Development and validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous determination of phthalates and bisphenol a in serum, urine and follicular fluid

Anna Pia Dima [a], Lucia De Santis [b], Cristina Verlengia [c], Francesco Lombardo [a], Andrea Lenzi [a], Monica Mazzarino [d], Francesco Botrè [d,e,f,*,1], Donatella Paoli [a,1],
on behalf of the Italian Society of Embryology, Reproduction, Research (SIERR)

[a] Laboratory of Seminology – Sperm Bank "Loredana Gandini", Department of Experimental Medicine, "Sapienza" University of Rome, Italy
[b] IVF Unit, San Raffaele Scientific Institute, Vita-Salute University, Milan, Italy
[c] UOSD Centro PMA Sant' Anna – ASL, Roma 1. Rome, Italy
[d] Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Largo Giulio Onesti 1, 00197 Rome, Italy
[e] Department of Experimental Medicine, "Sapienza" University of Rome, Italy
[f] REDS – Research and Expertise on AntiDoping Science, ISSUL – Institute des Sciences du Sport, Université de Lausanne, Synathlon – Quartier Centre, 1015 Lausanne, Switzerland

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ABSTRACT

Phthalates and bisphenol A interfere with the synthesis, secretion, transport, binding, metabolism, and excretion of endogenous hormones and, for this reason, are classified as endocrine disruptors.

We are here presenting an analytical method for the simultaneous detection of six phthalates metabolites and bisphenol A in different biological fluids (urine, serum and follifular fluid) by liquid chromatography coupled to tandem mass spectrometry. The quantification was carried out in negative electrospray ionization mode using selected reaction monitoring as acquisition mode. Different extraction protocols, using either solid phase or liquid/liquid extraction, were comparatively evaluated to optimize the sample preparation procedure. Solid-phase extraction was chosen as it ensured the best recovery and overall sensitivity. The method was successfully validated: recovery varying in the range 71 ± 2%–107 ± 6% depending on the target analyte and the matrix considered, *intra-assay* and *inter-assay* precision $\leq$ 12% for follicular fluid, $\leq$11% for serum and $\leq$ 10% for urine and accuracy $\leq$ 115% for follicular fluid, $\leq$113% for serum $\leq$ 115% for urine , linearity with $R^2 > 0.99$, with the exception of MEP (recovery 64 ± 8%, *intra-assay precision* $\leq$ 20%, *inter-assay* precision $\leq$ 16% for follicular fluid). The actual applicability of the method developed and validated in this study was assessed by the analysis of real samples, including 10 specimens of follicular fluid, serum and urine samples, that showed the presence of phthalates metabolites and Bisphenol A, and confirming that the newly developed method can be applied in the routine clinical laboratory for the identification and quantitation of these endocrine-disrupting chemicals.
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1. Introduction

The endocrine-disrupting chemicals (EDCs) are defined by the U.S. Environmental Protection Agency (EPA) as a class of exogenous agents that can interfere with the synthesis, secretion, transport, binding, metabolism, and excretion of endogenous hormones, altering, therefore, hormonal homeostasis and reproductive function [1].

The main mechanisms throughout EDCs act at the level of the reproductive system are related to their interaction with hormone receptors and the interference with steroidogenesis and hormonal metabolism [1,2]. Numerous studies reported several negative outcomes on human health and reproduction originating from the exposure to Bisphenol A (BPA) and phthalates, and therefore they were classified as EDCs.

Studies on rats and mice showed that BPA has estrogenic properties due to the binding affinity with estrogenic receptors

(ERs) alfa and beta competing with estradiol [3,4]. In addition, BPA was also shown to lower both the sperm count and the sperm quality parameters [5,6]. BPA is the principal monomer used in the production of most common forms of polycarbonate, which being almost unbreakable, is used for a large number of children's products, bottles, sports equipment, medical and dental devices, and wherever there are needed characteristics of hardness and resistance. It is also a component of epoxide resins that they are found as the internal coating in most food and beverage materials [7].

Phthalates instead showed anti-androgenic effects due to their ability to inhibit the synthesis of testosterone in Leydig cells and their prolonged intake, even if at apparently very low doses, can be linked to infertility due to its capacity of altering semen quality [8,9]. Since these compounds are contained in products of common use in daily life, humans are constantly exposed to their presence. The intake can occur through multiple routes: ingestion, inhalation, contact with skin, intravenous route, and biological transfer from the placenta and maternal milk [10]. Indeed, phthalates are a family of man-made compounds used in the plastic industry as plasticizer agents or as components of polymers to improve their flexibility and modelling. Their production is largely widespread because of their use in products of all kind: beauty and infant products, medical devices, coating of some medications, pharmaceutical, and cosmetically products, as solvents in perfume and pesticides and finally in the preparation of nail polish, adhesives and varnishes [11,12].

Although there is already a considerable amount of experimental evidence on the negative effects of phthalates and BPA on male reproduction, the information is still incomplete concerning their effects in females, even though consolidated evidence proved adverse effects also on the female reproductive system. For indeed, the main effects of these compounds involve the folliculogenesis [12,13], which is the evolution of primordial follicles to primary, pre-antral and antral follicles, before they become mature: phthalates and BPA are reported to interfere with the correct developing of follicles leading to fertility problems and other disorders like polycystic ovary syndrome and endometriosis [14]. Furthermore, levels of phthalates and BPA have been associated with elevating risks of implantation failure in women undergoing *in vitro* fertilization (IVF) [4].

For the above reasons, it would be extremely useful to detect the levels of these compounds in the ovary, both to allow population biomonitoring studies and to correlate their presence with the insurgence of specific diseases or syndromes. Levels of BPA, phthalates and their metabolites in urine [15–24], serum [17,18,25–27], and other matrices, like semen and meconium [28], have already been measured by previous investigators; while very few studies have instead been carried out to assess their presence and quantitation in the follicular fluid (FF) [8,25,29]. Indeed, FF is the microenvironment surrounding the oocyte and somatic cells and it is critical for oocyte health; consequently, FF is perhaps the biological matrix that best reflects the exposure status of the ovary, and therefore the most suitable one to monitor the effects on female reproduction and failure infertility [29].

We are here presenting the development and validation of an ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous determination of BPA and six phthalates metabolites not only in urine and serum but also in follicular fluid. The six phthalate metabolites here considered are the following: mono-ethyl phthalate (MEP), mono-n-butyl phthalate (*n*-MBP), mono-benzyl-phthalate (MBzP), mono-ethyl-hexyl phthalate (MEHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); they all originate from the most common phthalates diesters, and for this reason, they were classified by the US

Environmental Protection Agency (USEPA) as six chemicals priority pollutants [12,29].

We have pre-evaluated and optimized all the experimental conditions, specifically considering the sample preparation of the biological samples, as well as the chromatographic and mass spectrometric conditions.

