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RESEARCH PAPER

# Determination and separation of bisphenol A, phthalate metabolites and structural isomers of parabens in human urine with conventional high-pressure liquid chromatography combined with electrospray ionisation tandem mass spectrometry

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**Abstract** Phthalates, bisphenol A (BPA) and parabens (PBs), organic chemicals widely used in everyday products, are considered to be endocrine disruptors. We propose a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the determination of seven phthalate metabolites, six PBs and BPA in human urine. All three categories of the above endocrine disruptors were simultaneously extracted from 1 mL of human urine using solid phase extraction. In addition, with a conventional reversed phase LC column, we achieved for the first time the separation of three pairs of structural isomers, namely iso-/*n*-butyl paraben, propyl paraben and monobutyl phthalate. LC-MS/MS was operated and tested in both electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). ESI was selected for the analysis due to its superior stability and repeatability. The method limit of detection (mLOD), achieved for a single set of high-performance LC conditions, ranged from 0.01 to 0.84 ng/mL for phthalate metabolites, from 0.06 to 0.24 ng/mL for PBs and was 2.01 ng/mL for BPA. Derivatisation of BPA with dansyl chloride lowered its mLOD to 0.007 ng/mL. Blank contamination was non-detectable. The present method was successfully applied for the analysis of the above-mentioned compounds in 80 male human urine samples.

**Keywords** Urinary biomarkers · Bisphenol-A · Parabens · Phthalate metabolites · Dansyl chloride derivatisation · HPLC-ESI-MS/MS

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## Introduction

Endocrine disruptors (EDs) are a group of organic compounds, which cause serious alterations to the normal hormone function in humans and wildlife [1]. EDs interfere with hormone biosynthesis, metabolism or action resulting in a deviation from normal homeostatic control or reproduction in humans [2]. They disrupt the endocrine system by competing with naturally occurring hormones, such as estradiol, or by altering the synthesis and metabolism of these hormones [3]; in addition, there is evidence of reproductive toxicity in laboratory animals and possible health effects in humans [4]. Bisphenol-A (BPA), parabens (PBs) and phthalates are well recognised EDs. Six billion pounds of BPA are produced each year worldwide, and over 220,000 lbs of this compound are released yearly into the atmosphere [5]. Phthalates, with over 18 billion pounds used each year, represent one of the world's high production chemical families [6] and PBs, which are used in over 13,200 formulations in nearly all type of cosmetics [7]. Human exposure to these chemicals is occurring through the environment, food intake and the use of products containing them, through inhalation, dermal contact and ingestion [8–12].

Phthalate esters (1,2-diesters) have a variety of common uses. High molecular weight (HMW) phthalates are used in plastic as softeners, and low molecular weight (LMW) phthalates are used in personal care products and pharmaceuticals [13–15]. Previous animal tests and epidemiological studies have associated exposure to phthalates with detrimental effects to reproductive and developmental health, as well as increased risk to cancer [8–10]. Phthalates normally follow a metabolic pathway in at least two steps, a hydrolysis (phase I) where the phthalate diester is hydrolysed into the primary metabolite monoester phthalate and is followed (phase II) by a conjugation in order to form the more hydrophilic glucuronidated metabolite [16].

The 2,2-bis (4-hydroxyphenyl) propane or bisphenol-A (BPA) is used in industry for the production of many pesticides, resins and polycarbonate plastic. BPA can be found in food and beverage processing and in many products like dental sealants, personal care products, baby bottles, building materials, flame retardant materials, optical lenses, materials for the protection of window glazing, DVDs and household electronics [4, 17, 18]. Human exposure to BPA is linked to heart diseases, diabetes, liver abnormalities, reproduction adverse effects and alterations in the thyroid [19, 20]. BPA is excreted mainly via urine in its free form or in its more hydrophilic glucuronide/sulphate conjugate form [4, 21].

PBs are a group of alkyl esters of *p*-hydroxybenzoic acid. They have low cost of production and demonstrate high chemical stability, inertness and low acute toxicity [1]. These characteristics made them desirable in industry, as antimicrobial preservatives against mould and yeast, in cosmetics, in pharmaceuticals and in food and beverage processing [7]. PBs occur also naturally in food, wine and plants [22]. In vitro studies indicate that PBs induce the growth of MCF-7 human breast cancer cells and influence the expression of oestrogen-dependent genes [23, 24]. In general, PBs are partially hydrolysed by esterases to *p*-hydroxy-benzoic acid and produce glycine/glucuronide/sulphate conjugates, with increased water solubility that are more amenable to urinary excretion than are the free species [22, 25].

In order to assess the exposure of humans to phthalates, PBs and BPA, measurement of the urinary concentration of free species and their conjugates is essential [26–29]. Several methods suitable for measuring phthalate metabolites, BPA, or PBs have been published, but none of them measure all three compound classes [30–35]. Furthermore, only ultra performance liquid chromatography (UPLC) has been reported to sufficiently separate the structural isomers of propyl and butyl paraben [30].

We aimed to develop a suitable chromatographic method for assessing human exposure to the above-mentioned important EDs. Our main goals were (i) to develop a common clean-up procedure for phthalate metabolites, PBs and BPA, present in human urine samples; (2) to separate the structural isomers of propyl paraben, butyl paraben and monobutyl phthalate metabolite, using conventional high performance liquid chromatography (HPLC), instead of UPLC columns and pumps; and (3) to succeed the lowest possible detection limits for all the above-mentioned EDs. We thus achieved high sensitivity, selectivity and the capability to analyse large numbers of samples in reasonable times, making the method eminently suitable for epidemiological studies. In order to test the applicability and appropriateness of the present method, we applied it for the analysis of phthalate metabolites, BPA and PBs in a large number of male urine samples.

