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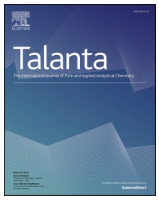

In-situ acetylation followed by liquid-liquid extraction and gas chromatography – mass spectrometry for the determination of bromophenols in urine

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ABSTRACT

A novel and simple method combining *in-situ* acetylation, liquid-liquid extraction and gas chromatography-mass spectrometry (GC-MS) has been developed for the quantification of 10 bromophenols in urine, used as biomarkers of exposure to polybrominated diphenyl ethers. The analytical process involves an enzymatic hydrolysis of the bromophenol glucuronide fraction followed by an aqueous derivatization of the phenol group with acetic anhydride. A subsequent liquid-liquid extraction of the sample with hexane allows the injection of the organic layer, using a programmed temperature vaporizer, into a gas chromatograph coupled to a single quadrupole mass spectrometer. Quantification is performed by the standard addition method. Limits of detection are in the pg mL^{-1} range. Trueness, assessed in terms of percentages of recovery, varies between 100 % and 118 % in synthetic urine and between 79 % and 117 % in human urine. Precision, assessed at two different levels, 0.25 ng mL^{-1} and 2.5 ng mL^{-1}, shows values of relative standard deviation below 14 % both in intra- and inter-day studies for both matrices. The method has been applied to the analysis of seven urine samples, measuring concentrations higher than the LOQ in three of them. These levels are in agreement with others found in literature, but they have been obtained by applying a much simpler and faster protocol. In addition, the replacement of silylating reagents by acetic anhydride, to derivatize the phenol moiety, provides a greener alternative to other GC-MS procedures published up to date.

1. Introduction

Brominated flame retardants (BFRs) are a class of synthetic compounds that are added to a great variety of consumer products, such as textiles, plastics, building materials, or electronic goods to reduce their flammability and comply with fire safety regulations [1–3]. Among BFRs, polybrominated diphenyl ethers (PBDEs) were widely used over decades until the early 2000s [4]. Their use as additives, *i.e.*, not chemically bound to the containing material, facilitates their leakage into the surrounding environment, from where they are subjected to long-range transport due to their environmental persistence [5]. This, together with their hydrophobicity, has promoted their constant bioaccumulation in both human and wildlife [6], garnering significant concern due to their known endocrine disruption effects [7,8]. Although PBDE mixtures were formally recognized as persistent organic pollutants by the Stockholm Convention in 2009 [9], and gradually withdrawn from manufacture and importation in the European Union and the US along 2003–2013 [10], human exposure still occurs due to their release from elder consumer goods and the intake of dust or contaminated food [11].

Assessing population exposure to PBDEs has been usually performed by quantifying selected BDE congeners, together with their hydroxylated and methoxylated metabolites, in human breast milk or blood [12–17]. Other human tissues, such as lung, liver, or adipose tissues have also been used [18–21]. However, sampling of tissues is an invasive approach that requires specialized health-care workers, and sampling of milk, although non-invasive, is restricted to lactating women, thus not reflecting general population exposure [22]. Alternatively, sampling of urine can be regarded as a non-invasive approach clearly appropriate for large-scale risk assessment studies. Phase II metabolites, *i.e.* glucuronidated and sulphated analogues of hydroxylated BDEs and bromophenols (BPs), have been proposed as suitable urinary biomarkers of exposure to PBDEs [7]. Actually, glucuronide and sulphate conjugates of 2,4-dibromophenol (2,4-DBP) and 2,4,6-tribromophenol (2,4,6-TBP)

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have been quantified in urine samples paired to blood plasma samples positive in PBDEs, hydroxylated BDEs, and/or methoxylated BDEs [23]. Total amounts of hydroxylated BDEs or BPs (summing up their free and conjugated forms, after an enzymatic hydrolysis of the latter) were measured in neonatal urine and correlated to the presence of hydroxylated BDEs in mother placenta and breast milk [24]. In another study, aimed to reveal the potential relationship between exposure to PBDEs and urinary BPs profiling, Feng et al. quantified up to 4 BP congeners in urine samples [25]. More recently, Lin et al. addressed the determination of hydroxy-BDEs and BPs in urine [26] and of PBDEs and BPs in paired serum, hair, and urine samples [27] as biomarkers of occupational exposure to PBDEs.

In the studies of Lin et al. [26,27], BPs were extracted from urine by a solid-phase extraction (SPE) stage, and subsequently separated and detected by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). The combination of SPE and HPLC-MS/MS provides low limits of detection and highly selective methods. However, it is expensive and, particularly if the SPE is performed off-line, time-consuming. Alternatively, Chen et al. [24] and Feng et al. [25] applied a liquid-liquid extraction (LLE) of the analytes followed by the analysis of the extract by gas chromatography-mass spectrometry (GC-MS), resulting in two analytical methods more accessible than SPE-HPLC-MS/MS to most laboratories. In both cases, authors submitted the sample to several cycles of extraction, evaporating the extracts to dryness prior to their reconstitution in *N,O*-bis--trimethylsilyl trifluoroacetamide (BSTFA, a derivatizing agent aimed to replace the active hydrogen of the phenol group by a silyl group). Although undoubtedly effective, the need of performing several LLE cycles, together with consecutive steps of extract evaporation and analyte silylation (which requires heating for 0.5–1 h) makes these procedures long, tedious, and susceptible to a decreased reproducibility. Moreover, silylating agents are toxic, require the use of anhydrous conditions, and imply the injection of a reactive mixture onto the column [28].

To address these issues, here, and for the first time in urine, we propose an *in-situ* aqueous acetylation of BPs prior to the extraction of the resulting derivatives in an organic solvent. After enzymatic hydrolysis (to quantify the total amount of BPs, including free and conjugated forms), samples are treated with acetic anhydride and submitted to a single LLE cycle. The selection of the appropriate extraction conditions, including working under supersaturation of NaCl, is crucial to allow the direct injection of the extract into the GC-MS system through a programmed temperature vaporizer (PTV). Unlike other LLE-based methods published up to date [24,25], neither further evaporation-reconstitution steps nor subsequent tedious cleaning protocols are needed, resulting in a very simple and fast procedure with similar performance.

