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# Quantitative analysis of unconjugated and total bisphenol A in human urine using solid-phase extraction and UPLC–MS/MS: Method implementation, method qualification and troubleshooting

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ABSTRACT

The aim of the presented investigation was to document challenges encountered during implementation and qualification of a method for bisphenol A (BPA) analysis and to develop and discuss precautions taken to avoid and to monitor contamination with BPA during sample handling and analysis. Previously developed and published HPLC–MS/MS methods for the determination of unconjugated BPA (Markham et al. Journal of Analytical Toxicology, 34 (2010) 293–303) [17] and total BPA (Markham et al. Journal of Analytical Toxicology, 38 (2014) 194–203) [20] in human urine were combined and transferred into another laboratory. The initial method for unconjugated BPA was developed and evaluated in two independent laboratories simultaneously. The second method for total BPA was developed and evaluated in one of these laboratories to conserve resources. Accurate analysis of BPA at sub-ppb levels is a challenging task as BPA is a widely used material and is ubiquitous in the environment at trace concentrations. Propensity for contamination of biological samples with BPA is reported in the literature during sample collection, storage, and/or analysis. Contamination by trace levels of BPA is so pervasive that even with extraordinary care, it is difficult to completely exclude the introduction of BPA into biological samples and, consequently, contamination might have an impact on BPA biomonitoring data. The applied UPLC–MS/MS method was calibrated from 0.05 to 25 ng/ml. The limit of quantification was 0.1 ng/ml for unconjugated BPA and 0.2 ng/ml for total BPA, respectively, in human urine. Finally, the method was applied to urine samples derived from 20 volunteers. Overall, BPA can be analyzed in human urine with acceptable recovery and repeatability if sufficient measures are taken to avoid contamination throughout the procedure from sample collection until UPLC–MS/MS analysis.



## 1. Introduction

Bisphenol A (BPA[1]) is a high production volume compound. It is mainly used as a monomer to make polymers for various applications including food-contact applications. Human exposure to BPA is investigated in a number of biomonitoring studies and shown to be low and mainly due to BPA that migrates into food from polymers [1,2,13].

Some studies report measurable concentrations of unconjugated BPA in urine and other biological fluids and conclude

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[1] Abbreviations: BPA: the unmetabolized, bioactive parent form also called in the literature "free" or aglycone. Total BPA: the sum of all BPA from unconjugated BPA plus all conjugated metabolites. BPA-G: the conjugated metabolite BPA-glucuronide. BPA-S: the conjugated metabolite BPA-sulfate.

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incomplete pre-systemic clearance [4–8]. Comprehensive toxicokinetic studies with labelled methyl-d6-BPA (d6-BPA) to avoid confounding with background contamination clearly confirm that virtually complete pre-systemic clearance of orally administered BPA occurs in humans by metabolism of unconjugated BPA to BPA-glucuronide (BPA-G) [3,4,9–12], which is a biologically inactive form [13].

Based on the periodic exposure, quantitative elimination via urine and the short half-life of 4–5 h, urine is considered to be the appropriate specimen to assess exposure to BPA [3,13–19]. There are different analytical methods reported in the literature to measure unconjugated and total BPA in urine. Because the sample preparation and analytical methods in many of the biomonitoring papers are poorly described, it usually cannot be ascertained if the methods used are fully validated. Important parameters are frequently not reported, such as, if there was appropriate use of solvent standards, blanks, matrix controls, and fortified matrix controls to assure data quality, or if specimen collection and storage devices were screened to confirm the absence of BPA. Contamination by trace levels of BPA is so pervasive that even with extraordinary care, it is difficult to completely exclude the introduction of BPA into biological samples [13,17–20].

Adequate generation of biomonitoring data requires validated and high-quality analytical methods, qualified laboratory personnel, and strict quality control/quality assurance laboratory practices [1]. The use of GC–MS and LC–MS/MS methods for analysis enhanced sensitivity and selectivity and facilitated replacement of less-specific methods such as enzyme linked immunosorbent assays (ELISA), which suffer from cross-reactivity with the major BPA metabolite, BPA-glucuronide [3,21].

This publication describes the transfer of an LC–MS/MS method previously developed to measure unconjugated and total BPA in urine [17,20] to another laboratory. The study indicates pitfalls encountered during method implementation and qualification and discusses troubleshooting and precautions taken to avoid and to monitor contamination with BPA during sample handling and analysis.

## 2. Materials and methods

### 2.1. Chemicals and materials

$^{12}$C BPA (purity 99.7%), citric acid, *tert*-butyl methylether (*t*-BME) and β-glucuronidase (type HP-2, product number: G7017; >100,000 units/ml; with a secondary activity of sulfatase ≤7500 units/ml) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). An authentic standard of BPA-mono-ß-(D)-glucuronide sodium salt (BPA-G sodium salt), (90.1 %) was obtained from UFC Ltd., Manchester, England. An authentic standard of $^{13}C_{12}$-BPA-G, Lot TA0627 V, 95.6% chemical purity, 100% isotopic purity was obtained from Sigma–Aldrich, Inc., Isotec Inc., Miamisburg, OH. $^{13}C_{12}$-BPA (100 μg/ml solution, 99%) and the internal standard $^{12}$C BPA-$d_8$ (chemical purity 100%; isotopic enrichment 98%) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Sodium hydroxide and ammonia solution (25%) were from Merck (Darmstadt, Germany). Methanol, ammonium acetate, acetonitrile and dichloromethane were purchased from Biosolve (Valkenswaard, The Netherlands). The water used was obtained from a Millipore water purification system (Merck Millipore, Darmstadt, Germany). All chemicals used were of best quality. Solid phase extraction (SPE) cartridges (Oasis HLB 200 mg/5 ml; LP glass) were obtained from waters chromatography BV (Etten-Leur, The Netherlands). Glass amber autosampler vials (Grace) and caps (blue PP screw top, red PTFE/white silicone; Grace) were used.

### 2.2. Precautions

Glassware was used whenever possible and cleaned with dichloromethane to eliminate any potential background of BPA. $^{13}C_{12}$-BPA served as the "surrogate" analyte for $^{12}$C BPA to aid in problem solving in case of $^{12}$C BPA background contamination. For the same reason, $^{13}C_{12}$-BPA-G served as the "surrogate" analyte for $^{12}$C BPA-G. To demonstrate that the UPLC system was not contaminated with trace amounts of $^{12}$C BPA and to avoid false positive responses in the UPLC–MS system, blank solvent (acetonitrile/water, 50/50) was analyzed at the start of each analytical run and between the urine samples.