2. Materials and methods

2.1. Chemicals and reagents

Certified standards of MEP (100 μg/mL in methyl-*tert*-butyl-ether, MTBE), *n*-MBP (100 μg/mL in MTBE), MBzP (100 μg/mL in MTBE), MEHP (100 μg/mL in MTBE), MEOHP (100 μg/mL in MTBE), MEHHP (100 μg/mL in MTBE), BPA (powder purity ≥ 99%) and the isotope labelled internal standards (IS) $^{13}C_{12}$-BPA (100 μg/mL in acetonitrile) and $^{13}C_4$MEOHP, (100 μg/mL in MTBE), were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). HPLC-grade reagents, including acetonitrile, methanol, MTBE, isopropanol (IPA), ethyl acetate (EtAc), chloroform ($CHCl_3$), were purchased from Sigma Aldrich (St Louis, MO, USA). The ultra-purified water used was of Milli-Q-grade (Millipore Italia, Vimodrone, Milano, Italy). The enzyme β-glucuronidase (from *E. coli*) used for the enzymatic hydrolysis of conjugates was purchased from Roche (Monza, Italy). Phosphate-buffered saline (PBS, pH 7,4), Bovine Serum Albumin (BSA, 96%) and *Surine negative control* (used as surrogate urine matrix) were purchased from Sigma Aldrich (St Louis, MO, USA). Buffers at pH 3.5 (ammonium acetate/hydrochloric acid), 7.0 (potassium phosphate monobasic/sodium phosphate dibasic) and 9.0 (Boric acid/sodium hydroxide/potassium chloride) were purchased from Carlo Erba (Milan, Italy). Oasis® HLB cartridges (3 mL, 60 mg) were obtained from Waters Corporation (Milford, Massachusetts, United States).

2.2. Working solutions, calibration solutions, in-house quality controls

Working solutions were prepared by diluting each stock solution (100 μg/mL) of phthalates metabolites and BPA (1 mg/mL in MTBE) in methanol at a concentration of 10 μg/mL. The internal standard working solution was instead prepared by diluting the IS stock solutions (100 μg/mL) in methanol at the final concentration of 0.5 μg/mL.

Due to the lack of phthalates and BPA-free matrices, calibrators were prepared in surrogate matrices consisting of a 4% (w/v) BSA solution in PBS for serum and FF, and in the *Surine negative control* purchased from Sigma Aldrich for urine [30,31].

The standard calibration solutions of eight concentration levels (0.625, 1.25, 2.5, 5, 10, 25, 50, 100 ng/mL) for both phthalates metabolites and BPA were prepared by adding aliquots of working solutions (10 μg/mL) to the above mentioned surrogate matrices. The in-house quality control (IQCs) samples were prepared using pooled serum, FF and urine from volunteers donors to assess the performance of the system (*intra- and inter assay precision and accuracy*).[31] The samples were analysed before to ensure that phthalate and BPA levels were lower than the lowest IQC level desired. The IQCs were prepared by daily spiking aliquots of working solutions (10 μg/mL) to pooled samples to obtain the desired levels (5, 25 and 100 ng/mL), and the concentrations were calculated by subtracting the signals of analytes already presents in the samples. All the working and calibration solutions were stored at −20 °C until their use.

2.3. Sample preparation

1 mL of follicular fluid or serum was transferred into glass tubes for proteins precipitation: 1 mL of acetonitrile was added, and the samples were mixed for 5 min and then centrifuged for 2 min; the supernatant was then transferred into a new glass tube and evaporated to dryness at 40 °C under nitrogen stream. The protein precipitation step is not necessary in the case of urine samples.

Urine samples, as well as the protein-free follicular fluid and serum sample extracts, obtained as described above, were prepared as follows: 1 mL of sample was mixed with 750 μL of buffer (pH 7.0), 20 μL of the internal standard mixture (ISs: $^{13}C_4$-MEOHP and $^{13}C_{12}$-BPA to a final concentration 10 ng/mL) and 50 μL of β-glucuronidase from *E. coli*. Samples were then incubated for 1 h at 55 °C. After the enzymatic hydrolysis step, the samples were loaded onto the OASIS® HLB cartridges, previously conditioned with 1 mL of methanol and 1 mL of ultra-purified water. The cartridges were then washed with 3 mL of ultra-purified water. The fraction containing the target analytes was finally eluted using 3 mL of methanol directly into glass tubes and evaporated to dryness at 40° C under nitrogen stream.

The residues from the SPE extraction procedure were reconstituted in 50 μL of mobile phase (initial composition), and an aliquot of 6 μL was injected in the UHPLC-MS/MS system.

2.4. UHPLC-MS/MS conditions

All the analyses were performed on a UHPLC (Infinity 1290 II by Agilent Technologies, Santa Clara, CA, USA) equipped with a G7116B autosampler and a G7120A 1290 high-speed pump, coupled to a triple quadrupole tandem mass spectrometric detector (6495 Triple Quad LC/MS by Agilent Technologies Santa Clara, CA, USA) equipped with an electrospray ionization source (ESI).

The chromatographic separation was carried out using a C18 Zorbax Eclipse Plus Column (2.1 mm i.d. × 50 mm length, 1.8 μm particle size) and ultra-pure water (solvent A) and a mixture of methanol/acetonitrile (1:1 v/v) (solvent B) as mobile phase. The gradient program started at 5% B, and after 2 min increasing to 95% of B in 9 min. The column was flushed for 2 min at 95% B and finally re-equilibrated at 5% B for 2 min. The flow rate was set to 0.3 mL/min. The column temperature was set at 40 °C. A washing program was used between each injection to prevent contamination of the needle, using isopropanol and water as washing solvents. The total runtime was 14 min.

Ionization was carried out in negative electrospray mode. Optimized parameters were as follows: gas temperature of 150 °C, a capillary and a nozzle voltage of 3000 and 1500 V respectively, drying gas flow was 15 L/min, and sheath gas flow of 12 L/min. The nebulizer gas was set at 35 psi, and the sheath gas temperature at 375 °C. The experiments were performed using selected reaction monitoring (SRM) as acquisition mode, based on the preliminary characterization of the mass spectrometric profile of all the target analytes and selecting the most appropriate diagnostic ion transitions for each of them (Table 1). Nitrogen (purity > 99.5%) was used as collision gas at 7.8 mPa, obtained from a dedicated nitrogen generator system (Cinel s.r.L. model Zefiro 60, CPS Analytica, Milano, Italy). All aspects of the instrumental control, method setup parameters, sample injection, sequence operation, and data analysis were controlled by the proprietary software (Agilent "Mass Hunter" version B.08.02, Agilent Technologies, Santa Clara, CA, USA).

2.5. Method validation

The newly developed analytical method was validated for specificity, limit of quantification (LOQ), linearity, accuracy, intra- and inter-assay precision, recovery, robustness, ion suppression/enhancement, carryover, and dilution linearity according to the FDA guidelines for endogenous compounds [30–32].

Details on the validation procedures are given in the following paragraphs.

2.5.1. Specificity

Being the analytes under investigation normally present in the matrices tested, during the analysis of the samples, the identity of each analyte tested was confirmed by monitoring retention time and relative abundance of the characteristic ion transitions selected for each compound.

2.5.2. Limit of quantitation

For the determination of the limit of quantitation (LOQ), surrogate matrices spiked with the compounds under investigation at a concentration of 5 ng/mL was used. Serial dilutions were made, and the LOQ was reported as the lowest concentration at which a compound could be quantified in all the samples tested, showing with the ion transition selected for the quantitative analysis a signal-to-noise ratio greater than 20.

2.5.3. Calibration linearity

8-point calibration curves from 0.625 to 100 ng/mL were used for the calibration of FF, serum and urine samples, as we expected the concentrations of the target compounds lower in FF samples and higher in serum and urine, according to previous reports [15,17,19,21,23,27].

2.5.4. Accuracy

Five different aliquots of the IQC samples were analysed, and accuracy was calculated using the following formula:

% = 100 × (measured concentrations of spiked samples-endogenous concentrations)/Nominal concentrations [31].

2.5.5. Intra-assay precision

Five aliquots of each IQC sample were analysed during the same day.

2.5.6. Inter-assay precision

Five aliquots of each IQC sample were analysed in three different days.