2. Materials and methods

2.1. Chemicals and reagents

The following target BPs were selected based on their previous positive detection in urine according to the studies of Feng et al. [25], Chen et al. [24], and Lin et al. [26]: 2-bromophenol (2-BP), 4-bromophenol (4-BP), 2,4-dibromophenol (2,4-DBP), 3,4-dibromophenol (3,4-DBP), 2,4,5-tribromophenol (2,4,5-TBP), 2,4,6-tribromophenol (2,4,6-TBP), and 2,3,4,6-tetrabromophenol (2,3,4,6-tetraBP). In addition, four non-previously detected BPs were considered: 3-bromophenol (3-BP), 2,3-dibromophenol (2,3-DBP), 2,5-dibromophenol (2,5-DBP), and 2,3,4,5,6-pentrabromophenol (pentaBP).

Standards of 2-BP (99.6 % purity), 3-BP (99.46 % purity), 2,4-DBP (98 % purity), 2,6-DBP (99.6 % purity), 2,4,6-TBP (99.5 % purity), and pentaBP (99.6 % purity) were supplied by LGC standards (Teddington, Middlesex, UK). Standards of 4-BP (98 % purity), 2,3-DBP (98 % purity), 2,5-DBP (98 % purity), 2,4,5-TBP (98 % purity), and 2,3,4,6-tetraBP were supplied by Toronto Research Chemicals (Toronto, ON, Canada). A Wasserlab Ultramatic water purification system (Noain, Spain) was used to produce ultra-high-quality water (UHQ). β-Glucuronidase enzyme from *Helix pomatia* (Type H-5, lyophilized powder, ≥400,000 units/g solid), creatinine, hexane (GC residue analysis grade), toluene (HPLC grade), methanol (MeOH, HPLC grade), *tert*-butyl methyl ether (MTBE, GC residue analysis grade), ethyl acetate (GC residue analysis grade), sodium acetate (99 %), potassium hydrogen carbonate (99.5 %), potassium hydrogen phosphate, potassium dihydrogen phosphate, acetic acid (99.8 %), pyridine (anhydrous, 99.8 %), acetyl chloride (>99.9 %), and acetic anhydride (>99 %) were supplied by Sigma-Aldrich (Steinheim, Germany). Sodium chloride, magnesium sulphate, ammonia solution in water (25 %), and hydrochloric acid (37 %) were supplied by Scharlau (Barcelona, Spain). Potassium chloride, calcium chloride, and urea were supplied by Panreac (Barcelona, Spain).

2.2. Standard solutions, enzyme suspension, and synthetic urine preparation

Stock solutions (5000 μg mL^{-1}) of each standard were prepared in MeOH and stored in the dark at −20 °C until use. From individual solutions, several mixed stock solutions of the 11 BPs (5.0–0.5 μg mL^{-1}) were prepared in MeOH and also stored at −20 °C. Before use, these solutions were left to warm up to room temperature.

The enzymatic suspension of β-glucuronidase (from *Helix pomatia*) was prepared by precisely weighing a specific quantity of lyophilized powder and suspending it in a known volume of 1 M acetic/acetate buffer at pH 5.5. This process aimed to attain a final suspension exhibiting a glucuronidase activity of 100,000 units per mL of suspension [26].

Synthetic urine was prepared as described in Ref. [29], dissolving precise amounts of sodium chloride, potassium chloride, calcium chloride, urea, magnesium sulphate, and creatinine in an aqueous solution containing ammonia and hydrochloric acid.

2.3. Human urine collection and treatment

First morning urine samples were collected from seven healthy adult individuals that had previously provided written informed consent. Samples were collected in disposable sterile collection cups and preserved at −20 °C until analysis (usually within 24–72 h).

Upon thawing at room temperature, samples were vigorously vortexed at maximum speed for 1 min to ensure a thorough homogenization. Their creatinine content was measured by the Jaffe method [30]. For the LLE-GC-MS analysis, 7.0 mL of sample and 3.5 mL of acetic acid/sodium acetate buffer (1 M, pH 5.5) were mixed in a 11 mL glass centrifuge tube. Thirty-five microliters of the working β-glucuronidase suspension (100,000 units mL^{-1}) were added and the solution was incubated at 37 °C overnight. After centrifugation at 2950 *g* for 5 min, 9.0 mL of the supernatant were treated with 150 μL of acetic anhydride. The mixture was vortexed for 5 min to ensure an adequate interaction between the derivatization reagent and the BPs. Finally, 3.0 g of NaCl and 0.5 mL of hexane were added; the mixture was vigorously vortexed for 5 min, and centrifuged at 2950 *g* for another 5 min. The resulting organic phase was transferred to a glass insert in a vial for its subsequent injection into the GC-MS system.

2.4. Instrumental configuration

An MPS2 Multi-Purpose Sampler (Gerstel, Mülheim an der Ruhr, Germany) was used to inject 40 μL of the extract into a programmable temperature vaporizer (PTV, CIS-4, Gerstel) equipped with a liner filled with deactivated glass wool (71 mm × 2 mm I.D., Gerstel CIS-4). Injection was performed in solvent vent mode using the following parameters: initial temperature of 70 °C, maintained for 0.80 min with a vent flow of 50 mL min^{-1} (2.0 psi); following solvent venting, the split

valve was closed and the liner underwent rapid heating (at a rate of 12 °C s^{-1}) up to 275 °C; the injection time was set to 1.5 min; subsequently, the temperature was held for 5 min post-injection, while the split valve remained open to enable cleaning with a split vent purge flow of 100 mL min^{-1}. Finally, liquid CO_2 (Air Liquide) was utilized to restore the initial temperature.

The GC system used was an Agilent 7890A model (Agilent Technologies, San Jose, CA, USA) equipped with an HP-5MS UI capillary column (30 m × 0.25 mm, 0.25 μm; J&W Scientific, Folsom, CA, USA). Helium N50 (99.9999 % pure, Air Liquide) served as carrier gas at a flow rate of 1.0 mL min^{-1}. Initially, the oven temperature was set at 60 °C, maintained for 2 min; next, the temperature was ramped up at 15 °C min^{-1} until 310 °C, and held for 1 min. The entire chromatographic run was 19.67 min. An Agilent 5978C inert XL MSD quadrupole mass selective detector (MSD) equipped with an electronic ionization source (70 eV) was used for detection and quantification. Operating temperatures were set at 230 °C for the ion source, 150 °C for the quadrupole, and 280 °C for the transfer line. Analyses were performed in Selected Ion Monitoring (SIM) mode with a solvent delay of 5.00 min. Characteristic *m/z* ratios (one quantification and two qualifier *m/z* ratios per analyte) were selectively monitored using a dwell time of 10 ms. For data acquisition, the MSD ChemStation, Ver. E.02.00.493 software from Agilent Technologies was used. The identification process relied on the NIST_98 (NIST/EPA/NIH Mass Spectral Library, version 2.0) database.