### 2.3. Preparation of reference standard stock solutions

The stock solutions of $^{12}$C BPA and the internal standard $^{12}$C BPA-$d_8$ were prepared by dissolving 10 mg of the substance into 100 ml acetonitrile. The stock solutions of the $^{12}$C BPA-G sodium salt and $^{13}C_{12}$-BPA-G were prepared by dissolving 10 mg of the substance into 100 ml acetonitrile/water (50/50, V/V). Preparation of $^{12}$C BPA stock solutions was performed in a separate room, different from the analytical laboratories, to avoid contamination.

### 2.4. Calibration standards

The calibration standards were prepared by spiking acetonitrile/water (50/50, V/V) with $^{12}$C BPA and $^{13}C_{12}$-BPA at concentration levels 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, and 25 ng/ml. A 100 μl aliquot of the internal standard solution (1 μg/ml acetonitrile/water (50/50, V/V)) was added to the calibration standards (10 ml). After preparation, the calibration standards were stored at 2–10 °C for up to several months.

### 2.5. Calibration procedure

Calibration standards were not processed through SPE columns and analyzed at the start and end of each analytical run. All 20 calibration results (10 levels, in duplicate) were used to construct one calibration curve. Linear calibration curves were obtained by plotting the quotient of the peak area of $^{12}$C BPA or $^{13}C_{12}$-BPA divided by the deuterated internal standard against the standard concentration. The blank solvent with internal standard was not included in the calibration curve. The $^{12}$C BPA concentration in the blank solvent was low (<0.02 ng/ml) and, consequently, not subtracted from the $^{12}$C BPA signals obtained from the calibration standards.

### 2.6. Sample collection

Blank urine from volunteers (TNO Triskelion) was collected in coded glass beakers without personal information. All volunteers gave informed consent for their donations. All glassware used for sample handling was pre-rinsed with dichloromethane to eliminate trace amounts of $^{12}$C BPA. The blank urine was stored at ≤−18 °C until analysis. Pooled urine was prepared by mixing urine from several volunteers.

### 2.7. Sample preparation

The urine samples were pre-treated using the method described by Markham et al. [17,20]. In short:

*Determination of unconjugated BPA*: 5 ml urine was transferred into a 15 ml glass tube, 10 μl internal standard solution (1 μg/ml) was added and the sample was intensively agitated. The SPE cartridge was conditioned with 4 ml *t*-BME, 3 ml methanol and 5 ml water, and the prepared urine sample was loaded. The urine sample ran through the SPE cartridge without vacuum. Subsequently,

5 ml water was added to the SPE cartridge (without vacuum). The cartridge was washed with 3 ml of 10% methanol/2% ammonium hydroxide in water, dried with (medium) vacuum for 5 s, washed with 5 ml of water, dried with (medium) vacuum for 5 s, washed with 3 ml of 50% methanol in water, dried with (medium) vacuum for 5 s, washed with 3 ml of dichloromethane and dried with high vacuum for 60 s. The SPE cartridge was placed onto a 10 ml glass tube. BPA was eluted with 4 ml *t*-BME at atmospheric pressure. The extract was evaporated to dryness under a gentle stream of nitrogen at 40 °C in a water bath. The residue was dissolved in 1 ml of 50% acetonitrile in water. The sample solution was transferred to an autosampler vial.

*Determination of total BPA*: 5 ml urine was transferred into a 15 ml glass tube, 10 μl internal standard solution (1 μg/ml) was added and the sample was intensively agitated. 5 ml of buffer solution (pH 4.5–5.0, prepared by dissolving 18.4 g citric acid and 6.5 g sodium hydroxide into 1000 ml water in a glass bottle) was added and the sample was mixed. 50 μl ß-glucuronidase (6086 units) was added. The sample was intensively agitated after which the sample was incubated in a shaking water bath for 16 h at 37 °C. The SPE cartridge procedure was followed as described above for unconjugated BPA.

Recent comprehensive toxicokinetic studies with methyl-d6-BPA (d6-BPA) report total BPA concentrations in serum and urine to be higher than the sum of BPA and BPA-G [10,11]. Thayer et al. exposed adult humans to 100 μg/kg bw d6-BPA by oral administration and conducted blood and urine analysis over a three day period. The authors used a *Helix pomatia* glucuronidase/sulfatase mixture to generate total BPA and reported virtually quantitative recovery of total administered d6-BPA in urine (84–109%). In urine samples aggregated from all subjects d6-BPA-G represented 87 ± 6.9% of the total d6-BPA excreted and d6-BPA-S was 3 ± 2.3%. The authors tentatively identified mixed sulfate/glucuronide bis-conjugate in both urine and serum. The hydrolysis method used in our study was optimized to hydrolyze BPA-G, the main BPA metabolite, and utilized >5000 units/sample *H. pomatia* type-2 β-glucuronidase (HP-2) and incubation for 16 h at 37 °C [20]. Since HP-2 β-glucuronidase expresses up to 7.5% secondary activity of sulfatase (≤350 units/sample) it is assumed, but not experimentally verified, that BPA-S and any BPA bis-conjugates would be hydrolyzed to BPA under the conditions used. For future experiments it should be considered to verify complete BPA-S and bis-conjugates hydrolysis with authentic standards.

### 2.8. Method qualification procedures

#### 2.8.1. Determination of unconjugated BPA in urine

For the determination of unconjugated BPA, pooled human urine (5 ml) was spiked with $^{12}$C BPA and $^{13}C_{12}$-BPA at six concentration levels ranging from 0.1 to 5.0 ng/ml. Five individual samples of each level, including blank urine, were pre-treated (SPE) and individually measured.

#### 2.8.2. Determination of total BPA in urine

For the determination of total BPA, pooled human urine samples (5 ml) were spiked with $^{12}$C BPA and $^{13}C_{12}$-BPA (6 levels; 0.2–10 ng/ml) to demonstrate appropriate recovery rates. Each level, including blank urine, was individually pre-treated (enzymatic treatment and SPE) and investigated in 5 independent samples, and each sample was analyzed individually with UPLC–MS/MS.