2.5.7. Recovery

For the extraction recovery, five different aliquots of surrogate matrices were fortified with the compounds under investigation at three concentration levels (10, 25 and 100 ng/mL) and extracted according to the optimized protocol (positive surrogate matrices) together with five different aliquots of not fortified surrogate matrices (blank surrogate matrices). The organic layer of the blank surrogate matrices were then spiked with the compounds under investigation at the same concentrations of the positive surrogate matrices before the evaporation. To both sets of samples, 20 μL of the IS working solution (0.5 ng/mL) were added into the organic layer before the evaporation. Recovery was calculated by comparison of mean peak area ratios of the analyte and the IS in samples fortified prior to and after pre-treatment.

This test was also performed on real matrices (FF, serum, and urine). Three concentration levels of the target analytes used to fortify the pre and the post-extraction samples were chosen (10, 25 and 100 ng/mL). Recoveries obtained from real matrices and surrogate matrices were compared [31].

2.5.8. Robustness

The robustness of the method was evaluated by analysing surrogate matrices spiked with the compounds under investigation at

Table 1

Retention time, precursor ion, product ions and collision energies (CE) of all the target analytes considered in this study. The values in bold refer to the ion selected for the quantification.

Analyte	RT (min)	Precursor ion (m/z)	Product ions (m/z)	CE (eV)
MEP	1.0	193	147; 149; **121**, 77	14; 6; 6; 14
MBP	4.7	221	121; **77**; 75; 71; 69	10; 22; 34; 10; 6
MBzP	5.2	255	107; 105; **77**	20; 8; 20
MEHHP	5.7	293	**145**; 121; 77	14; 14; 42
MEHP	8.2	277	**127**; 121; 77	18; 18; 30
MEOXP	5.5	291	143; **121**; 77	14; 18; 34
BPA	7.8	227	**212**; 133	18; 26
$^{13}C_{12}$-BPA	7.8	239	**224**; 139; 99	18; 26; 66
$^{13}C_4$-MEOXP	5.5	295	143; **124**; 79	14; 18; 34

the low level of IQCs (5 ng/mL). The measurements were carried out once a week for three weeks, randomly changing the column and the operator involved either in the instrumental analysis and/or in the preparation of the samples. The results were finally compared to that obtained using the real matrices.

2.5.9. Ion suppression/enhancement

The ion suppression/enhancement effects during the chromatographic run caused by matrix interference were also investigated. Specifically, different aliquots of surrogate matrices and solvent only were analysed with continuous co-infusion of the target analytes (1 μg/mL at a flow rate of 7 μL/min) via T-connector.

2.5.10. Carryover

The potential sample-to-sample carryover of the target analytes during processing or instrumental analysis was assessed. Three blank samples were assayed right after several samples containing the compounds under investigation at a concentration of 1 μg/mL in order to double-check the absence of peaks at the expected retention times of the target analytes in the chromatograms of the blank samples.

2.5.11. Dilution linearity

The concentration of some urine phthalates metabolites exceeds the upper limit of the calibration curves and must be diluted. The accuracy and precision of five aliquots of pooled samples of FF, serum and urine spiked with a quantitative of analytes to give a concentration above the upper limit of calibration linearity and then diluted 1:10, were evaluated.

3. Results and discussion

3.1. Optimization of the UHPLC-MS/MS conditions

3.1.1. MS conditions

Instrumental parameters in MS and MS/MS were optimized by injecting the standard solution of the analytes under investigation at a concentration of 1 μg/mL (Table 1). The full-scan MS analysis was first performed, both in positive and negative ionization mode, to identify the molecular ion. The best response was obtained in negative mode for the deprotonated molecular ion [M−H]$^-$ at m/z 193 for MEP, at m/z 221 for MBP, m/z 255 for MBzP, m/z 293 for MHHP, m/z 277 for MEHP, m/z 227 for BPA, m/z 291 for MEOXP, m/z 295 for $^{13}C_4$-MEOHP, m/z 239 for $^{13}C_{12}$-BPA.

The method was then optimized by evaluating different mass spectrometric parameters, including source temperature, product ions, and collision energies. The MS/MS experiments were carried out at a range of collision energies from 5 to 60 eV to obtain information about the dissociation patterns of the compounds under investigation and to select representative product ions to be used to set up the SRM acquisition method (Fig. 1).

Based on the obtained spectrum, it was hypothesized that the main fragmentation pathways for the MEOXP occur at the level of the ester bond, leading to the formation of the fragments ions at m/z 143, and at m/z 121 corresponding to benzoate [16]. A less abundant fragment ion was formed at higher collision energy at m/z 77. These two fragments (m/z 121 and 77) are the most characteristic ions in common with all the phthalates metabolites. In addition to them, two additional ions at m/z 147 and 149 were formed for MEP, the first corresponding to phthalic anhydride; three addition fragments for MBP at m/z 69, 75, and 71, likely originating from the cleavage at the level of the n-butyl chain; two other ions at m/z 105 and 107 for MBzP, deriving from the cleavage at the level of the ester bond; and finally, the fragments at m/z 145 and 127 for MEHHP and MEHP respectively, corresponding to the side chain originating from the cleavage at the level of the ester bond.

For BPA, instead, only two fragments were obtained (Fig. 1b): at m/z 212, due to the loss of one of the methyl groups, and at m/z 133, originating from the loss of one of the phenols, confirming the data reported in a previous study [33]. The same fragmentation, with different hydrogens/deuterium ratios, were obtained for $^{13}C_4$ MEOXP (m/z 124, 79 and 143) and for $^{13}C_{12}$ BPA (m/z 224,139 and 99). For each analyte considered at least two diagnostic ion fragments were selected (Table 1).

3.1.2. Chromatographic conditions

Different chromatographic conditions (gradient and mobile phases composition) were preliminarily evaluated to maximize chromatographic performance in term of peak shapes, resolution, and minimal background interferences. As the organic phase, acetonitrile and methanol were compared: the first gave better peak shape, the second a better peak separation of the analytes considered from the matrix interferences. A compromise was reached by choosing an organic phase composed of methanol/acetonitrile (1:1 v/v). In addition, as previous investigators reported [21], we also found that no acidic mobile phase additives can be used due to BPA ionic suppression. Concerning the initial composition of the gradient, the most problematic compound to retain was MEP, the best compromise between chromatographic retention and analysis run time was obtained starting at 5% of B. Fig. 2 shows the extracted ions chromatogram of a water sample spiked with all the compounds here considered, analysed by using the chromatographic conditions optimized in this study. As it can be seen, all the analytes were retained, with proper chromatographic resolution and peaks shape.

