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Bisphenol A, 4-t-octylphenol, and 4-nonylphenol determination in serum by Hybrid Solid Phase Extraction–Precipitation Technology technique tailored to liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and optimized for the simultaneous determination of bisphenol A, 4-t-octylphenol and 4-nonylphenol in human blood serum. For the first time, the electrospray ionization (ESI) parameters of probe position, voltage potential, sheath gas flow rate, auxiliary gas flow rate, and ion transfer tube temperature were thoroughly studied and optimized for each phenol by a univariate approach. As a consequence, low instrumental limits of detection were reported, demonstrating at 0.2 ng/mL (in solvent matrix) excellent injection repeatability (RSD < 14.5%) and a confirmation peak for all target phenols. Extraction and purification of serum was performed by the novel Hybrid Solid Phase Extraction–Precipitation Technology technique (Hybrid SPE-PPT). The limits of detection in human blood serum were 0.80, 1.3 and 1.4 ng/mL for BPA, 4-t-OP and 4-NP, respectively.

1. Introduction

Bisphenol A (BPA), 4-t-octylphenol (4-t-OP) and 4-nonylphenol (4-NP) are man-made alkylphenols (APs), and important xenoestrogens (Fig. S-1) [1–3]. BPA is one of the highest volume produced chemicals in the world and it is mostly used as an intermediate in the industrial production of resins (i.e. polycarbonate, epoxy, polysulphone and phenolic) [1]. Consequently, BPA is commonly found in a variety of everyday use items [1]. Its use has been documented in dental sealants, nail polishes, food packaging, flame-retardant materials [1], paper products [4], and lately even in paper currencies [5]. The other two APs, 4-t-OP and 4-NP, were extensively used until recently in the European Union (E.U.) and in the United States (U.S.) as intermediates in the production of phenolic resins and non-ionic detergents (alkylphenol ethoxylates) [2,3,6]. Today, all three chemicals are widespread and detected in a variety of environmental and biological media worldwide [6–8]. As xenoestrogen compounds, they bind to the estrogen receptors (ERs) and act competitively toward to natural hormones (i.e. 17β-estradiol); and even though their affinity is weak [1–3,9], reproductive and developmental toxicity studies have shown effects to aquatic organisms and animals [2,3,10–12]. However, the exact endocrine disruption properties on human population still remain controversial [8,13–15].

These chemicals are determined individually or simultaneously with other xenobiotic compounds mainly by gas chromatography (GC) coupled to mass spectrometric detection (MS or tandem MS) and by liquid chromatography (LC) coupled to mass spectrometric (MS or tandem MS), electrochemical (ECD) or fluorescence (FLD) detection [8]. Singularly, BPA is often determined in biological media with immunoassays [8]. However, LC–MS and LC–MS/MS techniques combine superiority over sensitivity, selectivity and precision even in low level concentration samples [8]. Thus far, a vast number of LC–MS and LC–MS/MS bioanalysis methods incorporated automated and/or on-line Solid Phase Extraction (SPE) sample preparation protocols [15–24] in order to minimize the contamination of the biosample from environmental background, and perceptibly decrease the lab bench work. However up to the present

Abbreviations: BPA, bisphenol A; 4-t-OP, 4-t-octylphenol; 4-NP, 4-nonylphenol (mixture of isomers); APs, alkyl phenols; EDCs, endocrine disrupting compounds; Hybrid SPE-PPT, Hybrid Solid Phase Extraction–Precipitation Technology technique; CCD, central composite design.

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day, the time consuming issue of SPE has not been dealt effectively enough.

Herein this work, an innovative rapid LC–ESI(-)MS/MS method was developed incorporating minimum sample preparation time with minimum solvent consumption for the simultaneous determination of BPA, 4-t-OP and 4-NP (technical mixture) in human serum. The backbone of the project consisted of two novel elements: The first element was the optimization of the electrospray ionization parameters of BPA, 4-t-OP and 4-NP by a multivariate approach in order to achieve optimal instrumental sensitivity; and the second element was the development of an optimized extraction protocol based on the interesting alternative of Hybrid Solid Phase Extraction–Protein Precipitation Technology technique, also known as Hybrid SPE-PPT. So far, there are no reports on these two topics of phenolic EDCs determination.

On the application of the Hybrid SPE-PPT methodology, the serum sample was first subjected to centrifugation, after the addition of a protein precipitation agent, isolating the sample from the endogenous gross amounts of proteins. Thereafter, the obtained supernatant fluid from the centrifugation step was directly subject to SPE using the Hybrid SPE cartridge. Making use of this cartridge, the SPE step demanded less than a minute for completion avoiding the time consuming steps of "classic SPE" that often need leniently a matter of hours for completion, and consequently correlate to low sample throughput, higher costs of analysis and higher workload. The Hybrid SPE cartridge is consisted of a zirconium packed-bed/low porosity filter/0.2 μm hydrophobic frit assembly. Phospholipids are retained specifically to the zirconium sorbent and the remaining proteins from the centrifugation step are retained non-specifically to the low porosity filter of the assembly [25]. The obtained eluent is even filtered through the cartridge frit, and thereafter it is ready for analysis. Ultimately, three processes are performed simultaneously in one. To our far knowledge, this technique is basically recommended for pharmaceutical applications [26,27], and has never been applied before for analysis of xenobiotics.

Throughout this work, LC–MS/MS matrix effects and losses generated during sample preparation were dealt by using bisphenol A-d16 (BPA-d16), 4-t-octylphenol-d2 (4-t-OP-d2) and 4-(3,6-dimethyl-3-heptyl)phenol–3,5-d2 (4-NP-d2) as the internal standards (ISs) (Fig. S-1). The analytical parameters of the ESI based method were shown.

2. Experimental

2.1. Materials and reagents

Standards of BPA (≥99%), BPA-d16 (100%), 4-t-OP (100%), 4-t-OP-d2 (100%), 4-NP (100%) and 4-NP-d2 (100%) were purchased from Sigma–Aldrich (Sheboygan Falls, WI, U.S.). Methanol (MeOH) and acetonitrile (ACN) of LC–MS grade were purchased from Merck (Darmstadt, Germany). Formic acid (HCOOH; 98% v/v), ammonium formate ($HCOONH_4$) of LC–MS grade, and acetone [$(CH_3)_2CO$] for residue analysis were purchased from Fluka (Buchs, Switzerland). Water was purified by a Milli-Q grade water system (Millipore, Bedford, MA, U.S.). Hybrid SPE cartridges (30 mg/1 cc) were supplied by Sigma–Aldrich (Sheboygan Falls, WI, U.S.).

