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ABSTRACT

With the objectives of both generating bisphenols (BPs) conjugates occurrence data in food from animal origin but also investigating the origin of associated contamination, the present study deals with the development of an efficient analytical method aiming at monitoring both BPA and BPS conjugated metabolites in food from animal origin. The objective of such monitoring is to determine the origin of BPs contamination (FCM or animal contamination). The targeted compounds were BPA-monoglucuronide (BPA-1G), BPA-diglucuronide (BPA-2G), BPA-monosulfate (BPA-1S), BPA-disulfate (BPA-2S) and BPS-monoglucuronide (BPS-1G). The developed standard operating procedure includes a preliminary solid-liquid extraction step followed by two successive solid phase extraction (SPE) stages, using successively a non-polar phase and a strong cation exchange polymer. Quantification was achieved according to both the isotopic dilution and surrogated quantification methods, using ^{13}C-BPA-1G and BPA-d_6-1S as internal standards. Linearity was validated (R^2>0.99) for each molecule within the concentration range [0 – 10] µg kg^{-1}. Detection limits ranged from 0.02 µg kg^{-1} (BPA-1G in muscle, BPA-1S and BPA-2G in liver) to 0.50 µg kg^{-1} (BPA-2S in muscle). The strategy was then proven on liver samples collected from pregnant ewes subcutaneously exposed to BPA during 105 days, at 50 µg kg^{-1} per day. BPA-1G, BPA-2G and BPA-1S were detected and quantified at a concentration of 3.81 µg kg^{-1}, 0.80 µg kg^{-1} and 0.09 µg kg^{-1}, respectively. The analytical method was finally implemented on fifty unpacked food samples from animal origin in which significant free BPA concentrations were previously measured. Since no metabolites of BPA could be measured (<LOD), it means that such free BPA present in the samples originates from direct contact of the food item with a material containing BPA.

KEYWORDS

Bisphenol A, Bisphenol S, conjugated metabolites, foodstuffs, UHPLC-MS/MS

1 Introduction

Bisphenol A (BPA) is a high production volume industrial chemical used in the production of polycarbonate and epoxy resins which have a large panel of applications, such as plastic food containers and epoxy food-can coatings. Its migration from packaging to food is well documented [1-5]. Currently authorized as food contact material within the European Union, the European Food Safety Authority (EFSA) has recently set a regulatory specific migration limit at 50 µg kg^{-1} [6] and many countries, in the world, have adopted restrictions regarding the use of BPA in baby products or in food contact materials, in particular in the case of infant feeding bottles [7].

Estrogenic properties of BPA have been highlighted in both animals and humans, together with a range of adverse effects on brain, behavior, mammary glands, metabolism, obesity and reproduction [8-15]. Therefore, potential alternatives or BPA substitutes have been considered; among them, bisphenol F (BPF) and mostly, bisphenol S (BPS) are commonly chosen as monomer in the production of epoxy resins used as food contact material, while adverse effects of both BPS and BPF have been reported in several articles [16-21]. In parallel, biomonitoring data, dealing with the total (enzymatic hydrolysis) bisphenol analogues determination have been recently published [22-25], showing that BPA and BPS are the predominant bisphenol chemicals in biological samples.

Metabolism and kinetics of BPA in humans have been exhaustively studied by Dekant et *al.* and Völkel et *al.* who identified BPA monoglucuronide (BPA-1G) and BPA monosulfate (BPA-1S) as predominant BPA conjugated metabolites in urine [26-28]. They also described both rapid biotransformation and excretion of BPA. Similar metabolism mechanisms have been described leading for BPS and BPF to the following conjugated metabolites: BPF-1G, BPF-1S, BPS-1G and BPS-1S [29-31].