## Materials and methods

### Analytical standards, reagents and consumables

Mono-ethyl phthalate (mEP), $^{13}C_4$-labelled mEP, mono-*n*-butyl phthalate (mnBP), $^{13}C_4$-labelled mnBP, mono-iso-butyl phthalate (miBP), mono-benzyl phthalate (mBzP), $^{13}C_4$-labelled mBzP, mono-2-ethyl-hexyl phthalate (mEHP), mono-2-ethyl-5-hydroxy-hexyl phthalate (mEHHP), $^{13}C_4$-labelled mEHHP, mono-2-ethyl-5-oxo-hexyl phthalate (mEOHP), $^{13}C_4$-labelled mNP, 4-methylumbelliferrone, $^{13}C_4$-labelled 4-methylumbelliferrone and $D_{16}$-bisphenol-A ($D_{16}$-BPA) were obtained from Cambridge Isotope Laboratories (USA). 4-Methylumbelliferryl glucuronide, $^{13}C_6$-labelled MPB, $^{13}C_6$-labelled EPB, $^{13}C_6$-labelled n-PPB, $^{13}C_6$-labelled *n*-BPB, dansyl chloride, formic acid (for MS, 98 %), solvents (Chromasolv grade for HPLC acetonitrile, ethyl acetate, acetone and methanol) and ammonium hydroxide (28 %*w*/*v* in water) were purchased from Sigma Aldrich (Germany). Methyl paraben (MPB), ethyl paraben (EPB), *n*-propyl paraben (n-PPB), *n*-butyl paraben (nBPB), iso-butyl paraben (iso-BPB), bisphenol-A (BPA) and iso-propyl paraben (iso-PPB) were purchased from AccuStandard (USA). Glacial acetic acid was purchased from Carlo Erba (Italy) and orthophosphoric acid (85 %*w*/*v* in aqueous solution) from Riedel de Haen (Switzerland). Ammonium acetate and monosodium phosphate (reagent grade) were provided by Fluka (Germany). *Escherichia coli* β-glucuronidase (140 U/mL) was purchased from Roche (Germany). SPE cartridges (Nexus, 60 mg sorbent/3 mL reservoir and 200 mg sorbent/6 mL reservoir) were acquired from Varian (USA). High-purity water (18.2 MΩ cm electrical resistivity) was produced by PURELAB ultra ionic purification system (ELGA, USA).

### Preparation of standards

All standard solutions were stored sealed at −20 °C in Teflon-capped bottles. Phthalate metabolites and 4-methylumbelliferrone standards (native and labelled)

obtained in solutions (100 μg/mL in methyl-*tert*-butyl ether or acetonitrile). 4-Methylumbelliferryl glucuronide standard stock solution was prepared in water at 1000 μg/mL. Dansyl chloride standard solution was prepared in acetone at 12.5 mg/mL. PBs, BPA and $D_{16}$-BPA stock solutions were prepared in methanol at 250 μg/mL. Working solutions were prepared at concentrations of 1 μg/mL in 1:1 methanol/water for mass spectrometer optimisation and at 2–8 μg/mL in synthetic urine [31] for spiking samples and calibration curves. Quantitative analysis was based on peak area measurements as ratios with the peak area of their corresponding internal standard. For phthalate metabolites, isotopically labelled analogues of native compounds were used as internal standards, except for miBP, mEOHP and mEHP where $^{13}C_4$-mnBP and $^{13}C_4$-mEHHP were used because the labelled analogues were not commercially available to us for the period of the study. For PBs analysis $^{13}C_6$-analogues of methyl, ethyl, *n*-propyl and *n*-butyl parabens were used as internal standards. For BPA analysis, $D_{16}$-BPA was used as the internal standard. Calibration curve solutions, blank, recovery and quality control (QC) samples were prepared in synthetic urine.

## Instrumentation

All analyses were performed on a liquid chromatography–tandem mass spectrometry (LC-MS/MS) system consisting of an reversed phase HPLC (RP-HPLC) chromatograph coupled to a mass spectrometer. Sample injections were performed via a Surveyor Autosampler (Thermo Finnigan, USA).

The chromatographic separation of PBs-BPA-phthalate metabolites was achieved using a Surveyor LC system (Thermo Finnigan, USA), equipped with a BetaSil Phenyl (3 μm, 100×2.1 mm) analytical column from Thermo Scientific (USA).

Dansylated-BPA/$D_{16}$-BPA were analysed with a PerfectSil $C_8$ (3 μm, 125×2.1 mm, MZ-Analytical, Germany) analytical column. The mass detection was achieved with a TSQ Quantum triple quadrupole mass spectrometer equipped with both electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) source (Thermo Finnigan, San Jose, USA). The system was controlled by the Xcalibur software, which also was used for the data acquisition, analysis and quantitation.

## Mass spectrometry conditions

The mass spectrometer was operated in the selected reaction monitoring (SRM) mode. Source collision induced dissociation (source CID), and tube lens voltage were set at optimum values for each SRM. Collision gas was Ar at 2.0 mTorr.