An issue of the utmost relevance was to minimize the risk that samples to be analysed could be accidentally contaminated by the presence in the environment of MBP, MEHP and BPA. Traces of these analytes were indeed detected in the solvents. For this reason, a trapping column (2.1 × 50 mm, particle size 1.8 μm) between the pumps and the autosampler was installed to separate the peaks due to the compounds contained in the solvents used for the preparation of the mobile phases from those actually present in the samples [15] (Fig. 3A–C).
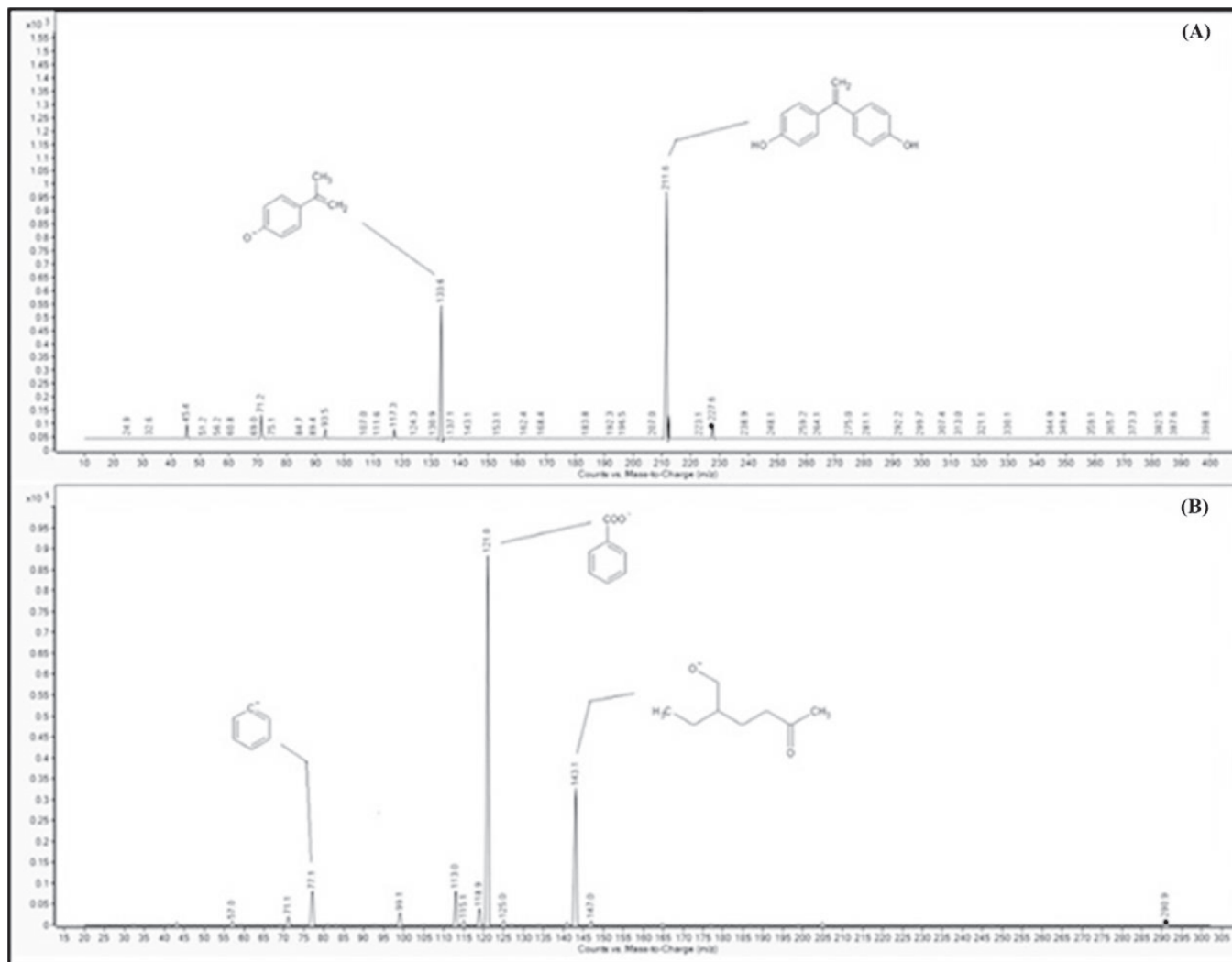
Fig. 1. Product ion spectra of BPA (a) and MEOXP (b), hypothesized structures of product ions for both in panel a and b.
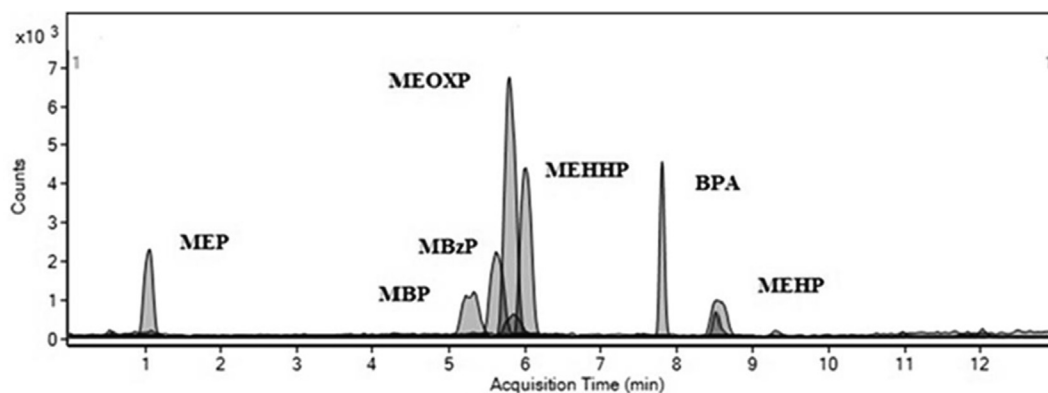

Fig. 2. Extracted ion chromatogram of a water sample spiked with all the analytes at concentration of 0.625 ng/mL.

