were supplied by Sigma–Aldrich (Madrid, Spain). Monochloro, dichloro and trichloro bisphenol A (Cl-BPA, $Cl_2$-BPA, $Cl_3$-BPA) were synthesized in our laboratory [34].

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 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 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. 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 [35] was used for statistical and regression analysis (linear mode).

### 2.3. Sample collection and storage

Placenta samples were collected during the different deliveries in the Maternity Unit of San Cecilio University Hospital of Granada (Spain). Each placenta was accurately examined and weighed, then a triangular portion, that included maternal and fetal sides as well as central and peripheral parts, was taken. Each portion 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. 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 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

Fifteen grams of placenta tissue samples were accurately weighed and placed in beakers. Spiked samples were prepared by adding 120 μL of methanol, every 1.5 g of placental tissue, containing the analytes (BPA and its chlorinated derivatives) at the different concentration levels. The mixtures were accurately mixed and in order to remove the added methanol, the 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 and 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 then the mixture

**Table 1**
Selected MRM transitions selected and optimized potentials: declustering potential (DP), focusing potential (FP), entrance potential (EP); collision energy (CE) and collision cell exit potential (CXP).

| Parameters | BPA | Cl-BPA | $Cl_2$-BPA | $Cl_3$-BPA | $Cl_4$-BPA | BPA-$d_{16}$ |
|---|---|---|---|---|---|---|
| Transitions | 227.2 → 212.2[a] | 261.2 → 182.1[a] | 295.1 → 244.1[a] | 329.1 → 250.1[a] | 365.0 → 314.2[a] | 241.2 → 142.0[a] |
| | 227.2 → 132.9[b] | 261.2 → 246.0[b] | 295.1 → 215.9[b] | 329.1 → 278.0[b] | 365.0 → 286.1[b] | 241.2 → 223.0[b] |
| Dwell (ms) | 200 | 200 | 200 | 200 | 200 | 200 |
| DP (V) | −41 | −45 | −42 | −46 | −50 | −43 |
| FP (V) | −255 | −170 | −240 | −150 | −260 | −160 |
| EP (V) | −10 | −10 | −9 | −10 | −10 | −11 |
| CE (V) | −30 | −40 | −30 | −47 | −41 | −32 |
| CXP (V) | −30 | −18 | −25 | −31 | −31 | −20 |

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

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, containing 50 ng $g^{-1}$ of BPA-$d_{16}$ as internal standard, and 100 μL of 0.1% (v/v) ammoniacal aqueous solution and shaking 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 and 4 mm of 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). The 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 return to 60% in 0.5 min. Flow rate was 0.25 mL $min^{-1}$, injection volume 40 μL and the column temperature was maintained at 40 °C. Total run time was 7.0 min and the post-delay time for reconditioning the column with 60% B was 3.5 min.

### 2.5.3. Mass spectrometric conditions

APCI ionization was performed in the negative ion mode. The tandem mass spectrometer was operated in the multiple reaction monitoring mode (MRM) 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 (1 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 to 200 ms. The optimized parameters for each compound are listed together with the mass transitions in Table 1.

## 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 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.

Chromatographic separation is based on the procedure proposed by Shao et al. [36]. The main goal was to obtain an improvement in sensitivity, selectivity and peak shapes using shorter chromatographic times. We performed some modifications in the initial mobile phase which consisted in a gradient mixture of an ammoniacal aqueous solution (0.1%; v/v) as solvent A and pure methanol as solvent B. First, we analyzed the effect of substituting methanol for acetonitrile but no improvements were observed neither in peak shapes nor resolution, so methanol was selected for further experiments. Second, the pH of the mobile phase was also studied and formic acid and ammonia were tested as additives. We checked that the addition of ammonia to methanol improved the sensitivity and the peak shapes because of the best ionization of the compounds. 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. A linear gradient, as described in the previous section, was used.

Lastly, we also performed a study to improve 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 and we observed no extra broadening of the peaks even at maximum value. Accordingly, 40 μL was chosen as injection volume.