In addition, pooled human urine samples (5 ml) were spiked with $^{12}$C BPA-G and $^{13}C_{12}$-BPA-G (6 levels; 0.5–20 ng/ml) to determine quantitative BPA-G hydrolysis. Each level, including blank urine, was individually pre-treated (enzymatic treatment and SPE) and investigated in 5 independent samples, and each sample was analyzed individually with UPLC–MS/MS.

**Table 1**
UPLC gradient for the analysis of BPA.

| Time (min) | Flow rate (ml/min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|---|
| 0.0 | 0.3 | 90.0 | 10.0 |
| 0.5 | 0.3 | 90.0 | 10.0 |
| 5.0 | 0.3 | 35.0 | 65.0 |
| 6.0 | 0.3 | 35.0 | 65.0 |
| 6.1 | 0.3 | 5.0 | 95.0 |
| 7.0 | 0.3 | 5.0 | 95.0 |
| 7.1 | 0.3 | 90.0 | 10.0 |

### 2.9. Determination of unconjugated and total $^{12}$C BPA in individual urine samples

The qualified methods were applied to urine samples from 20 volunteers. For the determination of unconjugated $^{12}$C BPA, urine samples of the 20 individuals were processed (SPE) and analyzed in three different replicate samples. In the same analysis run, calibration standards were analyzed as well as pre-treated water, pooled urine and pooled urine spiked with $^{12}$C BPA and $^{13}C_{12}$-BPA (0.5 ng/ml).

For the determination of total $^{12}$C BPA, urine samples of the 20 individuals were processed (enzymatic treatment and SPE) and analyzed in three different replicate samples. In the same analysis run, calibration standards were analyzed as well as pre-treated water, pooled urine and pooled urine spiked with $^{12}$C BPA-G and $^{13}C_{12}$-BPA-G (1 ng/ml).

### 2.10. Ultra performance liquid chromatography–tandem mass spectrometry

All samples were analyzed using an Acquity UPLC system (Waters Corporation) connected to a XEVO-TQS mass spectrometer (Waters). The analytical column was Acquity UPLC®BEH $C_{18}$, (2.1 × 100 mm, 1.7 μm). The column temperature was 50 °C. A trap column (XBridge C18; 2.5 μm, 2.1 × 50 mm) was installed between the mixing chamber and the analytical column to separate BPA contamination that may originate from the UPLC system. The temperature of the trap column was not controlled (room temperature). The injection volume was 10 μl. The mobile phases A and B consisted of 1 mM ammonium acetate solution and acetonitrile, respectively. Table 1 shows the UPLC gradient.

Electrospray ionization in the negative mode was performed using multiple reaction monitoring (MRM). Table 2 shows the ions

**Table 2**
MRM conditions for $^{12}$C BPA, $^{13}C_{12}$-BPA and BPA-$d_8$.

| Compound | Parent (*m*/*z*) | Daughter (*m*/*z*) | Cone voltage (*V*) | Collision energy (eV) | Remark |
|---|---|---|---|---|---|
| $^{12}$C-BPA | 227.10 | 132.90 | 48.0 | 22.0 | Qualifier |
| $^{12}$C-BPA | 227.10 | 211.95 | 48.0 | 18.0 | Quantifier |
| BPA-$d_8$ (IS) | 235.15 | 136.90 | 48.0 | 24.0 | Qualifier |
| BPA-$d_8$ (IS) | 235.15 | 220.00 | 48.0 | 18.0 | Quantifier |
| $^{13}C_{12}$-BPA | 239.10 | 138.95 | 48.0 | 26.0 | Qualifier |
| $^{13}C_{12}$-BPA | 239.10 | 224.00 | 48.0 | 20.0 | Quantifier |

monitored for $^{12}C$ BPA, $^{13}C_{12}$-BPA and BPA-$d_8$. The dwell time was 0.060 s. Mass spectrometry data were collected (6.0 min), processed and evaluated using MassLynx software version 4.1 (Waters).

## 3. Results and discussion

### 3.1. Acceptance criteria

For the spiked urine samples, the acceptance criteria were set at an accuracy of 80–120%, and a precision (CV) of 20%. To monitor contamination during sample preparation at least 3 blank water samples were pre-treated and analyzed in each analytical run. The acceptance criterion for the blank SPE processed water samples was a BPA concentration <0.1 ng/ml. For the calibration curve, the acceptance criterion was a minimum of 75% of calibration standards within ±15% relative error of the nominal concentration (±20% at LLOQ) and a coefficient of determination better than 0.995.

#### 3.1.2. Analyte confirmation

As outlined in Markham et al. [20] an additional criterion of MS–MS transition ratio was added to these data to improve the method specificity. This ratio is defined as the average of the confirmation ion transition response divided by the quantitation ion transition response observed for the solvent standards analyzed in a given sample set; 12C BPA (*m*/*z* 212 quantifier and *m*/*z* 133 confirmation), 12C BPA-d8 (*m*/*z* 220 quantifier and *m*/*z* 137 confirmation), 13C BPA (*m*/*z* 224 quantifier and *m*/*z* 139 confirmation). The acceptance criteria for the sample transition ratio were ±20% of the average standard transition ratio. A higher sample transition ratio indicates potential issues in the sample measured (e.g., quenching).

### 3.2. Implementation of the method

The method for the analysis of BPA in human urine published by Markham et al. [17,20] was implemented and slightly adjusted: ultra performance liquid chromatography (UPLC, Acquity, Waters) was used instead of HPLC, and a different triple quadrupole mass spectrometer (XEVO-TQS, Waters) was used. During the early implementation of the method several pitfalls were encountered (Tables 3A and 3B):

- Blank water, pre-treated with SPE, showed a $^{12}C$ BPA signal far above the acceptance limit of 0.1 ng/ml, whereas the $^{12}C$ BPA signal in non-pretreated water was below 0.1 ng/ml; it was concluded that contamination occurred during sample pretreatment (SPE).
- A high $^{12}C$ BPA signal was observed in pooled blank urine; this contamination was assumed to occur either during sample collection or SPE work-up.
- Non-pretreated blank solvents (water, acetonitrile) showed a measurable $^{12}C$ BPA signal. Contamination of the UPLC system (mobile phase, injection vials) was suspected.

To avoid $^{12}C$ BPA contamination from sample collection until UPLC–MS analysis several special measures were taken: all glass materials including glass beakers for collection of urine samples were pre-rinsed with dichloromethane to eliminate trace amounts of $^{12}C$ BPA. For the same reason, water used for SPE and for the preparation of mobile phase and buffer was extracted with dichloromethane before use. In addition, a trap column was installed between the mixing chamber and the analytical column to separate $^{12}C$ BPA that may originate from the UPLC system from $^{12}C$ BPA in the samples.

