Talanta 125 (2014) 284–292

Contents lists available at ScienceDirect

Talanta

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

# Reliable quantification of bisphenol A and its chlorinated derivatives in human urine using UPLC–MS/MS method

N. Venisse [a,b,*], C. Grignon [a,b], B. Brunet [a,b], S. Thévenot [a,b], A. Bacle [a,b], V. Migeot [a,b], A. Dupuis [a,b]

[a] University of Poitiers, CNRS-UMR 7285 IC2MP, School of Medicine and Pharmacy (Department of Analytical Chemistry, Pharmaceutics and Epidemiology), 6 rue de la Milétrie, 86034 Poitiers Cedex, France
[b] University Hospital of Poitiers, Biology-Pharmacy-Public Health Pole, 2 rue de la Milétrie, 86021 Poitiers Cedex, France

ARTICLE INFO

*Article history:*
Received 9 December 2013
Received in revised form
20 February 2014
Accepted 25 February 2014
Available online 12 March 2014

*Keywords:*
Bisphenol A
Bisphenol A chlorinated derivatives
Urine
LC–MS/MS

ABSTRACT

Bisphenol A (BPA), a widespread man-made chemical classified as an endocrine disruptor, is increasingly considered as a major cause of concern for human health. Chlorine present in drinking water may react with BPA to form chlorinated derivatives (ClxBPA), which have demonstrated a heightened level of estrogenic activity. If many epidemiological studies report that more than 90% of people have detectable BPA levels in their urine, then no such study has been undertaken regarding ClxBPA. The purpose of this work is to propose a highly sensitive and accurate analytical method adapted to large-scale biomonitoring studies aimed at assessing exposure to BPA and ClxBPA through the use of human urine. To achieve this, we have comprehensively validated a method using salting-out assisted liquid/liquid extraction (SALLE) coupled to UPLC–MS/MS and isotope dilution quantification, to measure unconjugated BPA and ClxBPA in human urine according to the accepted guidelines. Deutered BPA as well as deutered 2,2′-DCBPA was used as internal standards. The matrix calibration curve ranged from 0.05 to 1.60 ng $mL^{-1}$ and from 0.5 to 16.0 ng $mL^{-1}$ for ClxBPA and BPA respectively, and provided good linearity ($r^2 > 0.99$). This method was precise (the intra- and inter-day coefficients of variation were $<20\%$ at three different concentrations: 0.05 ng $mL^{-1}$, 0.2 ng $mL^{-1}$, 0.8 ng $mL^{-1}$ and 0.5 ng $mL^{-1}$, 2 ng $mL^{-1}$, 8 ng $mL^{-1}$ for ClxBPA and BPA, respectively) and accurate (bias ranged from $-13\%$ to $+12\%$). The limit of quantification, validated at 0.05 ng $mL^{-1}$ and 0.5 ng $mL^{-1}$ for ClxBPA and BPA respectively when using 300 μL of urine, was found to be suitable for the concentration existing in real samples. The matrix effect and the BPA cross-contamination were also investigated in this study. The analytical method developed in this study is in accordance with the requirements applicable to biomonitoring of BPA and ClxBPA in human urine.



## 1. Introduction

Bisphenol A (BPA) is of major concern to environmental public health due to its demonstrated toxicity and its high potential with regard to human exposure [1,2]. BPA is frequently used in production of many commonly available consumer products [3]. BPA toxicity has been intensively investigated over the past decade, and it is now widely considered to have an estrogenic effect. Extensive literature has shown adverse effects on animals following exposure to even a low dose of BPA, including developmental and reproductive toxicity, altered body weight, cancers and abnormally early puberty [4,5]. As for adverse health effects in humans, they are still being debated, and are suspected in diabetes, cardiovascular diseases and with regard to reproductive systems [6].

BPA has been found throughout the environment: in air, dust, sewage, consumer products, food, drinking water and tickets [7]. Numerous studies have focused on possible BPA exposure from dietary sources via food and packaging material or from work contact in industries producing or using BPA [8]. Moreover, water can be a potential route of exposure to BPA since it is continuously introduced into the aquatic environment by means of industrial, agricultural and municipal effluents. Occurrences of BPA have been widely reported in various aqueous media around the world [9]: surface water, drinking water from drinking water treatment plants, bottled water and household tap water [10–12].

Given the fact that in most drinking water treatment plants, routine operations are concluded by a chlorination step, the formation of chlorinated derivatives of BPA (ClxBPA) (mono-, di-, tri- and tetra-chlorobisphenol A) in drinking water is to be

* Corresponding author at: Laboratoire de Toxicologie et Pharmacocinétique, CHU de Poitiers, 2 rue de la Milétrie, 86021 Poitiers, France. Tel.: +33549444980; fax: +33549443973.
*E-mail address:* nicolas.venisse@chu-poitiers.fr (N. Venisse).

http://dx.doi.org/10.1016/j.talanta.2014.02.064
0039-9140/

expected [13]. Furthermore, various forms of ClxBPA (mono-, di-, tri-, tetra-chloro-BPA) have been detected in wastewater from waste paper recycling plants [14,15] as well as in drinking water [16]. Last but not the least, estrogenic activity in ClxBPA (mono-, di-, tri-chloro-BPA) may be higher than in parent compounds [17–19].

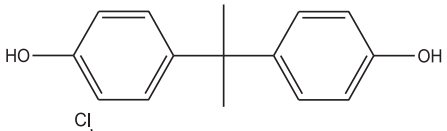
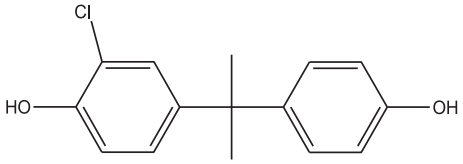
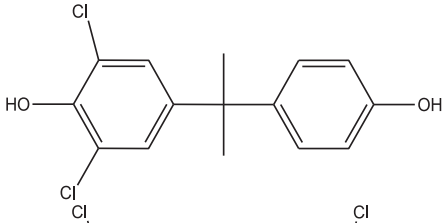
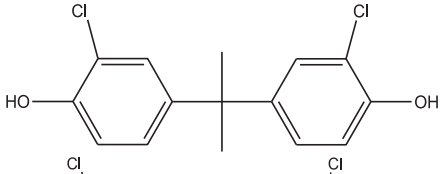
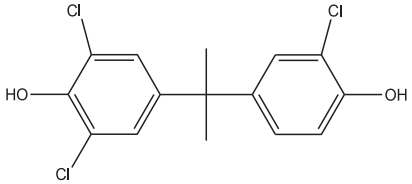
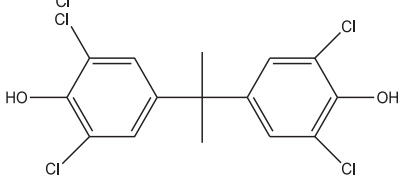
Human biomonitoring (i.e. measurement of micropollutants in biological fluids and tissues) has been proven to be at least as valuable as environmental measures in the estimation of human exposure to environmental contaminants [20]. However, biological monitoring of human exposure to pollutants presents several analytical challenges, particularly on account of the very low concentrations of substances to be measured in complex biological matrices. Among the various analytical tools available, tandem mass spectrometry is considered the most specific, accurate and precise detection method used to measure trace levels of environmental chemicals, especially in complex biological matrices [21].