### 3.2. Mass spectrometric analysis

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 analyzed. APCI negative mode was selected because of its higher sensitivity for all the compounds. $[M-H]^-$ 227.2, 261.2, 295.1, 329.1 and 365.0 *m/z* were the selected precursor ions for BPA, mono-, di-, tri- and tetrachloro-BPA respectively. Bisphenol A-$d_{16}$ was used as internal standard, the one with 241.2 *m/z*. Fig. 1 shows the MRM mode chromatogram of a standard mixture of the target compounds in a spiked placental tissue sample (10.0 μg $kg^{-1}$ of each compound) in SCAN-APCI negative mode.

Two product ions (two reactions) were monitored, one for quantification and the other for confirmation. For the 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.

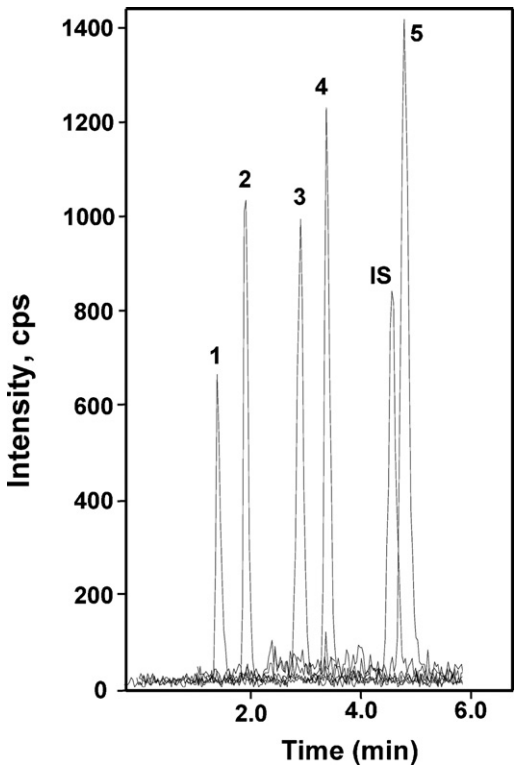


**Fig. 1.** MRM mode chromatogram of a standard mixture of the target compounds in a spiked placental tissue sample in MRM mode (10.0 ng g$^{-1}$ of each compound). Peak identification: (IS) Internal Standard (1) $Cl_4$-BPA; (2) $Cl_3$-BPA; (3) $Cl_2$-BPA; (4) Cl-BPA; (5) BPA.

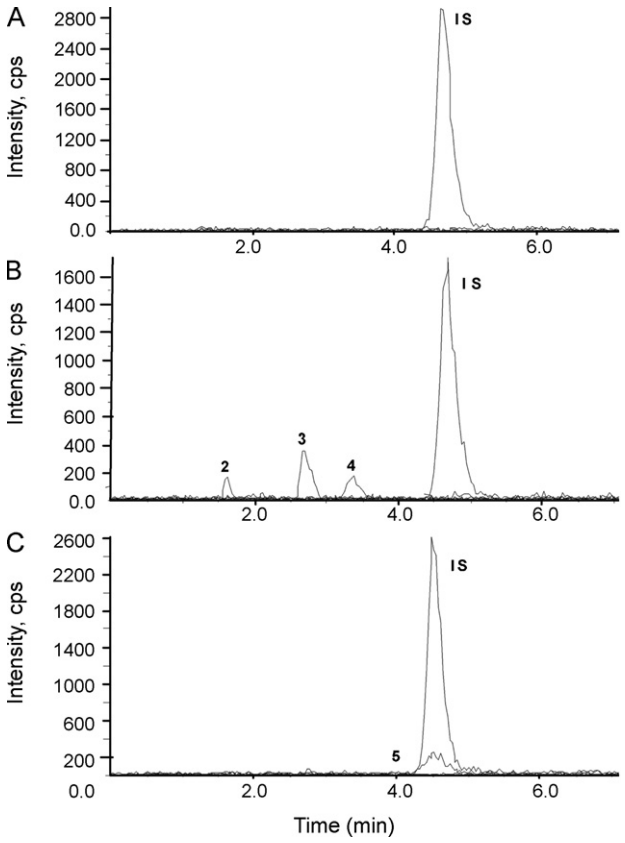


**Fig. 2.** MRM mode chromatograms of three women natural placental tissue samples. (A) Not contaminated; (B) Contaminated with $Cl_3$-BPA, $Cl_2$-BPA and Cl-BPA; (C) Contaminated with Bisphenol A.