For PBs–BPA–phthalate metabolites, ESI in negative mode was chosen as the ionisation source. Sheath gas pressure (32–45 arbitrary units, au) and auxiliary gas pressure (20 au) were $N_2$ and as with spray voltage (4000–4400 V) and Lens 0 offset (1.0–1.3 V), optimum values were set at each time segment. Ion transfer capillary temperature was set at 330 °C.

The monitored SRMs of the studied compounds and their isotopically labelled internal standards are presented in Table 1. Dwell time was set at 0.1–0.2 s, except for BPA, in which case, it was set at 1.5 s.

Dansylated-BPA was measured with ESI in the positive mode. Sheath gas pressure (35 au) and auxiliary gas pressure (20 au) were $N_2$. Spray voltage was set at 4000 V and Lens 0 offset at 0.4 V. Ion transfer capillary temperature was set at 300 °C. The SRMs are depicted in Table 1. Dwell time was set at 0.75 s for quantitation ion, 0.55 s for confirmation ion and 0.2 s for $D_{16}$-BPA ion.

## HPLC conditions

Injection volume was 20 μL, and autosampler settings were as follows: flush volume, 1600 μL; wash volume, 1600 μL; flush speed, 100 μL/s; and as wash/flush solvent, methanol/

**Table 1** Selected reaction monitored for phthalate metabolites, parabens, BPA, dansylated-BPA and their isotopically labelled analogues

| Analyte | Precursor ion (*m*/*z*) | Product ion (*m*/*z*) | Collision energy (eV) |
|---|---|---|---|
| MPB | 151.1 | 92.1 | 14 |
| $^{13}C_6$-MPB | 157.1 | 98.1 | 14 |
| EPB | 165.1 | 92.1 | 30 |
| $^{13}C_6$-EPB | 171.1 | 98.1 | 30 |
| nPPB/iso-PPB | 179.1 | 92.1 | 40 |
| $^{13}C_6$-nPPB | 185.1 | 98.1 | 40 |
| nBPB/iso-BPB | 193.1 | 92.1 | 33 |
| $^{13}C_6$-nBPB | 199.1 | 98.1 | 30 |
| mEP | 193.1 | 77.1 | 10 |
| $^{13}C_4$-mEP | 197.1 | 79.1 | 10 |
| mnBP/miBP | 221.1 | 77.1 | 10 |
| $^{13}C_4$-mnBP | 225.1 | 79.1 | 10 |
| mEHHP | 293.2 | 121.1 | 10 |
| $^{13}C_4$-mEHHP | 297.2 | 124.1 | 10 |
| mEOHP | 291.2 | 121.1 | 10 |
| mBzP | 255.2 | 105.1 | 10 |
| $^{13}C_4$-mBzP | 259.2 | 107.1 | 10 |
| mEHP | 277.2 | 134.1 | 10 |
| BPA | 227.2 | 212.2 | 18 |
| D16-BPA | 241.2 | 223.2 | 19 |
| 4-methylumbelliferrone | 177.1 | 133.1 | 26 |
| $^{13}C_4$-4-methylumbelliferrone | 179.1 | 135.1 | 26 |
| Dansylated BPA quantitation SRM | 695.5 | 171.1 | 40 |
| Dansylated BPA confirmation SRM | 695.5 | 235.1 | 38 |
| Dansylated $D_{16}$-BPA | 709.5 | 170.1 | 35 |

water 1:1 was used. For PBs–BPA–phthalate metabolites, we modified the gradient used by Silva et al. [32] as depicted in the Electronic Supplementary Material (ESM) Table S1. Flow rate was set at 350 μL/min. For dansylated-BPA analysis, the applied gradient (200 μL/min flow rate and 0.1 % formic acid as mobile phase additive) is presented in ESM Table S2.

## Sample preparation

After collection, samples were stored at −18 °C and were thawed overnight at 4 °C before analysis. Treatment and clean-up of the samples was based on previous work [33] but modified as follows: Urine samples (1 mL) were transferred to a Falcon tube (polypropylene, 15 mL) and spiked with 100 ng $^{13}C_4$-labelled phthalate metabolites and $^{13}C_4$-labelled 4-methylumbelliferrone, 20 ng $^{13}C_6$-labelled PBs and 200 ng 4-methylumbelliferryl glucuronide. The hydrolysis step, with use of *E. coli* or *Helix pomatia* β-glucuronidase, has been reported and evaluated in numerous publications [34, 36, 37]. We have used the *E. coli* β-glucuronidase hydrolysis as follows: *E. coli* β-glucuronidase buffer (prepared daily, per sample: 10 μL *E. coli* β-glucuronidase and 250 μL ammonium acetate buffer, 1 M in aqueous solution, pH 6.5) was added to the urine samples, and hydrolysis was completed at 37 °C for 90 min. After enzymatic hydrolysis completion, 1 mL of ammonium hydroxide buffer (0.15 %*w*/*v* $NH_4OH$ in 1:1 acetonitrile/water) was added to the samples, which were loaded onto the 60 mg solid phase extraction cartridge. The eluents of the first cartridge (60 mg) were acidified with 3 mL monosodium phosphate buffer (0.14 M $NaH_2PO_4$, aqueous solution, at pH 2) and loaded onto the second solid phase extraction cartridge (200 mg). The eluents from the 200 mg cartridge were discarded. Both cartridges were eluted with 3 mL acetonitrile and 3 mL ethyl acetate each. The eluents of both cartridges (12 mL in total) were combined and evaporated to dryness with a rotational vacuum concentrator RVC 2-25 (Martin Christ, Germany) (60 °C, 20–45 mbar, 150 min for 18 samples). The residues were dissolved in 0.4 mL of water and transferred to a 2-mL autosampler glass vial with a 0.4-mL volume insert. After phthalate metabolites–PBs–BPA LC-MS analysis, in order to enhance BPA detection limit, 160 μL 7 % *v*/*v* aquatic ammonium hydroxide and 40 μL dansyl chloride 12.5 mg/mL in acetone were added to the autosampler vials containing the samples (200 μL, the rest was discarded), and with 0.5 h heating at 65 °C, dansylation was completed, and samples were re-analysed with LC-MS. Dansylated-BPA structure is presented in Fig. 1. In order to normalise the variability in urine density, an aliquot of 0.5 mL for each urine sample was analysed to determine the creatinine by concentration using the Olympus 2700 immunoassay system (Beckman Coulter, USA).

