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RESEARCH PAPER

A high selective and sensitive liquid chromatography–tandem mass spectrometry method for quantization of BPA urinary levels in children

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Abstract A selective and highly sensitive liquid chromatography–tandem mass spectrometry method has been developed and validated for determination of Bisphenol A (BPA) in human urine using labeled d_6-BPA as internal standard. BPA was purified from human urine by affinity chromatography on solid extraction AFFINIMIP® Bisphenol A cartridges, based on molecularly imprinted polymers. After purification, the samples were analyzed on a Phenomenex Kinetex 100× 4.6 mm, 2.6 μm particle PFP reversed-phase HPLC column, coupled to a triple quadrupole mass spectrometer by an electrospray ion source. Analyses were performed in the multiple reaction monitoring mode and negative ionization; the product ions at 133.2 and 212.1 m/z for BPA and at 138.2 and 215.0 m/z for d_6-BPA were monitored to assess unambiguous identification. The linearity of the detector response was verified in human urine over the concentration range 0.100–200 ng/mL. The detection limit was calculated as 0.03 ng/mL and the limit of quantification of the method is 0.10 ng/mL. This LC/ESI-MS/MS method was in-house validated evaluating specificity, trueness, within-day and between-days precision. The mean recoveries of BPA from spiked urine samples were higher than 94 % and good reproducibility (relative standard deviations≤8.1 %) was observed. The developed method was applied to a pilot study involving 105 children, aged from 6 to 14 years (16 normal weight and 89 obese children), from the Regione Campania (Southern Italy). The aim of this study was to determine the concentrations of BPA in urine of children and possible correlations with childhood obesity.

Keywords MIP cartridge · Biological fluids · Bisphenol A · Liquid chromatography–tandem mass spectrometry

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Introduction

The organic compound Bisphenol A (BPA), with an annual production of 2–3 million tons, is a fundamental building block in the synthesis of plastics and epoxy resins. BPA is classified as an endocrine disruptor. It has been associated with adverse reproductive and developmental effects [1–5]. There is much evidence on the endocrine effects played by BPA and the European Food Safety Authority, in agreement with the US Environmental Protection Agency, established the maximum acceptable dose for BPA at 50 μg/kg body weight/day [6–9]. Infants and children fed with liquid food are among the most

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exposed, and those fed with food stored in polycarbonate containers can introduce up to 13 μg of BPA per kg bw per day [10–15].

To assess the relevance of human exposure to BPA by large-scale studies and to evaluate correlations with some widespread pathologies such as obesity, highly reliable extraction and accurate quantification methods have been developed to measure this compound in urine. Urine is a target matrix for biological monitoring of human exposure to BPA, especially in children, because it is readily available in sufficient amounts for analysis. Moreover, most of BPA is excreted in urine, where the concentrations are also higher than in blood, due to the relatively rapid metabolism and excretion pathway [16–19]. In fact, the ingested BPA is metabolized in the liver to its glucuronidated form (i.e., BPA glucuronide or BPAG) and excreted in urine, with a half-life in the range from 4 to 43 h. Because BPAG does not bind to the estrogen receptor, glucuronidation is considered as a mechanism of detoxification in humans [20]. However, BPAG can be deconjugated by β-glucuronidase, which is present at high concentrations in intestine, liver, kidney, and placenta [20]. The conversion of BPAG to BPA increases the potential risk of reactivation of BPA-induced effects. BPA has been detected in fat, suggesting that the compound bio-accumulates in lipids and other physiologic compartments [21–23] and can interfere with human hormones, creating health problems. Then, even if urinary BPA concentrations solely reflect recent consumption, it is likely to be a "noisy" indicator of chronic exposure.

Different methods have been developed and used to monitor this contaminant in human [24–31], but the BPA analysis is still an open challenge for analytical chemistry. Owing to the complex matrices and the low concentrations of BPA (parts per billion, ppb), extensive sample preparation (extraction, clean-up, concentration, derivatization, etc.) is required prior to the analysis by gas chromatography or liquid chromatography (LC), coupled to several detectors such as mass spectrometer (MS), diode array, and fluorescence spectrophotometer.

Herein we describe the development and validation of a high selective and sensitive quantitative method for BPA determination in urine by liquid chromatography coupled to electrospray tandem mass spectrometry on a triple quadrupole (LC/ESI-MS/MS). This method is based on the rapid and cost-effective urine sample clean-up and concentration in a single step, by solid-phase extraction (SPE) on molecularly imprinted polymer (MIP) cartridges specific for BPA. In the literature there are no data describing the application of this kind of affinity chromatography for quantification of urinary BPA levels [32]. MIP cartridges show high selectivity, due to the presence of specific sites for binding of the analyte, improving the crucial clean-up step, thus reducing the presence of matrix components, and consequently increasing method sensitivity. Determination of BPA by tandem mass spectrometry allowed us to obtain unambiguous identification by two product ions and a quantification limit lower than those resulting from other spectrophotometric detectors. By spiking samples with isotope-labeled BPA at the beginning of the sample extraction step, matrix effect, loss of analyte, variations in extraction volume, can be minimized, thus increasing method precision and accuracy.