**Table 3A**
Precautions taken to avoid and to monitor contamination of $^{12}C$ BPA during sample handling and analysis.

| No. | Precautions |
|---|---|
| 1 | The milli-Q water used for all experiments (including mobile phase) was extracted with dichloromethane to eliminate trace amounts of BPA in the water. |
| 2 | All glass materials, including the collection flasks for urine samples, were pre-rinsed with dichloromethane. |
| 3 | A trap column was installed between the mixing chamber and the analytical column to separate BPA, that may originate from the UPLC system (minor contamination), from the BPA in the samples. |
| 4 | UPLC gradient was slowed down (and the cone voltage for the internal standard was adjusted). |
| 5 | Preparation of $^{12}C$ BPA stock solutions and sample pre-treatment combined with UPLC–MS/MS were performed in separate laboratories to avoid contamination. |
| 6 | Before and between the UPLC–MS analysis of urine samples multiple blank solvent (acetonitrile/water, 50/50) injections were performed to reassure that the UPLC system was BPA-free, and to avoid false positives. |
| 7 | In each run, at least 3 blank water samples were pre-treated (SPE) together with the urine samples to make sure that no BPA was introduced during sample pre-treatment. |
| 8 | Unconjugated BPA and total-BPA should be measured for each sample in parallel; a substantial fraction of unconjugated BPA (e.g., >1%) is likely to indicate contamination of urine and/or hydrolysis during sample preparation and/or measurement. |

After the implementation of the unconjugated BPA method in human urine another issue occurred: a large variability of the $^{12}C$ BPA and $^{13}C_{12}$-BPA recovery rates from pooled urine samples was observed after the SPE procedure including enzymatic hydrolysis (total BPA method). Apparently, the internal standard (BPA-$d_8$) did not properly correct for recovery variations due to a matrix effect; an endogenous compound in the pooled urine that eluted at the same retention time as the internal standard may have affected the ionization of the internal standard. By adjusting the UPLC gradient procedure, the matrix effect disappeared and the recovery of $^{12}C$ BPA and $^{13}C_{12}$-BPA from pooled urine was improved.

In addition to the above mentioned special measures more general precautions were taken as well, i.e. (1) the preparation of the $^{12}C$ BPA stock solution and sample pre-treatment combined with UPLC–MS analysis were performed in separate laboratories to avoid contamination, (2) in each analytical run at least three blank water samples were pre-treated together with the urine samples to make sure that no $^{12}C$ BPA was introduced during sample pre-treatment, (3) blank solvent (acetonitrile/water, 50/50) was analyzed at the start of each analysis run and between the urine sample extracts to demonstrate that the UPLC system was not contaminated with

**Table 3B**
Pitfalls encountered during implementation and qualification of the SPE-UPLC–MS/MS methods.

| Observation | Precautions taken (see Table 3A) |
|---|---|
| All (non-pre-treated) blank solvents (water, acetonitrile) contained $^{12}C$ BPA due to contamination of the UPLC-system. | 1, 2, 3, 5, 6 |
| Blank water contained BPA after SPE; blank water was contaminated during SPE procedure. | 1, 2, 5, 7 |
| Blank urine sample contained BPA after SPE; blank urine sample was contaminated during sample collection or during SPE procedure. | 1, 2, 5, 7, 8 |
| Large variation of recovery after SPE including enzymatic treatment (total BPA method); the BPA-d8 (IS) signal did not compensate for matrix effect in pooled urine samples. | 4 |

**Table 4**
Validation results of unconjugated BPA in pooled human urine. Pooled human urine was spiked with $^{12}$C-BPA and $^{13}C_{12}$-BPA at six concentration levels. Each level was pre-treated (SPE). The mean unconjugated $^{12}$C-BPA concentration in pooled blank urine was 0.043 ng/ml (CV: 20.0%; $n = 5$).

| Spiked $^{12}$C-BPA $n = 5$ (ng/ml) | Mean $^{12}$C-BPA conc. (ng/ml) | CV (%) | Corrected[a] $^{12}$C-BPA conc. (ng/ml) | Accuracy $^{12}$C-BPA (%) | Mean accuracy $^{12}$C-BPA (%) |
|---|---|---|---|---|---|
| 0.0 | 0.043 | 20.0 | NA | NA | NA |
| 0.10 | 0.14 | 9.1 | 0.094 | 85–115 | 94 |
| 0.20 | 0.24 | 2.1 | 0.20 | 99–104 | 101 |
| 0.50 | 0.53 | 3.5 | 0.48 | 92–102 | 97 |
| 1.0 | 1.0 | 2.4 | 0.96 | 93–100 | 96 |
| 2.0 | 2.0 | 1.6 | 2.0 | 98–102 | 100 |
| 5.0 | 5.1 | 1.4 | 5.0 | 99–103 | 101 |
| Spiked $^{13}$C-BPA $n = 5$ (ng/ml) | Mean $^{13}$C-BPA conc. (ng/ml) | *CV* (%) | | Accuracy $^{13}$C-BPA (%) | Mean accuracy $^{13}$C-BPA (%) |
| 0 | 0 | *NA* | | NA | NA |
| 0.1 | 0.09 | 1.8 | | 89–92 | 90 |
| 0.2 | 0.19 | 1.9 | | 91–95 | 93 |
| 0.5 | 0.48 | 2.7 | | 91–98 | 95 |
| 1 | 0.95 | 2.2 | | 91–96 | 95 |
| 2 | 2 | 1.9 | | 97–102 | 100 |
| 5 | 5 | 1.4 | | 99–102 | 100 |

[a] The accuracy of $^{12}$C-BPA in spiked urine samples was calculated after subtraction of the mean signal in pooled blank urine.

trace amounts of $^{12}$C BPA and (4) only dichloromethane cleaned glass materials were used for sample handling (plastic materials were avoided whenever possible).