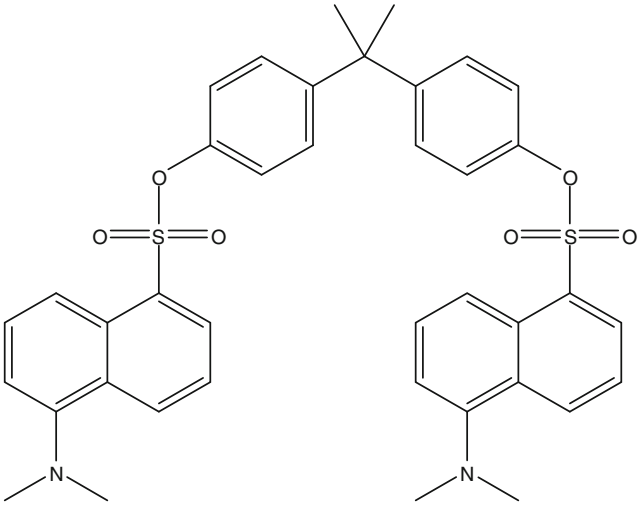

**Fig. 1** Dansylated BPA structure

## Analytical performance

The following parameters were evaluated for the analytical performance of the method: isotopic purity of labelled compounds, recovery and blank levels, method limit of detection/quantitation (mLOD/mLOQ), method limit of detection/quantitation (mLOD/mLOQ), linearity, accuracy and repeatability. Matrix effects cannot be calculated accurately due to the variability of urine density among samples. For this reason, an average matrix effect influence was taken into account for the analyses, using synthetic urine in calibration curves, blanks, recovery and quality control samples. In order to check the isotopic purity of labelled compounds, we analysed an aqueous solution (200 ng/mL) of each standard, three times. To determine the method recovery, 1 mL of synthetic urine (spiked with native analytes: 1, 5 and 50 ng for PBs/BPA and 5, 50 and 100 ng for phthalate metabolites) was analysed three times for each level, and internal standards were added before HPLC-MS analysis. To determine possible contaminations during analysis (blank levels), 1 mL of synthetic urine was analysed three times. Instrument limits of detection (iLOD) and quantitation (iLOQ) were set at signal to noise (S/N) ratios equal to 3 and 10, respectively. iLOD and iLOQ were calculated using a calibration curve in synthetic urine in order to take into account signal suppression due to matrix effect. The mLOD and mLOQ were calculated by adjusting iLOD and iLOQ, respectively, with the method recovery value and sample condensation factor using the following equations: [mLOD]=[iLOD]/[sample condensation factor×recovery] and [mLOQ]=[iLOQ]/[sample condensation factor×recovery]. The linearity of the method ($R^2$) was calculated using the linear equation of calibration curve for each analyte. In order to evaluate the repeatability of the method, a pooled urine sample, spiked with native analytes (1, 5 and 50 ng for PBs/BPA and 5, 50 and 100 ng for phthalate metabolites), was

aliquoted and analysed five times for each level. The accuracy of the method was calculated by analysing quality control QC samples ($N$=5 for each level), which were prepared by spiking the same amounts of native compounds as in repeatability test to synthetic urine.

### Application to real samples

Eighty urine samples were collected from men, working in the city of Heraklion (Island of Crete, Greece). The volunteer samples differed into groups based on their professional activity, namely chemists/biologists working in laboratory ($N$=28); professors of Chemistry and administration personnel ($N$=18); bank clerks ($N$=9); hairdressers ($N$=12); and miscellaneous others ($N$=13). It has to be mentioned that the above samples were collected in the frame of the EU funded ENVIROGENOMARKERS project (FP7-ENV-2008-1, Grant Agreement No. 226756), whose goal was to determine only phthalate metabolites. Thus, *E. coli* β-glucuronidase was chosen as the hydrolysis enzyme. The samples were initially analysed in order to measure phthalate metabolites and PBs. One injection was performed per sample, and the mobile phase contained 0.1 % acetic acid. Then, the samples were treated with dansyl chloride in order to enhance BPA detection limits and were reanalysed with LC-MS (one injection/sample). In every set ($N$=30) of urine samples, one blank and two quality control (QC) samples were analysed. Samples that exceeded calibration range were diluted with synthetic urine and extracted/analysed again.