Urine has been proposed in assessment of human exposure due to both the possibility of non-invasive sampling and to the relatively large volumes available in the framework of large-scale biomonitoring programs. As a consequence, several papers have reported on methods of BPA quantification in human urine. In contrast, there does not exist a large amount of published data on quantification of ClxBPA and as far as we know, only a limited number of studies have been dedicated to their determination in biological media such as adipose tissue [22], placenta [23], and human breast milk [24,25]. Unlike BPA, determination of ClxBPA in human urine has been the subject of only one published study [26].

The development and application of chemical measurement methods require implementation of rigorous quality assurance/quality control procedures. This is especially the case in the field of biomonitoring applied to health risk assessment for which the data provided should contribute to the drawing up of governmental rules and regulations. Several international institutions have proposed an appropriate framework along with recommendations well-suited for the development of analytical methods applied to biological media [27,28]. However, most of the methods reported in the literature fail to provide sufficiently detailed data on these critical points. Concerning BPA, special attention has got to be paid in order to avoid cross-contamination during the different steps of the assessment procedures [25]. And once again, it bears mentioning that most of the published data do not contain enough detailed information to ensure that no BPA contamination has occurred.

Given the analytical challenge represented by ultratrace determination of BPA and BPA contamination arising from collection

**Table 1**
Chemical structure of BPA and ClxBPA.

| Compound | Structure |
|---|---|
| BPA |  |
| 2-Chloro-4-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-phenol<br>CBPA |  |
| 2,6-Dichloro-4-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-phenol<br>2,6-DCBPA |  |
| 2-Chloro-4-[1-(3-chloro-4-hydroxy-phenyl)-1-methyl-ethyl]-phenol<br>2,2′-DCBPA |  |
| 2,6-Dichloro-4-[1-(3-chloro-4-hydroxy-phenyl)-1-methyl-ethyl]-phenol<br>TCBPA |  |
| 2,6-Dichloro-4-[1-(3,5-dichloro-4-hydroxy-phenyl)-1-methyl-ethyl]-phenol<br>TTCBPA |  |

procedures, urinary concentrations of "total BPA" (conjugated and unconjugated) have been proposed as a means of monitoring BPA exposure [29]. However, since conjugated BPA is rapidly excreted in urine and it does not display any estrogenic activity, an issue has been raised as regards to exposure assessment using total BPA, and that is yet another reason why every effort should be made to develop a method efficiently facilitating determination of unconjugated BPA in the media under consideration.

Taking all these issues into account, the aim of this work was to develop a highly sensitive and accurate method to determine unconjugated BPA and ClxBPA, in human urine, using salting-out assisted liquid/liquid extraction (SALLE) coupled to ultrahigh performance liquid chromatography–isotope dilution tandem mass spectrometry method (SALLE–UPLC–MS/MS).

## 2. Materials and methods

### 2.1. Chemicals and reagents

BPA (CAS 80-05-7) and internal standard (IS) BPA-$d_{16}$ (CAS 96210-87-6) were obtained from Sigma-Aldrich Inc. (St. Louis, USA). ClxBPA and corresponding internal standard (2,2′-DCBPA-$d_{12}$) were custom-synthesized by @rtMolecule (Poitiers, France). The ClxBPA (CBPA, 2,6-DCBPA, 2,2′-DCBPA, TCBPA and TTCBPA) were obtained from bisphenol A or its suitably protected precursor with regard to phenol function by direct chlorination using sulfuryl chloride [25]. The purity obtained for these compounds was $>98\%$. The chemical structures of these compounds are shown in Table 1.

The methanol and acetonitrile used during analysis were of residual pesticide analysis grade or LC–MS grade and were supplied by Carlo Erba (Val de Reuil, France). The water was of very high analytical grade quality (Optima®). Ammonium formate $>99.995\%$ was obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). All the solvents and reagents were tested to ensure that they were free of contamination from target compounds.

### 2.2. Preparation of reagent and standard solutions

The salting-out reagent (10 M ammonium formate) was prepared by dissolving 31.53 g of ammonium formate in a 50 mL volumetric flask with ultrapure water.

A 200 mg $L^{-1}$ methanol stock solution of each compound (BPA, CBPA, 2,6-DCBPA, 2,2′-DCBPA, TCBPA, TTCBPA) was stored at −20 °C. Extemporaneously, the initial stock solutions were diluted in methanol/water 50/50 (v/v) to obtain working standard solutions at 0.5, 1, 2, 4, 8 and 16 ng $mL^{-1}$ and at 5, 10, 20, 40, 80 and 160 ng $mL^{-1}$ for ClxBPA and BPA, respectively. The final standard concentrations in urine were 0.05–1.60 ng $mL^{-1}$ and from 0.5 to 16.0 ng $mL^{-1}$ for ClxBPA and BPA respectively. Internal standard solutions containing BPA-$d_{16}$ and 2,2′-DCBPA-$d_{12}$ were prepared in methanol/water 50/50 (v/v) at 80 and 8 ng $mL^{-1}$, respectively from initial stock solution (200 mg $L^{-1}$).

### 2.3. Sample preparation

Fresh human urine, collected in laboratory glassware from anonymous donors, was used for the preparation of calibration standards and quality controls (QCs). Extraction of BPA and ClxBPA from human urine was performed in a 5 mL glass tube. Thirty microliters of working standard solutions and 30 μL of IS solution were added to 300 μL of urine samples and homogenized by shaking. After that, 600 μL of acetonitrile was added and the samples were vortexed for 30 s. Salting-out reagent (150 μL of 10 M ammonium formate) was added and vortexed for 30 s. Tubes were then centrifuged for 10 min and 400 μL of the upper organic layer was transferred in clean glass tube and evaporated to dryness at room temperature, under a gentle nitrogen stream. Residues were dissolved in 100 μL of water. Finally, 30 μL of extract was injected onto the LC–MS/MS apparatus.