3.2. Sample preparation optimization

Different extraction protocols, liquid/liquid extraction (LLE), and solid-phase extraction (SPE) were evaluated to obtain good recoveries for all the analytes under investigation. In detail, for the LLE the following solvents were comparatively evaluated: MTBE, ethyl-acetate, chloroform, a mixture of chloroform/iso-propanol/MTBE (8:1:1 v/v/v); 7 mL of organic solvent were added, and the samples were mixed for 6 min on a mechanical shaker; after centrifugation, the organic layer was separated and evaporated to dryness at 40 °C under nitrogen stream. For both LLE and SPE different pH values adding three different buffer solutions (3.5, 7.0, and 9.0) before the hydrolysis step, were compared. A better recovery was observed using the buffer solution at pH 7.0 for
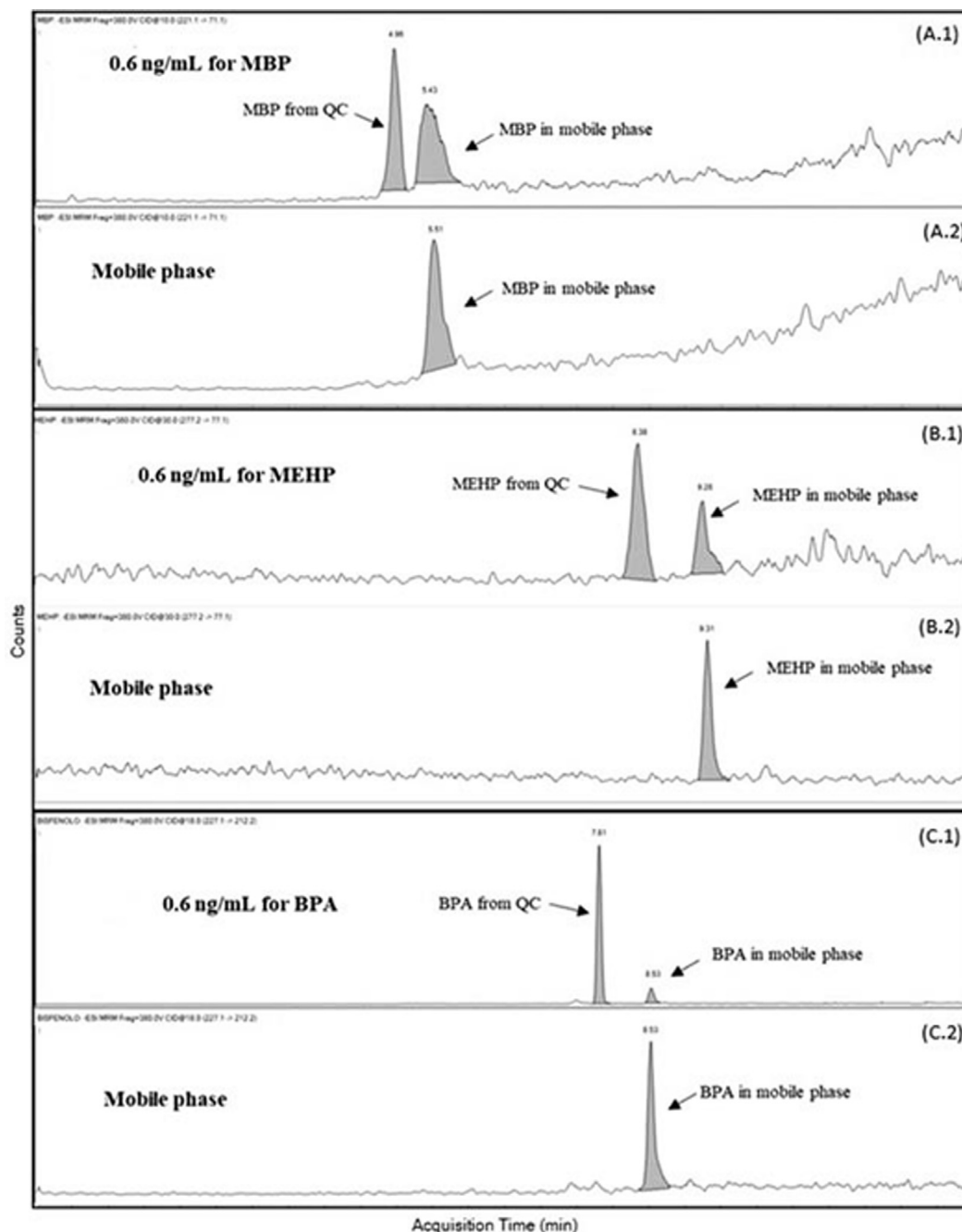
Fig. 3. MBP: extracted ion chromatogram (EIC) obtained from the injection of a standard solution at concentration of 0.6 ng/mL (A.1) and by flushing the mobile phase (A.2). MEHP: extracted ion chromatogram (EIC) obtained from the injection of a standard solution at concentration of 0.6 ng/mL (B.1) and by flushing the mobile phase (B.2). BPA: extracted ion chromatogram (EIC) obtained from the injection of a standard solution at concentration of 0.6 ng/mL (C.1) and by flushing the mobile phase (C.2).

both LLE and SPE extractions, probably due to the weak acidic characteristics of the analytes (Fig. 4). The use of the Oasis® HLB cartridges allowed the best recovery results, with range values between 71 ± 3% and 105 ± 6% for surrogate serum/FF and 71 ± 2 and 107 ± 6 for surrogate urine, exception for MEP, which showed a lower recovery (64 ± 10% and 64 ± 8% for surrogate serum/FF and urine), against the extractions with other solvents (0–66 ± 8% for surrogate serum/FF and 2–68 ± 5% for surrogate urine in MTBE; 0–73 ± 9% for surrogate serum/FF and 0–70 ± 5% for surrogate urine in EtAc; 0–36 ± 9% for surrogate serum/FF and 0–34 ± 6% for surrogate urine in CHCl3; 0–66 ± 10% for surrogate serum/FF and 2–6 7 ± 10% for surrogate urine in NE).

3.3. Method validation

The newly developed and optimized method was validated for qualitative and quantitative interpretation of data in the concentration range of 0.6–100 ng/mL.

3.3.1. Specificity
In the analyses performed on more than 10 serum, urine, or follicular fluids samples, the analytes under investigation were always correctly detected and identified monitoring the retention time and the relative abundances of characteristic ion transitions and comparing with that of calibrators or IQCs running in the same
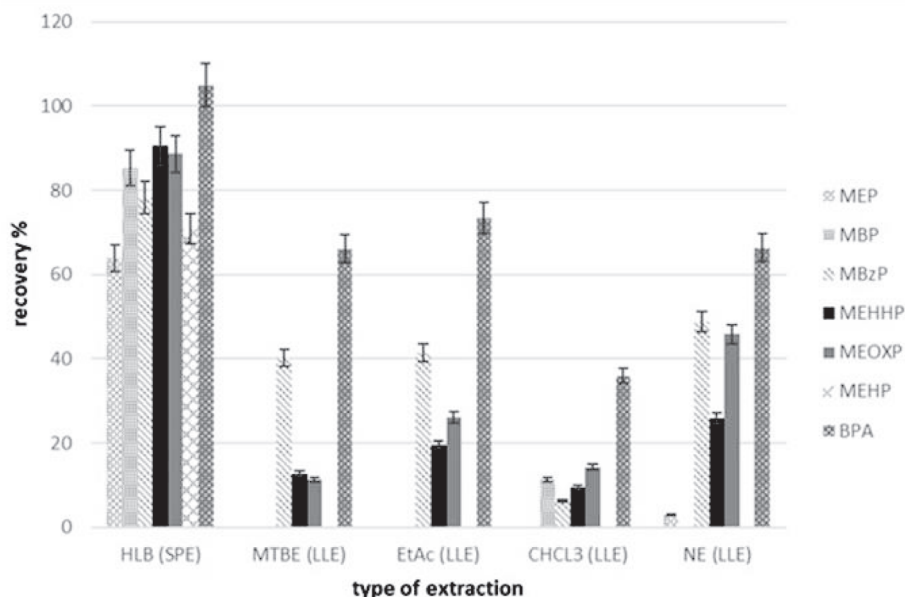
Fig. 4. Influence of different extraction conditions on recoveries of phthalates metabolites and BPA in surrogate serum/FF. (NE) stands for mixtures of chloroform with isopropanol and MTBE.

batch: RSD% of the retention times and of the relative abundances of the characteristic ion transitions selected were lower than 0.5 and 15% respectively.

3.3.2. Limit of quantitation
Results have shown a LOQ comprised in the range of 0.30–1.25 ng/mL, depending on the target compound (Table 2).

3.3.3. Calibration Linearity
Good correlation coefficients (R^2) in the range of 0.998–0.999 were found for all compounds in surrogate serum and urine samples with a linear curve fit type (weighted normal), except for MEP, whose R^2 was 0.997 (Table 2). In Fig. 5 are shown the 8-levels calibration curves prepared in surrogate serum of BPA and MBP.

3.3.4. Accuracy
Accuracy was evaluated from the mean values of the calculated concentrations of the IQCs (5.00, 25.0, and 100 ng/mL) analysed in the three different days. Accuracy was between 85 and 115% for all the compounds under investigation (Table 3).

3.3.5. Intra-assay precision
Within-run repeatability was measured by analysing 5 different aliquots of the IQCs (5.00, 25.0, and 100 ng/mL) during the same day. RSD% of the calculated concentrations was below 15% for all the analyte, except for MEP (RSD% $\leq$ 20%) (Table 3).

3.3.6. Inter-assay precision
Between-run repeatability was measured by analysing 5 different aliquots of the IQCs (5.00, 25.0 and 100 ng/mL) in three different days. RSD% of the calculated concentrations was below 15% for all the analyte except for MEP (RSD% $\leq$ 16%) (Table 3).