The method was validated evaluating the linearity of detector response, the limit of quantification, trueness, precision, analyte stability, and specificity; then, it was applied to study the exposure to BPA in some children by measuring the levels in their urine. Recently, BPA exposure has been shown to disrupt multiple metabolic mechanisms [33], suggesting that it in environmentally relevant doses may increase the body mass and therefore contribute to obesity in humans [34–36]. This possibility has been explored in adults and only recently in children [37]. Thus improved analytical methods are needed to a better understanding and a more concrete picture into the exposure of the most vulnerable segment of the human population.

Experimental

Materials

BPA (purity grade 98 %), β-glucuronidase/sulfatase from *Helix pomatia* (EC 3.2.1.31) and reagents were purchased from Sigma Aldrich (Sigma-Aldrich, Milano, Italy). HPLC-grade reagents, including water (residue<0.00005 %, resistivity>18 MOhm (T=25 °C), TOC<2 ppb), methanol (MeOH, assay>99.9 %, residue<0.0001 %), and acetonitrile (ACN, assay>99.9 %, residue<0.0001 %) were purchased from Romil (ROMIL Ltd, UK). The MIP cartridges purchased from Polyntell (Polyntell SA, Paris, France) were AFFINIMIP® SPE Bisphenol A in glass tube. Frozen human urines from ten volunteers were shipped in glassware and stored at −20 °C. These urine samples were used for development and validation of the method.

Preparation of standard reference materials and quality control samples

A BPA stock standard solution at 1.0 mg/mL was prepared in MeOH. In a similar manner, a stock standard solution of d_6-BPA, used as internal standard (IS), was prepared at 1.0 mg/mL in MeOH. Both standard stock solutions were stored in dark glass vials at −20 °C until use. The intermediate standard solutions were prepared from the stock solutions by serial dilution with MeOH/water (1/1, v/v). In a concentration range from 0.10 to 200 ng/mL, eight-point calibration curve standards were prepared fresh daily. Four levels of quality control samples (QCs) in urine containing 0.10 ng/mL (LLOQ-QC, lowest level quality control), 1.0 ng/mL (L-QC, low quality control), 10 ng/mL (M-QC, middle quality control), and

100 ng/mL (H-QC, high quality control) of BPA were prepared by spiking blank urine with standard solutions for determination of trueness (mean recoveries) and within-day and between-days precision. The standard solution volume not exceeds the 2 % total volume of blank urine.

Extraction and clean-up of urine samples

Since the analytical standards of the conjugated forms of BPA (including BPAG and BPA sulfates) are not commercially available, BPA analysis has been focused on the measurement of "total BPA". So, BPA purification from urine was performed in two steps: the enzymatic de-conjugation of BPA-glucuronide/sulfate adducts, then the solid-phase extraction by AFFINIMIP SPE columns. Urine samples were thawed at room temperature and vortexed for 30 s to ensure homogeneity. Three milliliters urine sample were added with 1 mL of sodium acetate buffer 0.1 M at pH 5.0, with 100 μL intermediate IS solution at 1 μg/mL and 20 μL of enzyme solution at 1.0 mg/mL in sodium acetate buffer 0.1 M at pH 5.0, then mixed thoroughly by vortex. The enzymatic reaction was carried out for 2 h in a thermostatic bath at 37 °C.

At each working session, a method blank containing 3 mL of a mix 1:1 MeOH/water (*v*/*v*) added with 1 mL of sodium acetate buffer 0.1 M at pH 5.0, with 100 μL intermediate IS solution at 1 μg/mL and 20 μL of enzyme solution at 1.0 mg/mL in sodium acetate buffer 0.1 M at pH 5.0, was also introduced; in this way, we monitored possible BPA contamination due to reagents/solvents and/or urine samples during all the analyses. Calibration curve standards and QCs were also prepared by adding 100 μL intermediate IS solution (1 μg/mL); moreover, in the QCs samples 20 μL of enzyme solution was added. The QCs and method blank samples were also de-conjugated and purified under the same conditions of the urine samples.

After the enzymatic de-conjugation reaction, the samples were loaded onto AFFINIMIP cartridges, previously conditioned with 5 mL of MeOH containing 2 % acetic acid, 5 mL ACN, and 5 mL of HPLC-grade water, according to the manufacturer instructions. After sample loading, the cartridges were washed with 4 mL of water, 4 mL of water/ACN (40:60 *v*/*v*), and eluted with 3 mL MeOH. The eluent was collected and subsequently concentrated by Eppendorf Concentrator at 45 °C, up to a final volume of approximately 0.5 mL. The sample is diluted to 1.0 mL final volume with HPLC-grade water. A 20-μL aliquot of the sample extract was injected for LC/ESI-MS/MS analysis. Reagent blanks, method blanks, calibration curve standards, and QCs were analyzed with the urine samples.

