Development and validation of an LC-MS/MS method to quantify metabolites of phthalates, including di-2-ethylhexyl terephthalate (DEHTP), and bisphenol A in human urine

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Complete List of Authors:	Frigerio, Gianfranco; University of Milan, Department of Clinical Sciences and Community Health Campo, Laura; La Fondazione IRCCS Ca' Granda Ospedale Maggiore di Milano Policlinico Mercadante, Rosa; University of Milan, Department of Clinical Sciences and Community Health Santos, Patricia; Universidad de Salamanca, Departamento de Química Analítica, Nutrición y Bromatología Missineo, Pasquale; La Fondazione IRCCS Ca' Granda Ospedale Maggiore di Milano Policlinico Polledri, Elisa; University of Milan, Department of Clinical Sciences and Community Health Fustinoni, Silvia; University of Milan, Department of Clinical Sciences and Community Health
Keywords:	LC-MS/MS, human biomonitoring, urinary metabolites, phthalates, bisphenol A
Abstract:	Rationale. Phthalates and bisphenol A are endocrine-disrupting chemicals (EDCs) spread into the environment. Recently, their use has been partially restricted, and less toxic compounds, such as di-2-ethylhexyl terephthalate (DEHTP), have been placed on the market. The aim of this work was to develop and validate a method for the simultaneous quantitation of bisphenol A and urinary metabolites of phthalates, including DEHTP. Methods. An isotopic dilution HPLC-ESI-MS/MS method for the determination of bisphenol A (BPA), monobenzyl phthalate (MBzP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP), mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP), monoethyl phthalate (MEP), and mono-n/i-butyl phthalates (MnBP/MiBP) in human urine was developed. A complete validation was carried out including the evaluation of accuracy using standard reference material and an external verification exercise. The method was applied to 36 non-occupationally exposed adults. Results. Limits of quantitation ranged from 0.02 (MECPP) to 1 µg/L (MnBP and MiBP). Relative standard deviations below 10% indicated a

	suitable precision; accuracy, evaluated using standard reference material, ranged from 74.3% to 117.5%; isotopically labelled internal standards were suitable for the correction of the matrix effect. The accuracy was confirmed by the successful participation in an external verification exercise. Levels of investigated chemicals in subjects were in line with those previously reported. Conclusions. An LC-MS/MS assay for the simultaneous measurement of BPA and phthalate metabolites in human urine was developed and validated; it is useful to investigate exposure in epidemiological studies involving the general population.

Development and validation of an LC-MS/MS method to quantify metabolites of phthalates, including di-2-ethylhexyl terephthalate (DEHTP), and bisphenol A in human urine

Gianfranco Frigerio[1], Laura Campo[2], Rosa Mercadante[1], Patricia Martín Santos[3], Pasquale Missineo[2], Elisa Polledri[1], Silvia Fustinoni[1,2]*

[1] Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy

[2] Environmental and Industrial Toxicology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

[3] Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Ciencias Químicas, Universidad de Salamanca, Salamanca, Spain

*Corresponding author.

Via S. Barnaba, 8 - 20122 Milan, Italy

e-mail: silvia.fustinoni@unimi.it

phone: +39 02 503 20116

Abstract

Rationale. Phthalates and bisphenol A are endocrine-disrupting chemicals (EDCs) spread into the environment. Recently, their use has been partially restricted, and less toxic compounds, such as di-2-ethylhexyl terephthalate (DEHTP), have been placed on the market. The aim of this work was to develop and validate a method for the simultaneous quantitation of bisphenol A and urinary metabolites of phthalates, including DEHTP.

Methods. An isotopic dilution HPLC-ESI-MS/MS method for the determination of bisphenol A (BPA), monobenzyl phthalate (MBzP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP), mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP), monoethyl phthalate (MEP), and mono-n/i-butyl phthalates (MnBP/MiBP) in human urine was developed. A complete validation was carried out including the evaluation of accuracy using standard reference material and an external verification exercise. The method was applied to 36 non-occupationally exposed adults.

Results. Limits of quantitation ranged from 0.02 (MECPP) to 1 µg/L (MnBP and MiBP). Relative standard deviations below 10% indicated a suitable precision; accuracy, evaluated using standard reference material, ranged from 74.3% to 117.5%; isotopically labelled internal standards were suitable for the correction of the matrix effect. The accuracy was confirmed by the successful participation in an external verification exercise. Levels of investigated chemicals in subjects were in line with those previously reported.

Conclusions. An LC-MS/MS assay for the simultaneous measurement of BPA and phthalate metabolites in human urine was developed and validated; it is useful to investigate exposure in epidemiological studies involving the general population.

Keywords

LC-MS/MS; human biomonitoring; urinary metabolites; phthalates; bisphenol A

1. Introduction

During the last century, the production of industrial chemicals has greatly increased, with a consequent use and dispersion in the environment. The following exposure of the general population raises concern for the possible adverse effects; particularly, those exerted by persistent organic pollutants acting as endocrine disruptors (EDCs), due to their potential ability to interfere with the physiological function of hormones.[1-3]

Phthalates are among compounds which have showed to act as EDCs, with estrogenic and androgenic disrupting properties.[3] They are used in several products, such as food packaging, plastic medical tubing, adhesives, synthetic leather, toys, personal care products, and as plasticizer in polyvinyl chloride plastics.[4,5] Among phthalates, di-2-ethylhexyl phthalate (DEHP) has been used in a huge variety of products including food packaging and medical products.[5] However, because of its potential adverse effects, the use of DEHP has been restricted, in particular in toys and childcare articles.[6] Di-2-ethylhexyl terephthalate (DEHTP) is chemically related to DEHP and it has been used as a substitute to DEHP as a safer alternative[7] and, for this reason, the human exposure to this compound may be on the rise.[7-9] Others phthalates, recognized to be ECDs, used in food packaging, personal care products, and medication excipients are butylbenzyl phthalate (BBzP), diethyl phthalate (DEP), di-n/i-butyl phthalate (DnBP/DiBP).[4] Similarly to DEHP, the use of these compounds have been restricted.[6]