### 2.4. UPLC–MS/MS analysis

The concentrations of BPA and ClxBPA were determined using an UPLC–MS/MS system consisting of an UPLC system Acquity® H Class (Waters, Milford, USA), coupled to a Xevo® TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA).

The UPLC column was an ACQUITY CSH™ C18 (1.7 μm particle size, 2.1 × 100 mm, Waters, Milford, USA) and the mobile phase consisted of pure water (A) and methanol (B) delivered in the gradient mode at a flow rate of 350 μL/min. The gradient was programmed as follows: 30% B for 0.5 min; linearly increased to 90% B from 0.5 to 7.0 min; linearly increased to 99% B from 7.0 to 7.5 min and then maintained at 99% from 7.5 to 12.5 min, went back to 30% B from 12.5 to 13 min and maintained at this proportion from 13 to 15.5 min. The temperature of the chromatography column was maintained at 40 °C in a column oven.

The MS–MS detector was equipped with an electrospray ionization (ESI) interface, operating in negative ionization mode. Quantitative analysis was carried out in the multiple reaction monitoring mode (MRM), using two specific combinations of a precursor–product ion transition for each compound. The first ion transition is used for quantification while the second one is used for confirmation. Precursor–product transitions along with their corresponding collision energies and cone voltages are shown in Table 2. MS/MS detector conditions were set as follows: source temperature 150 °C; desolvation temperature 550 °C, cone gas flow 150 L $h^{-1}$, desolvation gas (nitrogen) 800 L $h^{-1}$; collision gas (argon) 0.15 mL $min^{-1}$ and capillary potential 1.5 V.

### 2.5. Method validation

BPA contaminations may arise from laboratory accessories, reagent, extraction procedure, or the apparatus. In order to avoid contamination, only laboratory glassware, teflon seals and high-quality solvent and reagent were used throughout the study. Selectivity of the method was assessed by analyzing response from blank urine. The absence of interfering compounds was accepted where the response of blank urine was less than 20% of the lower limit of quantification (LLOQ) [27]. Linearity of the chromatographic response was assessed on five different days using standard

**Table 2**
MS/MS parameters of BPA and ClxBPA.

| Compound | Retention time (min) | MRM (m/z) | Cone voltage (V) | Collision energy (V) |
|---|---|---|---|---|
| BPA | 6.04 | 227.1–212.0 | 60 | 18 |
| | | 227.1–133.0 | 60 | 22 |
| BPA-$d_{16}$ | 5.93 | 241.2–223.1 | 70 | 20 |
| | | 241.2–142.1 | 70 | 26 |
| CBPA | 6.64 | 261.0–182.0 | 50 | 30 |
| | | 261.0–210.1 | 50 | 24 |
| DCBPA | 7.22 | 295.1–216.1 | 58 | 28 |
| | | 295.1–244.0 | 58 | 24 |
| 2,2′-DCBPA | 7.22 | 295.1–166.9 | 26 | 24 |
| TCBPA | 7.69 | 329.0–249.9 | 56 | 32 |
| | | 329.0–277.9 | 56 | 24 |
| TTCBPA | 8.15 | 364.9–313.9 | 76 | 26 |
| | | 364.9–285.9 | 76 | 32 |
| 2,2′-DCBPA-$d_{12}$ | 7.14 | 307.1–225.1 | 58 | 32 |
| | | 307.1–253.0 | 58 | 24 |

curves including 6 calibration points prepared by spiking human blank urine. Slope and coefficients of determination as well as the difference between the back calculated concentrations and the theoretical concentrations of the calibration standards (also called residuals and expressed as a percentage of the theoretical value) were reported. The residuals should be within ±15% of the nominal value, except for the LLOQ (±20%) [27]. Trueness and precision were determined by analysis of QCs prepared using blank human urine spiked at three different concentrations (0.5 ng $mL^{-1}$, 2 ng $mL^{-1}$, 8 ng $mL^{-1}$ and 0.05 ng $mL^{-1}$, 0.2 ng $mL^{-1}$, 0.8 ng $mL^{-1}$ for BPA and ClxBPA, respectively) using separately prepared stock solutions. Between-run analyses were performed using data from 13 runs on 13 different days. For validation of within- ($n=5$) and between-run ($n=13$) trueness, the mean concentration should be within ±15% of the nominal value, except for the LLOQ (±20%) [27]. For validation of within- ($n=5$) and between-run ($n=13$) precision, the coefficient of variation (CV) should not exceed 15%, except for the LLOQ (20%) [27]. Sample concentrations were determined for each compound using the corresponding spiked urine standard curve calibration. Calibration curves were constructed using compounds/IS peak area ratio versus compound concentration. BPA-$d_{16}$ and 2,2′-DCBPA-$d_{12}$ were used as an internal standard for BPA and ClxBPA, respectively. The LLOQ, set at the level of the lowest calibration standard, was fully validated using QCs prepared using blank human urine samples spiked at this low concentration [27]. The limit of detection (LOD) was calculated as three times the standard deviations (3SD) of five replicate analyses, using the lowest urine calibration standard (0.5 and 0.05 ng $mL^{-1}$ for BPA and ClxBPA, respectively).

Matrix effects were assessed using two different methods. Firstly, the post-column infusion method [30] was used for the initial comparison of two extraction methods: SALLE and solid-phase extraction (SPE) with OASIS cartridge according to the generic method proposed by Waters [31]. For that purpose, target compounds were infused separately into the LC stream and an extracted blank human urine sample was simultaneously injected onto the LC column under previously described chromatographic conditions. The signal of the infused analyte in MRM mode was recorded and compared to the signal obtained without concomitant injection of blank sample. For final validation, matrix effects and recovery were quantitatively assessed according to the method described by Matuszewski et al. [32]. Matrix factor (MF) was defined as the ratio of the peak area in presence of matrix (blank matrix spiked after extraction with target compounds) to the peak area in absence of matrix (pure solution of target compounds). Recovery was the ratio of the peak area of standards (blank matrix spiked before extraction with target compounds) to the peak area of blank samples spiked after extraction. Recovery and matrix effect were evaluated at low (1 and 0.1 ng $mL^{-1}$ for BPA and ClxBPA, respectively) and high (8 and 0.8 ng $mL^{-1}$ for BPA and ClxBPA, respectively) levels of concentration.