Fig. 2 shows an example of chromatogram of a placental tissue sample.

### 3.3. Extraction procedure

We tested an ultrasonic probe using a Branson digital sonifier and a vortex-mixer as extraction techniques. 1.5 g of placental tissue were placed in an 8 mL glass vial and homogenized with 1.5 mL of water shaking for 1 min on a vortex. Then, 3 mL of ethyl acetate were added in order to carry out both the extractions: shaking on vortex for 10 min or applying ultrasonic radiation for 10 min at 70% amplitude. Recoveries obtained under these conditions were similar for both procedures (from 55 to 78% for vortex extraction and from 52 to 80% 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, 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 using pure water. So placenta samples were homogenized with pure water.

### 3.4. Analytical performance

For calibration purposes, seven concentration levels were prepared. A seven concentration level calibration curve was built. The calibration standards were prepared adding 120 μL of methanol containing the analytes every 1.5 g of placental tissue. Each level of concentration was made in triplicate so for each concentration we weighted 6.0 g of placental tissue in a glass and added 480 μL of methanol containing the appropriated concentration of the analytes. The samples were stirred until they recovered original weight to remove the added methanol. Then we weighted 1.5 g of placental tissue in triplicate in 8 mL glass vials to apply the extraction procedure previously explained. Each calibration sample was analyzed twice.

Calibration curves were constructed using analyte/internal standard peak area ratio *versus* concentration of analyte. Calibration graphs were made using MRM mode. Bisphenol A-$d_{16}$ (at 50 ng g$^{-1}$) was used as internal standard.

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. The *t*-Student 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 *F*-Snedecor test. The *t*-Student 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 2 shows the analytical parameters obtained.

**Table 2**
Analytical and statistical parameters.

| Parameter[a] | BPA | Cl-BPA | $Cl_2$-BPA | $Cl_3$-BPA | $Cl_4$-BPA |
|---|---|---|---|---|---|
| $n$ | 42 | 42 | 42 | 42 | 42 |
| $a$ | −0.010 | 0.037 | 0.073 | 0.001 | 0.010 |
| $s_a$ | 0.005 | 0.013 | 0.006 | 0.008 | 0.005 |
| $b$ (g ng$^{-1}$) | 0.151 | 0.099 | 0.072 | 0.056 | 0.019 |
| $s_b$ (g ng$^{-1}$) | $2 \times 10^{-4}$ | $6 \times 10^{-4}$ | $3 \times 10^{-4}$ | $3 \times 10^{-4}$ | $2 \times 10^{-4}$ |
| $R^2$ (%) | 99.98 | 99.97 | 99.98 | 99.96 | 99.90 |
| $s_{y/x}$ | 0.021 | 0.048 | 0.022 | 0.024 | 0.011 |
| DL (ng g$^{-1}$) | 0.2 | 0.3 | 0.3 | 0.4 | 0.6 |
| QL (ng g$^{-1}$) | 0.5 | 1.0 | 1.0 | 1.4 | 2.0 |
| LDR (ng g$^{-1}$) | 0.5–50.0 | 1.0–50.0 | 1.0–50.0 | 1.4–50.0 | 2.0–50.0 |

[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; DL, detection limit; QL, quantification limit; LDR, linear dynamic range.