LC/ESI-MS/MS analysis

A Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific Inc, Italy) was used in this study. The chromatographic separation was carried out using a 100×4.6-mm Kinetex 2.6 μm PFP stainless steel HPLC column, equipped with a guard column (Phenomenex, Italy). The mobile phase consisted of water (A) and methanol (B). No additives, such as formic acid, acetic acid, ammonium acetate, or ammonia, were added to mobile phase because we also found, according to the literature [31, 38, 39], that they can cause severe signal suppression for BPA. Chromatography was run at room temperature by linear gradient elution; the analysis started with 30 % B for 1 min, then followed by a gradient from 30 to 95 % B in 1 min holding at 95 % B for 3 min. Finally, the mobile phase B was decreased to 30 % in 1 min and equilibrated at 30 % for further 3 min. The flow rate was 0.5 mL/min. The injection volume was 20 μL.

The HPLC system was coupled to a triple quadrupole instrument (API 2000; AB Sciex, Germany) equipped with a TurboIon electrospray source. Analytes were detected in negative ion mode at vaporization temperature of 450 °C and ion electrospray voltage of −4.5 kV. Other parameters were set at: −40 V for declustering potential (DP), −10 V for entrance potential (EP), and −35 eV for collision energy; 6 psi N_2 was used as collision gas, setting the curtain gas at 40 psi. The samples were analyzed in multiple-reaction monitoring (MRM) mode; the following selective ion transitions were monitored: m/z 227.1→m/z 212.1 (quantifier) and m/z 227.1→m/z 133.2 (qualifier) for BPA; m/z 233.1→m/z 215.0 (quantifier) and m/z 233.1→m/z 138.2 (qualifier) for d_6-BPA.

The data were handled by the Analyst™ software version 1.5.1 (ABI Sciex). BPA identification was based on the retention time of both quantifier and qualifier product ions; the presence of the BPA qualifier ion allowed for unambiguous confirmation of the compound. The peak area ratios of BPA and IS were reported vs BPA standard concentrations, and calibration curves were calculated by linear regression; BPA concentration in urine samples was calculated by interpolation of calibration curves, as well as the measured concentrations of standards and quality control samples.

Method validation

The method was in-house validated evaluating detector response linearity, within-day and between-days precision, trueness by mean recoveries, and ruggedness for slight changes, using four spiked QC samples.

The detector response linearity was evaluated by analyzing five calibration curves calculated by eight BPA standards within the concentration range 0.10–200 ng/mL. Each calibration curve was analyzed individually by using weighed ($1/x$) linear regression. The limit of detection (LOD) was calculated as the concentration showing a signal-to-noise ratio=3 in the method blank sample, at the same retention time of BPA. The concentration of the lowest BPA standard on the calibration curve was accepted as the lower limit of quantification (LLOQ), allowing also for confirmation by the presence of the qualifier ion. The

deviation of recalculated concentrations of standards from nominal levels was below ±15.0 %.

For determining the within-day trueness and precision, five replicate analyses of urine samples spiked with BPA were performed in the same day; five spiked samples at LLOQ-QC, L-QC, M-QC, and H-QC concentrations were run. The precision of the method was determined by calculating the relative standard deviation (%RSD) at each spiking level. The deviation at each concentration level from the nominal concentration was expected to be below ±15.0 %. The between days trueness and precision were calculated by analyzing five spiked samples at LLOQ-QC, L-QC, M-QC, and H-QC concentrations during three consecutive working days.

Mean recoveries and possible matrix effects were evaluated at high, middle, and low levels of urine quality samples in six replicates. Mean percentage recovery was calculated by comparing the mean area response of samples spiked before extraction to that of samples spiked at the same levels after extraction, at each concentration. The recovery of IS was similarly estimated at its working concentration. To assess if any co-eluting compound from the same sample matrix causes ion suppression or enhancement of the analyte signal, matrix effect (%) was assessed by comparing the mean area response of samples spiked after extraction with the mean area of neat standard solutions injected directly into LC/ESI–MS/MS system.

Ruggedness of the method for slight changes was evaluated by comparing the work of two analysts and using different columns of the same brand and model. The ruggedness experiment should meet the acceptance criteria for within-batch trueness and precision.

Time stability of BPA in the samples

Stability experiments were carried out to verify if BPA amount undergoes a loss in stock standard solutions and in urine samples at high, middle, and low concentrations, under different conditions. Short-term stability at room temperature (24 °C) for 6 h, short-term stability in refrigerator (2–4 °C) for 24 h and long-term stability in spiked samples stored at −20 °C for 30 days were assessed by comparing the peak area response of BPA and IS with those of fresh BPA stock standard solutions. BPA stability was assessed considering the deviation from nominal value was less than ±10 %.