3.3.7. Recovery
All the compounds evaluated were extracted with a recovery ranging from 71 ± 3 to 105 ± 6% for surrogate serum/FF and from 71 ± 2–107 ± 6% for surrogate urine (Table 2) Lower recoveries

were found for MEP (64 ± 10% for surrogate serum/FF and to 64 ± 8% for surrogate urine). A good comparison was found between the extraction of the analytes from surrogate matrices and real matrices with RSD% of the recoveries below 15.

3.3.8. Robustness
The measurements were carried out once a week for three weeks, randomly changing the column and the operator involved either in the instrumental analysis and/or in the preparation of the samples. Variation of both qualitative and quantitative data was recorded: the variability was < 15% in terms of the measured concentrations and of the relative abundance of the diagnostic MS/MS ion transitions, while the variability of the retention times was always < 1%.

Similar results were obtained using real matrices.

3.3.9. Ion suppression/enhancement
The test for ion suppression/enhancement effects by post-column split-infusion of analytes yielded no significant matrix effect (lower than 35%) at the retention times of the analytes used, while surrogate matrices were injected [30,34].

3.3.10. Carryover
The analysis of blanks (surrogate matrices) after surrogate matrix samples spiked with target analytes whose concentrations were at least ten times higher than their LOQ values showed no carryover effect. In addition, the configuration of the auto-sampler offered minimal or even zero carryovers to all analyses.

3.3.11. Dilution linearity
The accuracy of the FF, serum and urine samples diluted with their relative surrogate matrices was below 15% of the nominal concentration and precision below 10%.

3.4. Application to real samples

To test the validity of the analytical procedure developed and validated in this study, 10 samples of FF, 10 of serum and 10 of

Table 2
Calibration linearity, limit of quantification and recoveries obtained with SPE extraction procedure.

| Analyte	Calibration linearity	Surrogate serum/FF			Surrogate urine		
	Range (ng/mL)	R^2	LOQ (ng/mL)	Recovery %	R^2	LOQ (ng/ mL)	Recovery %
MEP	0.625–100	0.997	1.25	64 ± 10	0.998	1.25	64 ± 8
MBP	0.625–100	0.999	1.25	85 ± 9	0.999	1.25	85 ± 6
MBzP	0.625–100	0.999	0.3	78 ± 6	0.999	0.3	80 ± 6
MEHHP	0.625–100	0.998	0.3	90 ± 4	0.998	0.3	92 ± 4
MEOXP	0.625–100	0.999	0.3	89 ± 7	0.999	0.3	90 ± 5
MEHP	0.625–100	0.999	1.25	71 ± 3	0.999	1.25	71 ± 2
BPA	0.625–100	0.999	1.25	105 ± 6	0.999	1.25	107 ± 6
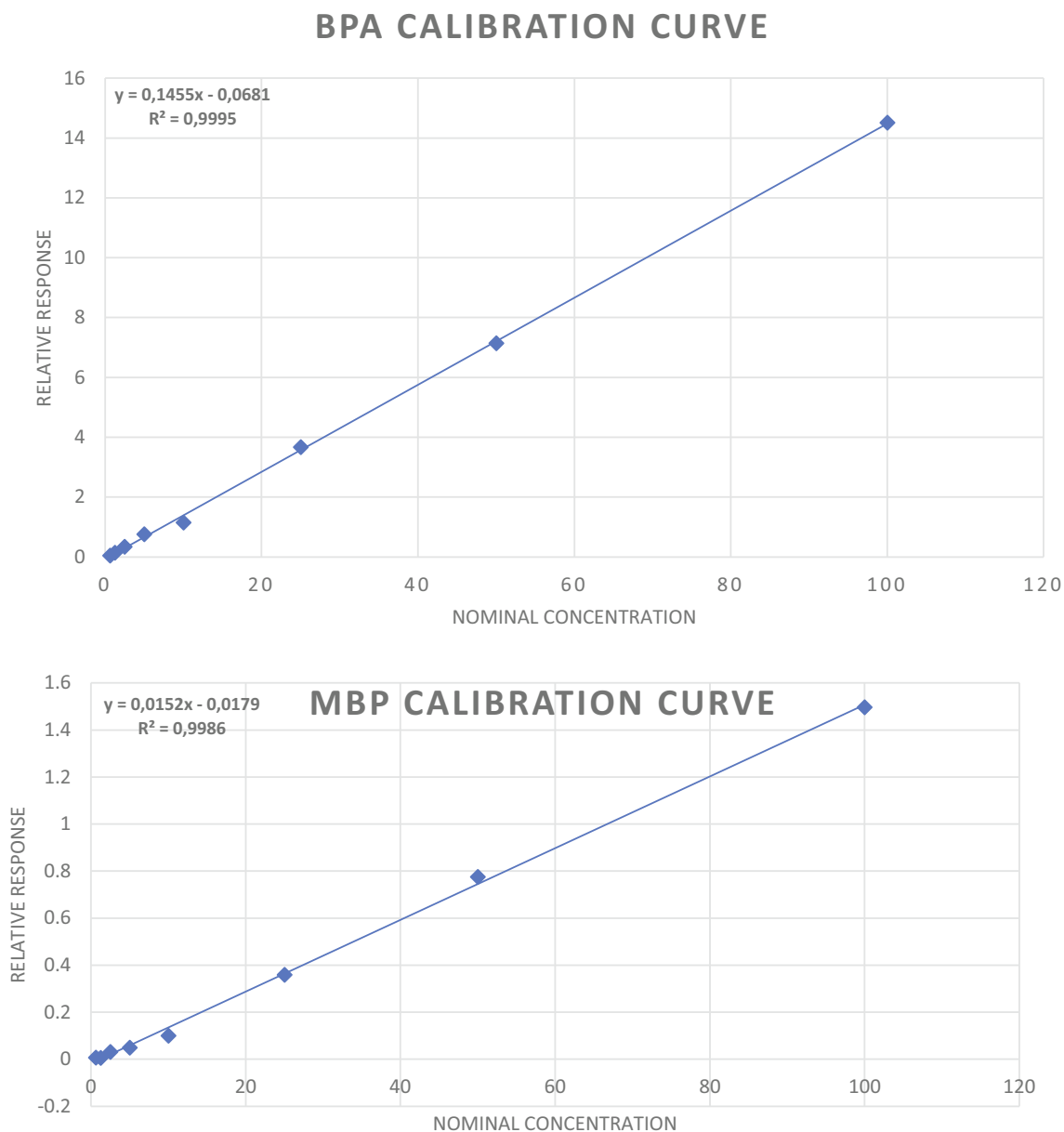
Fig. 5. Calibration curve at eight-point levels in the concentrations range of 0.625–100 ng/mL for BPA (top) and MBP (bottom).

urine from different female subjects undergoing fertility treatments were analysed for the presence/concentration of the phthalates metabolites and BPA. All samples were collected at the Reproductive Physiopathology Centre – Sant'Anna Hospital of Rome. The study was approved by the local Ethical Committee (Polyclinic "Umberto I" Ethics Committee). Written informed consent was obtained from all study participants.

Table 4 presents the summary of mean concentration levels and frequency of positivity of 6 phthalate monoester metabolites and BPA. The phthalates metabolites that were detected in all three

Table 3
Intra and inter-assay precision and accuracy at IQCs for FF, serum and urine for all analytes under investigation.