### 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 [37].

#### 3.5.1. Linearity

A concentration range from the minimal quantified amount up to two higher orders of magnitude (QL to 50 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 ($P_{lof}$ %) [38]. $R^2$ values ranged from 99.90 to 99.99% 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 50 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. A good separation was obtained under the described conditions and BPA and chlorinated derivatives eluted at 1.06 min, 1.33 min, 2.36 min, 3.06 min, and 4.69 min respectively. Internal standard appears at 4.50 min. These findings suggest that the spectrometric conditions ensured a high selectivity of the LC–MS/MS method.

**Table 3**
Recovery assay, precision (repeatability) and accuracy of target compounds in placental tissue.

| Compound | Spiked (ng g$^{-1}$) | Found ± SD (%, RSD)[a] | Rec. (%) | $t_{calc}$ | $P$ (%) |
|---|---|---|---|---|---|
| BPA | 5.0 | 4.9 ± 0.3 (4.9) | 99 | 1.22 | 23.8 |
| | 15.0 | 14.9 ± 0.5 (3.4) | 98 | 0.35 | 73.1 |
| | 30.0 | 29.9 ± 0.7 (2.5) | 99 | 0.85 | 40.8 |
| Cl-BPA | 5.0 | 4.8 ± 0.4 (8.1) | 97 | 1.74 | 10.0 |
| | 15.0 | 15.1 ± 0.5 (3.2) | 97 | 1.02 | 32.3 |
| | 30.0 | 30.0 ± 0.6 (2.2) | 100 | 0.32 | 75.3 |
| $Cl_2$-BPA | 5.0 | 4.9 ± 0.2 (4.5) | 98 | 1.70 | 10.7 |
| | 15.0 | 14.9 ± 0.5 (3.2) | 100 | 0.69 | 49.9 |
| | 30.0 | 29.9 ± 0.5 (1.8) | 99 | 0.29 | 77.3 |
| $Cl_3$-BPA | 5.0 | 5.1 ± 0.3 (5.1) | 101 | 1.10 | 28.6 |
| | 15.0 | 14.7 ± 0.4 (2.9) | 100 | 2.01 | 6.1 |
| | 30.0 | 30.2 ± 0.6 (1.9) | 101 | 1.66 | 11.5 |
| $Cl_4$-BPA | 5.0 | 4.8 ± 0.3 (5.1) | 97 | 1.99 | 6.3 |
| | 15.0 | 15.0 ± 0.5 (3.6) | 105 | 0.14 | 88.7 |
| | 30.0 | 30.4 ± 0.7 (2.4) | 101 | 2.02 | 6.0 |

SD, standard deviation; RSD (%), relative standard deviation.
[a] Mean value of 18 determinations (ng g$^{-1}$)

#### 3.5.3. Precision and accuracy

The precision and accuracy data for the analytical procedures are shown in Table 3. Intra-day and inter-day precision (% RSD) of the method were lower than 5% and were within the acceptable limits to meet the guidelines for bioanalytical method validation which is considered to be ≤20%. To evaluate the precision of the assay, laboratory reproducibility and repeatability were estimated at three different concentrations for each compound (5, 15 and 30 ng g$^{-1}$). Placental tissue samples were spiked, extracted and analyzed in triplicate. 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 relative standard deviation (RSD), are summarized in Table 3. Precision and accuracy data indicated that the methodology to extract the compounds from placental tissue is highly reproducible and robust.

#### 3.5.4. Sensitivity

A fundamental aspect which needs to be examined in the validation of any analytical method is its limits of detection and quantification in order to determine if an analyte is present in the sample. The DL is the minimum amount of analyte detectable in the sample while the QL 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 [39]. 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. [40]. 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 $W_b = 6\sigma = 2.548\, W_{0.5h}$, where $W_{0.5h}$ is the half-width of the peak. Extrapolation of the graph of $W_{0.5h}$ at different concentrations of analyte can give us an adequate 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 $t_R \pm 0.5W_{b0}$ 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. Detection and quantification limits which are better adjusted to a statistical evaluation are implemented in Table 2.