Matrix serum standards were prepared from serum pool samples. Three pools were available and stored in polypropylene tubes of 15 mL (120 mm × 17 mm, Sardstedt, Nümbrecht, Germany) at −20 °C. Each pool sample was obtained from ten anonymous individuals. Detailed stock solution preparation is described in Supplementary Material (SM).

2.2. Apparatus

The chromatographic separation was carried out using a UHPLC Thermo Accela pump incorporating a column thermostat, a degasser and an autosampler (San Jose, U.S.). The tandem mass spectrometer system was a Thermo TSQ Quantum Access triple quadrupole mass analyzer. The tested C18 columns were Thermo Hypersil Gold C18 (50 mm × 2.1 mm, 1.9 μm; porous particles), Grace C18 (50 mm × 2.0 mm, 1.5 μm; porous particles), Ascentis C18 (75 mm × 2.1 mm, 2.7 μm; fused-core particles), and Kinetex C18 (100 mm × 2.1 mm, 2.6 μm; fused-core particles). Phenomenex C18 (4.0 mm × 2.0 mm, 5 μm) was used as guard column. A 12-port Visiprep DL (Disposable Liners) SPE vacuum manifold with the Visidry drying attachment was supplied by Sigma–Aldrich (Sheboygan Falls, WI, U.S.). Disposable liners (from PTFE) were used to decrease the possibility of cross contamination during SPE and solvent evaporation. Nitrogen gas (N_2) was applied for evaporation of the solvents. Colorless Eppendorf tubes of 1.5 mL (Sarstedt, Nümbrecht, Germany) were used during sample preparation. Conical autosampler vials of 1.5 mL with an assembled screw cap (with hole and PTFE/silicone septum) and vial inserts (size 200 μL) were supplied by Sigma–Aldrich (Sheboygan Falls, WI, U.S.). Statistical treatment was performed with STATGRAPHICS Centurion XV software package (Stat Point, Inc., Version 2002) and Excel (Microsoft, 2010). Raw data handling was performed with Xcalibur 2.0.6 software.

2.3. Method development

The development of an ESI method can be a challenging task since a number of interdependent parameters are involved affecting the electrospray "Taylor Cone" formation, and consequently the electrospray response in terms of sensitivity and signal stability [28]. Recently, Maragou et al. used a simple strategy plan in order to achieve optimal LC–MS/MS response [29]. This strategy plan was used herein as a guide, contributing this knowledge to BPA, 4-t-OP and 4-NP analysis (SM).

Six electrospray ionization parameters of the target analytes were optimized according to a full factorial experimental design that required specified loop injection experiments. The voltage potential, probe positioning (V-distance), micrometer positioning (Y-distance), sheath gas flow rate, auxiliary gas flow rate and ion transfer tube temperature were the optimized parameters. A schematic diagram of the ionization source and the ion focusing path of the used LC–ESI-MS/MS instrumentation were shown by Maragou et al. [29]. The applied full factorial experimental design was performed consecutively in two parts. In the first part, a screening experiment was realized in order to define the most critical parameters and joint effects in response. In the second part, multilevel experiments were performed in order to obtain the optimal value for each examined parameter. A rotatable and orthogonal central composite design (CCD) 2^n + star point was applied for n parameters, and the response variables were the $[M-H]^-$ peak areas of the target analytes (SIM, Single Ion Monitoring). For the development of the chromatographic system, a number of mobile phase mixtures were tested, and the performance of the four aforementioned C18 columns was evaluated.

Under ESI operation, optimization of the in-vial solvent composition and the study of Hybrid SPE-PPT technique were performed in order to conclude in a fully optimized sample preparation protocol. For the development of the Hybrid SPE-PPT protocol, fourteen precipitating protein agents were evaluated for serum: plain MeOH; MeOH containing 0.1, 0.5 and 1% w/v $HCOONH_4$; MeOH containing 0.1, 0.5 and 1% v/v HCOOH; 85: 15% v/v ACN/MeOH; 85: 15% v/v ACN/MeOH containing 0.1, 0.5 and 1% w/v $HCOONH_4$; and 85: 15% v/v ACN/MeOH containing 0.1, 0.5 and 1% v/v HCOOH. The

Table 1
Precursor–product ions, collision energies and tube lens voltage values.

APs	Precursor ions $[M-H]^-$ (m/z)	Fragment ions (m/z)	Collision energy (V)	Tube lens offset (V)
BPA	227.0	211.8[a]	19	87
		132.9	28	87
BPA-d16	241.0	222.8[a]	21	87
		142.0	33	87
4-t-OP	205.2	133.3[a]	24	88
		134.3	20	88
4-t-OP-d2	207.2	134.8[a]	33	105
		135.8	23	105
4-NP	219.2	133.3[a]	35	98
		147.1	34	98
4-NP-d2	221.2	135.0[a]	37	114
		149.7	18	114

[a] Quantification ion.

small percentage of MeOH (15% v/v) in ACN combinations was used for better solubilization of the $HCOONH_4$ salt, and therefore, for comparison purposes a similar approach was followed for the rest ACN-based precipitating agents. Furthermore, the optimal volume of the extracted serum and the optimal ratio of serum/precipitation agent were determined.

2.4. LC–MS/MS analysis

Chromatographic separation was performed using Ascentis C18 and the column temperature was set at 25 °C. The proposed precursor–product ions of all compounds (target analytes and ISs) which were obtained from direct infusion and loop injection experiments are illustrated (Figs. S-2–S-7). Two characteristic product ions were selected for quantifying and confirming each target analyte. The precursor–product ions, the collision energies and the tube lens voltage values that were set for the ESI method are presented in Table 1.