## Results and discussion

### Optimisation of mass spectrometry

ESI is the widely used ionisation technique due to its enhanced sensitivity, the low flow rates it requires and its capability to ionise a wide range of analytes [31, 34, 37, 38]. Although sensitivity was at same levels with APCI, the need for frequent maintenance of this ionisation source (cleaning/replacing corona needles, replacing sample tube) and the significantly larger solvent consumption led us to use ESI. A preliminary optimisation of the mass spectrometer parameters took place with direct infusion of each compound at 10 μL/min flow rate via a syringe pump. Optimisation was repeated, after HPLC method development, and each compound was re-optimised using the chromatographic conditions (flow rate and solvent type), existing at its retention time. Mobile phase flow (via HPLC pump) and analyte solution flow (via syringe pump) were connected with a T-junction and were driven to the mass spectrometer. Despite the automatic optimisation capability of TSQ Quantum, in order to achieve more accurate results, we performed this step manually. In order to achieve appropriate detection limits for phthalate metabolites, PBs and BPA with our mass spectrometer (TSQ Quantum, model acquired in 2003), we chose to follow one SRM per analyte. Furthermore, we have tested and optimised two SRMs per analyte without observing co-eluting peaks in any sample. For dansylated-BPA, two SRMs were monitored.

**Table 2** Comparison of instrument/method limits of detection/quantification for different conditions

| | iLOD–iLOQ (ng/mL) | | | mLOD–mLOQ (ng/mL) | | |
|---|---|---|---|---|---|---|
| Analyte | iLOD–iLOQ with 0.1 % acetic acid (ng/mL) | iLOD–iLOQ without acetic acid (ng/mL) | iLOD–iLOQ with dansylation (ng/mL) | With acetic acid in mobile phase | Without acetic acid in mobile phase | With dansylation |
| mEP | – | – | – | 0.40–1.33 | – | – |
| mnBP | – | – | – | 0.25–0.83 | – | – |
| miBP | – | – | – | 0.41–1.37 | – | – |
| mBzP | – | – | – | 0.02–0.07 | – | – |
| mEHP | – | – | – | 0.84–2.80 | – | – |
| mEHHP | – | – | – | 0.01–0.03 | – | – |
| mEOHP | – | – | – | 0.18–0.60 | – | – |
| MPB | 0.28–0.93 | 0.14–0.47 | – | 0.12–0.40 | 0.06–0.20 | – |
| EPB | 0.13–0.43 | 0.12–0.40 | – | 0.06–0.20 | 0.06–0.18 | – |
| iso-PPB | 0.41–1.37 | 0.23–0.77 | – | 0.24–0.80 | 0.13–0.45 | – |
| nPPB | 0.33–1.10 | 0.19–0.63 | – | 0.15–0.50 | 0.09–0.29 | – |
| iso-BPB | 0.15–0.50 | 0.07–0.23 | – | 0.08–0.27 | 0.04–0.13 | – |
| nBPB | 0.15–0.50 | 0.08–0.27 | – | 0.07–0.23 | 0.04–0.12 | – |
| BPA | 4.43–14.76 | 0.16–0.53 | 0.008–0.026 | 2.01–6.69 | 0.07–0.24 | 0.007–0.024 |

Optimisation of HPLC

For PBs-BPA-phthalate metabolites and with ESI as ionisation source, in addition to the selected BetaSil Phenyl column, we also tested a PerfectSil 120 Phenyl (3 μm, 100× 2.1 mm; MZ-Analytical, Germany) and a Gemini $C_{18}$ (3 μm, 100×2 mm; Phenomenex, USA) HPLC columns, which did not provide adequate separation and peak shapes. Methanol was tested as the mobile phase; although for PBs and BPA analysis, the results were similar to those obtained using ACN; for phthalate metabolites, the separation and peak shapes were unsatisfactory. With APCI, a pair of tandemly connected Hypersil ODS (5 μm, 250×4.6 mm, MZ-Analytical, Germany) HPLC columns were tested, with both