Determination of BPA concentration in urine of obese children

Urine samples, from children aged between 6 and 14 years, were provided by the Obesity Center of the Children's Hospital of the Second University of Naples. The study was described in detail to all participant parents and all gave informed consent. All samples were stored at −20 °C prior to analysis. The urine samples were prepared and analyzed according to the procedures previously described. To verify that there was no carry-over effect, and to monitor method linearity, the solvent blanks and the standard solutions for calibration curve were injected before and after all sample batches. In particular, each batch of samples included also four QCs and three method blanks. When BPA was detected in method blanks, the corresponding value was subtracted from the concentration of all the samples in that batch.

For each subject, body weight was measured by a balance beam scale, the child being undressed, height was measured by a Harpenden stadiometer, and body mass index (BMI) was calculated by dividing the body mass for the height square. Children were defined as obese if the BMI exceeded the 95th percentile for age and sex according to the Italian charts [40].

Results and discussion

Optimization of LC/ESI–MS/MS conditions

To optimize mass spectrometer ionization and fragmentation conditions, the best potential values for DP, EP, and collision gas flow were set, by direct infusion of both BPA and d_6-BPA standard solutions at 10 μg/mL. Negative ionization mode showed the best signal response for precursor ions at m/z 227.1 and m/z 233.1, respectively; the collision-induced fragmentation was optimized to obtain two characteristic product ions in relative intensities comparable to the data reported in literature [30, 37, 38], for both BPA and d_6-BPA. The dwell time was set at 200 ms to allow good peak point collection. Identification and quantitation were performed using MRM mode to monitor the transitions m/z 227.1→m/z 212.1 (quantifier) and m/z 227.1→m/z 133.2 (qualifier) for BPA and m/z 233.1→m/z 215.0 (quantifier) and m/z 233.1→m/z 138.2 (qualifier) for IS.

Then, the chromatographic separation was optimized, trying various combinations of water/organic phase and additives, such as formic acid, acetic acid, ammonium acetate, or ammonia, on different reversed-phase columns, including Macherey-Nagel Nucleodur Sphinx RP (250×4.6 mm, 5 μm), Macherey-Nagel Nucleodur C18 HTec (100×2.1 mm, 3 μm), Phenomenex Synergi Fusion—RP 80A (50×2.0 mm, 4 μm), Phenomenex Kinetex PFP 100A (100×3.0 mm, 2.6 μm), and Dionex Acclaim Phenyl—1 (50×3.0 mm, 3 μm). Acetonitrile and methanol were compared as the organic component of the mobile phase. The column Kinetex PFP 100A from Phenomenex and a mobile phase containing water and methanol, showed the best peak shape, adequate analyte retention times, and baseline resolution between BPA and the IS, under linear gradient elution. We observed that addition of buffer salts or ion pairing acids did not improve separation but dramatically reduced sensitivity. The flow rate was also optimized at 0.5 mL/min to obtain a total run time of 9 min.

Table 1 Mean percentage recoveries from differnt SPE cartridges

Cartridge	Mean percentage recovery (%)		
	Spiked concentration of BPA (ng/mL)		
	1	10	100
Sep-Pak Light Florisil (Waters)	85.7	79.2	77.2
PP Chromabond HR-X (Macherey-Nagel)	89.1	87.5	91.3
Affinimip SPE Bisphenol A (Polyntell)	102.6	94.7	97.6
Strata-X (Phenomenex)	90.5	89.5	87.6

Optimization of BPA purification

When a compound is present at low concentrations in a complex matrix, a clean-up step before determination is crucial to improve sensitivity, reliability, and specificity by removing interfering compounds and concentrating the analyte. To this aim, we tried solid-phase extraction procedures that were widely described in the literature [26, 38, 39, 41, 42]. Different sorbents (silica and/or polymer sorbent suitably functionalized) are commercially available, and many solvents can be combined, to work in normal or reversed-phase chromatography, hydrophobic interaction, and ion exchange. For these reasons, this technique is highly versatile, allowing for an efficient recovery of the analytes also if they are present in traces. Moreover, the availability of Affinimip SPE cartridge allowed us to test also affinity chromatography, using specific column developed to retain BPA.

We tested different SPE columns that are Sep-Pak Light Florisil cartridge (Waters), PP Chromabond HR-X (Macherey-Nagel), and Affinimip SPE Bisphenol A, both in glass and in polypropylene tubes (Polyntell), Strata-X (Phenomenex).

Initially, the cartridges were equilibrated with MeOH and/or ACN and washed with water according to procedures from manufacturers reported in the respective data sheets. Different solvents were compared, but MeOH was chosen as the