ESI chromatographic separation was carried out using a gradient elution of MeOH and Milli-Q grade water as binary mobile phase at a flow rate of 100 μL/min. The run started with 50 (% v/v) MeOH which was held for 1.50 min. Then, a gradient elution started with 90 (% v/v) MeOH and increased linearly to 100% MeOH in 8.00 min. 100% MeOH was held for 5.50 min (until the 15.00 min) and reverted to 50% MeOH for 5.00 min for a total run time of 20.00 min. The ESI was applied at a potential of −2500 V. The probe was fixed at the 0.40 inches (by the unit of measure of the Thermo instrument) position of the micrometer for Y-distance and at the position D for V-distance. The sheath gas (N_2) flow rate was set at 42 Arbitrary Units (A.U.), the auxiliary gas (N_2) flow rate was set at 15 A.U. and the ion transfer capillary temperature was set at 300 °C. The skimmer offset potential was set at 0 V. The data acquisition values were set at 0.120 s for scan speed, 0.500 m/z for scan width, 0.70 FWHM (Full Width at Half Maximum) for resolving power and 10 A.U. for the chromatography filter (noise reduction).

2.5. Sample preparation

Pool serum sample was thawed, equilibrated to room temperature and vortex mixed for 1 min. Then, aliquots of 150 μL of serum were transferred into the Eppendorf tubes of 1.5 mL. All blanks and samples were spiked with a known amount of ISs before extraction. Matrix-spiked samples were fortified with a known amount of ISs and an appropriate amount of target analytes prior to the extraction of samples (referred to as pre-extraction matrix spikes). 450 μL of MeOH were added and samples were vortex mixed for 1 min. Centrifugation followed for 10 min at 4000 × g (rpm), and thereafter the supernatant was collected and passed through the Hybrid-SPE cartridge. The extracts, approximately 450 μL in volume, were received into the conical autosampler vials. The eluents were evaporated to near-dryness under a gentle stream of nitrogen gas (N_2). Then, 5 μL of $(CH_3)_2CO$ were added and the eluents were diluted up to 150 μL with a mixture of MeOH/Milli-Q water (75:25% v/v), and set to the autosampler tray for analysis. For the determination of recoveries and matrix effects, ISs and target analytes were spiked into final extracts (referred to as post-extraction matrix spikes).

2.6. Method validation

Instrumental calibration of the ESI method was verified by injecting matrix matched calibration standards (pre-extraction matrix spikes). Matrix calibration curves were constructed by measuring pre-extraction matrix spikes that were spiked for BPA and 4-t-OP at concentrations of 0.75–15.0 ng (calibration points: 0.75, 1.5, 3.0, 7.5, 11.3, 15.0 ng) and for 4-NP at concentrations of 0.75–60.0 ng (calibration points: 0.75, 3.0, 7.5, 15.0, 30.0, 45.0, 60.0 ng). For the ESI method, the method limit of detection (LOD) for each target analyte was defined as the concentration of the analyte in pre-extraction matrix spiked that was equal to three times the average level of the baseline background close to the respective peak. The ESI method limit of quantification (LOQ) for each target analyte was defined as the concentration that was ten times the same background level.

For reproducibility experiments (inter-day precision), a sample was spiked at two fortification levels, and six replicate analyses ($N = 6$) were prepared for each level in-between two different days ($k = 2$). The low fortification level was 3.0 ng and the high fortification level was 15.0 ng. The accuracy (trueness) was evaluated through recovery experiments at the aforementioned fortification levels of the target analytes. In both fortification levels, absolute (abs.) and relative (rel.) recoveries (rec.) % were calculated in six ($N = 6$) replicate analyses. Furthermore, the matrix effect (ME%) was quantified by comparing instrumental responses of the post-extraction matrix matched standards spiked (at 3.0 and 15.0 ng) with those of the external calibration standards prepared in solvent MeOH/Milli-Q water (75:25% v/v).

3. Results and discussion

3.1. Optimization of the electrospray parameters

All ESI experiments were performed under a mobile phase flow rate of 100 μL/min. The six electrospray ionization parameters were examined through screening experiment at two levels, low and high level (Table S-1). A two-block full factorial design ($2^6 + 1$ center point for each block) was created, proposing experiments in a randomized order. The Standardized Pareto Chart–Main Effects Plot for every chemical were obtained as illustrated in Fig. 1.