In humans, phthalates are predominately biotransformed via hydrolysis of esters, to their respective monoester derivatives. In the presence of a long alkyl chain, such as for DEHP and DEHTP, this can undergo oxidation to form hydroxyl derivatives that can be further oxidized to carboxylic acids, if the oxidation involves the terminal carbon atom. Carboxylic acid moieties can be conjugated to glucuronide and/or sulphate conjugates and finally excreted in urine.[10] For this reason, phthalate exposure can be assessed using urine as biospecimen.[11] In particular, the two major DEHP metabolites are mono-2-ethyl-5-carboxypentyl phthalate (MECPP) and mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP)[10] while the analogous, recently identified, DEHTP metabolites are mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP) and mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP).[12,13] The searchable metabolites in urine of BBzP, DEP and DnBP/DiBP are monobenzyl phthalate (MBzP), monoethyl phthalate (MEP) and mono-n/i-butyl phthalates (MnBP/MiBP), respectively.[10]

Another recognised EDC is bisphenol A (BPA), which is widely used for the manufacture of polycarbonate plastic and other products, among which food containers, bottles, medical equipment, electronic devices, and toys.[4,14,15] Due to its extensive use, BPA is ubiquitous in the environment and can enter the human body

mainly through contaminated food, drinking water and dust.[16] Once in the human body, its main biotransformation is the conjugation with glucuronic acid,[17] which is then excreted in urine. For this reason, the most common method to assess human exposure to BPA is measuring urinary concentrations of both free and conjugated BPA.[18]

Human biomonitoring is a useful approach to evaluate the exposure to these EDCs. Several methods have been developed in order to quantify bisphenol A and the most common urinary metabolites of phthalates.[11,19-23] Only recently, a few new methods have been set up for the determination of metabolites of alternatives phthalates such as DEHTP.[24-26] However, to how knowledge, no comprehensive method to determine BPA and metabolites of phthalates including DEHTP has been reported. The aim of this work was to develop and validate a suitable analytical assay for the simultaneous quantification of exposure biomarkers of bisphenol A and phthalates recognised to be ECDs, along with the novel alternative phthalate DEHTP.

2. Experimental

2.1. Chemicals

For the preparation of standard solutions, bisphenol A (BPA) (purity: 98%), bisphenol A-d8 (BPA-d8) (chemical purity: 98%, isotopic purity: 98.4%), monobenzyl phthalate (MBzP) (purity: 98%), monobenzyl phthalate-d4 (MBzP-d4) (chemical purity: 96%, isotopic purity: 98.8%), mono-2-ethyl-5-carboxypentyl phthalate (MECPP) (purity: 95%), monoethyl phthalate (MEP) (purity: 98%), monoethyl phthalate-d4 (MEP-d4) (chemical purity: 98%, isotopic purity: 98.8%), monoisobutyl phthalate (MiBP) (purity: 98%), monoisobutyl phthalate-d4 (MiBP-d4) (chemical purity: 98%, isotopic purity: 98.9%), mono-n-butyl phthalate (MnBP) (purity: 98%), and mono-n-butyl phthalate-d4 (MnBP-d4) (chemical purity: 98%, isotopic purity: 99.2%) were purchased from Toronto Research Chemicals (Ontario, Canada). Moreover, mono-2-ethyl-5-carboxypentyl phthalate-d4 (MECPP-d4) (purity: ≥ 95%), mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP) (purity: ≥ 95%), mono-2-ethyl-5-carboxypentyl terephthalate-d4 (MECPTP-d4) (purity: ≥ 85%), mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP) (purity: ≥ 95%), and mono-2-ethyl-5-hydroxyhexyl terephthalate-d4 (MEHHTP-d4) (purity: ≥ 95%) were purchased form Cansyn Chem Corp (Toronto, Canada). Molecular structures of these chemicals are shown in Table 1. Analytical grade acetonitrile, acetic acid, aqueous ammonia (30%), methanol, and the enzyme β-glucuronidase from *Escherichia coli* K 12 were purchased from Sigma-Aldrich (Milan, Italy). Purified water was obtained using a Milli-Q Plus ultrapure water system (Millipore, Milford, MA, USA).

2.2. Standard solution preparation

Each analytical standard (both native and isotopic labelled) was weighted and dissolved in methanol in order to obtain stock solutions at the concentration of 1 mg/mL. Isotopically labelled standards were diluted in water in order to obtain a mixture at the final concentration of 1 mg/L for each chemical (internal standards working solution). Stock solutions of native standards were diluted and mixed in water in order to obtain a working solution of native standards at the final concentration of 0.5 mg/L for BPA, 1 mg/L for MBzP, MECPP, MECPTP, and MEHHTP, and 5 mg/L for MEP, MiBP, and MnBP. This solution was further diluted in water in order to obtain calibration curve and quality control (QC) solutions.

Ten solutions at different concentrations were prepared for the calibration curve, along with an unadulterated (blank) sample of water. Analyte concentrations in these solutions ranged from 0.01 to 50 µg/L for BPA; from 0.02 to 100 µg/L for MBzP, MECPP, MECPTP, and MEHHTP, and from 0.1 to 500 µg/L for MEP, MiBP, and MnBP.

Independent concentrations for QC were 1.56 and 12.5 µg/L for BPA; 3.13 and 25 µg/L for MBzP, MECPP, MECPTP, and MEHHTP; 15.63 and 125 µg/L for MEP, MiBP, and MnBP.

Stock and aliquoted working solutions of native and internal standards were stored at -20°C in the dark.

2.3. Sample preparation

1 mL of each sample (or blank, calibration curve level, and QC) was added with 500 µL of ammonium acetate buffer (1 M, pH 6.5) and with 5 µL of the internal standards working solution. 30 µL of β-glucuronidase from *E. coli* K 12 (diluted 1:1 with the buffer) was then added, the solution was vortexed and incubated at 37°C for 2.5 hours. After incubation, 50 µL of pure acetic acid was added and the mixture was vortexed again. The mixture was directly loaded onto an Oasis® Prime HLB 3cc (60 mg) extraction cartridge (Waters S.p.A., Sesto San Giovanni, Milan, Italy) connected to a vacuum chamber. The cartridge was first washed with 2 mL of an aqueous solution containing 30% of methanol and 1% of acetic acid, and then the analytes were eluted with 1.5 mL of pure methanol. The obtained eluate was evaporated to dryness under a gentle nitrogen flow while kept at 40°C. Finally, 100 µL of water was added to the residue to reconstruct a solution, and the tube was thoroughly vortexed and placed in the autosampler for the analysis.