### 2.6. Method application

In view of assessing the suitability of the developed method 10 human urine samples were collected from donors. All of the samples were obtained under strictly controlled collection. In order to avoid contamination of target compounds, urine was collected directly in laboratory glass beaker and sampled in glass tubes, without using any device, materials or gloves. All samples were kept frozen at −20 °C until analysis.

## 3. Results and discussion

A LC–MS/MS method using salting-out assisted liquid/liquid extraction (SALLE) was developed and fully validated. SALLE has already been successfully applied and validated for the LC–MS/MS determination of xenobiotics in urine samples [33] but what we describe here is its first use for the extraction of BPA and BPA chlorides. In our study, similar extraction yields were obtained across target compounds (range 33–45%). Though the recovery was moderate it remained constant over the concentration range (Table 3). Previous studies using SALLE have found similar extraction yields with sulfonamide compounds [34] or benzimidazole fungicides [35]. SALLE efficiency depends upon solvent type and volume, sample pH, and vortex time [35]. Our experimental conditions have been chosen in order to optimize ClxBPA extraction from urine: ammonium acetate increased BPA recovery whereas ammonium formate increased ClxBPA recoveries; therefore this latter was retained as a salting-out reagent. Increasing acetonitrile volume and/or vortex time did not lead to better extraction efficiency. SALLE presents significant benefits over conventional LLE and SPE including short sample preparation time, reduction in organic solvent consumption and low cost. Importantly for BPA trace analysis, the main advantage of SALLE is to avoid the contamination with target compound that may arise from the use of SPE cartridges, unless they are in glass. Finally, SALLE can be applied for the simultaneous extraction of compounds presenting different physico-chemical properties, for example different partition coefficients [36].

SALLE provides cleaner extracts than protein precipitation due to a true phase separation between the aqueous phase (urine) and the organic solvent (acetonitrile). In our method phase separation was obtained using a highly concentrated solution of ammonium formate that was chosen for its compatibility with ESI source [37]. However, in order to avoid the introduction of salts and weakly retained substances into the MS system, the first minute of the effluent was directed to waste. As shown in Figs. 1 and 2, SALLE led to lower matrix effects than SPE using Waters OASIS® cartridges (generic method [31]). At the retention times of target compounds, the signal dropped dramatically with OASIS® (Fig. 2), leading to poor sensitivity, whereas with SALLE, ion suppression or enhancement remained low to moderate. Quantitative analysis of matrix effects confirmed that with SALLE ion suppression was low (CBPA, DCBPA, 2,2′-DCBPA) to moderate (BPA, TCBPA, TTCBPA) (Table 4). When present, matrix effects were compensated by the use of stable isotope labeled-internal standards (SIL-IS). In contrast with the only other published method describing ClxBPA assay [26], our method used a specific SIL-IS for the quantification of ClxBPA. Matrix effect is a particularly neglected aspect of the current published BPA LC–MS/MS assays in urine since only one [38], out of several papers [26,39–43], has reported results from ME study.

**Table 3**
Mean recovery (%) of BPA and ClxBPA from urine using the salting-out method ($n=5$).

| | 1 ng $mL^{-1}$ (BPA) or 0.1 ng $mL^{-1}$ (ClxBPA) | | | | | | 8 ng $mL^{-1}$ (BPA) or 0.8 ng $mL^{-1}$ (ClxBPA) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA |
| **Mean** | 34.5 | 41.2 | 41.3 | 45.1 | 38.7 | 38.1 | 33.0 | 36.5 | 39.9 | 39.9 | 39.8 | 36.6 |
| **SD** | 20.0 | 8.2 | 8.0 | 17.3 | 9.2 | 13.6 | 16.6 | 6.7 | 8.2 | 8.0 | 11.6 | 9.2 |

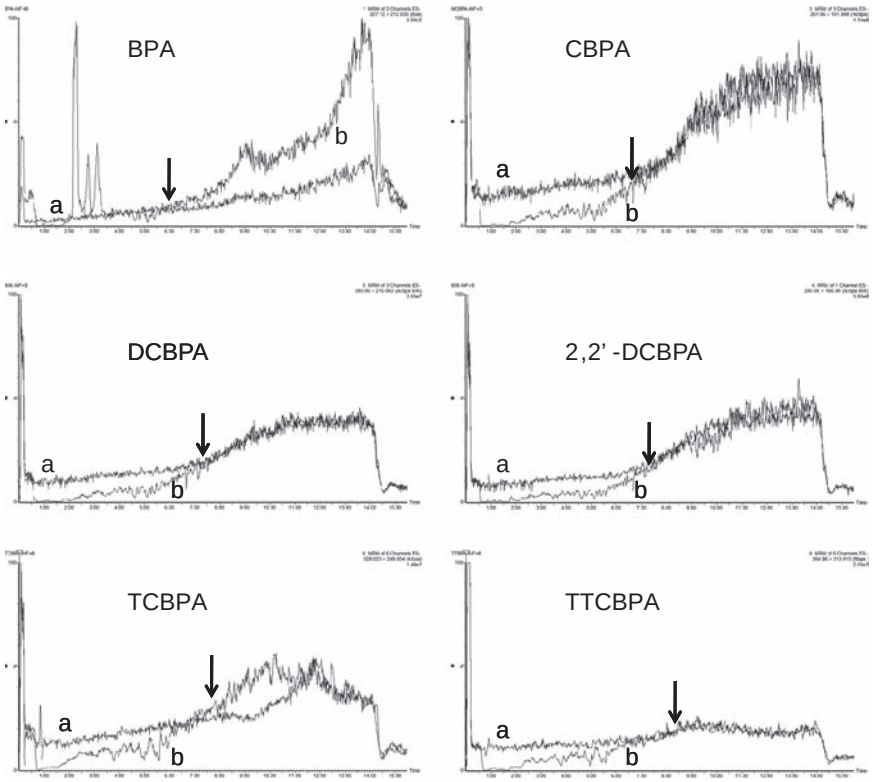



**Fig. 1.** Post-column infusion chromatograms illustrating the matrix effects on the UPLC–MS/MS response of target compounds using SALLE. (a) Response of target compounds using post-column infusion alone (control experiment). (b) Assessment of matrix effects using post-column infusion and concomitant injection of extracted blank human urine samples. Arrows indicate theoretical retention times of target compounds.