2.5. Quantification and method validation

Analytes were quantified by the standard addition method (see sections 3.5 and 3.6 for further details). To assess linearity and matrix effects, two calibration curves were prepared: one in synthetic urine and the other one in an analyte-free human urine sample. For synthetic urine, 6.0 mL of sample and 3.03 mL of buffer (to account for the buffer plus the enzymatic suspension volume in real urine) were poured in eight centrifugal tubes and spiked with progressively higher analyte concentrations: 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00 and 10.0 ng mL^{-1}. These solutions were submitted to the standard sample preparation protocol: acetylation with 150 μL of acetic anhydride followed by LLE in 0.5 mL of hexane with 3.0 g of NaCl. For human urine, eight aliquots were prepared as detailed in section 2.3, including the enzymatic hydrolysis and centrifugation (prior-to-acetylation) steps. In both matrices, a non-spiked sample was prepared (9^{th} tube) to check for potential contamination issues (procedural blank in synthetic urine) or for the natural presence of BPs in the human sample (which turned out not to show any BP above the limit of detection, LOD). Determination coefficients (R^2) were obtained after plotting analyte peak areas versus their theoretical concentrations in the calibration extracts. Matrix effects were evaluated by comparing the slopes of the calibration curves in synthetic and real urine by means of a Student *t*-test (significance level 0.05 %).

Limits of quantification (LOQs) and LODs were calculated as the analyte concentration providing a signal-to-noise ratio of 3 (LOD) and 10 (LOQ). Trueness was evaluated from recoveries, expressed as percentages and calculated as the ratio between the measured concentration and the theoretical spiked concentration (0.25 ng mL^{-1}). Precision was assessed from the relative standard deviation (%RSD) obtained from the analysis of samples spiked at two different levels: 0.25 ng mL^{-1} and 2.5 ng mL^{-1}. Intra-day precision was estimated from the analysis of five samples of each level within the same day. Inter-day precision was estimated from the analysis of five samples of each level in two different days over two weeks.

Procedural blanks (non-spiked synthetic urine samples) were extracted and analysed every day to ensure the absence of contamination issues during the whole analytical procedure. Solvent blanks (injections of clean hexane) were analysed between samples and at the beginning and the end of every set of samples to check for potential memory effects in the instrument.

3. Results and discussion

3.1. Enzymatic hydrolysis

Quantifying the total amount of BPs excreted in urine, in both free and conjugated forms, requires a first hydrolysis step to deconjugate the potential glucuronidated and sulphated BPs occurring in the sample. The unavailability of commercial conjugated BPs prevented the optimization of the hydrolysis procedure; hence, this was conducted following the conditions established by Lin et al. [26]: enzymatic hydrolysis with β-glucuronidase from *Helix pomatia* (exhibiting both glucuronidase and sulphatase activity) by addition of 500 units of enzymatic activity per mL of urine, and incubation at 37 °C overnight.

3.2. In-situ acetylation of BPs

Initially, individual standards of the underivatized analytes were prepared in ethyl acetate and injected into the GC-MS system. However, either no signals or very weak signals were observed. Thus, an aqueous acetylation prior to the LLE was proposed as derivatization strategy with a dual purpose: (i) to enhance the volatility of BPs; and (ii) to improve their partitioning to the organic phase. Various well-known acetylation reagents and derivatization conditions, typically consisting of a carboxylic acid derivative in basic medium, were tested. First, 50 μL of acetyl chloride and 50 μL of pyridine were added to 1.0 mL of UHQ-water containing 1 μg mL^{-1} of all the analytes. The mixture was vortexed for 5 min and the analytes were liquid-liquid extracted in 1.0 mL of ethyl acetate. Under these conditions, only signals corresponding to the acetylated derivatives were observed. To avoid the use of pyridine, 1.0 mL of 0.4 M of potassium hydrogen carbonate in UHQ was used as basic medium, but the reaction did not work. Replacing acetyl chloride by acetic anhydride (in 1.0 mL of 0.4 M of potassium hydrogen carbonate) provided chromatographic signals very similar to those achieved with pyridine and acetyl chloride, demonstrating the effectiveness of the reaction in this case.

The influence of lowering the pH was further assessed with 1 M of potassium hydrogen phosphate/dihydrogen phosphate buffer at different pHs: 7.5, 6.5 and 5.5. In all cases, 1.0 mL of buffer was spiked with all the analytes and acetic anhydride, vortexed for 5 min, and liquid-liquid extracted in 1.0 mL of ethyl acetate (n = 3). No significant differences were observed between the signals obtained at different pHs (data not shown). Thus, 5.5, the pH value at which the urine is buffered for the enzymatic hydrolysis, was selected. Phosphate buffer was then replaced by acetic acid/acetate buffer 1 M, the one recommended, and used, to prepare the β-glucuronidase suspension. The minimum volume required to ensure a correct sample buffering was tested with three different urines (initial pH values: 5.7, 6.5, and 6.8), confirming the need of a urine-to-buffer ratio of 2:1 [26].

The optimal volume of acetic anhydride for the acetylation reaction was investigated with two distinct urines spiked with all the analytes. Different volumes (10 μL, 25 μL, 50 μL, and 100 μL) of acetic anhydride were added to 1.5 mL aliquots of hydrolysed sample (comprising 1.0 mL of urine and 0.5 mL of buffer), and the samples were processed as stated above (n = 3). Analytes with higher pK_a values, such as mono- and di-BPs, showed an increased signal as the volume of acetic anhydride increased. Conversely, analytes with lower pK_a values (tri-, tetra-, and penta-BP), displayed the opposite trend (Figure S1 in the Supplementary Material). As a compromise, 25 μL was selected as the final volume of acetic anhydride per 1.5 mL of hydrolysed sample (*i.e.* per 1.0 mL of urine).