2.4. LC-MS/MS analysis

The instrumentation used to achieve the separation and the determination of compounds consisted of a high-pressure liquid chromatograph Agilent 1260 (Agilent Technologies, Cernusco Sul Naviglio, Italy)

coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500; AB Sciex, Monza, Italy) with an electrospray ionization source. The autosampler temperature was set at 10°C and the injection volume was set at 2 µL. The column used was an Accucore Phenyl-X (150 mm x 2.1 mm, 2.6 µm) (Thermo Fisher Scientific, Rodano, Italy) along with a pre-column Accucore Phenyl-X (10 mm x 2.1 mm, 2.6 µm) (Thermo Fisher Scientific, Rodano, Italy). The mobile phase was composed by a linear gradient with two mobile phases; the A phase was composed by water with 0.02% acetic acid, while the B phase was composed by acetonitrile with 0.02% acetic acid. The gradient was programmed as follows: 0-0.5 min, 20% B isocratic; 0.5-4 min, from 20% to 40% B; 4–7 min, 40% B isocratic; 7-11 min, from 40% to 100% B; 11-16 min, 100% B isocratic; 16–16.1 min, from 100% to 20% B; and 16.1–22 min, 20% B isocratic. The flow rate was set at 300 µL/min and the column was kept at 40°C.

The mass spectrometer operated in scheduled selected reaction monitoring (SRM) mode, with a retention time window of 240 s. The target scan time was set at 1 s. The polarity was set to negative and the following conditions were used: gas 1 (air) pressure, 40 psi; gas 2 (air) pressure, 65 psi; curtain gas (N_2) pressure, 35 psi; heater temperature, 550°C; ion spray voltage, −4500 V; and entrance potential, 10 V. For each analyte, the precursor ion/product ion pair and the collision energy were manually optimized through direct infusion. The precursor ion was always the deprotonated molecule $[M - H]^-$. The two most intense SRM transitions were recorded for each native analyte; the most intense transition was used for quantitation and the other transition was used for qualification (Table 1). For each isotopically labelled standard, the ion transition corresponding to the analogous of the related native standard was recorded; in every case, it was the most intense one.

The Analist® software (version 1.6.3; AB Sciex) was used to prepare the method and the batches for analysis, while the MultiQuant™ software (version 3.0.8664.0; AB Sciex) was used for quantification.

2.5. Method validation

A complete validation of the method was performed according to FDA guidelines.[27] Furthermore, the considerations raised by González et al. for evaluating the matrix effect were taken into account.[28]

2.5.1. Sensitivity

For each analyte, a calibration curve built with low concentration levels were used to estimate the limit of quantitation (LOQ). In particular, the following formula was used:

$$LOQ = (3SEq + q)/m,$$

where SEq is the standard error of the intercept, q is the intercept, and m is the slope of the linear calibration curve. [29]

2.5.2. Precision and accuracy

Precision and accuracy were determined by analysing QC solutions. Intra-day precision was evaluated by calculating the relative standard deviation (RSD) of peak area ratios of five replicates injected on the same day. Inter-day precision was determined by calculating RSD on five different days. Accuracy was calculated as the percent ratio between the concentration calculated using the calibration curve and the actual value of the prepared solution.

2.5.3. Accuracy with standard reference materials

Accuracy of the method was also verified analysing two standard reference materials for organic contaminants in urine, developed by the National Institute of Standards and Technology: SRM3672 and SRM3673.[30] These samples reported reference values for BPA, MBzP, MECPP, MEP, MiBP, and MnBP. Reference values were converted from µg/kg to µg/L using the density of urine value reported in the work of Schantz and co-workers (1.019 kg/L), and then accuracy was calculated as described above.

2.5.4. Selectivity and carryover effect

Selectivity was evaluated analysing the blank sample, while carryover effect was tested analysing the same blank sample after an analysis of the highest concentration level of the calibration curve. The presence of interfering peaks with the same transitions and retention times of the considered compounds was investigated.

2.5.5. Matrix effect, recovery, and process efficiency

The experiments for the evaluation of matrix effect, recovery, and process efficiency were set up taking into account the guidelines reported in the literature.[28,31] Urine samples were donated from five different healthy volunteers, and, for each urine sample, three different sets of samples were prepared: water spiked with standards before the sample preparation (W), urine spiked with standards before the sample preparation (U-pre) and urine spiked with standards after the sample preparation (U-post). The three different validation parameters were calculated as follows:

Matrix effect (ME) (%) = (U-post / W) * 100

Recovery (REC) (%) = (U-pre / U-post) * 100

Process efficiency (PE) (%) = (U-pre / W) * 100

Two different concentrations, corresponding to QCs, were evaluated for each parameter and the peak area ratio values in spiked urine were subtracted with values found in healthy volunteers' urine before calculation.

2.5.6. Calibration curve

The ranges of concentrations used to build the calibration curve are reported in "Standard solution preparation" section. Each solution was analysed at least twice in each sequence. For each analyte, the calibration curve was built by plotting the concentrations on the x axis, and the ratios between the chromatographic peak areas of native compound and those of the relative internal standard on the y axis. A weighted least-squares linear regression, with a weighting factor of 1/x, was then interpolated.

2.5.7. Stability

The stability of considered compounds was evaluated as short-term and long-term stability. Short-term stability was tested comparing signals of analytes of QC solutions kept in the autosampler (at 10°C) for 2 weeks with those of the same fresh-prepared solutions. Analogously, long-term stability was tested keeping the solutions at -20°C for more than 6 months and after four freeze-thaw cycle.