Several approaches have been reported for the determination of BPA-1G, BPA-2G [32], BPA-1S [32, 33] and BPA-2S [34], BPS-1G [35] in biological matrices, such as in plasma, serum and/or urine. Sample preparation procedures are usually based on solid phase extraction (SPE) such as solid-phase mixed-mode reverse-phase/anion-exchange extraction [32, 36], a combination of two successive SPE cartridges such as an amino-propyl silica-based cartridge (NH_2) and a reverse-phase C_{18} [34], a polymeric reversed-phase sorbent with hydrophilic-lipophilic balance properties (HLB) [33], on-line SPE [35]. Finally, LC-MS/MS is commonly used as detection technique using the negative electrospray ionization [28, 32,

34-36] and the Selected Reaction Monitoring (SRM) mode. All reported analytical methods enable the quantification of BPA-1G which is identified as the main conjugated metabolites of BPA. Its quantification is generally performed according to the isotopic dilution method using to the labeled standard ^{13}C-BPA-1G while other targeted metabolites quantification is carried out using ^{13}C-BPA-1G, BPS-1G-d_8 and/or BPA-d_6-1S as surrogated internal standards. Therefore, direct determination of conjugated metabolites of BPA in biological matrices, such as blood, serum or urine has been investigated in the framework of human exposure studies [32-34, 36-39]. Nevertheless, no analytical method focused simultaneously on the four conjugated metabolites of BPA, no more than conjugated metabolites of BPS. Additionally, these studies deal with biological matrices, and most of the reported method are implemented for urine samples.

Regarding foodstuff investigations, numerous data of BPA concentrations have been reported for several years, among them, results obtained in the framework of the second French total diet study (TDS2) dealing with a large scale of food items[40-42]. In this study, significant BPA concentration levels have been reported in non-canned foodstuffs, especially in food of animal origin. Therefore, the question of the origin of BPA contamination was raised: Is the contamination *ante-* or *post-mortem*? The answer could be found if the presence of conjugated metabolites of BPA is confirmed. While, to our knowledge, no analytical strategy has been already published for the direct analysis of conjugated metabolites of BPs in food, an analytical method targeting direct determination of these compounds, namely, BPA-1G, BPA-2G, BPA-1S, BPA-2S and BPS-1G in a wide range of foodstuff items of animal origin, was undertaken in the present work. An adequate strategy was developed and optimized for the determination of the five targeted metabolites using UHPLC-MS/MS. The corresponding analytical performances were assessed and the method was implemented to food samples in order to determine the origin of BPA contamination.

2 Material and methods

2.1 Materials, reagents and reference substances

Acetonitrile and water (HPLC gradient-grade quality) were purchased from Carlo Erba (Val de Reuil, France) and from Panreac (Castellar del Vallès, Spain), respectively. Cyclohexane, acetic acid, formic acid, methanol and both ammonium acetate and ammoniac were obtained

from VWR (Fontenay-sous-Bois, France). Both solid phase extraction columns, i.e. Chromabond HR-X and SAX Cuqax were purchased, respectively, from Macherey-Nagel (Hoerdt, France) and UCT (Bristol, United States).

Bisphenol S β-D-glucuronide (BPS-1G), Bisphenol A β-D-glucuronide (BPA-1G), Bisphenol A bis sulfate (BPA-2S), Bisphenol A bis β-D-glucuronide (BPA-2G), Bisphenol A mono sulfate (BPA-1S), $^{13}C_{12}$-Bisphenol A β-D-glucuronide (^{13}C-BPA-1G) and BPA-d_6 mono sulfate (BPA-d_6-1G) were obtained from Toronto Research Chemicals *Inc.* (Toronto, Canada). The chemical structures, molecular formulae and mono isotopic masses of the targeted molecules are shown in **Table 1**.

Individual stock standard solutions were prepared at a concentration of 100 ng μL^{-1} by dissolving an appropriate amount of each substance in the recommended solvent, i.e. methanol for BPA-1G, BPA-2G, ^{13}C-BPA-1G, BPA-1S and BPA-d_6-1S, and water for BPA-2S. Working solutions were obtained by tenfold successive dilution in the appropriate solvent at concentrations ranging from 10 ng μL^{-1} to 0.01 ng μL^{-1}. All the standard solutions were stored at 4°C, in the dark.