Finally, the potential adsorption of derivatized and non-derivatized BPs to cells and other particles precipitating after the freeze-and-thaw of urine was assessed. Two distinct urines were frozen and thawed, buffered, spiked with 100 ng mL^{-1} of all the analytes, and allowed to age for 15 h before enzymatic hydrolysis. For each sample, two sets of studies were conducted: in the initial set (n = 3), the hydrolysed sample

was centrifuged, and the derivatization was performed on 1.0 mL of the supernatant, followed by a LLE of the acetylated analytes in 1.0 mL of ethyl acetate. In the subsequent set (n = 3), the acetylation was performed prior to centrifugation, followed by the LLE of 1.0 mL of the supernatant in 1.0 mL of ethyl acetate. In both samples, all analyte signals were higher when the centrifugation was performed in the first place, preceding the aqueous acetylation (Fig. 1). This could be attributed to a lower consumption of acetic anhydride once the sediment has been removed, or to a reduced trend of non-acetylated BPs, as compared to their acetylated derivatives, to get adsorbed onto the urine precipitate. Consequently, the final adopted protocol consisted of freezing-and-thawing the samples, performing the enzymatic hydrolysis on an aliquot of raw urine, centrifuging it, removing the precipitate, and then proceeding with the acetylation process.

3.3. Sample preparation conditions

For the LLE of the acetylated BPs, different organic solvents (hexane, toluene, MTBE, and ethyl acetate) were assessed under conditions of sample supersaturation (50 % of sample mass) with NaCl and without the addition of NaCl. In both cases, 9.0 mL of buffer-diluted and hydrolysed urine (comprising 6.0 mL of urine, spiked with the target analytes at 2.5 ng mL^{-1}, plus 3.0 mL of buffer) were extracted with 0.5 mL of organic solvent. The maximum sample and solvent volumes were determined by the capacity of the centrifugal tubes used for the extraction and the centrifugation (11 mL). For most of the investigated solvents, the absence of salt prevented the clear separation of the two phases after the LLE and the recovery of the organic extract. Therefore, only the extracts obtained under NaCl supersaturation conditions were subjected to analysis.

For di-BPs and tri-BPs, ethyl acetate yielded the highest extraction efficiencies (Fig. 2). However, this solvent was unable to extract pentaBP, and while mono-BPs were presumably extracted, they could not be quantified due to the presence of several interferences at their retention times. A similar pattern was observed with MTBE (Fig. 3). Less polar solvents (toluene and hexane) exhibited cleaner chromatograms, although toluene still presented some interferences at the retention times of mono-BPs and di-BPs. Conversely, hexane did not only extract all BP derivatives (Fig. 2), but also provided cleaner chromatograms (Fig. 3). Only 2-BP could not be quantified in one of the samples due to a co-eluting interferent sharing its quantification and qualifier *m/z* ratios. This interference was repeatedly observed in urines from different individuals and, thus, 2-BP was finally excluded from the target set of analytes. In terms of extraction efficiencies, and compared to toluene, hexane provided lower values for mono-BPs, di-BPs, and 2,4,6-TBP; a comparable efficiency for 2,4,5-TBP; the double for tetra-BP, and it was the only solvent that successfully extracted pentaBP. Thus, it was selected as the solvent of choice for the LLE.

3.4. PTV-GC-MS analysis

Instrumental conditions were optimized by analysing an organic extract of the acetylated BPs obtained by the LLE of a UHQ-water sample spiked with 1 μg mL^{-1} of all the analytes.

The mass spectrometer acquisition mode was configured for Selected Ion Monitoring (SIM). Table S1 in the Supplementary Material shows the retention times and the *m/z* ratios selected for the quantification (in bold) and confirmation of the acetylated BPs. These *m/z* values agree with those compiled in the NIST 2.0 database for the acetylated derivatives of mono-, di- and tri-BPs. Since this database does not include acetylated 2,3,4,6-tetraBP and pentaBP, their identification was confirmed by analysing the fragmentation patterns in their mass spectra. For instance, the fragmentation of the CH_3CO moiety, observed in the spectra of acetylated mono-, di- and tri-BPs, is also seen for tetraBP and pentaBP (Supplementary Material, Figure S2). The dwell time varied between 10 and 50 ms; for all the analytes, best peak definition and most intense signals were observed with a value of 10 ms.

The oven temperature programme was optimized varying the initial temperatures between 60 °C and 110 °C and the final temperatures between 280 °C and 310 °C. Optimal conditions were as follows: initial temperature of 60 °C (held for 2 min) ramped to 310 °C (held for 1 min) at a rate 15 °C min^{-1}.

Since the injection of large volumes of organic extract enhances the mass of analytes reaching the mass spectrometer and, consequently, the method sensitivity, the solvent vent was selected as the injection mode to remove the excess of organic solvent inside the liner, before transferring the analytes onto the chromatographic column. Several factors influencing the efficiency of this injection mode were assessed: vent time, vent flow, injection time, and PTV initial temperature (vent temperature). Vent time was varied from 0.5 to 1.0 min; vent flow from 50 to 100 mL min^{-1}; injection time from 1.0 to 3.0 min; and PTV initial temperature from 50 to 100 °C, while the PTV final temperature (injection temperature) was maintained at the maximum recommended by the liner manufacturer, 275 °C. Optimal conditions were as follows: vent time 0.75 min; vent flow 50 mL min^{-1}; injection time 1.5 min; and PTV initial temperature 70 °C.