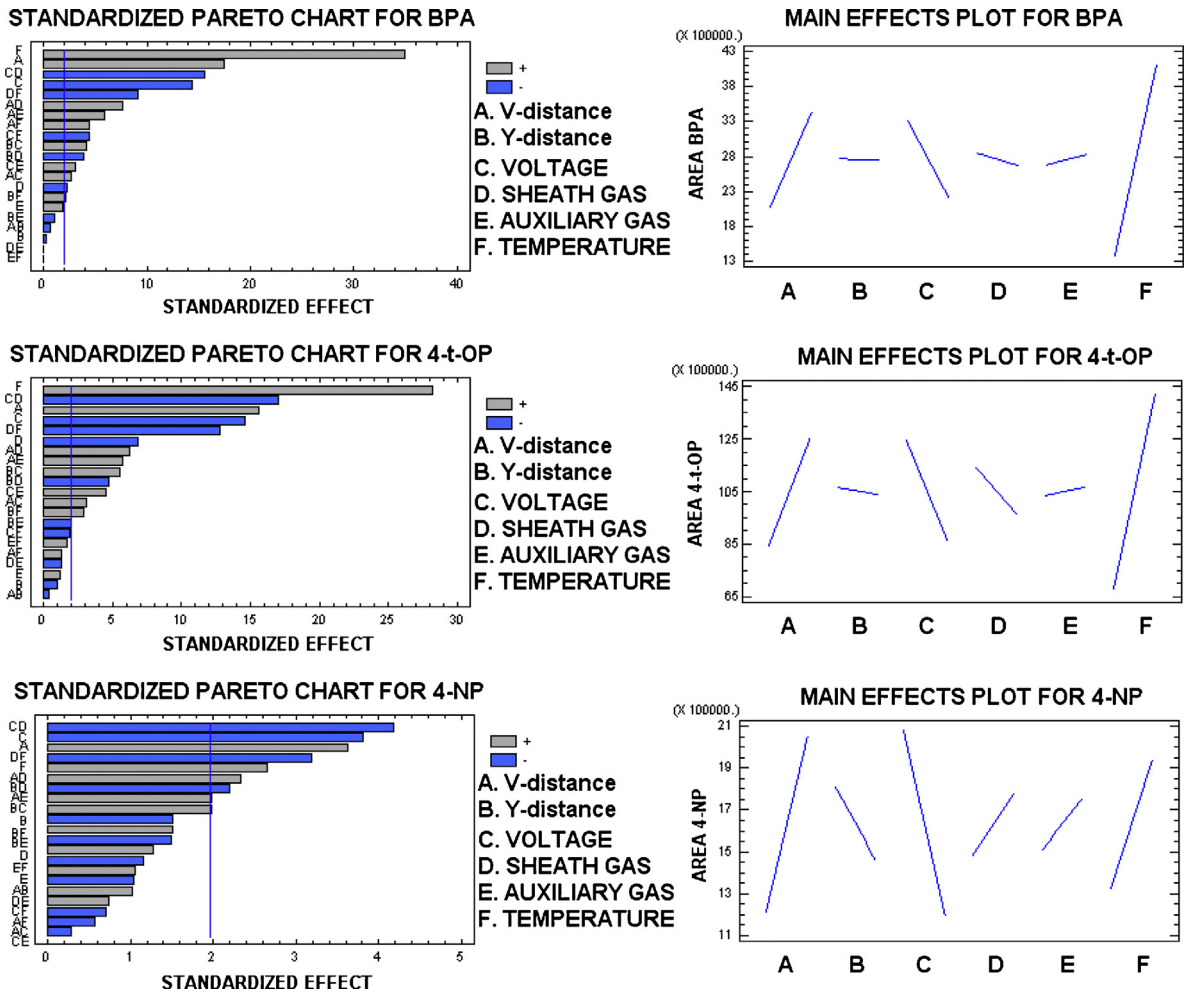

Fig. 1. Standardized Pareto Charts and Main Effects Plots from screening experiment.

The Standardized Pareto Chart shows each of the estimated effects in a decreasing order of magnitude. The length of each bar is proportional to the standardized effect, which is the estimated effect divided by its standard error. The bars which extend beyond the line correspond to effects which are statistically significant at the 95.0% confidence level. The Main Effects Plot demonstrates the estimated change in response when each of the factors is moved from the low to the high level of the design.

The screening design proposed a number of injections in different combinations of the parameters. However, due to the likelihood of potential instability in response, each combination of parameters could be examined more than once. Thus, 237 injections were performed and it was observed that the response of every chemical exhibited good correlation ($r \geq 0.85$) to the response of each of the other two chemicals (Fig. S-8), proving that all three alkylphenols share common optimal values for the ESI parameters. From the Main Effects Plots (Fig. 1), it was concluded that sensitivity was strongly influenced for all three target analytes by three specific parameters, also called "Critical Factors": the probe positioning (V-distance), the capillary voltage, and the ion transfer tube temperature. Optimal sensitivity for all compounds was reached at the highest value of the probe positioning (V-distance) at position D (which is the far distant V-distance position from the entrance cone), applying high ion transfer tube temperature values (>290 °C), and at the lowest assessed voltage potential of −2500 V. From the Standardized Pareto Charts, a number of joint effects were identified. The most important one was the interaction between the capillary voltage and the sheath gas pressure (joint effect CD). The capillary voltage is responsible for the formation of the "Taylor Cone" during ESI, whereas the sheath gas pressure is responsible for the stretching out of the "Taylor Cone". When these two factors are well-specified, the "Taylor Cone" is well-formed [28]. According to the CD joint effect, best response for all chemicals is obtained in-between the window of −3200 V ≥ capillary voltage ≥ −2500 V and 60 A.U. > sheath gas pressure > 20 A.U. From this point and onwards, the ESI optimization was completed through multilevel experiments by maintaining V-distance fixed at position D.

Despite the aforementioned finding for capillary voltage, the ionization of the analytes was evaluated furthermore under three capillary voltages of −2500, −3500 and −4000 V by performing three independent multilevel experiments, one for every assessed voltage potential. The applied CCD is demonstrated (Table S-2). A second-order model was fitted for each set of data that was obtained for every compound. The good fitness of the model was evaluated by the statistical R^2 of the ANOVA. The R^2 indicates the percentage of variability in peak area that has been accounted for by the fitted model. At the level of −4000 V, the R^2 was found 29.3% for BPA and 45.0% for 4-t-OP and 4-NP. At the level of −3500 V, the R^2 value was found 34.2% for BPA, 52.2% for 4-t-OP and 39.9% for 4-NP. Finally, the R^2 value was found 79.0% for BPA, 68.1% for 4-t-OP and 85.9% for 4-NP at the level of −2500 V. Eventually, the best fit of the model to the data was obtained at −2500 V, proving that a more stable response is obtained at this value. This finding was consistent with theory of droplet electrospray formation. As reported in literature, a stable negative electrospray operation is achieved setting the potential a few hundred volts higher than the onset