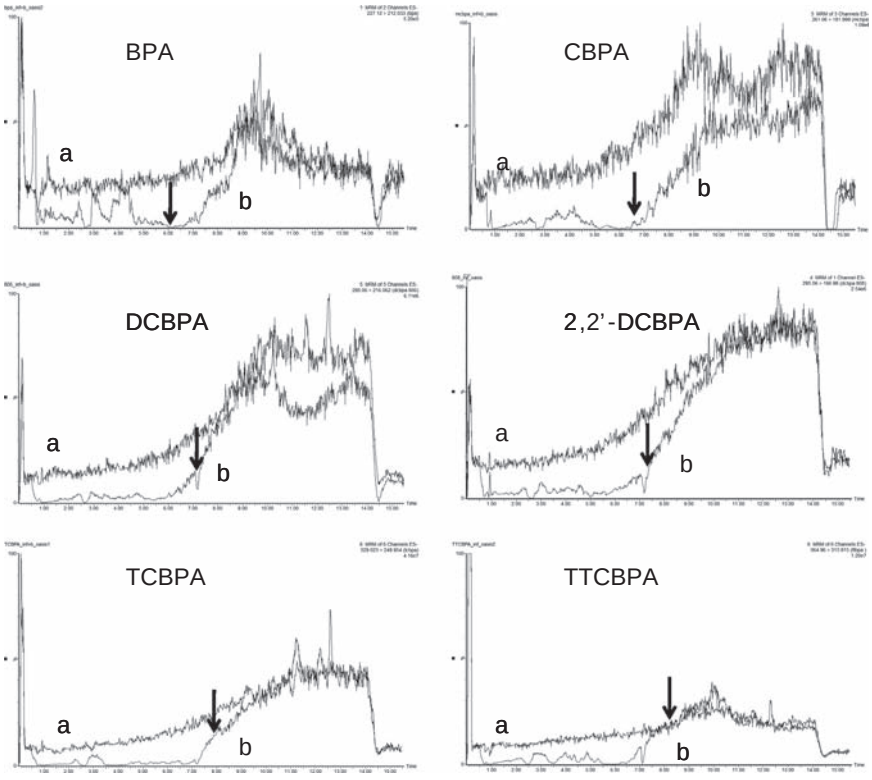



**Fig. 2.** Post-column infusion chromatograms showing the matrix effects on the UPLC–MS/MS response of target compounds using SPE. (a) Response of target compounds using post-column infusion alone (control experiment). (b) Assessment of matrix effects using post-column infusion and concomitant injection of extracted blank human urine samples. Arrow indicates theoretical retention times of target compounds.

Sample concentrations were determined for each compound using the corresponding spiked urine 6-level standard calibration curve which was processed with every batch of QCs and patients analyzed. In contrast, in the method developed by Liao and Kannan [26], quantification of the 6 target compounds was based on recovery of the internal standard, $^{13}C_{12}$-BPA and not on

**Table 4**
Mean matrix factor (MF) of target compounds ($n=5$ different batches of human urine).

| | 1 ng mL$^{-1}$ (BPA) or 0.1 ng mL$^{-1}$ (ClxBPA) | | | | | | 8 ng mL$^{-1}$ (BPA) or 0.8 ng mL$^{-1}$ (ClxBPA) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA |
| **Mean MF** | 0.78 | 0.95 | 0.95 | 0.88 | 0.51 | 0.41 | 0.67 | 0.93 | 0.86 | 0.99 | 0.61 | 0.37 |

**Table 5**
Mean slope of the linear regression model and mean coefficient of determination ($n=5$).

| | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA |
|---|---|---|---|---|---|---|
| **Mean slope (exp. 10$^{-5}$)** | 3.5 | 11.5 | 58.1 | 4.2 | 18.8 | 10.6 |
| **SD** | 0.5 | 1.2 | 6.9 | 0.4 | 2.8 | 1.7 |
| **Mean $r^2$** | 0.9973 | 0.9974 | 0.9985 | 0.9972 | 0.9982 | 0.9977 |
| **SD** | 0.0017 | 0.0018 | 0.0008 | 0.0017 | 0.0011 | 0.0004 |