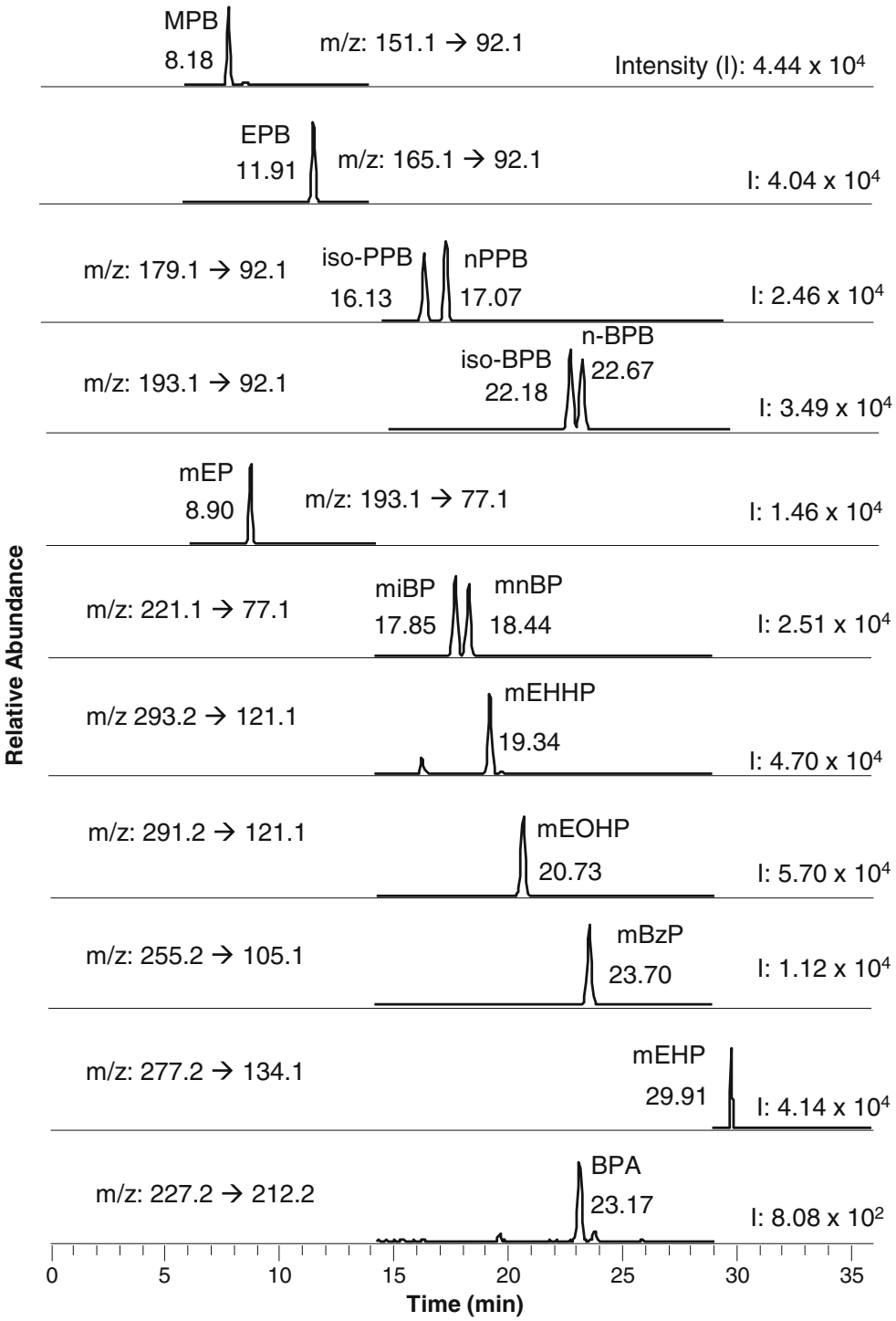


**Fig. 2** Pooled urine sample chromatogram for simultaneous phthalate metabolites/PBs/BPA analysis and their SRMs, peak intensities and retention times in minutes

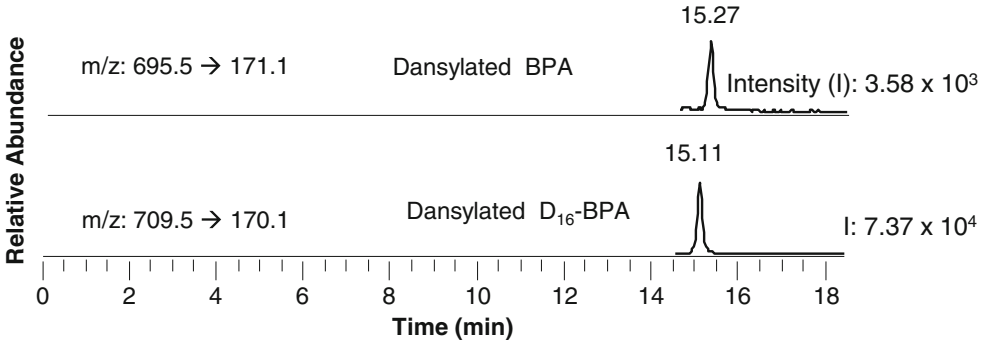


**Fig. 3** Urine sample chromatogram for dansylated BPA/$D_{16}$-BPA and their SRMs, peak intensities and retention times in minutes

ACN and methanol used as mobile phases. Although separation for PBs was similar to that achieved using a BetaSil Phenyl column with ESI, the gradient was longer, the system pressure and flow rate were significantly higher and the separation of phthalate metabolites was inadequate. The optimum results for the baseline separation of the structural isomers of BPB, PPB and mBP were accomplished with the BetaSil Phenyl HPLC column. The modified gradient (of a previously reported method [32]; see "HPLC conditions" under "Materials and methods") we used completed the separation of paraben stuctural isomers. The addition of 0.1 % acetic acid to the mobile phase was essential for the retention of phthalate metabolites and their proper separation [34, 37, 38]. Besides, the acidic mobile phase suppresses the analyte signal, in the negative ESI mode, and increases the iLOD for all studied analytes (Table 2) and particularly for BPA [34, 37, 38]. A chromatogram of a pooled urine sample, spiked with 100 ng of all analytes, is shown in Fig. 2.

For dansylated-BPA analysis, between PerfectSil $C_8$, BetaSil Phenyl and Gemini $C_{18}$ columns, the first demonstrated the best chromatographic performance (peak shape, S/N, matrix components separation). The new gradient that we developed is presented in ESM Table S2, and the chromatographic result of its use for the analysis of a real urine sample is depicted in Fig. 3.