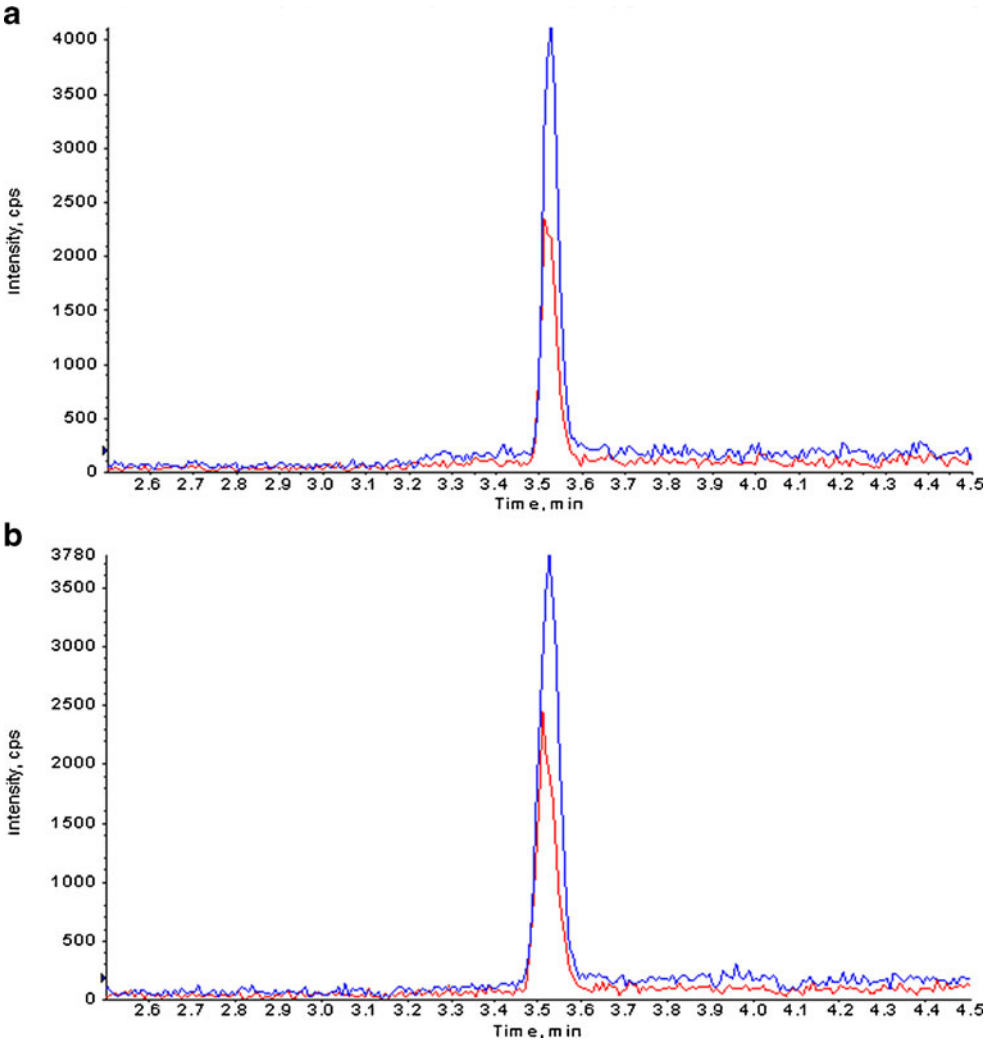

Fig. 1 LC-MS/MS chromatograms obtained from analysis of samples spiked before extraction procedure (**a**) and after extraction procedure (**b**) at BPA concentration of 1 ng/mL. The *blue line* refers to the transition *m/z* 227.1→*m/z* 212.1 (quantifier), while the *red line* to *m/z* 227.1→*m/z* 133.2 (qualifier)

Table 2 Mean percentage recoveries and matrix effect in spiked urine

QC	Spiked concentration of BPA (ng/mL)	Peak area ratio (mean±SD)		Mean percentage recovery (%)	Peak area ratio (mean±SD)		Matrix effect (%)
		BPA response of sample spiked before extraction	BPA response of sample spiked post extraction		Post-extraction spiked sample	Neat standard solutions	
L-QC	1	(1.98±0.15) E+04	(1.93±0.16) E+04	102.6	(1.46±0.10) E+04	(1.75±0.09) E+04	83.4
M-QC	10	(7.02±0.06) E+04	(7.41±0.08) E+04	94.7	(6.21±0.07) E+04	(6.98±0.05) E+04	89.0
H-QC	100	(4.56±0.05) E+05	(4.67±0.07) E+05	97.6	(4.26±0.04) E+05	(4.95±0.05) E+05	86.0

"eluting solvent". Once concentrated, to increase the volume of eluted solution from 0.50 to 1.0 mL with water was found out to reduce significantly matrix effects and resulted in lower quantification levels by improving BPA ion signals.