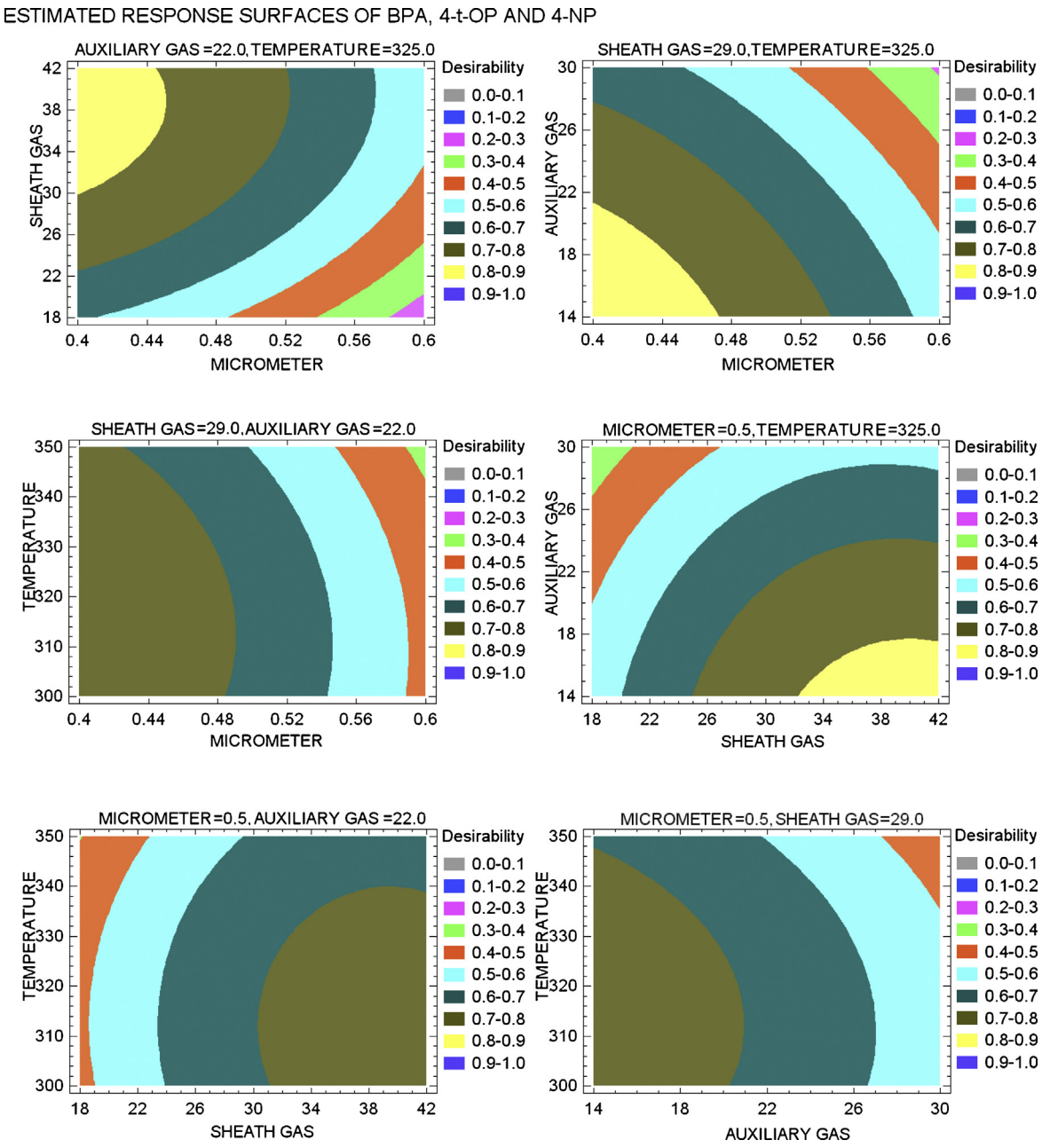

Fig. 2. Desirability plots at −2500 V.

voltage of the used mobile phase [28]. Herein, the onset voltage of the used mobile phase (MeOH/Milli-Q grade water 90:10 (% v/v) in the loop injection experiments) was calculated to be −2365 V, since the onset voltage of MeOH and Milli-Q grade water is −2200 V and −4000 V, respectively [28].

Once setting the capillary voltage at −2500 V, a multilevel experiment followed in the applied levels of a CCD (Table S-3). The experimental factors were the ion transfer tube temperature, the micrometer positioning (Y-distance), the sheath gas flow rate, and the auxiliary gas flow rate. From the above four parameters, only the ion transfer tube temperature was acknowledged as a critical factor from the screening experiment.

Multiple response optimization was performed for BPA, 4-t-OP, and 4-NP peak areas in order to achieve maximization of all three responses simultaneously, combined in one function (desirability plot). Desirability plots were created as a function of two examined parameters and by holding constant the other two parameters at their middle value level of the applied CCD. Thus, six desirability plots illustrate the corresponding estimated response surface as presented in Fig. 2.

The good fitness of the model was evaluated, and R^2 was found 89.8% for BPA, 93.9% for 4-t-OP and 82.5% for 4-NP. From the desirability plots it was demonstrated that the three phenols need intense conditions for desolvation during their electrospray ionization. In final analysis, optimal response was achieved by applying high capillary temperature and sheath gas flow rate, and by setting the probe position in the far distant position of V-distance from the entrance cone. Unlike probe positioning (V-distance), the micrometer positioning (Y-distance) was favored located very close to the entrance cone. This contradiction proves the different impact that distance has on sensitivity, depending on spatial dimensions. However, the micrometer positioning (Y-distance) was not set lower than 0.4 inches, since this value is located very close to the entrance cone, and an impact on signal stability would occur.