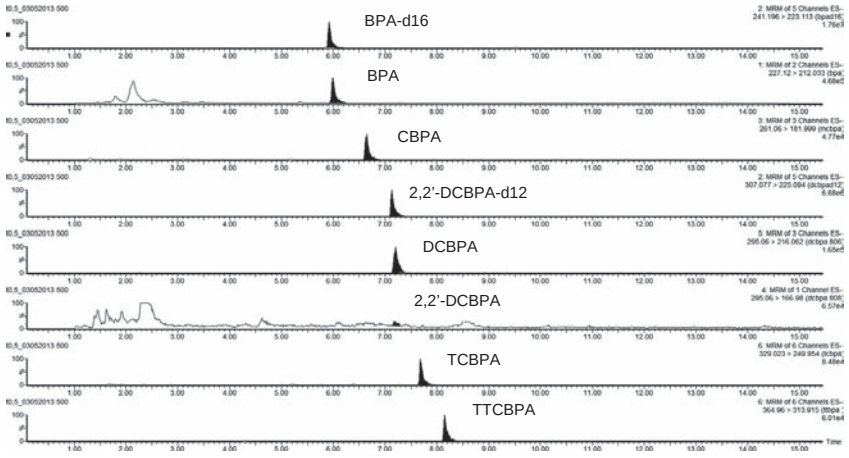



**Fig. 3.** Chromatogram of a urine calibration standard corresponding to the LLOQ.

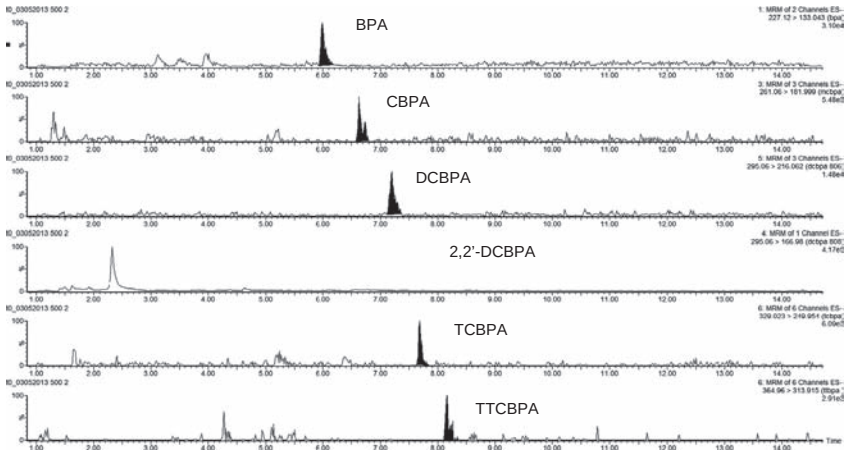



**Fig. 4.** Chromatogram of a blank urine sample.

calibration curve. Moreover, in the latter study, as already mentioned, no specific internal standard (i.e. a deuterated ClxBPA, as in our study) was available for chlorinated compounds. Linearity of our method was demonstrated over the concentration range (0.5–16.0 ng mL$^{-1}$ and from 0.05 to 1.6 ng mL$^{-1}$ for BPA and ClxBPA respectively): residuals remain between $\pm$15% of the nominal value at any concentration (data not shown) level and coefficients of determination were $>0.997$ for all target compounds (Table 5). These results are consistent with recommended bioanalytical practices [27]. Calibration curve slope parameters are also reported in Table 5.

Our chromatographic conditions allow the separation of all target compounds except for the two dichlorobisphenol A isomers (Fig. 3). If a specific transition was available for quantitation of 2,2′-

**Table 6**
Intra-day precision (expressed as CV%) and trueness (expressed as bias%) ($n=5$).

| | $C_{low}$ (0.5 ng mL$^{-1}$ (BPA) or 0.05 ng mL$^{-1}$ (ClxBPA)) | | | | | | $C_{medium}$ (2 ng mL$^{-1}$ (BPA) or 0.2 ng mL$^{-1}$ (ClxBPA)) | | | | | | $C_{high}$ (8 ng mL$^{-1}$ (BPA) or 0.8 ng mL$^{-1}$ (ClxBPA)) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA |
| **Mean (pg mL$^{-1}$)** | 458 | 55 | 49 | 50 | 47 | 49 | 2071 | 175 | 197 | 219 | 224 | 200 | 8371 | 855 | 841 | 792 | 870 | 837 |
| **SD** | 16 | 5 | 3 | 8 | 6 | 5 | 122 | 7 | 13 | 15 | 9 | 20 | 367 | 44 | 45 | 76 | 49 | 56 |
| **CV%** | 3 | 8 | 6 | 15 | 13 | 9 | 6 | 4 | 7 | 7 | 4 | 10 | 4 | 5 | 5 | 10 | 6 | 7 |
| **Bias%** | −8 | 10 | −3 | 1 | −6 | −2 | 4 | −13 | −1 | 9 | 12 | 0 | 5 | 7 | 5 | −1 | 9 | 5 |

**Table 7**
Inter-day precision (expressed as CV%) and trueness (expressed as bias%) ($n=13$).

| | $C_{low}$ (0.5 ng mL$^{-1}$ (BPA) or 0.05 ng mL$^{-1}$ (ClxBPA)) | | | | | | $C_{medium}$ (2 ng mL$^{-1}$ (BPA) or 0.2 ng mL$^{-1}$ (ClxBPA)) | | | | | | $C_{high}$ (8 ng mL$^{-1}$ (BPA) or 0.8 ng mL$^{-1}$ (ClxBPA)) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA |
| **Mean (pg mL$^{-1}$)** | 489 | 46 | 45 | 46 | 45 | 46 | 1947 | 196 | 197 | 193 | 191 | 196 | 7629 | 754 | 768 | 764 | 776 | 795 |
| **SD** | 85 | 9 | 5 | 9 | 5 | 9 | 113 | 20 | 7 | 20 | 19 | 30 | 589 | 56 | 47 | 60 | 71 | 118 |
| **CV%** | 17 | 19 | 10 | 20 | 12 | 19 | 6 | 10 | 4 | 10 | 10 | 15 | 8 | 7 | 6 | 8 | 9 | 15 |
| **Bias %** | −2 | −8 | −10 | −9 | −9 | −8 | −3 | −2 | −2 | −4 | −5 | −2 | −5 | −6 | −4 | −5 | −3 | −1 |

DCBPA, no specific transition providing sufficient intensity was found to be available for 2,6-DCBPA. Therefore quantification of both dichlorobisphenol isomers was based on the method described by Cariot et al. [25] by subtracting the amount of 2,2′-DCBPA from the total DCBPA obtained.

A signal was found at the retention times of target compounds in blank urine (Fig. 4). However the intensity of this signal remains low, representing less than 10% of the intensity of a peak at the LLOQ (Fig. 3). Therefore the contribution of this contamination to the quantification process could be considered negligible. Actually, it does not affect precision and trueness of the developed method as demonstrated in Tables 6 and 7. Intra- and inter-day CV and bias were $\leq \pm 20\%$ for the low level QC corresponding to the LLOQ and $\leq \pm 15\%$ for other QCs. These results were consistent with recommended bioanalytical practices [27]. Contamination could have occurred during LC–MS/MS analysis (autosampler carryover, etc.) or more likely during sample preparation. No signal could be detected when injecting samples consisting in mobile phase that had not been submitted to SALLE immediately after high standard sample, therefore carryover was considered negligible.

Only one other paper, so far, has been reported in the literature for the determination of BPA and ClxBPA concentrations in urine [26]. It depicts the achievement of similar LLOQ for ClxBPA (50 pg mL$^{-1}$) but was more sensitive for the quantification of BPA (10 pg mL$^{-1}$). However the authors determined their LLOQ on the basis of a signal-to-noise approach whereas our LLOQ was determined as the lowest concentration that can be quantified reliably i.e. with acceptable trueness and precision using spiked urine QCs. Our LLOQ for BPA is closer than those achieved by Chen et al. [39] or by Markham et al. [42] who also used spiked urine QCs for LLOQ validation. Our LODs were 48, 14, 9, 23, 18 and 14 pg mL$^{-1}$ for BPA, CBPA, DCBPA, 2,2′-DCBPA, TCBPA and TTCBPA, respectively.

Our method is suitable for the determination of unconjugated BPA and ClxBPA concentrations in urine. In the study cited above, Liao and Kannan [26] found in the urine of healthy volunteers, concentrations ranging from < LLOQ to 18.7 ng mL$^{-1}$, < LLOQ to 1.68 ng mL$^{-1}$, < LLOQ to 1.06 ng mL$^{-1}$ and < LLOQ to 0.675 ng mL$^{-1}$ for BPA, CBPA, DCBPA and TCBPA, respectively. In the USA population, total BPA urine concentrations range from 0.4 to 149 ng mL$^{-1}$, with a geometric mean of 2.6 ng mL$^{-1}$ [44]. In other studies, unconjugated BPA was found at concentrations ranging from < LLOQ to 2.5 ng mL$^{-1}$ [4].