### 3.3. Calibration results

The optimized method was applied to the analysis of calibration standards. Calibration of $^{12}$C BPA and $^{13}C_{12}$-BPA using linear regression with weighting factor 1/x was successfully performed in the range from 0.05 to 25 ng/ml. $^{13}C_{12}$-BPA was added to the calibration standards to compare the performance of the method for the two compounds, and to aid in problem solving in case of $^{12}$C BPA contamination. The relative error (%) was within the acceptance criteria for $^{12}$C BPA and $^{13}C_{12}$-BPA. Fig. 1A shows a mass chromatogram of blank solvent (acetonitrile/water, 50/50) spiked with internal standard (BPA-$d_8$) only (no $^{12}$C BPA and $^{13}C_{12}$-BPA were added to the solvent). Despite all measures taken to avoid $^{12}$C BPA in blank solutions, a very small $^{12}$C BPA signal was observed in the blank solvent (at 4.97 min) whereas the $^{13}C_{12}$-BPA signal was zero. Fig. 1B shows a mass chromatogram of the lowest calibration standard ($^{12}$C BPA and $^{13}C_{12}$-BPA concentration: 0.05 ng/ml). Although for $^{13}C_{12}$-BPA the signal-to-noise ratio was higher than for $^{12}$C BPA, due to the $^{12}$C BPA background, both calibration curves enabled reliable quantification in the range from 0.05 to 25 ng/ml.

### 3.4. Method qualification results

#### 3.4.1. Determination of unconjugated BPA in urine

The method qualification results of unconjugated BPA in pooled human urine are presented in Table 4. For each of the 6 validation levels the mean measured $^{12}$C BPA and $^{13}C_{12}$-BPA concentration and the coefficient of variation (CV) were calculated. The $^{13}C_{12}$-BPA signal in water and human urine was zero. For $^{13}C_{12}$-BPA the accuracy was 90–100% with a maximum CV of 2.7%.

The mean unconjugated $^{12}$C BPA concentration in blank pooled human urine was 0.043 ng/ml ($n = 5$); this $^{12}$C BPA concentration can be considered as background signal because the mean $^{12}$C BPA in pre-treated water was 0.052 ng/ml ($n = 5$). The accuracy of $^{12}$C BPA in spiked urine samples was calculated after subtraction of the mean $^{12}$C BPA signal in blank pooled urine. The accuracy of spiked $^{12}$C BPA in pooled human urine ranged from 94% to 101% (maximum CV of 9.1%). Based on these results it can be concluded that the method enabled reliable quantification of unconjugated $^{12}$C BPA and $^{13}C_{12}$-BPA in human urine at a Limit of Quantification (LOQ) of 0.1 ng/ml.

#### 3.4.2. Determination of total BPA in urine (spiked with BPA)

The results of the method qualification are presented in Table 5. The $^{13}C_{12}$-BPA signal in water and human urine was zero. For $^{13}C_{12}$-BPA the accuracy varied from 89% to 101%, indicating that without background signal the analytical method worked well.

The total $^{12}$C BPA concentration, i.e., unconjugated $^{12}$C BPA plus $^{12}$C BPA originating from conjugated metabolites, in pooled blank urine was 0.843 ng/ml ($n = 5$). This level was above the background level of $^{12}$C BPA in pre-treated water (0.063 ng/ml; $n = 5$). The accuracy of $^{12}$C BPA in spiked urine samples was calculated after subtraction of the mean $^{12}$C BPA background signal in pooled blank urine. The accuracy of spiked $^{12}$C BPA in pooled human urine was between 78% and 102% with good reproducibility. These results showed that even on top of the total $^{12}$C BPA in pooled "blank" urine (0.843 ng/ml) the spiked level of 0.2 ng/ml can be measured accurately. Therefore, the LOQ for total BPA in human urine was set at 0.2 ng/ml.

#### 3.4.3. Determination of total BPA in urine (spiked with BPA-G)

The total BPA concentration was determined in pooled human urine which was spiked with $^{12}$C BPA-G and $^{13}C_{12}$-BPA-G. During sample pre-treatment $^{12}$C BPA-G and $^{13}C_{12}$-BPA-G were enzymatically converted to $^{12}$C BPA and $^{13}C_{12}$-BPA, respectively. $^{13}C_{12}$-BPA-G served as the "surrogate" analyte for $^{12}$C BPA-G to compare the performance of the method for the two compounds, taking into account that potential contamination with $^{12}$C BPA might have an influence on the concentration and accuracy.

Fig. 2A shows the mass chromatogram of blank pooled human urine spiked with internal standard: no $^{13}C_{12}$-BPA was observed. The total $^{12}$C BPA signal was the sum of unconjugated $^{12}$C BPA plus $^{12}$C BPA originating from conjugated metabolites after enzymatic hydrolysis in pooled blank urine. Fig. 2B shows a representative mass chromatogram obtained from the analysis of pooled urine spiked with $^{12}$C BPA-G and $^{13}C_{12}$-BPA-G at 0.5 ng/ml.

The validation results are presented in Table 6. The $^{13}C_{12}$-BPA signal in water and human urine was zero. For $^{13}C_{12}$-BPA-G/$^{13}C_{12}$-BPA, the accuracy was 79–85%. The mean total $^{12}$C BPA concentration in pooled blank urine was 0.379 ng/ml ($n = 5$). The