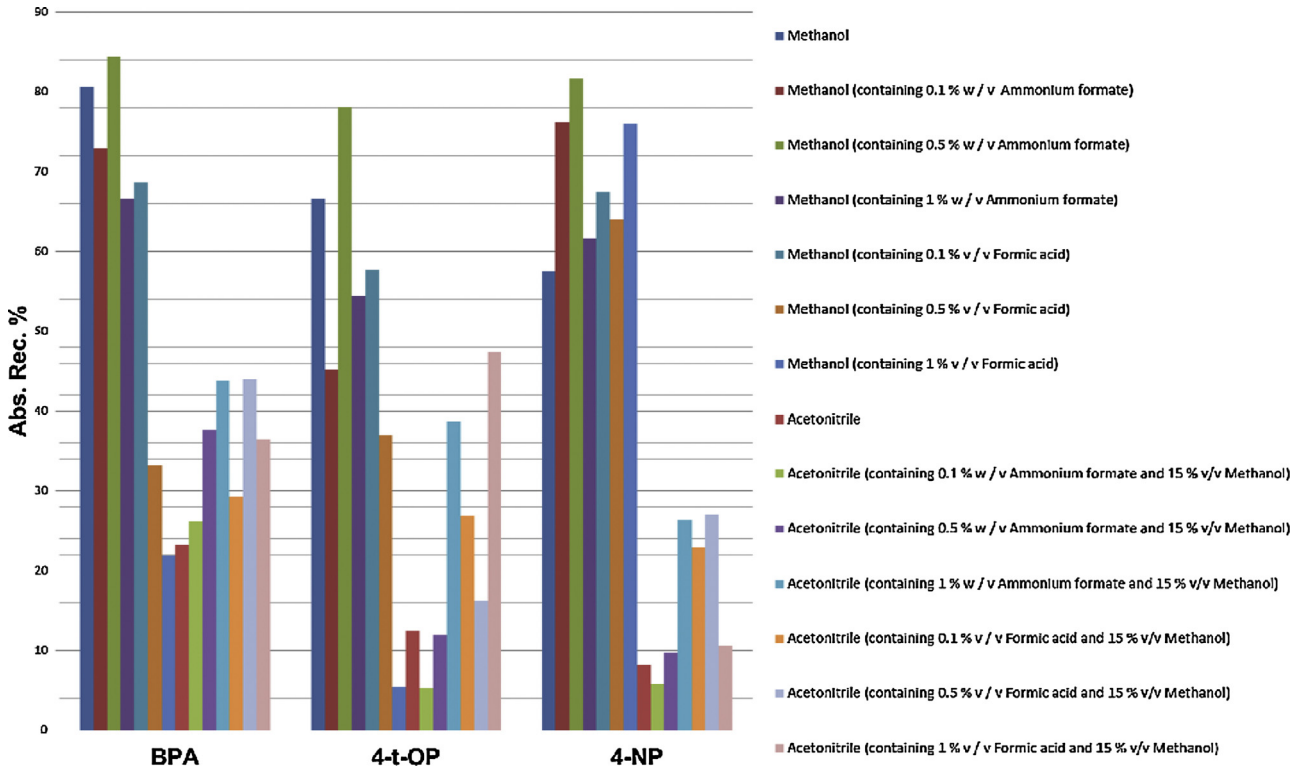

Fig. 3. Absolute recoveries (%) of BPA, 4-t-OP, and 4-NP with different precipitating agents.

The optimal values of electrospray were set on the ESI method (Section 2.4).

3.2. Optimization of the chromatographic parameters

The optimal mobile phase mixture for chromatographic separation was MeOH/Milli-Q grade water. The other two tested mixtures, MeOH/5 mM $HCOONH_4$ in Milli-Q grade water and MeOH/0.01 (% v/v) HCOOH in Milli-Q grade water, lowered perceptibly the response of chemicals. Seven mixtures of MeOH/Milli-Q grade water were tested for eluting the target analytes from the Thermo Hypersil Gold C18 column: 70:30, 75:25, 90:10, 95:5, 98:2, 99:1, and 100:0 (% v/v). No significant response signal differences were observed in relation to the percentage of MeOH used for elution of the target analytes.

The target analytes presented lower response with the porous C18 columns (Thermo Hypersil Gold C18 and Grace C18), whereas their response was 25% higher with the fused core C18 columns (Ascentis C18 and Kinetex C18). Due to the fused core technology, chemicals spend less time diffusing into the pores as they travel through the column. This shorter diffusion path allows faster mass transfer that results in less band broadening of the peak, and consequently higher responses are obtained [30]. For both columns, Ascentis C18 and Kinetex C18, the difference in pressure increased proportionally as the flow rate increased (Fig. S-9). Indicative, retention times of 4-NP at various flow rates with Ascentis and Kinetex column are presented (Table S-4); and SRM chromatograms of BPA, 4-t-OP and 4-NP with Thermo Hypersil Gold and Kinetex column are depicted in Figs. S-10 and S-11, respectively. Applying the same mobile phase flow rate on Ascentis and Kinetex, faster elution of chemicals with less pressure drop (Table S-5) was achieved by Ascentis column due to its lower length. Therefore from this point and onwards, method development was performed by Ascentis C18 column with the ESI chromatographic gradient that is depicted in Section 2.4.

In the in-vial presence of $HCOONH_4$ (0.005–0.5 (% w/v)), signal intensity of BPA was decreased and signal intensities of 4-t-OP and 4-NP were increased (SM). The signal intensities of 4-t-OP and 4-NP were favored in the in-vial presence of $HCOONH_4$, but were disfavored as aforementioned, in the presence of $HCOONH_4$ in the mobile phase. Moreover, reduction of response occurred for all three target analytes in the in-vial presence of HCOOH (0.05, 0.25 and 0.5 (% v/v)) (SM).

During sequential analysis, the in-vial use of $(CH_3)_2CO$ prevented the LC–MS/MS system of losing sensitivity and was proved beneficial for optimal ESI performance of the target chemicals (SM). The ion optics of the tandem MS instrument were kept clean during sequential analysis by frequently injecting a blank solvent solution that contained a high fraction of $(CH_3)_2CO$ (50% v/v) and by adding in-vial a small percentage of $(CH_3)_2CO$ (i.e. ~3.3% v/v) in solvent and matrix-matched standard solutions (more information of this effect can be found in the SM).

3.2.1. Hybrid SPE-PPT protocol

The fourteen precipitating agents were tested by applying the sample preparation procedure (Fig. S-12). The final extract (300 μL) was diluted up to 1200 μL prior to analysis in order to decrease matrix effects during protocol optimization. The final amount of the target analytes was 120 ng. For each precipitating agent, absolute recoveries (Fig. 3) and matrix effects (MEs%) (Fig. 4) were presented.

The recoveries of each target analyte exhibited good correlation ($r \geq 0.70$) to the recoveries of each of the other two target analytes (Fig. 3). In general, matrix enhancement of BPA and 4-t-OP responses was observed in serum with HCOOH combinations, whereas suppression of signal was observed with $HCOONH_4$ combinations (Fig. 4). The obtained matrix effects of 4-NP differed from those of BPA and 4-t-OP (Fig. 4). Using ACN as precipitating agent, strong HILIC interactions were introduced between the Hybrid SPE cartridge and the three phenols, especially for 4-t-OP and 4-NP, lowering perceptibly their recoveries (Fig. 3). The manufacturer of