#### 3.5.5. Recovery

Due to the absence of certified materials, we performed a recovery assay in order to validate the method. We use a blank spiked placenta, previously analyzed in order to ensure that it did not contain the analytes or they were below the limit of detection 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 from the standard calibration curve within the linear dynamic range and compared with the amount of analytes previously added to the samples. A recovery test ($t$-Student test) was used. The results are shown in Table 3. As P-values calculated in all cases were greater than 0.05 (5%), the null hypothesis appears to be valid, *i.e.*, recoveries are close to 100%. The results indicate the high extraction efficiency of the procedure.

**Table 4**
Method application to women placental tissue samples.

| Sample | Concentration (ng g$^{-1}$)* | | | | |
|---|---|---|---|---|---|
| | BPA | Cl-BPA | $Cl_2$-BPA | $Cl_3$-BPA | $Cl_4$-BPA |
| S01 | 15.8 | nd | nd | nd | nd |
| S02 | 6.4 | 7.1 | 21.5 | 11.5 | nd |
| S03 | nd | 9.1 | 20.9 | 9.9 | nd |
| S04 | nd | 9.7 | 21.2 | 9.4 | nd |
| S05 | nd | nd | nd | nd | nd |
| S06 | nd | nd | nd | nd | nd |
| S07 | 9.3 | 12.0 | 34.8 | 14.0 | nd |
| S08 | 9.5 | nd | nd | nd | nd |
| S09 | nd | nd | nd | nd | nd |
| S10 | nd | 21.4 | 58.8 | 31.2 | nd |
| S11 | 22.2 | 19.6 | 40.6 | 23.8 | nd |
| S12 | nd | nd | nd | nd | nd |
| S13 | 0.9 | nd | nd | nd | nd |
| S14 | nd | nd | nd | nd | nd |
| S15 | 8.0 | nd | nd | nd | nd |
| S16 | nd | 5.7 | 15.6 | 7.9 | nd |
| S17 | 34.9 | nd | nd | nd | nd |
| S18 | nd | 11.2 | 25.9 | 14.6 | nd |
| S19 | nd | nd | nd | nd | nd |
| S20 | 10.9 | nd | nd | nd | nd |
| S21 | nd | 5.2 | 12.7 | 4.9 | nd |
| S22 | nd | 6.5 | 15.6 | 8.9 | nd |
| S23 | nd | 9.5 | 25.0 | 12.0 | nd |
| S24 | nd | 5.9 | 14.5 | 3.9 | nd |
| S25 | nd | nd | nd | nd | nd |
| S26 | 1.1 | nd | nd | nd | nd |
| S27 | nd | nd | nd | nd | nd |
| S28 | nd | nd | nd | nd | nd |
| S29 | 1.3 | nd | nd | nd | nd |
| S30 | 1.6 | 10.9 | 22.4 | 10.3 | nd |
| S31 | nd | nd | nd | nd | nd |
| S32 | 0.7 | nd | nd | nd | nd |
| S33 | nd | 6.4 | 14.5 | 10.6 | nd |
| S34 | nd | 9.0 | 28.5 | nd | nd |
| S35 | nd | 8.3 | 18.6 | 8.6 | nd |
| S36 | 5.7 | 7.7 | 18.3 | 11.1 | nd |
| S37 | nd | nd | nd | nd | nd |
| S38 | nd | 8.7 | 22.4 | 10.6 | nd |
| S39 | nd | nd | nd | nd | nd |
| S40 | nd | nd | nd | nd | nd |
| S41 | 1.6 | nd | nd | nd | nd |
| S42 | nd | 7.8 | 22.4 | 10.5 | nd |
| S43 | nd | nd | nd | nd | nd |
| S44 | nd | 5.1 | 14.4 | 5.6 | nd |
| S45 | 1.2 | 11.0 | 27.7 | 11.3 | nd |
| S46 | nd | 12.1 | 24.1 | 11.1 | nd |
| S47 | 6.3 | nd | nd | nd | nd |
| S48 | nd | 6.2 | 14.3 | 5.1 | nd |
| S49 | nd | 10.8 | 25.2 | 13.4 | nd |

* Mean of three determinations. nd: not detected (<DL).