2.5.8. External verification

We took part in the Interlaboratory Comparison Investigations and External Quality Assurance Schemes (ICI-EQUAS) study, which is an inter-laboratory verification in the frame of the European Human Biomonitoring Initiative (HBM4EU).[32] Participating in the third round of the ICI-EQUAS study, two urine samples containing unknown levels (low and high) of MBzP, MECPP, MEP, MiBP and MnBP, and two urine samples containing unknown levels (low and high) of BPA were shipped to our laboratory. The results of our analyses were then compared to the results obtained from reference laboratories. Z-scores were calculated according to the following formula:

$$Z = (x - C) / \sigma_T$$

where x is the result submitted by the laboratory, C is the expert-assigned value, and σ_T is 0.25*C (a fit-for-purpose target standard deviation). $|Z| \leq 2$ was considered satisfactory, $2 < |Z| < 3$ was considered questionable, and $|Z| \geq 3$ was considered unsatisfactory.

2.6. Analytical sequence

A typical sequence consisted of the analysis of calibration curve levels, followed by the analysis of unknown samples along with the analysis of QC solutions and two sample duplicates for every 10 samples, followed by a second analysis of calibration curve levels.

2.7. Study subjects

The method was applied to the analysis of urine from 36 healthy volunteers enrolled in our Department at the University of Milan. The samples were collected in the period November 2014-March 2015. All subjects lived in Milan and were not occupationally exposed to phthalates or BPA. Among subjects, 14 males and 22 females were present, the mean age was 48.7 years (from 38 to 73), and the mean body mass index was 24.5 kg/m^2 (from 18.6 to 29.6). All subjects were non-smokers. Samples were collected and then stored at -20°C in glass vial until analysis.

2.8. Statistical analysis

Descriptive statistical analyses of the pilot study were performed using the software Rstudio (version 1.2.1335). For each analyte, a value corresponding to half of the LOQ was attributed when the concentration was not quantifiable. Median, 5th percentile, and 95th percentile were then calculated. Correlation between MECPTP and MEHHTP (both metabolites of DEHTP) were performed after removing observations with MEHHTP below the LOQ and expressing concentrations as nmol/L (nM). The Pearson's correlation coefficient r was calculated, along with a linear model having MEHHTP as dependent and MECPTP as independent variable.

3. Results

3.1. Validation

Fig. 1 shows the overlapped extracted ion chromatograms of a blank sample (Fig. 1A), a standard mixture in water (Fig. 1B), and a real urine sample (Fig. 1C).

3.1.1. Sensitivity

LOQ values are reported in Table 2 and ranged from 0.02 μg/L (MECPP) to 1 μg/L (MiBP and MnBP). The background contamination led to higher LOQ values for BPA, MiBP, and MnBP (see "3.1.5. Selectivity and carryover effect" section).

3.1.2. Precision and accuracy

Results for accuracy and precision are reported in Table 2. The RSD mean values for the intra-day precision ranged from 1.3% (QC low of MECPTP) to 7.3% (QC low of BPA), while the inter-day precision ranged from 2.1% (QC low of MECPTP) to 9.4% (QC high of MnBP). The accuracy, calculated as the percent

ratio between the measured and theoretical concentrations, ranged from 92.1% (QC low of BPA) and 109.2% (QC low of MiBP).

3.1.3. Accuracy with standard reference materials

The results obtained analysing SRM3672 and SRM3673 (with relative accuracy) were the following: BPA, 2.52 µg/L (81.1%) and 2.30 µg/L (114.9%); MBzP, 7.33 µg/L (85.9%) and 4.91 µg/L (84.7%); MECPP, 30.52 µg/L (85.1%) and 22.78 µg/L (74.3%); MEP, 113.14 µg/L (117.5%) and 90.17 µg/L (110.3%); MiBP, 5.33 µg/L (81.7%) and 4.99 µg/L (94.5%); MnBP, 8.92 µg/L (82.5%) and 9.55 µg/L (83.7%). Overall, results were acceptable since accuracy ranged from 74.3% to 117.5%.

3.1.4. Selectivity and carryover effect

Laboratory materials and solvents were verified in order to use those with the lowest background levels of analysed compounds. Background signals were always present for BPA, MiBP, and MnBP in blank samples, while no interference or background signal was detected for the other analytes. Background peaks for BPA, MiBP and MnBP, not eliminable even with repeated cleaning procedures, impacted the LOQ values, as reported in the "sensitivity" section. No carryover effect was found.

3.1.5. Matrix effect, recovery, and process efficiency

Table 3 shows the results from matrix effect, recovery and process efficiency. ME, calculated without considering the internal standards, were below 80% for most of the considered analytes (with a minimum of 42.5% for QC low of BPA) indicating that the matrix decreases the signal of these molecules. However, after the correction with the internal standards, ME ranged from 74.0% (QC low of BPA) to 144.7% (QC low of MEP), indicating that ISs are able to properly adjust results. The recovery of the method ranged from 66.5% (QC low of MECPTP) to 126.0% (QC low of BPA), without considering the ISs and from 82.0% (QC low of MEP) to 123.8% (QC low of BPA) after the correction with the ISs. Process efficiency, which measures the overall contribution of both matrix effect and recovery, ranged from 48.7% (QC low of MiBP) to 108.9% (QC low of MEP) without considering the ISs and from 84.5% (QC high of BPA) to 116.0% (QC low of MEP) after the correction with the ISs.

3.1.6 Calibration curve

For each linear regression, the coefficient of determination (R^2) was calculated. The values ranged from 0.9803 (MiBP) to 0.9970 (MEP), demonstrating optimal linearity for the concentration ranges investigated (Table 2).

3.1.7. Stability

The results of the short-term stability tests (expressed as ratio with the fresh-prepared solution) ranged from to 98.2% (QC low of MEHHTP) to 111.7% (QC high of MBzP) (Table 2), indicating the absence of degradation in the autosampler up to two weeks. Long-term stability ranged from 80.3% (QC low of MECPTP) to 116.7% (QC low of MEHHTP), indicating a good reproducibility over 6 months and after freeze-thaw cycles.

3.1.8. External verification

The results from the ICI-EQUAS study were satisfactory for all considered compounds. In particular, Z-scores were: BPA: -1.9 and -1.6 for low and high level samples; MBzP: -0.1 for high level sample (for the low level sample no Z-scores were calculated as it was below LOQ of expert labs); MECPP: -0.9 and -0.7 for low and high level samples; MEP: 0.5 and 0.6 for low and high level samples; MiBP: 0.0 and 0.2 for low and high level samples; MnBP: 0.3 and -0.1 for low and high level samples.