Glass Affinimip SPE Bisphenol A improved significantly method specificity, owing to the selectivity of BPA binding to the molecularly imprinted stationary phase. The mean recoveries of the analyte are very satisfactory, and higher than those measured testing other SPE cartridges based on different techniques such as absorption and reversed-phase chromatography (Table 1). Moreover, the mean recoveries from MIPs are higher than those described by most of other authors in the literature [26, 38, 39, 41]. In particular, the use of cartridges in glass should avoid possible release of BPA from plastic material of cartridge columns. The effective mean recoveries of BPA, without considering compensation due to the labeled IS, were observed to range from 94.7 to 102.6 % (Table 1), to be compared to mean values from 65 up to 98 % reported in the literature [26, 38, 39, 41]. Figure 1 show chromatograms obtained from LC/ESI–MS/MS analysis of urine samples. In particular, Fig. 1a refers to a urine sample spiked with a known concentration of our target analyte (1 ng/mL of BPA) prior to extraction procedure; while Fig. 1b refers to a urine sample spiked with a 1 ng/mL of BPA after the extraction procedure. The retention time of BPA was approximately 3.52 min. The presence of single sharp chromatographic peaks, for both quantifier and qualifier ions, accounts for high method selectivity, compared to other sorbents for clean-up [42]. By using MIP cartridges, a low baseline noise was also observed.

Method in-house validation

Method performances were evaluated at three different concentrations of BPA in urine. In Table 2 the analyte peak areas relative to the quantifier product ion are reported. Ion suppression of BPA signal was observed and a matrix effect was calculated in a range 83.4–89.0 %. As expected, this effect appears to be greater at the lowest concentration. Because isotope-labeled IS was used in this study and the results showed the same degree of ion suppression in IS, the quantification based on the analyte/IS response ratios allows to correct the matrix effect. This is the first advantage in the use MS-based methods but not fully applied for the BPA. Isotope-labeled BPA was used only in a few methods previously described [31, 38, 39, 43].

Matrix effect and mean recoveries were also investigated using different urine sample volumes (3, 10, 25, and 50 mL) spiked at 1 ng/mL BPA. Usually, the low concentrations were difficult to detect using initial small volumes of urine sample. So, increasing the urine volume loaded on cartridge increases the concentration factor of the analyte, but decreases the sensitivity due to interfering compounds. Moreover, larger sample volume and, consequently, BPA amount, can result in HPLC column overloading and peak tailing. By data reported in Fig. 2, 3 mL of urine was selected as the initial urine volume in this study. It was not possible to use a volume smaller than 3 mL because our LC-MS system was not able to detect concentrations below 0.1 ng/mL. The addition of 1 mL buffer acetate pH 5 in urine samples is required for enzymatic hydrolysis but also to reduce the turbidity of the sample, improving the flow rate during the SPE sample loading step.

The calibration curve obtained by plotting peak area ratio versus concentrations was linear over the 0.1–200 ng/mL calibration range (regression coefficient $R \geq 0.99$). The mean back-calculated concentrations of the standards were between 94 and 103 % of the theoretic values of analytes. The LOD and LOQ

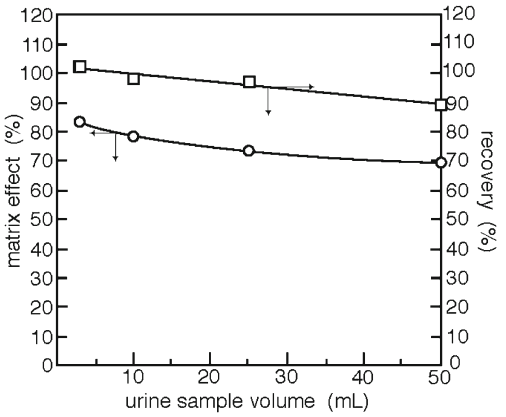

Fig. 2 Matrix effect and mean recoveries as a function of different urine sample volumes

Table 3 Comparison of within-day and between-days precision and trueness for BPA in spiked urine

QC	Spiked concentration of BPA (ng/mL)	Within day				Between days			
		n	Measured concentration (mean±SD) ng/mL	%RSD	% trueness	*n*	Measured concentration (mean±SD) ng/mL	%RSD	% trueness
LLOQ-QC	0.1	5	0.101±0.006	6.3	100.8	15	0.094±008	8.1	93.8
L-QC	1		1.028±0.061	6.0	102.8		1.008±0.068	6.8	100.8
M-QC	10		10.292±0.424	4.1	102.9		9.830±0.659	6.7	98.3
H-QC	100		102.740±4.871	4.7	102.7		99.700±5.608	5.6	99.7

were 0.03 and 0.1 ng/mL, respectively. By analyzing the standards prepared in water/methanol versus matrix matched standards in human urine, matrix effect on the calibration curve was evaluated, in agreement with the results of matrix effect and recovery above described. The slopes of calibration curves by standards prepared in human urine differ at most by 5 % from the slopes of calibration curves in water/methanol. For this reason the calibration curves were prepared in water/methanol.

Method blanks were prepared in water/methanol to capture possible environmental contamination of analytes from water or solvents, as well as contaminants released from material used for sample preparation (including tubes, pipette tips, and vials). When we detected trace levels of BPA in method blank samples, background subtraction was used to correct for this minor contamination.