**Table 8**
Concentrations of BPA and ClxBPA (pg mL$^{-1}$) measured in 10 human urine samples. LLOQ, lower limit of quantification; LOD, limit of detection; < LLOQ means that urinary concentration is between the LLOQ and the LOD. ND, not detected means that concentration is < LOD.

| Human urine sample no. | BPA | CBPA | DCBPA | 2,2′-DCBPA | TCBPA | TTCBPA |
|---|---|---|---|---|---|---|
| **1** | ND | 138 | 57 | ND | 201 | 447 |
| **2** | 1378 | < LLOQ | ND | ND | ND | ND |
| **3** | 536 | ND | ND | ND | ND | ND |
| **4** | 538 | 202 | 109 | < LLOQ | 368 | 1501 |
| **5** | ND | ND | ND | ND | ND | ND |
| **6** | < LLOQ | ND | ND | ND | ND | ND |
| **7** | ND | 57 | ND | ND | ND | ND |
| **8** | 635 | < LLOQ | < LLOQ | ND | ND | ND |
| **9** | ND | < LLOQ | < LLOQ | < LLOQ | < LLOQ | < LLOQ |
| **10** | ND | ND | ND | ND | 292 | 55 |

In order to illustrate the suitability of the developed method, 10 human urine samples have been analyzed (Table 8). Fig. 5 shows chromatograms corresponding to urine numbers 1 and 2. Unconjugated BPA and ClxBPA were not found in all urine samples in accordance with previous reports [4,26].

## 4. Conclusion

Fully-validated assays are mandatory in order to obtain reliable results for the monitoring of BPA and ClxBPA exposure. The method developed in this study uses SALLE as a sample clean-up process and provides ultrasensitive quantification allowing for reliable determination of unconjugated BPA and ClxBPA, while the reported LOD and LLOQ are altogether consistent with the

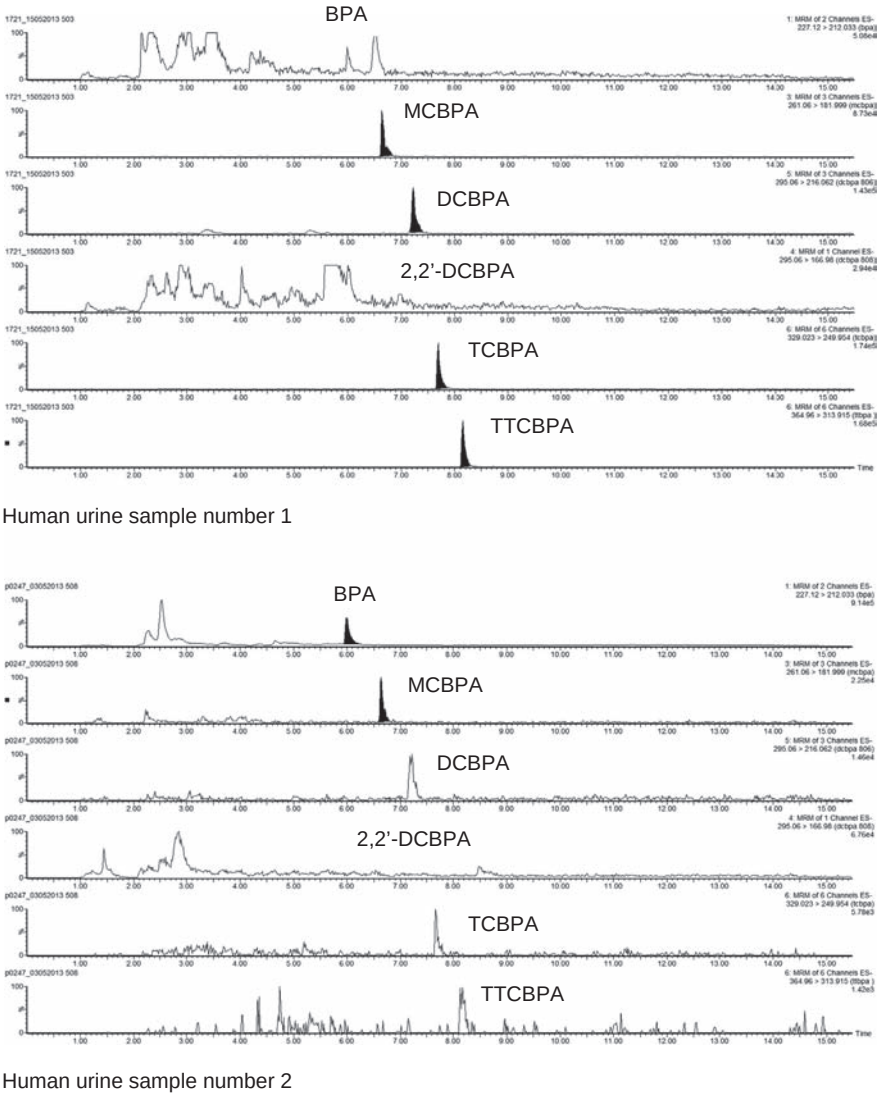



**Fig. 5.** Chromatograms of two human urine samples. In sample number 1, CBPA, DCBPA, TCBPA and TTCBPA were found. In sample number 2, only BPA and MCBPA were found.

concentrations observed in actual human urine. It has been validated according to current guidelines.

## Acknowledgments

We wish to thank Jeffrey Arsham for his highly helpful reading of our original text.