In order to increase reproducibility and zero carry-over effects in HPLC, a mixture of 1:1 methanol/water was chosen as syringe cleaning solvent, and syringe washes were modified as described in HPLC conditions" under "Materials and methods. To the best of our knowledge, there is only one report using a UPLC column for the separation of both paraben structural isomers (butyl and propyl) [30]. Another study [39] applied an unpublished method, which separates both propyl and butyl, although only few details are given. In the present study, we achieved for the first time to separate these three isomers using a conventional HPLC column and pump.

**Table 3** Analytical performance characteristics

| Compound | Linearity range ($R^2$>0.99) (ng/mL) | Recovery % ($n$=3, ±SD) | | | Accuracy (%) | | | Repeatability (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Low | Medium | High | Low | Medium | High | Low | Medium | High |
| mEP | 0.5–512 | 60.5±6.4 | 69.5±4.5 | 68.5±7.5 | 9.2 | 5.5 | 7.3 | 2.6 | 2.6 | 5.2 |
| mnBP | 0.5–512 | 80.7±7.8 | 87.2±7.8 | 70.6±8.2 | 5.7 | 6.6 | 4.2 | 2.0 | 7.0 | 3.5 |
| miBP | 0.5–512 | 73.9±9.6 | 76.7±5.4 | 67.3±7.4 | 9.0 | 3.9 | 3.1 | 6.2 | 2.7 | 4.2 |
| mBzP | 0.5–512 | 64.8±5.4 | 69.4±6.6 | 67.0±6.7 | 7.4 | 4.0 | 3.8 | 6.8 | 5.5 | 3.5 |
| mEHP | 1–512 | 43.5±5.2 | 40.8±1.8 | 41.5±16.0 | 20.8 | 19.3 | 5.2 | 7.9 | 2.8 | 5.7 |
| mEHHP | 0.5–512 | 64.2±7.8 | 62.1±1.8 | 66.6±7.5 | 9.1 | 7.6 | 6.3 | 5.2 | 5.7 | 5.6 |
| mEOHP | 0.5–512 | 59.1±5.1 | 79.6±7.9 | 68.3±7.1 | 15.6 | 8.5 | 7.0 | 6.9 | 7.6 | 6.2 |
| MPB | 0.25–512 | 95.0±8.6 | 99.8±4.3 | 91±3.4 | 19.5 | 17.8 | 10.1 | 11.0 | 2.8 | 1.2 |
| EPB | 0.25–512 | 99.0±8.2 | 103.2±2.6 | 88±6.7 | 12.7 | 10.3 | 13.1 | 8.0 | 6.0 | 1.5 |
| iso-PPB | 0.25–512 | 75.9±11.5 | 70.7±6.1 | 69±8.0 | 19.9 | 0.1 | 3.9 | 7.0 | 4.7 | 1.9 |
| nPPB | 0.25–512 | 89.8±4.6 | 95.4±4.3 | 86±6.9 | 5.1 | 3.9 | 3.7 | 15.0 | 1.4 | 3.4 |
| iso-BPB | 0.25–512 | 75.3±5.1 | 80.8±3.8 | 77±5.7 | 8.6 | 6.5 | 7.5 | 9.3 | 1.9 | 1.8 |
| nBPB | 0.25–512 | 69.8±4.8 | 87.7±2.7 | 83±5.6 | 15.2 | 1.1 | 1.1 | 8.6 | 1.8 | 1.5 |
| BPA (dansylated) | 0.05–64 | 75.2±4.2 | 78.9±4.3 | 88±5.6 | 3.1 | 1.9 | 1.4 | 2.4 | 2.6 | 1.6 |

*SD* standard deviation

**Table 4** Concentration values in nanograms per millilitre of urine for 80 urine samples

| | Median (ng/mL) | Maximum (ng/mL) | Average (ng/mL) | Detectability (%) |
|---|---|---|---|---|
| MPB | 5.5 | 3868.6 | 76.7 | 100 |
| EPB | 0.4 | 205.4 | 5.9 | 94 |
| iso-PPB | N.D. | 0.2 | N.D. | 1 |
| nPPB | 0.2 | 806.6 | 21.6 | 60 |
| iso-BPB | N.D. | 0.9 | N.D. | 13 |
| nBPB | N.D. | 163.0 | 2.2 | 15 |
| mEP | 100.3 | 3649.8 | 218.4 | 100 |
| miBP | 45.2 | 352.1 | 58.1 | 100 |
| mBP | 29.9 | 144.8 | 41.8 | 100 |
| mEHHP | 52.1 | 243.0 | 69.2 | 100 |
| mEOHP | 34.6 | 122.4 | 40.8 | 99 |
| mBzP | 11.3 | 205.3 | 15.6 | 100 |
| mEHP | 15.0 | 99.2 | 20.5 | 91 |
| BPA | 0.6 | 3.93 | 0.85 | 96 |

*N.D.* not detected

## Optimisation of sample preparation

Applying the modified clean-up procedure for phthalate metabolites (see "Sample preparation" under "Materials and methods"), PBs and BPA were also effectively retained (Table 3). Furthermore, to analyse more samples simultaneously and minimise manual intervention, evaporation to dryness was performed with the rotational vacuum concentrator. Derivatisation of phenolic hydroxyl groups with dansyl chloride is a well-known reaction in organic chemistry, and it has applied to enhance mass spectrometric sensitivity of urinary BPA [40, 41]. We used aqueous $NH_4OH$ to buffer the samples, instead of a non-volatile buffer [40, 41], in order to prevent blocking of ion transfer tube after a few injections. Basic pH does not affect BPA deprotonation at ESI source and therefore does not suppress its signal since it elutes at 15.1 min ($NH_4OH$ is not retained by the column) and the eluent contains 0.1 % formic acid. Dansyl chloride was added in excess (0.5 mg per sample) to ensure dansylation for any urine sample (variable concentrations of compounds with phenolic hydroxyls). To the best of our knowledge, this is the first common clean-up protocol reported for the analysis of phthalate metabolites, PBs and BPA.