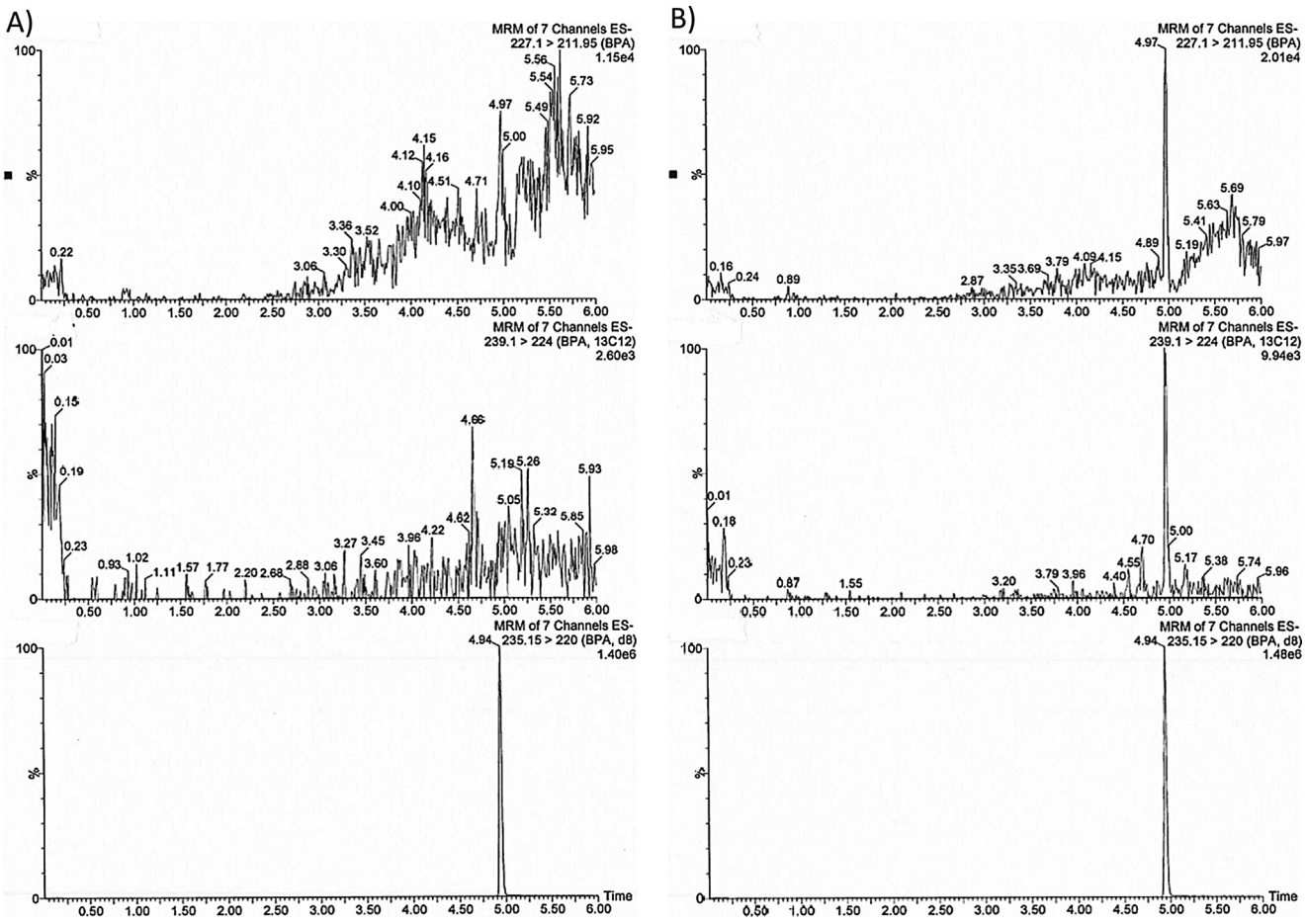


**Fig. 1.** (A) Mass chromatogram of blank solvent (acetonitrile/water, 50/50) spiked with internal standard (BPA-$d_8$, lower trace). No $^{12}C$ BPA (upper trace, low signal, retention time 4.97 min) and $^{13}C_{12}$-BPA (middle trace, no signal) were added to the solvent. (B) Mass chromatogram of a calibration standard acetonitrile/water (50/50) spiked with $^{12}C$ BPA (upper trace), $^{13}C_{12}$-BPA (middle trace) and BPA-$d_8$ (lower trace). The $^{12}C$ BPA and $^{13}C_{12}$-BPA concentration was 0.05 ng/ml.

**Table 5**
Validation results of total bisphenol A in pooled human urine. Pooled human urine was spiked with $^{12}C$-BPA and $^{13}C_{12}$-BPA at six concentration levels. Each level was pre-treated (SPE and β-glucuronidase treatment). The mean total $^{12}C$-BPA concentration in pooled blank urine was 0.843 ng/ml (CV: 12.4%; $n = 5$).

| Spiked $^{12}C$-BPA $n = 5$ (ng/ml) | Mean $^{12}C$-BPA conc. (ng/ml) | CV (%) | Corrected[a] $^{12}C$-BPA conc. (ng/ml) | Accuracy $^{12}C$-BPA (%) | Mean accuracy $^{12}C$-BPA (%) |
|---|---|---|---|---|---|
| 0.0 | 0.843 | 12.4 | NA | NA | NA |
| 0.20 | 1.0 | 2.0 | 0.16 | 71–93 | 78 |
| 0.50 | 1.3 | 3.5 | 0.51 | 88–109 | 101[b] |
| 1.0 | 1.8 | 1.6 | 0.94 | 89–96 | 94 |
| 2.0 | 2.8 | 3.9 | 2.0 | 90–103 | 100 |
| 5.0 | 5.9 | 1.6 | 5.0 | 97–102 | 100 |
| 10.0 | 11.0 | 1.2 | 10.2 | 99–103 | 102 |

| Spiked $^{13}C$-BPA $n = 5$ (ng/ml) | Mean $^{13}C$-BPA conc. (ng/ml) | *CV* (%) | Accuracy $^{13}C$-BPA (%) | Mean accuracy $^{13}C$-BPA (%) |
|---|---|---|---|---|
| 0 | 0 | *NA* | NA | NA |
| 0.2 | 0.18 | 12.8 | 77–107 | 89 |
| 0.5 | 0.49 | 3.6 | 94–103 | 99 |
| 1 | 0.96 | 1.4 | 95–98 | 96 |
| 2 | 2 | 3.7 | 92–101 | 98 |
| 5 | 5 | 0.8 | 98–100 | 99 |
| 10 | 10.1 | 1.6 | 98–103 | 101 |

[a] The accuracy of $^{12}C$-BPA in spiked urine samples was calculated after subtraction of the mean signal in blank urine.
[b] One outlier (807%) excluded from average.