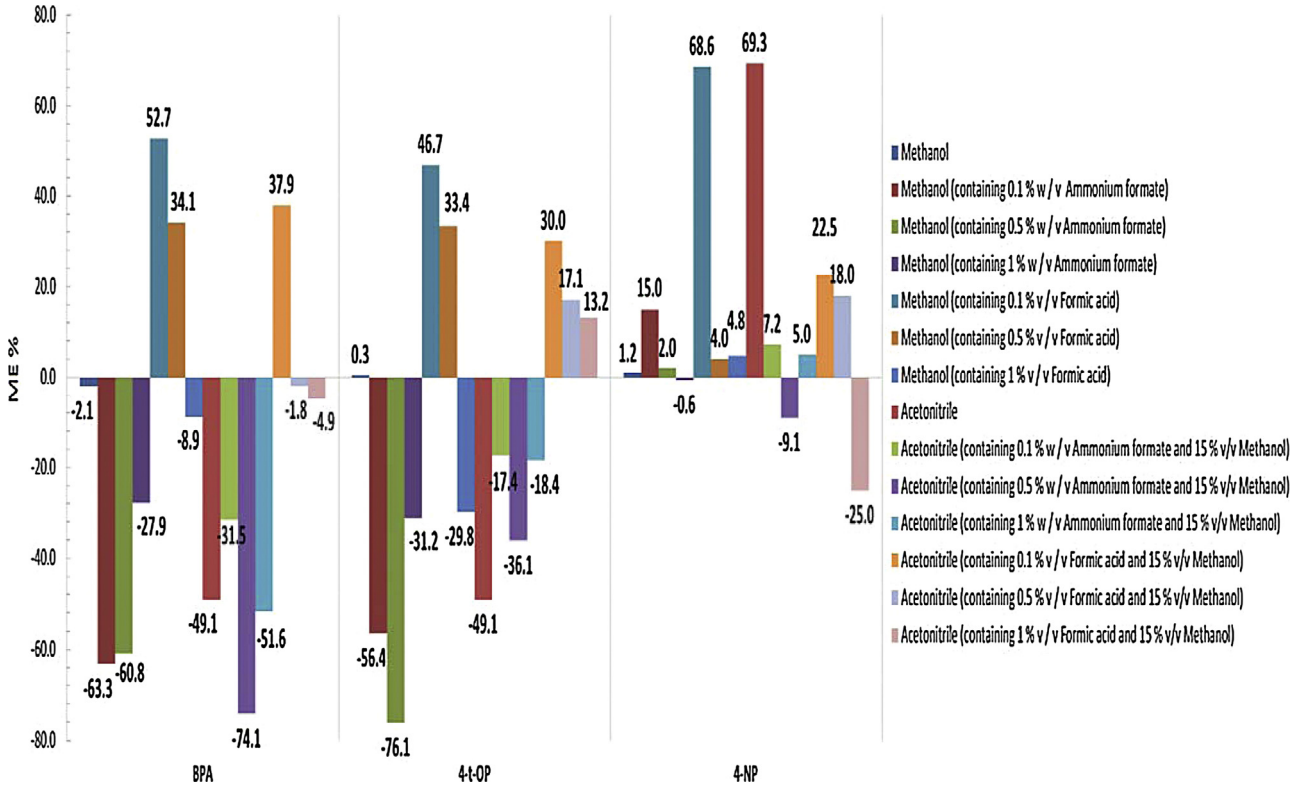

Fig. 4. Matrix effects (ME %) of BPA, 4-t-OP, and 4-NP in different precipitating agents.

the Hybrid SPE cartridge notes that when using ACN as precipitating agent, HILIC interactions are introduced due to secondary interactions derived from the silica surface of the cartridge [31]. Ultimately, plain MeOH was chosen as optimal precipitating agent for this bioanalytical application since it provided high recoveries with the least matrix effects.

The optimal quantity of serum used in the protocol was determined by testing 100, 150, 200, and 300 μL of serum (Fig. S-13). The transparencies of the extracts were compared. Cleaner extracts were obtained by using 100 or 150 μL of serum. When 200 and 300 μL of serum were used, turbid eluents were obtained due to the breakthrough of proteins. Further, the ratios of 1:1, 1:2 and 1:3 serum/MeOH were tested (Fig. S-14). The final eluent was turbid prior to analysis when the 1:1 and 1:2 serum/MeOH ratios were applied again due to the breakthrough of proteins, whereas the cleanest eluent was obtained with the 1:3 serum/MeOH ratio.

3.2.2. Method performance

The instrumental parameters (in solvent matrix) of linearity, LOD, LOQ, calibration curve and injection repeatability for each target analyte are presented (Tables S-6 and S-7). The method linearity (in serum matrix) obtained for all analytes was good in the investigated intervals with correlation coefficients higher than 0.991 (SM). The method LOD was 0.80 ng/mL for BPA, 1.3 ng/mL for 4-t-OP and 1.4 ng/mL for 4-NP. The LOQ was 2.7 ng/mL for BPA, 4.2 ng/mL for 4-t-OP and 4.7 ng/mL for 4-NP. The absolute recoveries (±RSD%) at the low and high concentration level were 62.7 (±7.5)% and 75.0 (±8.9)% for BPA, 59 (±25)% and 85 (±14)% for 4-t-OP, and 59 (±22)% and 64.3 (±9.7)% for 4-NP, respectively. The relative recoveries (±RSD%) at the low and high concentration level were 106.0 (±5.6)% and 124 (±10)% for BPA, 111 (±11)% and 121.0 (±7.9)% for 4-t-OP, and 97.0 (±6.3)% and 109.7 (±5.4)% for 4-NP, respectively. The reproducibility of the method (RSDm%) at the low concentration level was 14.3% for BPA, 10.9% for 4-t-OP and 13.5% for 4-NP. The reproducibility of the method (RSDm%) at the high concentration level was 12.8% for BPA, 8.3% for 4-t-OP, and 7.1% for 4-NP. The ME % was −93% for BPA, −67% for 4-t-OP, and −36% for 4-NP. For the monitoring of BPA-d16, the second ion fragment in abundance was selected (142.0 *m/z*, confirmation ion) as best for the method, since the most abundant product ion of 222.8 *m/z* presented high background noise This observation was made during the optimization of the Hybrid SPE-PPT protocol and the validation process (Fig. 5).

Instrumental LODs and LOQs (in solvent matrix) were slightly lower during winter time (Table S-7). To our far knowledge, this is the first reporting of low instrumental limits of detection (in solvent matrix) with an excellent injection repeatability (RSD < 14.5%) and a confirmation peak for each phenolic compound at the concentration level of 0.2 ng/mL (Figs. S-15–S-17). The ion ratios % in standard matrix and solvent solutions are presented in Table S-8. This achievement was attributed to the applied chemometrics for optimizing the electrospray parameters and the cutting-edge technology of the fused core columns.

Finally, it was observed that in low concentration standard matrix-match solutions, the quantification SRMs of BPA and 4-t-OP (and their ISs) maintained more interfering peaks than their respective confirmation SRMs.

3.2.3. Background contamination

The problem of developing/validating the aforementioned method was background contamination. From the reagent blanks it was concluded that the most important source of contamination originated from laboratory air. The most severe contamination for all three compounds was observed during the SPE and evaporation process. This was observed by far higher levels of magnitude for 4-NP. In order to control contamination, SPE and evaporation of samples was always performed one at a time under a hood. During the evaporation step, fifteen minutes were left to pass in-between successive sample evaporations in order to clean the supernatant air above the evaporation manifold for the next sample.