The within-day and between-days precision and trueness data were acquired for the QC samples at four different concentrations (H-QC, M-QC, L-QC, and LLOQ-QC levels) from five sets of samples, analyzed on three different days. All values are summarized in Table 3. The within-day precision (%RSD) was within 4.1–6.3 %. The values of within-day trueness were between 100.8 and 102.9 %. The between days precision and trueness were determined by pooling all validation data from all QC samples at each concentration level. The between-days precision (%RSD) was ≤8.1 %. The values of between-days trueness were in a range 93.8–100.8 %. All the results were very satisfactory, indicating the method is highly reliable.

Method ruggedness for minor changes was tested by evaluating performance of different analytes using different HPLC column lots. The run consisted of a calibration curve and a total of 18 spiked samples, including six replicate each of the low, medium, and high QC samples. The %RSD ranged from 1 to 5 % and the percentage of nominal values ranged from 96.2 to 102.3 %; these results fall within validation data previously described.

The stability of the standard stock solutions at 24 °C (room temperature), 2–4 °C and −20 °C was tested over 6 h, 24 h, and 30 days, respectively. The results are resumed in Table 4. The percentage differences between the stored at room temperature and fresh solutions were ≤8 %, indicating that degradation of analyte in urine is negligible within the time required for analyzing the samples. The BPA in urine had acceptable stability also under short-term storage at refrigerator (2–4 °C for 24 h) long-term storage condition (−20 °C for 30 days) since there were no relevant differences (≤4 %) in the mean analyte concentrations at each level of 1, 10, and 100 ng/mL compared with initial mean values.

Application of the method in healthy human subjects

Recent studies focused on the correlation between prolonged exposure to BPA during childhood and adolescence and an increased risk of obesity [35, 36, 44]. Starting from this consideration, the developed method has been applied to a study involving 105 children (16 normal weight and 89 obese children) residing in the Regione Campania (Southern Italy), aged from 6 to 14 years. Aim of this study was to estimate the amount of BPA in the urine of normal weight and obese children, in order to evaluate a possible association between exposure to this endocrine disruptor and childhood obesity.

From data collected with a standardized questionnaire, there were no relevant differences in maternal education, physical activity, socio-economic status, and diet type. We therefore assumed all participants to have normal physical activity and categorized them as having “excessive” caloric

Table 4 Stability of BPA in spiked urine under different storage conditions

QC	Spiked concentration of BPA (ng/mL)	BPA concentrations (ng/mL) (mean±SD) under different storage conditions		
		24 °C for 6 h	2-4 °C for 24 h	−20 °C for 30 days
L-QC	1	0.95±0.02	0.98±0.05	1.06±0.08
M-QC	10	9.59±0.22	9.62±0.52	9.95±0.31
H-QC	100	96.50±3.22	98.20±3.80	102.50±4.16