### 3.6. Method application

We used the proposed method to determine BPA and its chlorinated derivatives in 49 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 4. Fig. 2 shows an example of chromatograms corresponding to three of the analyzed samples.

BPA was detected in 20.4% ($n$ = 10/49) of the samples, in concentrations ranging from 5.7 to 22.2 ng g$^{-1}$. Some of the chlorinated derivatives were also detected: $Cl_3$-BPA in 49.0% ($n$ = 24/49) of the samples in concentrations from 4.0 to 31.2 ng g$^{-1}$; $Cl_2$-BPA in 51.0% ($n$ = 25/49) of the samples in concentrations from 12.7 to 58.8 ng g$^{-1}$ and Cl-BPA in 51.0% ($n$ = 25/49) of the samples in concentrations from 5.1 to 21.4 ng g$^{-1}$ while $Cl_4$-BPA was not found in any of the samples. The presence of BPA and/or its chlorinated derivatives depends on the exposure to these compounds as well as the different metabolism of each compound.

## 4. Conclusions

Determination and quantification of Bisphenol A and its chlorinated derivatives 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). BPA concentration found in placental tissue samples was at ng g$^{-1}$ levels as it has been previously reported by Schönfelder et al. [33]. However, we found concentrations from 0.7 to 34.9 ng g$^{-1}$ and them from 1.0 to 104.9 ng g$^{-1}$.

Studies on human exposure to BPA are needed to address the question of whether maternal exposure to BPA can lead to adverse health effects in the offspring. The method we propose here allows determining levels of free BPA and its chlorinated derivatives and might be used to perform exposure studies on the human populations.

## Acknowledgement

This work was supported by the Ministry of Science and Innovation of the Regional Government of Andalusia, Spain (Project of Excellence No. P09-CTS-4470).

## References

[1] Multi-Year Plan (FY2000-2012) for Endocrine Disruptor, Office of Research and Development, U.S. Environmental Protection Agency, 2003. Available from: http://www.epa.gov/osp/myp/edc.pdf.
[2] F. Paris, P. Balaguer, B. Terouanne, N. Servant, C. Lacoste, J.P. Cravedi, J.C. Nicolask, C. Sultan, Mol. Cell Endocrinol. 193 (2002) 43.
[3] A. Rivas, N. Olea, F. Olea-Serrano, Trends Anal. Chem. 16 (2005) 613.
[4] M.F. Fernández, B. Olmos, A. Granada, M.J. López-Espinosa, J.M. Molina-Molina, J.M. Fernández, M. Cruz, F. Olea-Serrano, N. Olea, Environ. Health Perspect. 115 (2007) 8.
[5] J.G. Bromer, Y. Zhou, M.B. Taylor, L. Doherty, H.S. Taylor, FASEB J. 24 (2010) 2273.
[6] V.L. Bosquiazzo, J. Varayoud, M. Muñoz-de-Toro, E.H. Luque, J.G. Ramos, Biol. Reprod. 82 (2010) 86.
[7] M.V. Maffini, B.S. Rubin, C. Sonnenschein, A.M. Soto, Endocrinology 25 (2006) 179.
[8] Document, 2007. Available from: http://www.bisphenol-a.org.
[9] E. Burridge, Eur. Chem. News 17 (2003) 14.
[10] A. Ballesteros-Gómez, S. Rubio, D. Pérez-Bendito, J. Chromatogr. A 1216 (2009) 449.
[11] T. Yamamoto, A. Yasuhara, Chemosphere 46 (2002) 1215.
[12] H. Fukazawa, M. Watanabe, F. Shiraishi, H. Shiraishi, T. Shiozawa, H. Matsushita, Y. Terao, J. Health Sci. 48 (2002) 242.
[13] R. Kuruto-Niwa, Y. Terao, R. Nozawa, Environ. Toxicol. Pharmacol. 12 (2002) 27.
[14] H. Takemura, J. Ma, K. Sayama, Y. Terao, B.T. Zhu, K. Simio, Toxicology 207 (2005) 215.
[15] A.V. Krishnan, P. Stathis, S.F. Permuth, L. Tokes, D. Feldman, Endocrinology 132 (1993) 2279.
[16] F.S. Vom Saal, C. Hughes, Environ. Health. Perspect. 113 (2005) 926.
[17] R.T. Zoeller, R. Bansal, C. Parris, Endocrinology 146 (2005) 607.
[18] Y.B. Wetherill, C.E. Petre, K.R. Monk, A. Puga, K.E. Knudsen, Mol. Cancer Ther. 7 (2002) 515.
[19] B.T. Akingbemi, C.M. Sottas, A.I. Koulova, G.R. Klinefelter, M.P. Hardy, Endocrinology 145 (2004) 592.
[20] European Food Safety Authority, EFSA J. 428 (2006) 1.
[21] Integrated Risk Information System (IRIS), Bisphenol A (CASRN 80-05-7), U.S. Environmental Protection Agency, Washington, DC, 1988. Available from: http://www.epa.gov/iris/subst/0356.htm.
[22] EEC, Commission Directive 2004/19/EC, Off. J. Eur. Commun. L71 (2004) 8.
[23] N.K. Wilson, J.C. Chuang, M.K. Morgan, R.A. Lordo, L.S. Sheldon, Environ. Res. 103 (2007) 9.
[24] R.E. Chapin, J. Adams, K. Boekelheide, L.E. Gray, S.W. Hayward, P.S. Lees, B.S. McIntyre, K.M. Portier, T.M. Schnorr, S.G. Selevan, J.G. Vander-