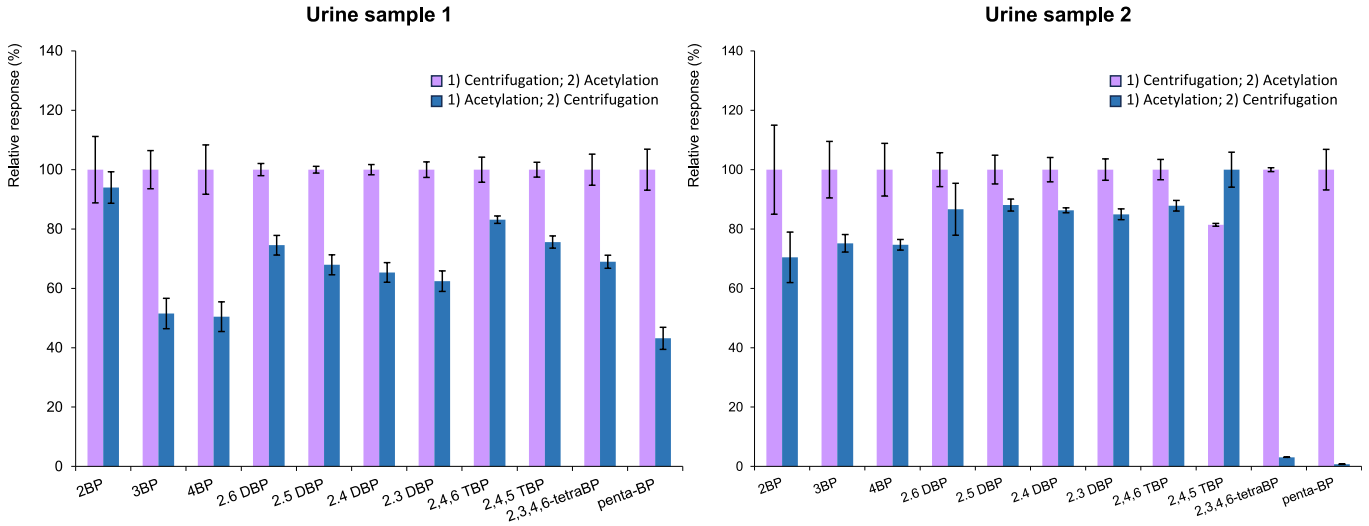

Fig. 1. Relative responses (expressed in percentage) of the 11 derivatized BPs when their acetylation is performed: (i) after sample centrifugation and precipitate removal; (ii) before sample centrifugation; two distinct urines, three replicates per urine and set of experiments.

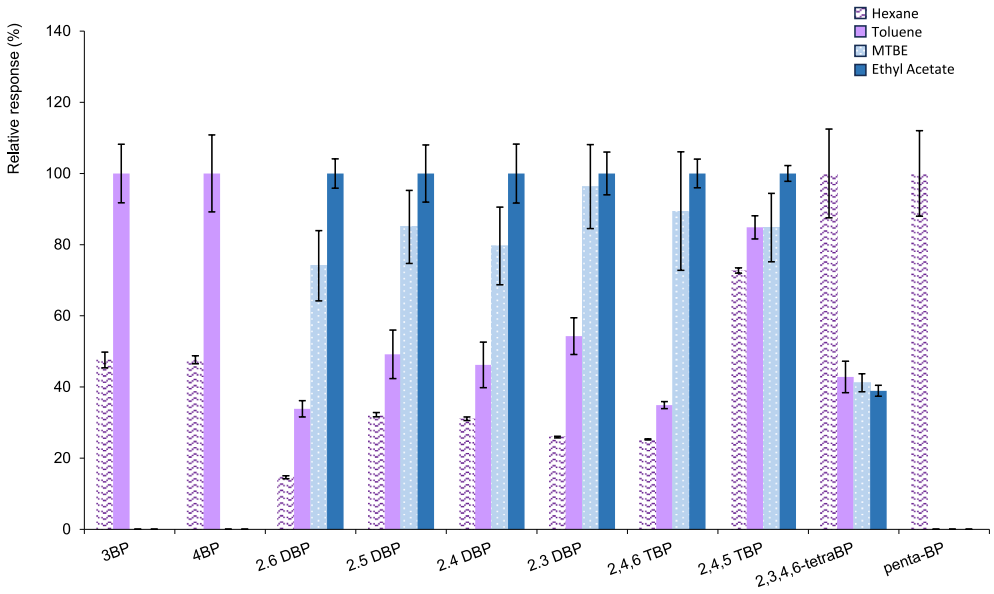

Fig. 2. Relative responses (expressed in percentage) of the 10 derivatized BPs in urine extracts in hexane, toluene, methyl *tert*-butyl ether (MTBE), and ethyl acetate; 9.0 mL of hydrolysed sample liquid-liquid extracted to 0.5 mL of solvent; three replicates per solvent.