3.2. Application of the method

Results of the application of the method to urine samples from healthy subjects are reported in Table 4. All samples were quantifiable for their levels of MBzP, MECPP, MECPTP, and MEP. 30 out of 36 (83%) samples were >LOQ for BPA, 33 (92%) for MEHHTP, and 34 (94%) for both MiBP and MnBP. Median values ranged from 0.27 µg/L (MEHHTP) to 117.08 µg/L (MEP), 5th percentile ranged from <LOQ (BPA) to 18.99 µg/L (MEP), 95th percentile ranged from 3.39 µg/L (MEHHTP) to 651.63 µg/L (MEP).

The Pearson's coefficient assessing the correlation between concentrations (nM) of MECPTP and MEHHTP was 0.73 (95% CI: 0.52 - 0.86, $P < 0.001$), while the slope of the linear regression having MEHHTP as dependent and MECPTP as independent variable was 0.10 (95% CI: 0.07 - 0.14, $P < 0.001$) (Fig. 2).

4. Discussion

In this work, we developed and validated an analytical method for the simultaneous determination of both BPA and the metabolites MBzP, MECPP, MECPTP, MEHHTP, MEP, MiBP, and MnBP, which are exposure biomarkers of phthalates BBzP, DEHP, DEHTP, DEP, DiBP, and DnBP.

The main advantage of the method is its ability to simultaneously quantify the urinary biomarkers of both BPA and phthalates, including the DEHTP, recently introduced as a less toxic alternative to traditional

phthalates and recently reported to be on the raise.[7,8] The choice of the metabolites to be used as biomarker was carefully made: indeed, the hydrolytic monoesters (MBzP, MEP, MiBP, and MnBP) are the most abundant metabolites of phthalates with a short alkyl chain (BBzP, DEP, DiBP, and DnBP), while long-chain phthalates (DEHP and DEHTP) are excreted mainly as oxidized monoesters[33] Toxicokinetics of DEHTP has been recently studied, and revealed that it is eliminated as mono-2-ethylhexyl terephthalate (MEHTP) only in negligible percentage of the oral dose (<0.1%), while the oxidized MECPTP and MEHHTP represent about 13% and 6%, respectively, of the oral dose.[13] We included both oxidized compounds in our method in order to be able to further investigate the behaviour of these two metabolites: indeed, Silva and co-workers reported interesting differences in the molar ratio of MECPTP and MEHHTP in urine samples collected at different times of the day and from subjects with different ages.[8] The choice of oxidised monoesters was also useful for the contamination issue: indeed, we detected a considerably low background level for MECPP, MECPTP, and MEHHTP, and consequently we could achieve low LOQ values for these compounds, while we observed a high background level especially for BPA, MiBP, and MnBP. We managed to reduce the entity of these contaminations applying additional short C18 columns, as suggested form a previous work:[34] one was applied after the HPLC pump A, in order to delay the contamination present in the aqueous phase, and one just before the autosampler, to delay all contaminations and having them eluted when organic phase reaches a higher percentage during the gradient program. Furthermore, the presence of contaminations was tested for each solvent implemented and the one with the lowest background level was chosen. Moreover, the minimization of possible contaminations was achieved through a special care in all steps of the sample preparation, as analysing the contamination derived from each type of plastic disposable used and avoiding the use of absorbent underpads. In spite of all these cares and the consideration reported in literature,[34,35] we still had a background level for BPA, MiBP, and MnBP which affected the LOQ values of these analytes.

An advantage of this analytical assay is its ability to quantify both phthalates and BPA in a single chromatographic run. Only a few methods able to simultaneously separate these chemically different compounds have been reported.[19,21,22] However, some considerations need to be addressed when developing such an approach. Because of the very low expected levels of BPA in the general population and its low signal intensity (especially in comparison with phthalates), we decided to focus our efforts in the optimization of the chromatographic method for this molecule. Indeed, we tested different acids at different concentrations in the mobile phases: although a stronger acid (e.g. formic acid) and a relative higher concentration is required for a better resolution of phthalates, we observed a loss in signal for BPA under these conditions. This finding was in agreement with previous works,[19,36,37] where it has been reported that the best sensitivity for BPA is obtained avoiding the organic acids in the mobile phase at all. In the end, we

set up a mobile phase with a relatively weak acid (acetic acid) and at a very low concentration (0.02%), in order to optimize the response of BPA and still retain the phthalates. Overall, we obtained a chromatogram in which the peak of BPA is well resolved and symmetric, while those of phthalates had an asymmetry (Fig. 1). Nevertheless, this affected neither quantitation nor accuracy of the method.

To perform the deconjugation of the analytes before the chromatographic analysis, the choice of the enzyme was made taking into consideration the advice reported by Koch and co-workers:[38] indeed, the use of β-glucuronidase from *E. coli* K 12 allowed a specific cleavage of glucuronide conjugates, while it has been reported that the use of enzymes with other enzymatic activities (e.g.: lipase or arylsulfatase) could lead to undesired modification of the analytes, such as the cleavage of the ester bond.[24] The use of the specific β-glucuronidase is appropriate for the deconjugation of phthalates, for which the glucuronide moiety is the only conjugate detected,[39,40] but it is not completely appropriated for BPA, that is also excreted as monosulfate.[41] However, the percentage of BPA excreted as sulfate is reported to be rather low, about 3%.[42] All things considered, we finally chose to use the β-glucuronidase from *E. coli* K 12 to optimize the deconjugation of phthalates and to achieve a good cleavage also for BPA, considering negligible the loss of signal associated with the lack of deconjugation of the BPA sulphate. The suitability of our decision was also confirmed by the results obtained in the external verification, which were acceptable, although lower if compared to the results obtained from reference laboratories.