## References

[1] European Union, European Workshop on the Impact of Endocrine Disrupters on Human Health and the Environment, Environment and Climate Research Programme DG XII European Commission Report EUR 17549, 1997.
[2] J.H. Kang, F. Kondo, Y. Katayama, Toxicology 226 (2006) 79–89.
[3] Plastics Europe, Application of Bisphenol A, 2007, On-line at: ⟨http://www.bisphenol-a-europe.org/uploads/applications%20of%20BPA%20Sept%2008.pdf⟩, (accessed 25.11.13).
[4] L.N. Vandenberg, I. Chahoud, J.J. Heindel, V. Padmanabhan, F.J. Paumgartten, G. Schoenfelder, Environ. Health Perspect. 118 (2010) 1055–1070.
[5] L.N. Vandenberg, T. Colborn, T.B. Hayes, J.J. Heindel, D.R. Jacobs Jr, D.H. Lee, T. Shioda, A.M. Soto, F.S. vom Saal, W.V. Welshons, R.T. Zoeller, J.P. Myers, Endocr. Rev. 33 (2012) 378–455.
[6] ANSES, Effet sanitaire du bisphenol A. Connaissances relatives aux usages du Bisphenol A. 2009-SA-0331 et 2010-SA-0197, 2011.
[7] C. Liao, K. Kannan, Environ. Sci. Technol. 45 (2012) 9372–9379.
[8] L.N. Vandenberg, R. Hauser, M. Marcus, N. Olea, W.V. Welshons, Reprod. Toxicol. 24 (2007) 139–177.
[9] S. Flint, T. Markle, S. Thompson, E. Wallace, J. Environ. Manag. 104 (2012) 19–34.
[10] A. Dupuis, V. Migeot, A. Cariot, M. Albouy-Llaty, B. Legube, S. Rabouan, Environ. Sci. Pollut. Res. Int. 19 (2012) 4193–4205.
[11] X. Li, G.G. Ying, H.C. Su, X.B. Yang, L. Wang, Environ. Int. 36 (2010) 557–562.
[12] V.A. Santhi, N. Sakai, E.D. Ahmad, A.M. Mustafa, Sci. Total Environ. 427–428 (2012) 332–338.
[13] H. Gallard, A. Leclercq, J.P. Croue, Chemosphere 56 (2004) 465–473.
[14] H. Fukazawa, K. Hoshino, T. Shiozawa, H. Matsushita, Y. Terao, Chemosphere 44 (2001) 973–979.
[15] H. Gallart-Ayala, E. Moyano, M.T. Galceran, J. Chromatogr. A 1217 (2010) 3511–3518.
[16] Z. Fan, J. Hu, W. An, M. Yang, Environ. Sci. Technol. 47 (2013) 10841–10850.
[17] H. Fukazawa, M. Watanabe, F. Shiraishi, H. Shiraishi, T. Shiozawa, H. Matsushita, Y. Terao, J. Health Sci. 48 (2002) 242–249.
[18] J.Y. Hu, T. Aizawa, S. Ookubo, Environ. Sci. Technol. 36 (2002) 1980–1987.
[19] H. Takemura, J. Ma, K. Sayama, Y. Terao, B.T. Zhu, K. Shimoi, Toxicology 207 (2005) 215–221.
[20] M.N. Bates, J.W. Hamilton, J.S. LaKind, P. Langenberg, M. O'Malley, W. Snodgrass, Environ. Health Perspect. 113 (2005) 1615–1621.
[21] L.L. Needham, D.G. Patterson Jr, D.B. Barr, J. Grainger, A.M. Calafat, Anal. Bioanal. Chem. 381 (2005) 397–404.
[22] M.F. Fernandez, J.P. Arrebola, J. Taoufiki, A. Navalon, O. Ballesteros, R. Pulgar, J. L. Vilchez, N. Olea, Reprod. Toxicol. 24 (2007) 259–264.
[23] I. Jimenez-Diaz, A. Zafra-Gomez, O. Ballesteros, N. Navea, A. Navalon, M. F. Fernandez, N. Olea, J.L. Vilchez, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2012) 3363–3369.

[24] V. Migeot, A. Dupuis, A. Cariot, M. Albouy-Llaty, F. Pierre, S. Rabouan, Environ. Sci. Technol. 47 (2013) 13791–13797.
[25] A. Cariot, A. Dupuis, M. Albouy-Llaty, B. Legube, S. Rabouan, V. Migeot, Talanta 100 (2012) 175–182.
[26] C. Liao, K. Kannan, Environ. Sci. Technol. 46 (2012) 5003–5009.
[27] European Medicines Agency, Commitee for Medicinal Products for Human Use, Guideline on Bioanalytical Method Validation, 2011.
[28] US Department of Health and Human Services, Food and Drug Aministration, Bioanalytical Method Validation, 2001.
[29] A.M. Calafat, Food and Agriculture Organization of the United Nations and World Health Organization, 2010.
[30] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 877 (2009) 2198–2207.
[31] Waters, Oasis HLB Method Development Guide. Generic SPE Method, 2003.
[32] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[33] E.G. Yanes, D.P. Lovett, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 909 (2012) 42–50.
[34] J. Liu, M. Jiang, G. Li, L. Xu, M. Xie, Anal. Chim. Acta 679 (2010) 74–80.
[35] Y. Wen, J. Li, F. Yang, W. Zhang, W. Li, C. Liao, L. Chen, Talanta 106 (2013) 119–126.
[36] S. Song, E.N. Ediage, A. Wu, S. De Saeger, J. Chromatogr. A 1292 (2013) 111–120.
[37] H. Wu, J. Zhang, K. Norem, T.A. El-Shourbagy, J. Pharm. Biomed. Anal. 48 (2008) 1243–1248.
[38] M. Chen, L. Tao, E.M. Collins, C. Austin, C. Lu, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 904 (2012) 73–80.
[39] M. Chen, P. Zhu, B. Xu, R. Zhao, S. Qiao, X. Chen, R. Tang, D. Wu, L. Song, S. Wang, Y. Xia, X. Wang, J. Anal. Toxicol. 36 (2012) 608–615.
[40] K. Inoue, M. Kawaguchi, Y. Funakoshi, H. Nakazawa, J. Chromatogr. B Anal. Technol. Biomed. Life. Sci. 798 (2003) 17–23.
[41] M.Z. Lacroix, S. Puel, S.H. Collet, T. Corbel, N. Picard-Hagen, P.L. Toutain, C. Viguie, V. Gayrard, Talanta 85 (2011) 2053–2059.
[42] D.A. Markham, J.M. Waechter Jr, M. Wimber, N. Rao, P. Connolly, J.C. Chuang, S. Hentges, R.N. Shiotsuka, S. Dimond, A.H. Chappelle, J. Anal. Toxicol. 34 (2010) 293–303.
[43] W. Volkel, N. Bittner, W. Dekant, Drug Metab. Dispos. 33 (2005) 1748–1757.
[44] A.M. Calafat, X. Ye, L.Y. Wong, J.A. Reidy, L.L. Needham, Environ. Health Perspect. 116 (2008) 39–44.