Compound	Spiked concentrations (ng/mL)	FF			Serum			Urine		
		Intra-assay RSD%	Inter-assay RSD%	Accuracy %	Intra-assay RSD%	Inter-assay RSD%	Accuracy %	Intra-assay RSD%	Inter-assay RSD%	Accuracy %
MEP	5	20	16	97	10	12	99	8	10	87
	25	6	8	87	8	10	112	5	2	115
	100	4	4	101	15	4	85	3	1	88
MBP	5	10	0.7	87	2	4	86	1	3	87
	25	4	1.2	99	1	2	85	0.9	0.4	90
	100	4	0.9	99	0.6	0.8	99	1	1	100
MBzP	5	2	0.6	101	2	1	113	3	6	101
	25	1	1	98	0.4	2	99	0.5	0.1	101
	100	0.5	0.5	99	2	7	85	8	10	88
MEHHP	5	0.6	1.2	104	0.4	0.1	85	1	2	88
	25	2	3.2	85	1	1	97	4	2	87
	100	1	2	100	0.5	0.3	100	0.2	0.2	100
MEOXP	5	2	2	111	0.0	2	108	2	7	101
	25	0.4	4	104	0.8	0.9	106	0.4	0.2	100
	100	4	6	106	1	1	102	0.4	0.6	99
MEHP	5	10	0.8	115	3	1	111	2	0.9	86
	25	5	1	97	5	11	87	8	5	85
	100	12	8	88	2	6	98	10	4	89
BPA	5	2	3	110	0.8	1	102	0.2	0.2	108
	25	0.4	1	100	1	0.9	99	0.8	0.9	101
	100	0.3	1	99	4	2	90	1	0.6	98

Table 4
Concentration range and frequency of positiveness for all the analytes under investigation in all the samples of FF, serum and urine.

Analyte	Follicular Fluid		Serum		Urine	
	Range (ng/mL)	Frequency (%)	Range (ng/mL)	Frequency (%)	Range (ng/mL)	Frequency (%)
MEP	2.3–142.2	78	52.0–107	90	62.8–17000	98
MBP	15.0–102	80	11.0–100	87	17.0–18124	97
MBzP	<LOQ	0	<LOQ	0	0.90–4.10	65
MEHHP	<LOQ	0	<LOQ	0	1.10–2.70	70
MEOXP	<LOQ	0	<LOQ	0	0.70–2.00	94
MEHP	1.30–29.0	100	7.6–13	100	5.00–8.00	98
BPA	<LOQ-2.0	43	<LOQ-3.1	89	1.80–7.80	100

biological matrices were MEP MBP, MEHP, and BPA, with the following frequencies, respectively, in FF, serum, and urine. MEP: 78% (ff), 90% (s) and 98% (u); MBP: 80%, 87% and 97%; MEHP: 100%, 100% and 98%; BPA: 43%, 89% and 100%. MBzP, MEOXP, MEHHP were detected only in urine samples, with levels lower than the LOQ in the other two matrices. MEP was in the range of concentration, respectively, of 2.3–142.2 ng/mL in follicular fluid, 52–107 ng/mL in serum, and 62.8–17000 ng/ mL in urine. MBP in the range of 15.1–102 in follicular fluid, 11–100 in serum and 17–18124 ng/mL in urine; MEHP in the range of 1.3–28.7 ng/mL (follicular fluid), 7.6–13 ng/mL (serum) and 5–8.1 ng/mL (urine); BPA in the range of < LOQ-2.0, <LOQ-3.1 and 1.8–7.8 ng/mL for FF, serum and urine respectively in accordance with previous studies [24,26]. Several samples of serum and urine needed to be diluted since the concentration of MEP and MBP were greater than the upper limit of linearity of the calibration curves. MBzP, MEOXP, MEHHP were found in addition to the aforementioned analytes in urine; MBzP in the range of 0.9–4.1 ng/mL, MEOXP 0.7–2.1 ng/mL MEHHP 1.05–2.7 ng/mL. The results are for the most of phthalates metabolite, in accordance with previous studies for the same matrices, except for MEP and MBP, whose levels in follicular fluids were found to be higher than in a previously reported study [8].

However, it has to be stressed out that the results obtained for the MEP cannot be considered quantitatively reliable since the method performance results have not respected the acceptance criteria. Fig. 6a–c report the extracted ion chromatograms of the analytes found in representative follicular fluid (Fig. 6a), serum (Fig. 6b), and urine (Fig. 6c) samples.