bergh, S.R. Woskie, Birth Defects Res. B: Dev. Reprod. Toxicol. 83 (2008) 157.
[25] L.N. Vandenberg, R. Hauser, M. Marcus, N. Olea, W.V. Welshons, Reprod. Toxicol. 24 (2007) 139.
[26] Y. Nie, Z. Qiang, H. Zhang, C. Adams, J. Chromatogr. A 1216 (2009) 7071.
[27] H. Gallart-Ayala, E. Moyano, M.T. Galcerán, J. Chromatogr. A 1217 (2010) 3511.
[28] L.K. Ackerman, G.O. Noonan, W.M. Heiserman, J.A. Roach, W. Limm, T.H. Begley, J. Agric. Food Chem. 58 (2010) 2307.
[29] E.M. Malone, C.T. Elliott, D.G. Kennedy, L. Regan, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 878 (2010) 1077.
[30] W. Volkel, N. Bittner, W. Dekant, Drug Metab. Dispos. 33 (2005) 1748.
[31] X. Ye, Z. Kuklenyik, L.L. Needham, A.M. Calafat, Anal. Chem. 77 (2005) 5407.
[32] X. Ye, L.J. Tao, L.L. Needham, A.M. Calafat, Talanta 76 (2008) 865.
[33] G. Schönfelder, W. Wittfoht, H. Hopp, C.E. Talsness, M. Paul, I. Chahoud, Environ. Health Perspect. 110 (2002) 703.
[34] J.L. Vílchez, M. del Olmo, A. González-Casado, A. Navalón, Patrones químicos monocloro, dicloro y tricloro derivados del Bisfenol-A, Spanish patent No. 2,190,852, 2005.
[35] Statgraphics Centurion XV, vs 15.1.02, 1982–2006, Statpoint Inc.
[36] B. Shao, H. Han, J. Hu, J. Zhao, G. Wu, Y. Xue, Y. Ma, S. Zhang, Anal. Chim. Acta 530 (2005) 245.
[37] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), 2001.
[38] Analytical Methods Committee, Analyst 119 (1994) 2363.
[39] L.A. Currie, Anal. Chim. Acta 391 (1999) 105.
[40] A. González-Casado, L. Cuadros-Rodríguez, E.A. Hernández, J.L. Vílchez, J. Chromatogr. A 726 (1996) 133.