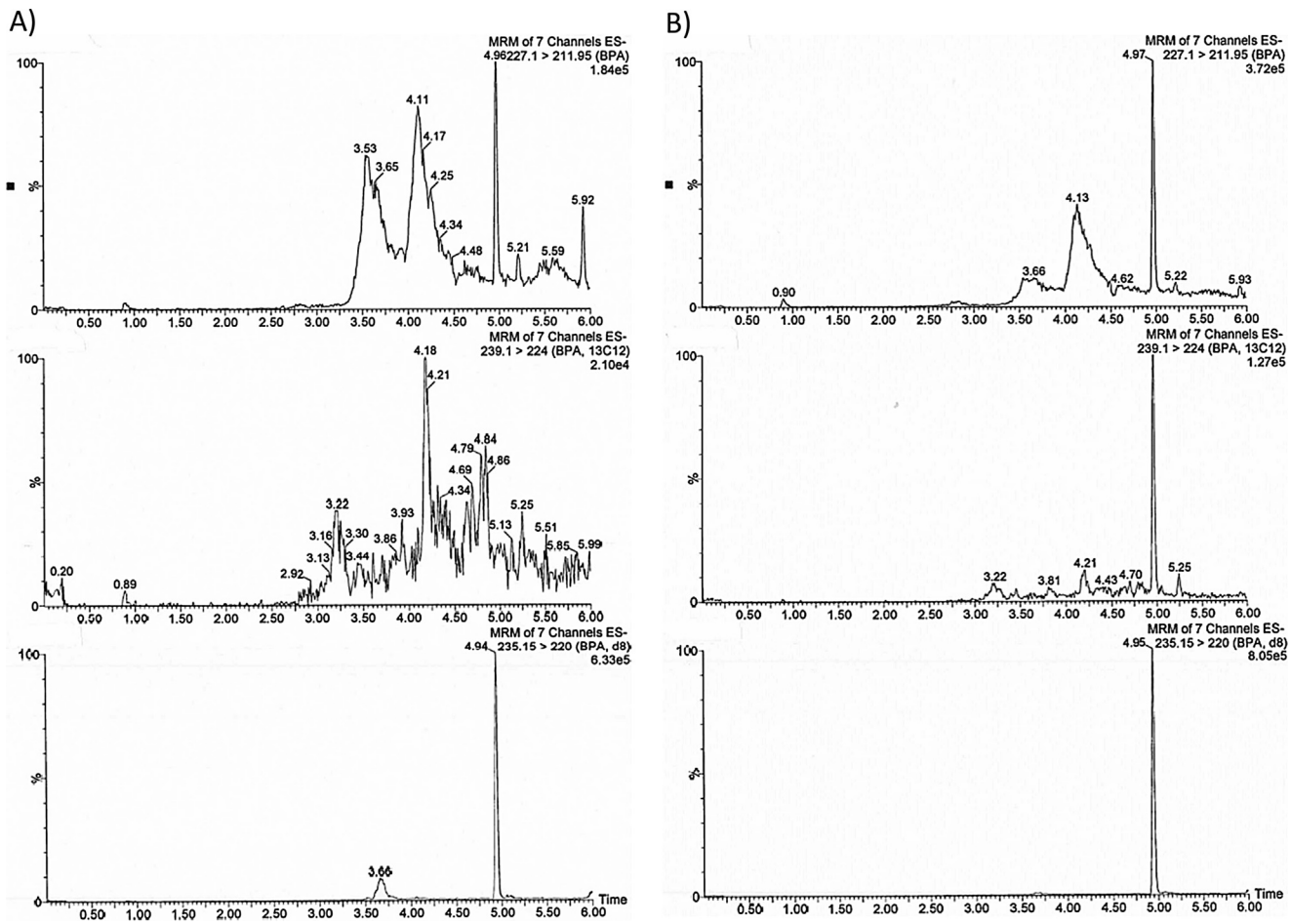


**Fig. 2.** (A) Mass chromatogram of pooled blank human urine with internal standard (BPA-$d_8$; lower trace) pre-treated with glucuronidase and SPE. The urine was not spiked with $^{12}$C BPA (upper trace, retention time 4.96 min) and $^{13}C_{12}$-BPA (middle trace). (B) Mass chromatogram of pooled human urine spiked with $^{12}$C BPA-glucuronide (0.5 ng/ml), $^{13}C_{12}$-BPA-glucuronide (0.5 ng/ml) and internal standard (BPA-$d_8$; lower trace). The $^{12}$C BPA-glucuronide and $^{13}C_{12}$-BPA-glucuronide were enzymatically converted to $^{12}$C BPA (upper trace, retention time 4.97 min) and $^{13}C_{12}$-BPA (middle trace, retention time 4.97 min).

**Table 6**
Validation results of total BPA in pooled human urine. Pooled human urine was spiked with $^{12}$C-BPA-glucuronide and $^{13}C_{12}$-BPA-glucuronide at six concentration levels. Each level was pre-treated (SPE and glucuronidase treatment) and analyzed in 5-fold. The mean $^{12}$C-BPA concentration in pooled blank urine was 0.379 ng/ml (CV: 4.4%; $n = 5$).

| Spiked $^{12}$C-BPA-gluc $n = 5$ (ng/ml) | Expected $^{12}$C-BPA conc. (ng/ml) | Mean $^{12}$C-BPA conc. (ng/ml) | CV (%) | Corrected[a] $^{12}$C-BPA conc. (ng/ml) | Accuracy $^{12}$C-BPA (%) | Mean accuracy $^{12}$C-BPA (%) |
|---|---|---|---|---|---|---|
| 0.0 | 0.0 | 0.379 | 4.4 | NA | NA | NA |
| 0.42 | 0.24 | 0.57 | 4.9 | 0.19 | 66–96 | 78 |
| 0.85 | 0.48 | 0.76 | 1.1 | 0.38 | 76–81 | 79 |
| 1.7 | 1.0 | 1.1 | 1.8 | 0.76 | 77–83 | 80 |
| 4.2 | 2.4 | 2.3 | 1.9 | 1.9 | 77–81 | 79 |
| 8.5 | 4.8 | 4.2 | 0.6 | 3.8 | 79–81 | 80 |
| 17.0 | 9.6 | 7.9 | 2.9 | 7.5 | 76–81 | 78 |
| Spiked $^{13}$C-BPA-gluc $n = 5$ (ng/ml) | Expected $^{13}$C-BPA conc (ng/ml) | Mean $^{13}$C-BPA conc. (ng/ml) | *CV* (%) | | Accuracy $^{13}$C-BPA (%) | Mean accuracy $^{13}$C-BPA (%) |
| 0 | 0 | 0 | *NA* | | NA | NA |
| 0.5 | 0.27 | 0.22 | 15.6 | | 58–89 | 79 |
| 0.99 | 0.55 | 0.45 | 2.6 | | 79–85 | 83 |
| 2 | 1.1 | 0.93 | 2.9 | | 81–87 | 85 |
| 5 | 2.7 | 2.3 | 1.6 | | 82–85 | 83 |
| 9.9 | 5.5 | 4.6 | 1.5 | | 82–85 | 84 |
| 19.9 | 11 | 9 | 1.4 | | 81–84 | 82 |

[a] The accuracy of $^{12}$C-BPA in spiked urine samples was calculated after subtraction of the mean signal in blank urine. The expected BPA concentration was calculated based on the molecular weight of BPA glucuronide and BPA as well as the purity.