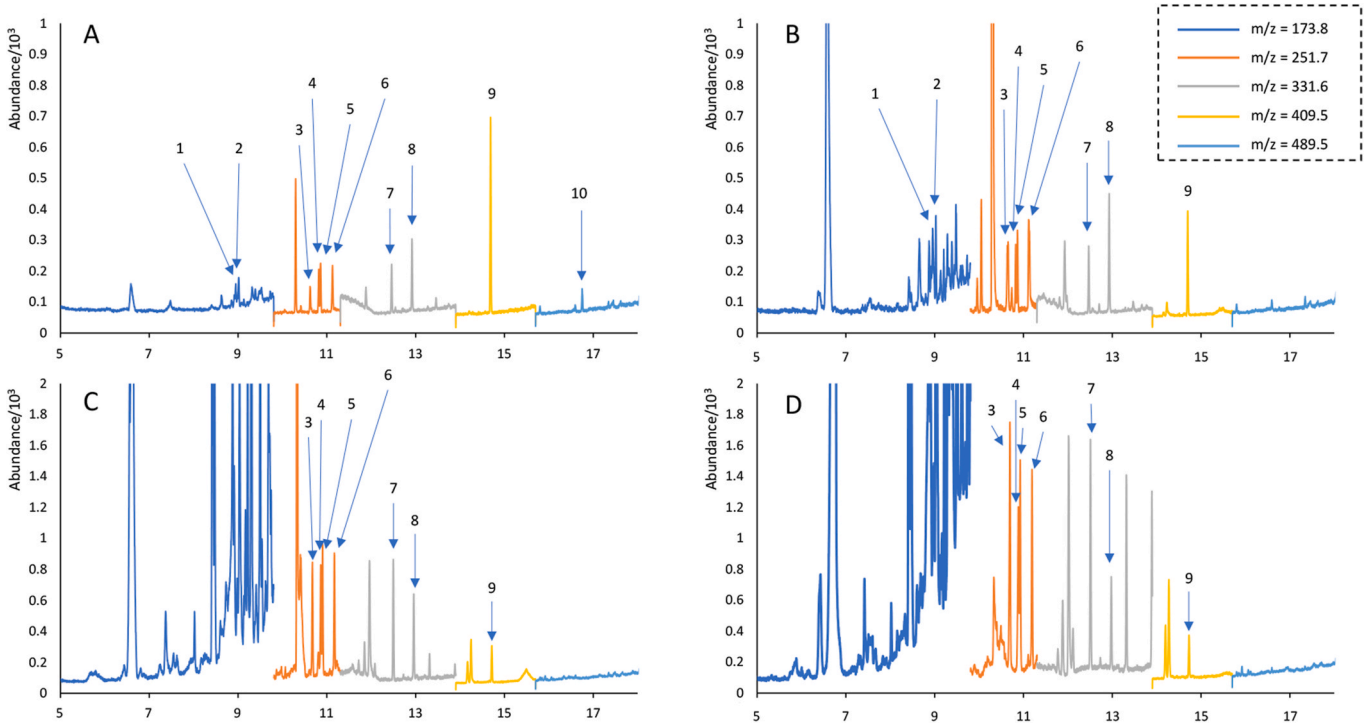

Fig. 3. Extracted ion chromatograms of a urine extract in (A) hexane, (B) toluene, (C) MTBE, and (D) ethyl acetate; 3-BP (1), 4-BP (2), 2,6-DBP (3), 2,5-DBP (4), 2,4-DBP (5), 2,3-DBP (6), 2,4,6-TBP (7), 2,4,5-TBP (8), 2,3,4,6-tetraBP (9), pentaBP (10).

Different types of liners were evaluated: empty baffled deactivated-glass liners and liners packed with different materials (glass wool and Tenax TA®). The latter yielded no signals after the GC-MS analysis, likely due to the degradation of all the analytes in the packing material. When comparing the signals obtained with the other two types of liners, the best results were achieved with the glass wool-packed liner (Figure S3).

Finally, the volume of the hexane extract to be injected was investigated in the range from 10 to 50 μL. A linear signal increase was observed from 10 to 40 μL, where a plateau started to be reached for all the analytes except for 2,3,4,6-tetraBP and pentaBP, which still presented a linear increase. As a compromise between enhanced signals and preserving the lifespan and usability of the liner packing material, 40 μL was selected as the optimal injection volume (Figure S4; for clarity, data are only presented for one representative BP of each type).

3.5. *Method validation, matrix effects, and blank assessment*

The optimized method was assessed in terms of linearity, LODs and LOQs, trueness, and intra- and inter-day precision. The analytical

performance results are compiled in Table 1 (results in synthetic urine) and Table 2 (results in real urine). All analytes exhibited good linear behaviour, with R^2 values between 0.9990 and 0.9997 in synthetic urine and between 0.9941 and 0.9993 in real urine. ANOVA tests were conducted to assess the validity of the results, and no lack of fit was observed in any case. To compare the slopes of the calibration curves in both matrices, a Student *t*-test (with a significance level of 0.05 %) was also performed. The obtained *p*-values were consistently lower than 0.05 in all cases, confirming the presence of matrix effect and the need of applying the standard addition method for quantification.

Fig. 4 shows the chromatograms of two synthetic urine and two human urine samples, spiked with the 11 analytes at two distinct levels (0.25 ng mL^{-1} and 2.5 ng mL^{-1}), and processed following the proposed method.

Recovery percentages, to assess method trueness, varied from 100 to 118 % in synthetic urine and from 79 to 117 % in human urine. In terms of intra-day precision, RSD values in synthetic urine were in the range of 2.5–8.9 % at the low level (0.25 ng mL^{-1}) and of 3.3–8.8 % at the high level (2.5 ng mL^{-1}). Inter-day RSD values were 4.5–13.7 % at the low level and 4.4–9.6 % at the high level. In human urine, RSD percentages for the intra-day analyses were between 4.7 and 7.2 % at the low level, and between 3.1 and 7.1 % at the high level; inter-day results varied in the range of 5.3–10.4 % at the low level and 3.1–9.7 % at the high level.

Except for pentaBP, LOD values varied from 5.8 to 17 pg mL^{-1} in synthetic urine and from 7.8 to 27 pg mL^{-1} in human urine. As it is discussed in section 3.7, these values are similar to or slightly higher than those reported in literature by a GC-MS method [25]. However, they are still low enough to allow the quantification of these analytes in urine samples (see section 3.6) [7]. For pentaBP, the LOD was 71 pg mL^{-1} in synthetic urine and 109 pg mL^{-1} in real urine. This analyte is the heaviest one and, hence, the least suitable for GC analysis due to its low volatility.

Finally, no analyte signals were observed neither in the procedural blanks nor in the solvent blanks, demonstrating the absence of contamination problems or memory effect.

3.6. Analysis of urine samples

To demonstrate its usability, the optimized method was applied to the analysis of urine collected from seven healthy adult individuals. Analyte concentrations and their confidence intervals, in ng mL^{-1} and normalized to creatinine content, are displayed in Table 3. As it is shown, three samples presented analyte levels above the LOQ of the method. One of them (sample from subject 1) presented quantifiable concentrations for six BPs: the two targeted mono-BPs (3-BP and 4-BP), three di-BPs (2,3-DBP, 2,4-DBP and 2,5-DBP) and 2,4,6-TBP. The latter was also quantified in the samples from subjects 2 and 3, and 4-BP was also measured above the LOQ in the sample from subject 3. In some other cases, analyte levels were above the LOD but below the LOQ and, thus, could not be quantified.

Compared to the concentrations reported in literature (Table S2 in the Supplementary Material), the values measured here for 4-BP and 2,4-DBP are in line with the levels measured in general adult population, but considerably lower than the concentrations measured in occupationally exposed workers (e-waste dismantling workers) [26,27]. Conversely, 2,4,6-TBP levels reported here (0.04–0.36 μg g^{-1} creatinine) are always lower than the values measured in general adult population [25,27].

3.7. Comparison with other studies

Table S3 compares the methodological conditions and analytical performance of other three analytical methods found in literature for the quantification of BPs in urine. One of them, applied in two different studies [26,27], involves an SPE step followed by the HPLC-MS/MS analysis of the extract. Since the SPE is performed off-line, and the extract solvent is evaporated to dryness before reconstitution, the sample preparation procedure is clearly more tedious than the simple one-cycle LLE performed here. Moreover, the requirement for commercial SPE cartridges and an HPLC-MS/MS instrument significantly increases the cost of the analysis. In terms of analytical performance, LODs and %R values are comparable to those reported here: 8–93 pg mL^{-1} versus 7.8–109 pg mL^{-1} (this work), and 64–128 % versus 79–117 % (this work).