A limitation of this method for its applicability on large epidemiologic studies is the relatively long time required for sample preparation, due to the several steps involved (incubation, SPE extraction, evaporation). However, we partially managed to speed it up using the novel SPE cartridges "Oasis PRiME HLB", which do not require neither conditioning nor equilibrations, therefore allowing to load the sample directly onto the cartridge. For the same reason, we decided to avoid the derivatization with dansyl chloride.[43]

Overall, the method underwent a complete validation, which demonstrated the suitability of the method. A strength of our work is the validation using standard reference materials,[30] along with the successful participation to an external verification exercised (ICI-EQUAS study), although this approach was not possible for MECPTP and MEHHTP, since these novel compounds were still not included either in the standard reference materials or in the ICI-EQUAS.

The method was applied to a pilot biomonitoring study and the results obtained are in agreement with those previously reported.[7,23,44] Regarding the metabolites of DEHTP, we confirm that MECPTP is the most abundant metabolite, with concentrations about 10-fold higher than those of MEHHTP. The ratio between the two metabolites was similar to the one in previous studies.[7-9]

In conclusion, the reported analytical assay can be considered a useful tool for the simultaneous biomonitoring of BPA and phthalates, including DEHTP, which is increasingly used as substitutes for DEHP. Since exposure data to terephthalates are limited, an assay like the one presented in this work may be useful to fill this void since it can be implemented to monitor both the potential increasing exposure to DEHTP along with the exposure to other phthalates and BPA, whose use has been restricted.

Declarations of interest

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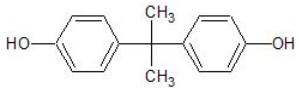
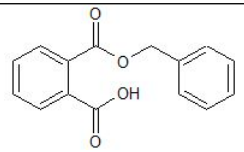
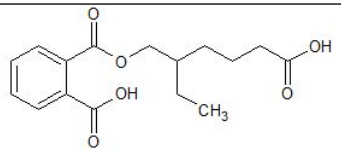
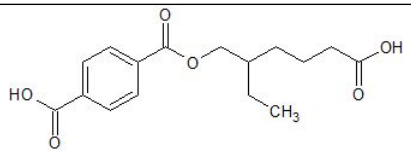
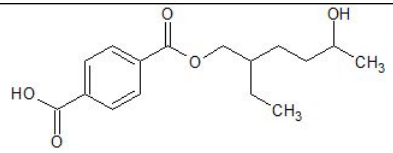
Tables

Table 1

List of the acronyms of investigated analytes, with molecular structures, molecular weight, CAS numbers and relative parent compounds. For each analyte, the SRM ion transitions used for quantitation (quant) and qualification (qual), are showed; moreover, the SRM ion transition of the isotopic labelled internal standard (IS) is showed. Finally, the retention time (RT) of each chemical and the collision energy (CE) used to obtain the SRM ion transition are provided.

Investigated analytes	Molecular structure	Molecular weight (Dalton)	CAS number	Parent compounds	SRM transitions (precursor ion/product ion) (m/z)		RT (min)	CE (V)
BPA		228	80-05-7	bisphenol A (BPA)	Quant.: Qual.: IS (-d8 isotope):	227.0/212.0 227.0/133.0 235.0/220.0	10.13 10.01	-25 -32 -26
MBzP		256	2528-16-7	butylbenzyl phthalate (BBzP)	Quant.: Qual.: IS (-d4 isotope):	255.0/77.0 255.0/106.9 259.0/77.0	14.59 14.52	-26 -18 -28
MECPP		308	40809-41-4	di-2-ethylhexyl phthalate (DEHP)	Quant.: Qual.: IS (-d4 isotope):	307.3/158.8 307.3/113.0 311.3/159.0	14.00 13.93	-17 -37 -17
MECPTP		308	1684398-42-2	di-2-ethylhexyl terephthalate (DEHTP)	Quant.: Qual.: IS (-d4 isotope):	307.2/164.8 307.2/121.0 311.3/168.7	14.40 14.36	-20 -31 -20
MEHHTP		294	1684398-38-6	di-2-ethylhexyl terephthalate (DEHTP)	Quant.: Qual.: IS (-d4 isotope):	293.2/120.9 293.2/76.9 297.2/125.0	13.82 13.77	-23 -40 -23

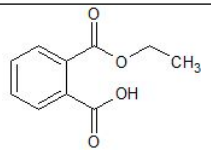
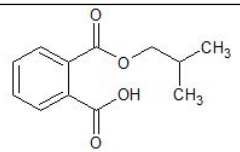
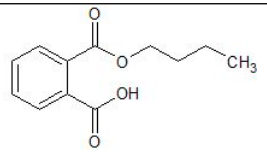
MEP		194	2306-33-4	diethyl phthalate (DEP)	Quant.: Qual.: IS (-d4 isotope):	193.0/77.0 193.0/121.0 197.0/81.0	10.38 10.22	-22 -14 -22
MiBP		222	30833-53-5	diisobutyl phthalate (DiBP)	Quant.: Qual.: IS (-d4 isotope):	221.0/77.0 221.0/133.8 225.0/81.0	13.37 13.29	-22 -18 -23
MnBP		222	131-70-4	di-n-butyl phthalate (DnBP)	Quant.: Qual.: IS (-d4 isotope):	221.0/77.0 221.0/71.0 225.0/81.0	13.57 13.50	-22 -18 -23

Table 2

Results from the method validation: limits of quantification (LOQ), calibration curves (investigated range and mean R^2), precision, accuracy, short- and long-term stability.