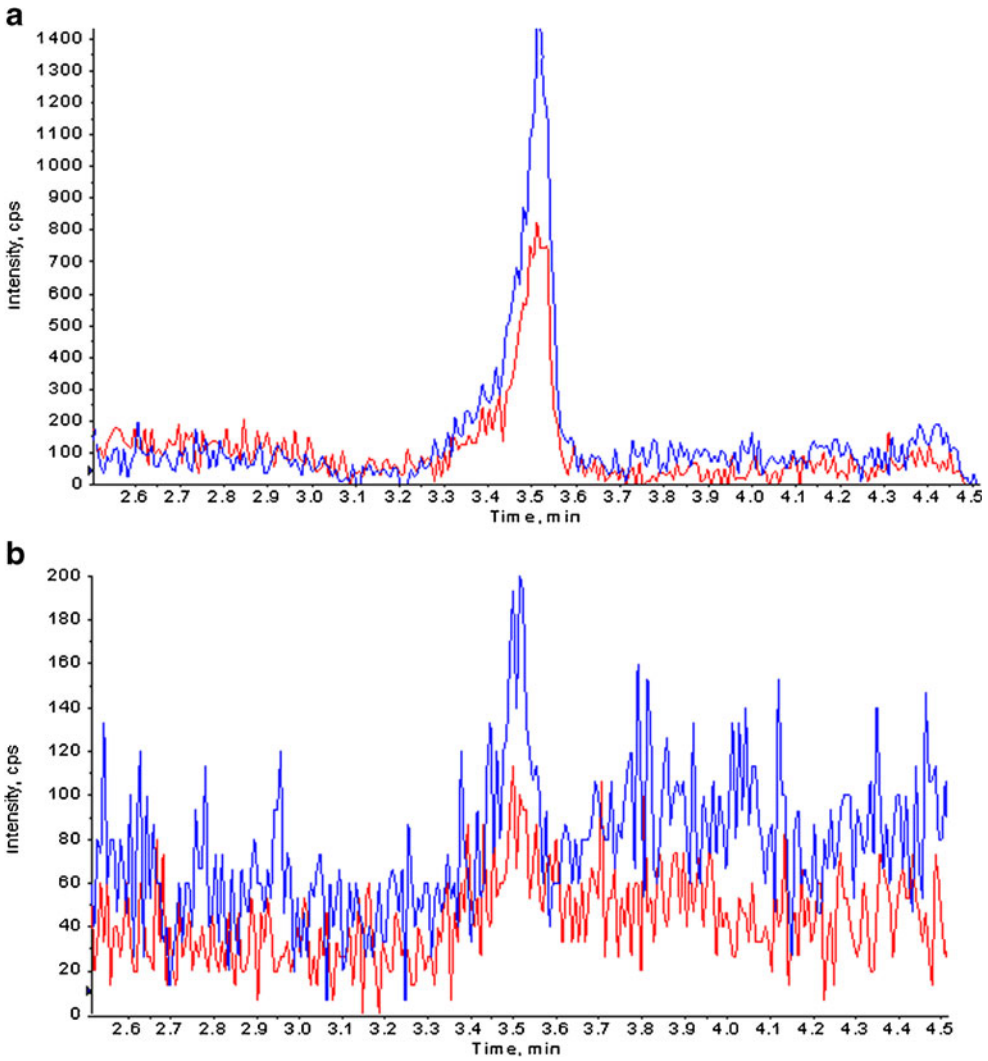

Fig. 3 **a** LC-MS/MS chromatogram obtained from analysis of extracted urine of children (BPA concentration of 0.38 ng/mL; signal to noise ratio (S/N) 13.9); **b** LC-MS/MS chromatogram of the method blank sample (BPA concentration <0; signal to noise (S/N) 1.9). The *blue line* refers to the transition *m/z* 227.1→*m/z* 212.1 (quantifier), while the *red line* to *m/z* 227.1→*m/z* 133.2 (qualifier)

intake based on the daily caloric guidelines set for children with normal physical activity.

BPA was detected in 95 % of the samples (from normal and obese patients) with total concentration ranging from 0.2 to 1.45 ng/mL. Figure 3a shows the LC-MS/MS chromatogram of a urine sample (measured BPA concentration at 0.38 ng/mL), while the Fig. 3b shows the LC-MS/MS chromatogram of the corresponding method blank. The mean value of concentration of BPA in the urine of obese children ($N=89$) is found to be equal to 0.72±0.23 ng/mL while, for the normal weight, mean value is found to be equal to 0.47±0.12 ng/mL (Fig. 4), acknowledging our small number ($N=16$).

Analyzing the data in relation to the sex difference (Fig. 4), the mean concentration of BPA in obese girls ($N=40$) is 0.65 ±0.13 ng/mL and is 41 % greater than that of normal weight (0.46±0.06 ng/mL). The mean concentration of BPA in obese boys ($N=49$) is 0.79±0.16 ng/mL and it is 65 % greater than that of normal weight (0.48±0.09 ng/mL).

The concentrations of BPA in obese boys, also, exceed more than 20 % the corresponding values in obese girls. Indeed, for normal weight children, the difference in BPA concentration in the two sexes is not relevant.

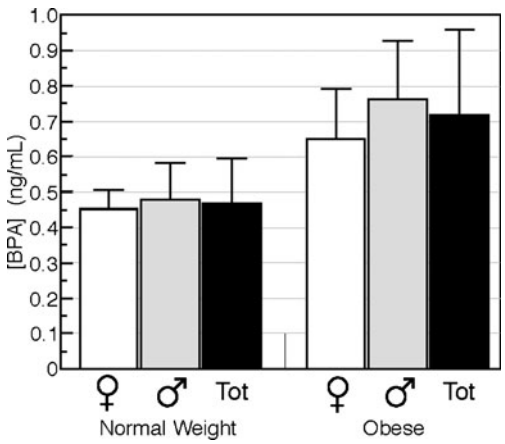

Fig. 4 The mean values of BPA concentration in urine of obese and normal weight children (boys and girls)

Conclusion

The proposed study describes a triple quadrupole LC/ESI-MS/MS method for measuring BPA levels in human urine. The method was in-house validated, and is simple, reliable, sensitive, accurate, and precise. The use of specific SPE cartridges for affinity chromatography purification allows obtaining high percentages of recovery while the use of the internal standard allows correcting for the matrix effects.

The high sensitivity of tandem mass spectrometry determination allows detecting low levels of BPA in urine samples of children, and makes the method an excellent analytical tool for routine analysis to assess human health risks due to low-dose BPA exposures.

The results suggest that children residing in Regione Campania (Southern Italy) have positive urinary concentrations of BPA. In addition, based on the analysis of one spot urine sample, obese children have higher urinary concentration of BPA compared to normal weight children. The study has several limitations including its small sample size and it should be considered as a pilot study aimed to elucidate children's exposure to BPA. Because urinary BPA was only measured at a single time point in this study, the potential for measurement error exists since exposure is likely to be variable over time. We do not know how and when the intake occurs, whether the sources of calories consumed differ between obese and normal weight children. Certainly, obese children may drink more canned or bottled beverages, or eat more canned food, and thus are potentially more exposed to BPA. This study is the first to document the BPA levels in Italian children urine and suggests the need for further studies with large sample size along with detailed clinic information to associate urinary BPA concentration with obesity and the correlated pathologies such as insulin resistance.

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