**Table 7**
Free $^{12}$C-BPA and total $^{12}$C-BPA concentrations in urine of 20 volunteers (sample code V1–V20). The mean $^{12}$C-BPA concentration of each volunteer was calculated from three replicates. To monitor background $^{12}$C-BPA levels, blank water was pre-treated and analyzed in each run. The reported free and total $^{12}$C-BPA concentrations were not corrected for background.

| Sample | Mean free $^{12}$C-BPA concentration (ng/ml; $n=3$) | CV (%) | Mean total $^{12}$C-BPA concentration (ng/ml; $n=3$) | CV (%) |
|---|---|---|---|---|
| Water | 0.097 | 24.6 | 0.108[a] | 82.4[a] |
| V1 | 0.046 | 13.4 | 1.740 | 2.4 |
| V2 | 0.054 | 12.9 | 1.082 | 5.3 |
| V3 | 0.051 | 11.7 | 4.930 | 8.9 |
| V4 | 0.051 | 21.7 | 0.243 | 13.2 |
| V5 | 0.051 | 14.5 | 1.564 | 5.2 |
| V6 | 0.119 | 19.8 | 6.812 | 5.1 |
| V7 | 0.119 | 7.7 | 0.663 | 2.8 |
| V8 | 0.037 | 21.6 | 0.247 | 10.5 |
| V9 | 0.069 | 12.2 | 6.678 | 8.0 |
| V10 | 0.035 | 20.5 | 0.553 | 28.8 |
| V11 | 0.057 | 13.5 | 4.172 | 3.9 |
| V12 | 0.040 | 24.2 | 1.064 | 1.7 |
| V13 | 0.036 | 23.4 | 0.409 | 20.3 |
| V14 | 0.038 | 15.1 | 0.664 | 10.1 |
| V15 | 0.041 | 25.3 | 1.064 | 2.0 |
| V16 | 0.039 | 24.7 | 0.975 | 1.4 |
| V17 | 0.075 | 22.3 | 2.930 | 4.9 |
| V18 | 0.069 | 4.6 | 0.677 | 8.9 |
| V19 | 0.072 | 21.5 | 0.660 | 12.9 |
| V20 | 0.072 | 22.4 | 1.558 | 8.5 |

[a] The $^{12}$C-BPA concentration in blank water was 0.056, 0.065, 0.075, 0.078 ng/ml and 0.267 ng/ml.

accuracy of $^{12}$C BPA from urine samples spiked with $^{12}$C BPA-G was calculated after subtraction of the mean signal in pooled blank urine. The accuracy of $^{12}$C BPA-G/$^{12}$C-BPA was 78–80% at all concentration levels. The somewhat lower accuracy obtained for $^{12}$C BPA-G/$^{12}$C-BPA may have been caused by the hygroscopic nature of the $^{12}$C BPA-G reference standard, used for spiking of pooled urine.

### 3.5. Application of the methods to individual human urine samples

The qualified methods were applied to urine samples of 20 volunteers. All 20 urine samples were analyzed with both methods: unconjugated $^{12}$C BPA was measured using SPE ($n=3$), and total $^{12}$C BPA was measured using enzymatic treatment and SPE ($n=3$). The unconjugated and total BPA concentration of the individual urine samples are presented in Table 7.

The unconjugated $^{12}$C BPA concentrations in the human urine samples ranged from 0.035 ng/ml to 0.119 ng/ml. As the mean $^{12}$C BPA concentration in pre-treated (SPE) water was 0.097 ng/ml ($n=3$) all mean $^{12}$C BPA concentrations measured in human urine samples were at or below 0.097 ng/ml and can be considered background. Only a few individual urine measurements showed unconjugated $^{12}$C BPA concentrations at or just above the background level and were also considered as background.

The mean total $^{12}$C BPA concentrations, i.e. unconjugated $^{12}$C BPA plus $^{12}$C BPA originating from conjugated metabolites, in the human urine samples varied from 0.243 ng/ml to 6.81 ng/ml. All total $^{12}$C BPA concentrations measured in the urine samples from the 20 volunteers were above the background level.

## 4. Conclusions

Trace-level quantitation of unconjugated and/or total $^{12}$C BPA (<1 ng/ml) in human urine and specimens is very challenging. Unconjugated BPA and total BPA should be measured in parallel and a comprehensive trouble shooting analysis should be performed in cases where substantial amounts (e.g., >1%) of unconjugated BPA are observed compared to total BPA [10,11]. Particular precautions must be taken to avoid and to monitor contamination of BPA with the appropriate blanks, controls and fortified controls during sample handling and analysis, since contamination might have a large impact on BPA biomonitoring data [3,15–19,22].

As mentioned earlier by Markham et al. [20] the use of a surrogate analyte, in this case $^{13}$C BPA-G is strongly recommended as it provides valuable information on method performance and aids in method troubleshooting as it is not affected by exogenous $^{12}$C BPA. During the study several pitfalls were encountered that resulted in high BPA background levels. Comprehensive troubleshooting was performed and precautions were taken to avoid and monitor $^{12}$C BPA contamination (see Table 3A) including:

- Water used for all experiments was extracted with dichloromethane to eliminate trace amounts of BPA.
- All glass materials were pre-rinsed with dichloromethane to eliminate trace amounts of BPA.
- A trap column was installed between the mixing chamber and the analytical column to separate BPA that originates from the UPLC system from the BPA in the samples.

Even by taking the above mentioned precautions into account, background contamination with BPA was frequently in the range of 0.01–0.02 ng/ml (solvent blank).

The method was applied to urine samples from 20 volunteers. Consistent with previously reported data in the literature, unconjugated BPA concentrations are at or below the background level and it can be concluded that no unconjugated BPA is present in any human urine sample measured in this study.

Total BPA concentration in urine (0.243 ng/ml–6.81 ng/ml) is consistent with earlier reports in large population studies confirming low exposure to BPA in the general population [1,13,23–26].

Overall, the HPLC–MS/MS method developed by Markham et al. [20] to determine unconjugated and total $^{12}$C BPA in urine was successfully transferred to another laboratory. Any analysis of BPA at sub-ppb levels must strive to eliminate and continually monitor for background contamination from all sources (collection to analysis) with the appropriate blanks, controls and fortified controls.

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