## Analytical performance

Recoveries were higher than 59.1 % for all studied metabolites and spiking levels except mEHP (41.5–43.5 %), which was not eluted effectively from the SPE cartridges possibly due to its high lipophilicity (Table 3). We consequently achieved method limits of detection at the picogram per millilitre to low nanogram per millilitre range. Blank contamination was not detectable. The linearity, for the expected concentration range (as presented in Table 3), was excellent ($R^2$>0.99). All isotopically labelled standards were found without detectable contaminations of native compounds. Matrix effects have been reported, for both APCI and ESI analyses, of the studied compounds [33, 34, 37]. In order to control matrix effects and to perform an accurate analysis, we have used isotopically labelled internal standards for most of the target compounds. Due to their limited commercial availability in the period of study, we used nine labelled internal standards for 14 target compounds. Repeatability experiments showed standard deviations (STD) <15.0 % and accuracy <20.8 % for all studied metabolites and spiking levels (Table 3). The chromatogram of a spiked pooled urine sample, analysed for phthalate metabolites–PBs–BPA, is shown in Fig. 2, and a urine sample, processed with dansyl chloride, is presented in Fig. 3.

## Application to real samples

We have applied our method to analyse free and glucuronated metabolites of phthalates, PBs and BPA in eighty urine samples of adult male subjects (see "Application to real samples" under "Materials and methods"). Enzymic hydrolysis completion was confirmed both by the absence of 4-methylumbelliferryl glucuronide and by the presence of 4-methylumbelliferrone in the processed samples. Reaction was successful in all analysed

**Table 5** Median concentration values in micrograms per gram of urinary creatinine for 80 samples

| | MPB | EPB | iso-PPB | nPPB | nso-BPB | nBPB | BPA | mEP | miBP | mBP | mEHHP | mEOHP | mBzP | mEHP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Chemists /biologists | 2.0 | 0.2 | N.D. | 0.1 | N.D. | N.D. | 0.4 | 62.5 | 26.1 | 20.7 | 41.9 | 27.8 | 9.2 | 10.9 |
| Professors/administrative personnel | 2.9 | 0.3 | N.D. | N.D. | N.D. | N.D. | 0.4 | 42.0 | 30.6 | 19.9 | 27.2 | 15.0 | 5.2 | 5.8 |
| Bank clerks | 3.5 | 0.3 | N.D. | N.D. | N.D. | N.D. | 0.3 | 64.4 | 40.9 | 27.3 | 22.2 | 15.4 | 4.7 | 10.6 |
| Hairdressers | 10.5 | 1.3 | N.D. | 0.5 | N.D. | N.D. | 0.3 | 146.8 | 29.4 | 20.2 | 27.4 | 15.7 | 9.0 | 13.4 |
| Various | 1.6 | 0.2 | N.D. | N.D. | N.D. | N.D. | 0.3 | 46.2 | 22.8 | 12.7 | 26.0 | 15.5 | 5.4 | 6.4 |

*N.D.* not detected

samples. In order to assure the determination of the above-mentioned metabolites, we performed the instrumental analysis of the samples twice: (i) with acetic acid addition to the mobile phase to determine phthalate metabolites and PBs and (ii) with dansylation of the samples in order to obtain the optimal sensitivity for BPA. It has to be underlined that, with newer MS instruments, significantly lower limits of detection can be achieved and dansylation might be avoided. Chen et al. [37], with an AB-SCIEX API 4000 LC-MS/MS modern instrument (use of 0.1 % acetic acid in acetonitrile/$H_2O$ as mobile phase and analysis of 0.2 mL volume sample), obtained mLOD for BPA in the order of 0.3 ng/mL. Urinary concentration data, with the detectability of analytes, are shown in Table 4. Phthalate metabolites were detected, almost in all samples, at significantly higher levels compared to BPA and PBs. Median values of creatinine-adjusted concentrations are shown in Table 5. We attempted to trace differentiations between the professional group exposures. The most interesting outcome is the particularly higher concentrations determined in the hairdressers group samples compared to the other groups, for mEP, MPB and EPB. This observation is possibly justified by the extensive daily use of cosmetic products by these professionals [7, 8].

## Conclusions

We have developed the first common clean-up procedure for the determination of phthalate metabolites, PBs and BPA. In addition, we elaborated an HPLC-ESI-MS/MS method, with chromatographic characteristics, suitable for the identification and quantification of seven phthalate metabolites, six PBs and BPA in human urine. This method provided a clear separation of *n*-/iso-structural isomers of butyl paraben and propyl paraben. To the best of our knowledge, this is reported for the first time using conventional HPLC columns. The described clean-up procedure and LC/MS method were successfully applied to human urine analysis allowing the determination of the reported analytes in a large number of samples.

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**Conflict of interest** The authors declare that they have no conflicts of interest.

**Compliance with ethical standards** All participants were provided written, informed consent for themselves after having received a complete description of the study, which was approved by the Ethics Committee of the University Hospital in Heraklion, Greece.

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