The other two analytical methods published for BPs in urine involve an LLE followed by the GC-MS analysis of the organic extract. The LLE is either a two-cycle [25] or a three-cycle procedure [24], but followed in both cases by a clean-up of the extract using silica gel cartridges, evaporation of the eluent solvent, and reconstitution. This, together with the silylation of BPs, which requires heating for 40–60 min, makes the sample preparation protocol much longer and potentially more affected by a higher irreproducibility than the one-cycle LLE, with no evaporation and no clean-up, optimized in this study. The chromatographic run time of the proposed method is also shorter: 19.7 min versus 37.5 min [25] and 48.5 min [24]. However, our procedure has been optimized for 10 analytes (originally 11) versus the 19 and 18 compounds considered elsewhere. In addition, we apply the standard addition method to correct matrix effects during analyte quantification. This entails the

Table 1
Analytical performance of the method in synthetic urine.

Compound	Linear equation	R^2	LOD (pg mL^{-1})	LOQ (pg mL^{-1})	Trueness (%)[a]	Intra-day precision (RSD, %)[b]		Inter-day precision (RSD, %)[c]	
						Low level[d]	High level[e]	Low level[d]	High level[e]
3-BP	$y = (363 \pm 4) \cdot 10^2 x + (1 \pm 2) \cdot 10^3$	0.9993	10	32	107	8.3	8.5	13.7	9.1
4-BP	$y = (350 \pm 3) \cdot 10^2 x + (1 \pm 2) \cdot 10^3$	0.9996	7.2	24	118	6.5	8.8	9.9	8.7
2,6-DBP	$y = (259 \pm 4) \cdot 10^2 x + (0 \pm 2) \cdot 10^3$	0.9990	17	57	104	7.0	5.9	10.6	7.8
2,5-DBP	$y = (542 \pm 6) \cdot 10^2 x + (0 \pm 2) \cdot 10^3$	0.9995	5.8	19	102	8.9	8.1	9.4	7.9
2,4-DBP	$y = (612 \pm 5) \cdot 10^2 x + (1 \pm 2) \cdot 10^3$	0.9997	6.0	20	107	7.0	7.1	9.6	9.6
2,3-DBP	$y = (458 \pm 4) \cdot 10^2 x + (1 \pm 2) \cdot 10^3$	0.9996	7.4	25	107	7.8	7.5	9.4	9.1
2,4,6-TBP	$y = (475 \pm 7) \cdot 10^2 x + (1 \pm 3) \cdot 10^3$	0.9990	10	33	106	7.9	6.7	7.1	8.1
2,4,5-TBP	$y = (760 \pm 10) \cdot 10^2 x - (2 \pm 4) \cdot 10^3$	0.9991	12	39	103	6.5	6.8	7.0	7.8
2,3,4,6-tetra-BP	$y = (896 \pm 13) \cdot 10^2 x + (0 \pm 5) \cdot 10^3$	0.9991	17	56	111	6.9	8.7	8.5	8.3
Penta-BP	$y = (352 \pm 5) \cdot 10^2 x - (1 \pm 1) \cdot 10^3$	0.9993	71	236	100	2.5	3.3	4.5	4.4

[a] Trueness expressed as recovery values, in %.
[b] Intra-day precision estimated from the analysis of five samples of each level within the same day.
[c] Inter-day precision estimated from the analysis of five samples of each level in two different days over two weeks.
[d] Concentrations in low level: 0.25 ng mL^{-1}
[e] Concentrations in level high: 2.5 ng mL^{-1}

Table 2
Analytical performance of the method in human urine.

Compound	Linear equation	R^2	LOD (pg mL^{-1})	LOQ (pg mL^{-1})	Trueness (%)[a]	Intra-day precision (RSD, %)[b]		Inter-day precision (RSD, %)[c]	
						Low level[d]	High level[e]	Low level[d]	High level[e]
3-BP	$y = (373 \pm 1) \cdot 10^2 x - (4 \pm 4) \cdot 10^3$	0.9955	9.0	30	91	5.7	6.6	7.9	6.5
4-BP	$y = (377 \pm 1) \cdot 10^2 x - (4 \pm 5) \cdot 10^3$	0.9948	8.1	27	99	7.0	7.0	10.4	8.4
2,6-DBP	$y = (182 \pm 7) \cdot 10^2 x - (4 \pm 5) \cdot 10^3$	0.9941	25	85	85	6.6	7.0	9.1	6.9
2,5-DBP	$y = (385 \pm 1) \cdot 10^2 x - (0 \pm 5) \cdot 10^3$	0.9949	24	81	87	6.9	4.9	5.6	7.2
2,4-DBP	$y = (467 \pm 2) \cdot 10^2 x - (0 \pm 6) \cdot 10^3$	0.9946	26	88	92	7.0	4.3	5.5	7.2
2,3-DBP	$y = (363 \pm 1) \cdot 10^2 x - (1 \pm 4) \cdot 10^3$	0.9944	27	89	93	5.9	3.9	5.7	9.7
2,4,6-TBP	$y = (317 \pm 8) \cdot 10^2 x - (1 \pm 3) \cdot 10^3$	0.9969	7.8	26	95	6.2	3.2	5.3	3.1
2,4,5-TBP	$y = (595 \pm 8) \cdot 10^2 x - (1 \pm 3) \cdot 10^3$	0.9993	20	67	79	6.6	3.1	7.1	3.2
2,3,4,6-tetra-BP	$y = (425 \pm 11) \cdot 10^2 x - (3 \pm 5) \cdot 10^3$	0.9961	10	34	88	4.7	7.1	6.7	9.1
Penta-BP	$y = (465 \pm 1) \cdot 10^2 x + (4 \pm 6) \cdot 10^2$	0.9979	109	362	117	7.2	5.6	7.2	6.1

[a] Trueness expressed as recovery values, in %.
[b] Intra-day precision estimated from the analysis of five samples of each level within the same day.
[c] Inter-day precision estimated from the analysis of five samples of each level in two different days over two weeks.
[d] Concentrations in level low: 0.25 ng mL^{-1}
[e] Concentrations in level high: 2.5 ng mL^{-1}