4. Conclusion

The LC-MS/MS method here presented allows the simultaneous detection of six phthalates metabolites and BPA in the same analytical run (i.e., using a single sample pre-treatment and instrumental protocol) and in different human biological fluids (serum, urine, and follicular fluid). The method shows a considerably ample linearity range, allowing the quantitation of the target analytes in a broad range of concentrations. All the targeted analytes were successfully validated, with the exception of MEP, whose method validation did not match fully the expected criteria. Future applications of this method may allow shedding further light on the association between female infertility and the presence of endocrine disruptors in the follicular fluid.
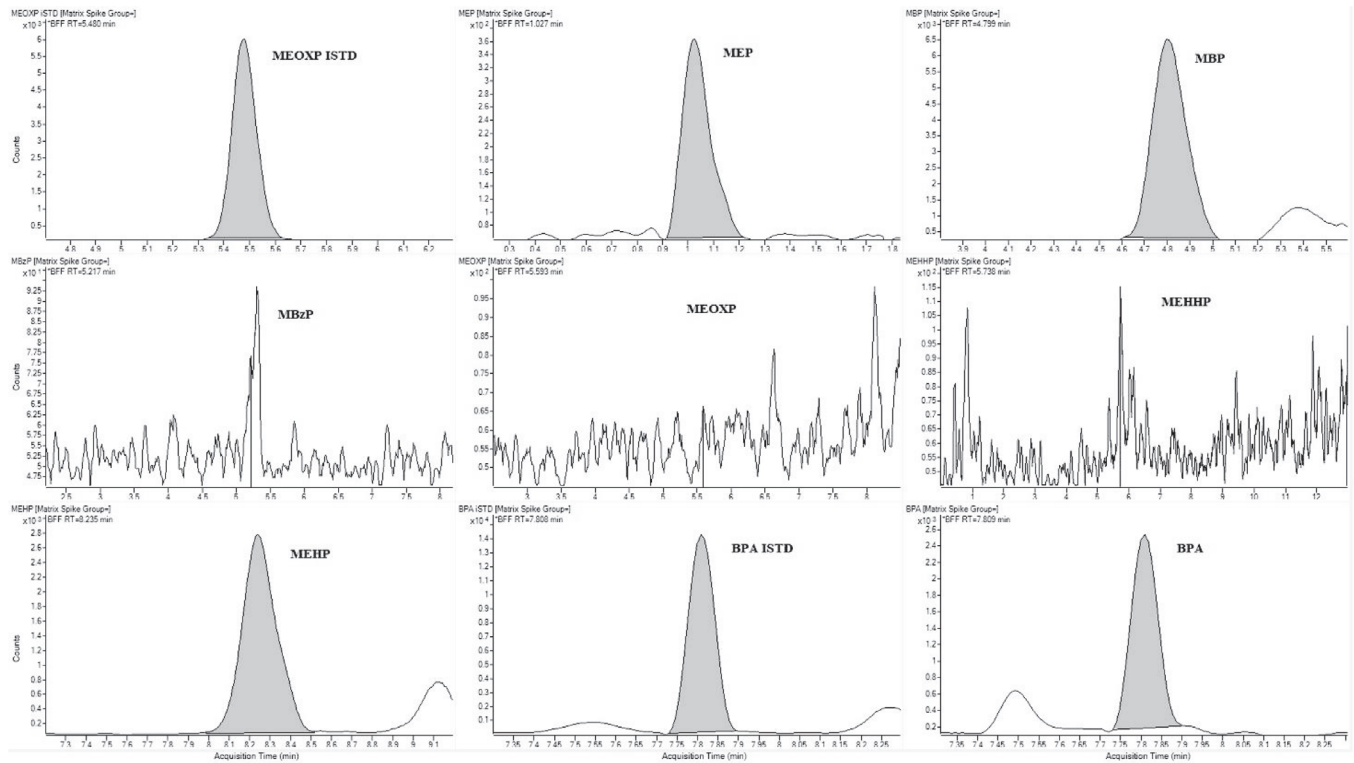
Fig. 6. Extracted ion chromatograms of the compounds under investigation and ISs obtained analysing a follicular fluid sample (a), a serum sample (b) and a urine sample (c) using the analytical procedure developed in this study.
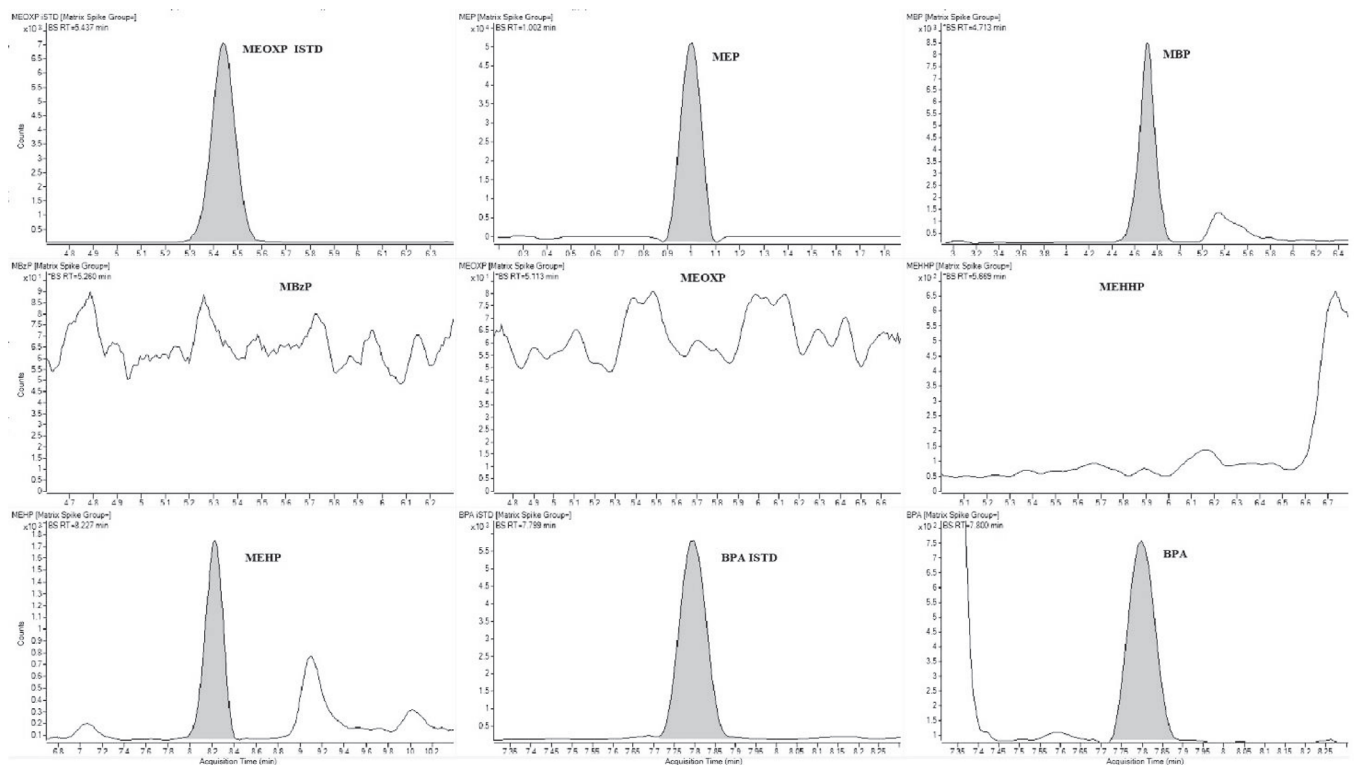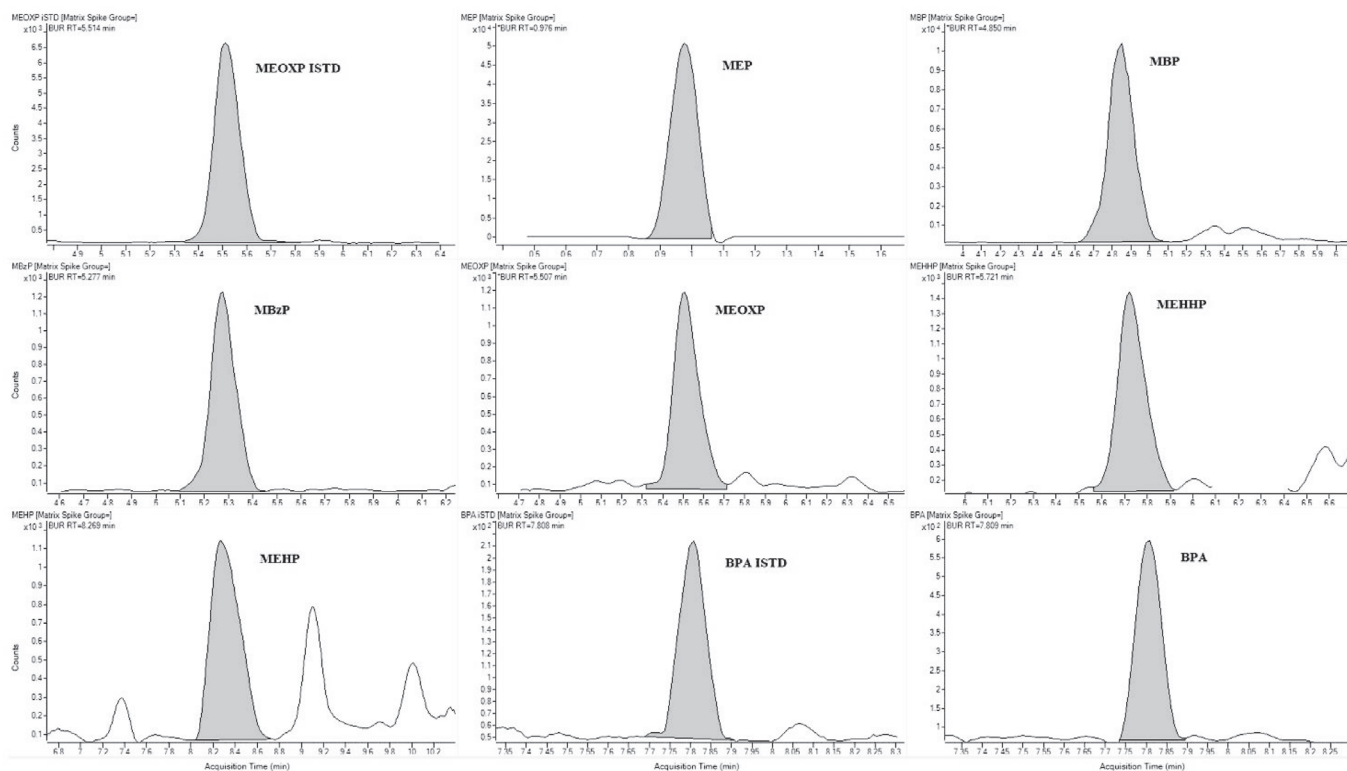

Fig. 6 (*continued*)

Fig. 6 (*continued*)

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.

Acknowledgements

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