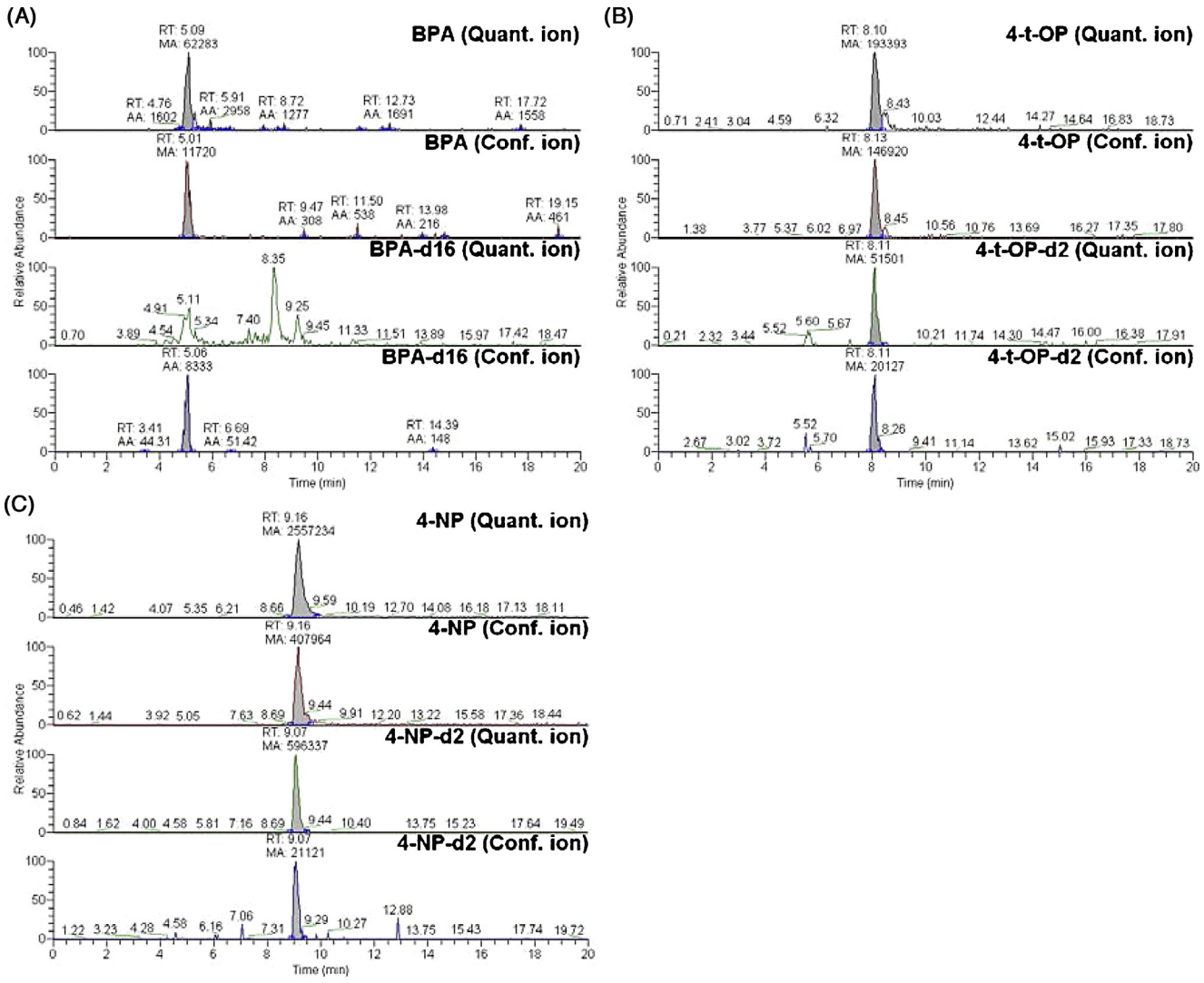

Fig. 5. (A) SRM chromatogram of serum sample spiked with 2 ng/mL BPA; (B) SRM chromatogram of serum sample spiked with 20 ng/mL 4-t-OP; and (C) SRM chromatogram of serum sample spiked with 200 ng/mL 4-NP.

The use of Hybrid SPE-PPT cartridges did not pose an important contamination source; nonetheless, two reagent blanks (plain Milli-Q water) were always carried out through all steps of sample preparation and instrumental analysis. If instrument background levels of the target analytes were found, they were eliminated before analyses by injecting sufficient blanks to clean the system (8–15 blanks were required). Additionally, to minimize build-up of the target analytes during mobile phase equilibration and to keep background levels constant, the time duration for which the system was kept under initial conditions was maintained as short as possible. Prior to daily use, we flushed the LC column with elution solvents [MeOH/ACN (50:50, % v/v) and plain $(CH_3)_2CO$] before initiating a sequence.

The analysis of actual samples was tempered by the contamination during sample collection (i.e. use of plastic blood bags) that could compromise the quality of analysis with serum matrices. At this stage, strict sample collection and storage condition guidelines for 4-t-OP and 4-NP are not available. Thus, it is deemed necessary to proceed in proposing such guidelines because trace level analysis of serum needs to be carefully executed to prevent contamination, especially for 4-t-OP and 4-NP, during sampling and storage. In closing, it is the authors' firm belief that the limited number of published biomonitoring studies on 4-t-OP and 4-NP (compared to the ones on BPA), is inevitably linked to the lack of control over contamination.

4. Conclusions

Researchers that work on LC–MS/MS analysis of these phenolic EDCs concede that there are difficulties on their electrospray ionization [8,32,33], but no published method so far has clearly stated this commonly known fact. However, the issue was effectively dealt by the coupling of the new generation fused core C18 column technology with chemometrics (full factorial experimental design) that led to impressive results toward ESI instrumental sensitivity and response stability.

In conclusion, this work suggests an impetus in a broad context of modern LC–MS/MS bioanalysis. The approach that was followed herein can surely be expanded in other LC–MS (or MS/MS) analytical applications. In the near future, sample preparation by the rapid Hybrid SPE-PPT technique will definitely exhibit interesting prospects in terms of different matrices analyzed and compounds determined.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.02.009.

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