Investigated analytes	Calibration curve			QC precision and accuracy				Stability	
	LOQ (µg/L)	Investigated ranges (µg/L)	Mean R^2 (n=5)	Spiked concentrations (µg/L)	Intra-day precision %RSD (Min-Max)	Inter-day precision %RSD	Accuracy %Theoretical (Min-Max)	Short-term stability (ratio% with fresh solution)	Long-term stability (ratio% with fresh solution)
BPA	0.50	LOQ - 50	0.9872	1.56	7.3 (1.0-12.2)	8.3	92.1 (87.2-95.8)	101.2	97.9
				12.5	4.9 (2.3-6.5)	5.3	94.2 (92.3-95.8)	106.3	105.1
MBzP	0.03	LOQ - 100	0.9956	3.13	2.5 (1.6-3.5)	4.1	100.4 (98.0-103.0)	109.6	89.5
				25	2.6 (0.7-4.5)	5.0	100.6 (96.4-103.6)	111.7	98.4
MECPP	0.02	LOQ - 100	0.9966	3.13	2.0 (1.6-2.5)	4.0	104.4 (97.8-114.0)	100.4	109.8
				25	1.8 (1.0-2.6)	2.4	100.1 (96.3-107.6)	105.9	103.6
MECPTP	0.11	LOQ - 100	0.9925	3.13	1.3 (0.4-2.3)	2.1	96.7 (91.2-100.7)	101.3	80.3
				25	1.4 (0.7-1.7)	2.8	98.7 (93.1-103.9)	101.1	84.8
MEHHTP	0.03	LOQ - 100	0.9953	3.13	1.6 (1.2-2.1)	4.5	98.6 (87.8-110.0)	98.2	116.7
				25	1.7 (0.8-3.0)	3.2	100.7 (95.5-109.0)	100.7	105.8

MEP	0.28	LOQ - 500	0.9970	15.63	2.7 (0.7-4.5)	4.5	101.9 (95.8-108.7)	102.3	107.7
				125	2.2 (0.7-4.0)	3.3	98.0 (94.6-101.5)	102.0	103.8
MiBP	1	LOQ - 500	0.9803	15.63	2.7 (1.7-3.6)	3.9	109.2 (102.7-117.6)	100.2	110.1
				125	3.1 (1.9-4.4)	7.9	95.0 (77.3-103.7)	101.7	101.0
MnBP	1	LOQ - 500	0.9906	15.63	4.3 (3.4-5.4)	5.0	105.4 (91.1-110.5)	101.0	108.1
				125	4.1 (3.0-5.0)	9.4	100.6 (81.8-110.5)	105.7	98.7

Table 3

Results from the method validation: evaluation of the matrix effect, recovery and process efficiency. Results are given as means ± standard deviations (SD) from the urine of five healthy volunteers.

Investigated analytes	Spiked concentrations (µg/L)	Matrix effect (ME) (%mean±SD)		Recovery (REC) (%mean±SD)		Process efficiency (PE) (%mean±SD)	
		without IS	with IS	without IS	with IS	without IS	with IS
BPA	1.56	42.5±9.6	74.0±13.3	126.0±29.4	123.8±26.3	55.5±17.2	100.1±12.9
	12.5	54.1±17.3	84.4±3.4	113.2±51.7	100.2±9.2	54.3±7.9	84.5±7.2
MBzP	3.13	63.2±14.8	91.8±21.6	86.0±10.3	105.8±18.1	54.0±13.1	94.1±8.2
	25	74.8±21.6	100.7±13.5	75.1±22.7	93.9±5.7	52.8±10.1	94.1±7.3
MECPP	3.13	76.7±31.3	83.8±18.2	87.2±27.6	114.1±19.7	62.4±22.7	93.4±13.8
	25	88.6±40.8	89.0±25.8	83.8±30.3	102.1±13.5	70.4±27.4	89.1±18.2
MECPTP	3.13	72.4±54.4	115.8±37.6	66.5±34.6	89.4±30.2	55.9±43.8	94.8±6.6
	25	97.8±46.8	104.9±26.1	75.4±40.7	96.6±14.8	65.7±17.5	98.3±7.4
MEHHTP	3.13	66.9±11.8	98.9±14.2	88.2±8.6	101.0±12.5	58.4±7.4	98.6±4.8
	25	76.3±12.0	104.8±13.0	78.5±24.0	99.3±7.7	57.8±11.0	103.3±6.5
MEP	15.63	111.6±54.6	144.7±31.9	76.5±14.4	82.0±15.1	108.9±62.4	116.0±9.7
	125	76.3±30.9	92.6±29.1	108.0±87.4	111.3±41.5	55.2±25.9	97.0±2.5
MiBP	15.63	53.9±3.3	82.6±24.0	82.3±19.3	107.4±49.7	48.7±17.8	99.2±31.3
	125	73.4±12.5	97.8±19.7	80.5±20.6	102.9±4.9	54.0±10.6	108.3±21.4
MnBP	15.63	60.5±6.4	97.1±19.5	92.3±8.0	110.4±25.4	55.5±4.5	103.4±4.7
	125	78.6±8.7	107.4±11.6	79.2±17.7	103.5±4.1	61.2±8.6	110.8±7.8

Table 4

Results of the application of the method to a pilot biomonitoring study on 36 adult subjects.