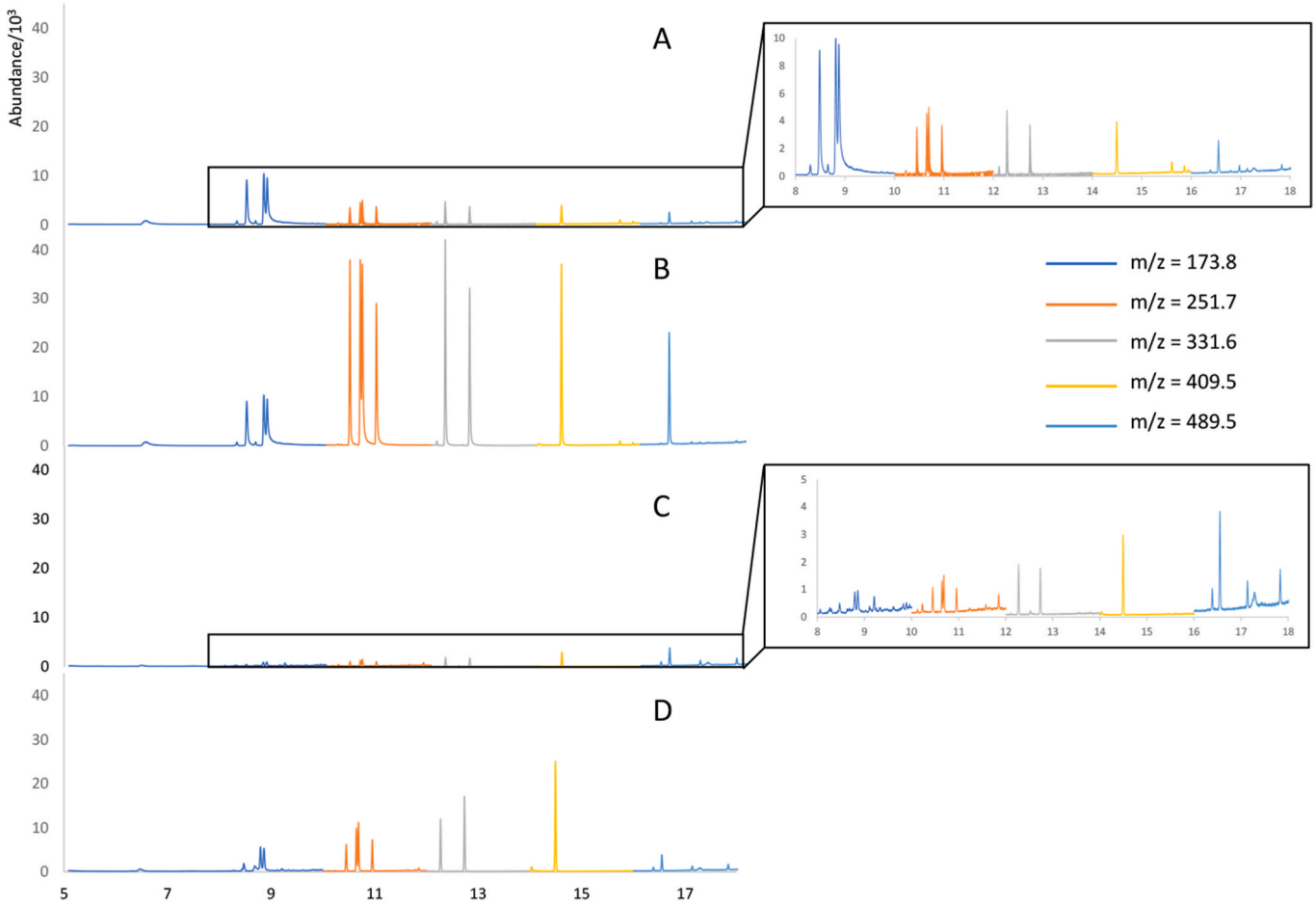

Fig. 4. Extracted ion chromatograms of two synthetic urine (A, B) and two human urine samples spiked at 0.25 ng mL^{-1} (A, C) and 2.5 ng mL^{-1} (B, D) with all the analytes and processed following the proposed methodology.

preparation and analysis of multiple spiked aliquots per sample and, ultimately, an increased analysis time compared to the quantification of individual aliquots by the internal standard method. Finally, the LODs provided by Feng et al. [25] are comparable to those achieved with this method (1.8–22.9 pg mL^{-1} versus 7.8–27 pg mL^{-1}, excluding pentaBP).

4. Conclusions

Herein we present the first quantification of bromophenols in human hydrolysed urine samples via an *in-situ* aqueous acetylation, followed by a single-cycle LLE process. The thorough selection of the extraction conditions enables the direct injection of the organic extract, with no further clean-up or evaporation, into the GC-MS system, eliminating the

Table 3
Concentrations measured in urine samples (in ng mL^{-1} and in μg g $creatinine^{-1}$).

	ng mL^{-1}			μg g $creatinine^{-1}$		
	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
3-BP	0.11 ± 0.03	<LOD	<LOD	0.07 ± 0.02	<LOD	<LOD
4-BP	0.15 ± 0.03	<LOD	0.03 ± 0.01	0.09 ± 0.02	<LOD	0.03 ± 0.01
2,6-DBP	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD
2,5-DBP	0.10 ± 0.03	<LOD	<LOD	0.06 ± 0.02	<LOD	<LOD
2,4-DBP	0.11 ± 0.03	<LOD	<LOD	0.07 ± 0.02	<LOD	<LOD
2,3-DBP	0.22 ± 0.04	<LOD	<LOD	0.13 ± 0.03	<LOD	<LOD
2,4,6-TBP	0.05 ± 0.02	0.031 ± 0.006	0.46 ± 0.04	0.037 ± 0.008	0.04 ± 0.01	0.36 ± 0.03
2,4,5-TBP	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD
2,3,4,6-tetra-BP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Penta-BP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

need for additional clean-up or evaporation steps. The injection is performed in solvent-vent mode through a PTV injector. Compared to other LLE-GC-MS methods in the literature, this procedure stands out for its simplicity and reduced analysis time despite the need of analysing multiple aliquots per sample (to correct matrix effect by the standard addition method). Furthermore, the optimized methodology exhibits good values of linearity, trueness, precision, and the LODs and LOQs are comparable to those reported in previous studies. These findings prove it as an innovative and efficient tool in the identification and quantification of BPs in urine, as biomarkers of exposure to brominated flame retardants. The validity of the method has been proved by the analysis of urine from seven adult individuals.

CRediT authorship contribution statement

Javier Peña: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Iria González-Mariño:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **José Luis Pérez Pavón:** Writing – review & editing, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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