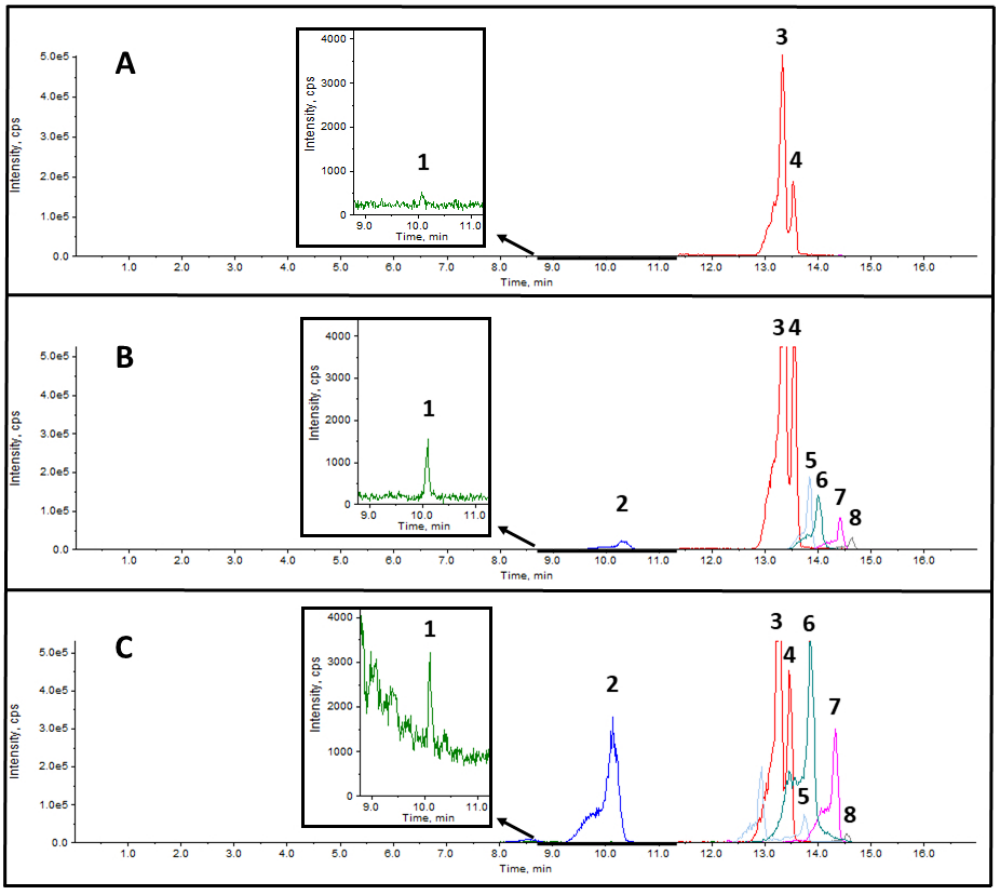
	Median (µg/L)	5th percentile (µg/L)	95th percentile (µg/L)	Number of samples >LOQ (%)
BPA	2.07	<LOQ	6.67	30 (83%)
MBzP	4.00	1.26	20.90	36 (100%)
MECPP	12.75	4.56	36.24	36 (100%)
MECPTP	3.73	0.85	35.25	36 (100%)
MEHHTP	0.27	0.02	3.39	33 (92%)
MEP	117.08	18.99	651.63	36 (100%)
MiBP	22.15	4.70	42.04	34 (94%)
MnBP	16.02	2.35	43.61	34 (94%)

Figure legends

Fig. 1. A: superimposed extracted ion chromatograms of a blank (water) sample; the estimated background concentrations were about 0.2, 4 and 1 µg/L for BPA, MiBP, and MnBP, respectively. **B:** superimposed extracted ion chromatograms of a standard mixture in water at concentrations of 1.56 µg/L for BPA; 3.13 µg/L for MBzP, MECPP, MECPTP, and MEHHTP; and 15.63 µg/L for MEP, MiBP, and MnBP. **C:** superimposed extracted ion chromatograms of a urine sample from a subject included in the study, with calculated concentrations of 4.67 (BPA), 4.60 (MBzP), 18.56 (MECPP), 25.41 (MECPTP), 1.89 (MEHHTP), 251.3 (MEP), 21.59 (MiBP), and 14.23 (MnBP) µg/L.

1, BPA; 2, MEP; 3, MiBP; 4, MnBP; 5, MEHHTP; 6, MECPP; 7, MECPTP; 8, MBzP.

Fig. 2. Scatter plot between urinary concentrations (nmol/L) of MECPTP and MEHHTP (metabolites of DEHTP) in urine samples of the 36 individuals belonging to the general population (excluding the three subjects with MEHHTP < LOQ). The line is the representation of the linear model and the shadow represents the interval of the relative standard error. Pearson's correlation coefficient and slope of the linear model are also given. Data are shown with a logarithmic scale on both x and y axis.



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1, BPA; 2, MEP; 3, MiBP; 4, MnBP; 5, MEHHTP; 6, MECPP; 7, MECPTP; 8, MBzP.

245x220mm (96 x 96 DPI)

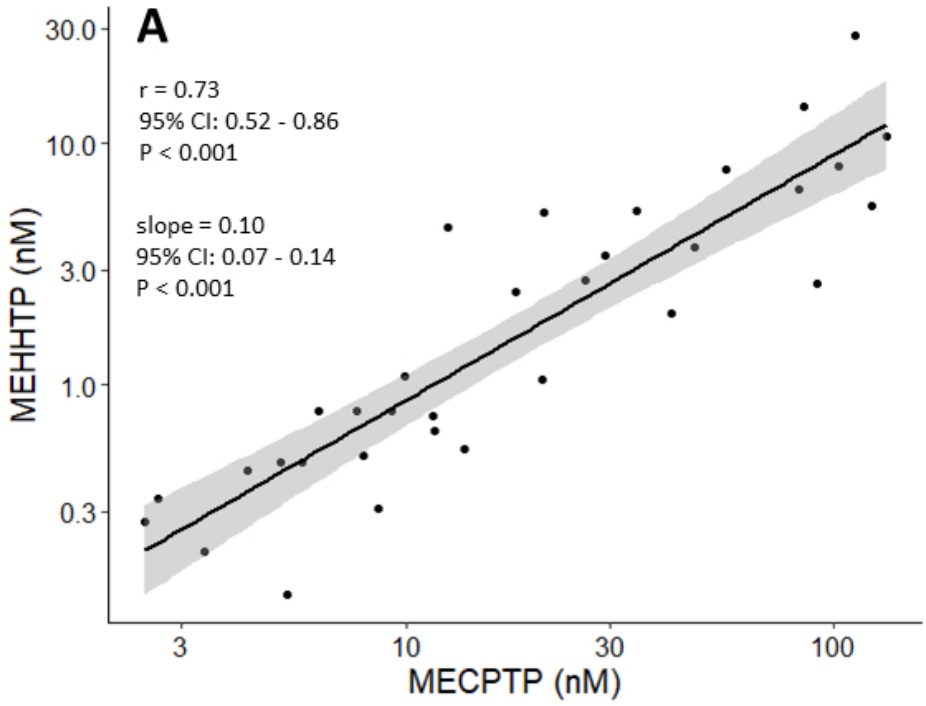

Fig. 2. Scatter plot between urinary concentrations (nmol/L) of MECPTP and MEHHTP (metabolites of DEHTP) in urine samples of the 36 individuals belonging to the general population (excluding the three subjects with MEHHTP < LOQ). The line is the representation of the linear model and the shadow represents the interval of the relative standard error. Pearson's correlation coefficient and slope of the linear model are also given. Data are shown with a logarithmic scale on both x and y axis.

160x120mm (96 x 96 DPI)