2.2 Foodstuffs of animal origin

Food items used for development, method performance evaluation and generation of preliminary data regarding conjugated metabolites of BPA and BPS detection in food, were collected in the framework of the second French Total Diet Study dealing with the investigation of BPA level in food items from animal origin [40, 41, 43]. Therefore, a wide range of matrices were included in the scope of the developed method, including various fish products, such as pollock, cod, salmon or hake, delicatessen meats, such as terrine, merguez sausage, raw/smoked or Parma ham, bovine/porcine/ovine meat, liver, muscle, turkey breast, roast pork and chicken. Samples were prepared according the procedure described by Sirot et *al.* [44] and were ground for homogenization using a food mixer.

2.3 Incurred liver sample

Liver samples were originating from a previous experiment looking at the effect of long term bisphenol A gestational exposure on the thyroid function in the pregnant ewe [45]. Animal procedures were carried out under agreement 311155515 for animal experimentation from the

French Ministry of Agriculture and validated by the local ethical committee for animal experimentation (project number: 2016 0323 10542323).

Briefly, pregnant ewes (n=5) were exposed to BPA, administered subcutaneously, at a dose of 50 µg kg^{-1} per day during 105 days. Plasma concentrations of BPA, BPAG and BPAS were assayed using a previously developed different method [37]. Results are reported in the study referenced above. Liver samples from the same area were collected in average 24h after the last BPA administration et were immediately snap frozen in liquid nitrogen. Samples were stored at -80°C until assay in the current study to confirm the identity of bisphenol metabolites.

2.4 Standard Operating Procedure

The analytical strategy was developed for the determination of BPA-1G, BPA-2G, BPA-1S, BPA-2S and BPS-1G in a wide range of food items from animal origin (**Figure 1**). The equivalent of 5 g of sample homogenized after a milling step was accurately weighted in polypropylene tube of 50 mL. Then, 25 µL of internal standards (^{13}C-BPA-1G and BPA-d_6-1S, 1 ng μL^{-1}) were directly added to the samples, mixed and left in contact with the matrix at least 2 h. Afterwards, 20 mL water/acetonitrile (50:50, v/v) were added to the fresh sample before shaking during 1 min with the vortex. The extraction mixture was kept around 12 h at room temperature (20°C) before centrifugation at 4500 rpm during 20 min. The supernatant was collected into two tubes and a second extraction was directly performed on the matrix without any contact duration using 10 mL of the same mixture of water /acetonitrile (50:50, v/v). The supernatants were combined and acetonitrile was evaporated under a gentle stream of nitrogen at 50°C. For clean-up purposes, two successive solid phase extractions (SPE) were carried out. The first SPE was performed using a polystyrene-divinylbenzene polymer with a moderate/high specific surface (>1000m^2 g^{-1}). The SPE cartridge was conditioned successively with 6 mL methanol and 6 mL water. After loading the extracted sample (around 10 mL after evaporation), the stationary phase was successively washed with 5 mL water and 8 mL cyclohexane. Then, a strong vacuum was applied and a first elution containing sulfate compounds was carried out using 4 mL acetonitrile. Afterwards, the stationary phase was washed another time with 10 mL acetonitrile and glucuronide molecules were eluted with 20 mL methanol. Both organic eluted fractions were evaporated to dryness under a gentle stream of nitrogen, at 50°C and were reconstituted in 5 mL water. These aqueous phases corresponding to glucuronide and sulfate fractions, were separately loaded onto a second

quaternary ammonium SPE SAX cartridge previously activated with 6 mL methanol and 6 mL water. After washing with 5 mL water and 10 mL methanol, the glucuronide fraction (G) was eluted using 10 mL methanol with 2% formic acid (v/v). An additional washing step was carried out using 10 mL methanol and sulfate fraction (S) was finally eluted using 10 mL methanol with 15% of ammonia 32% (v/v). The two collected fractions (G and S) were evaporated to dryness under a nitrogen stream and reconstituted each in 100 µL of water/acetonitrile (90:10, v/v) for injection. For one sample, solvent consumption was estimated to: 31 mL water, 29 mL acetonitrile, 72 mL methanol, 8 ml cyclohexane, 0.2 mL formic acid and 1.5 mL ammonia. In the experimental conditions implemented in our laboratory, an operator was able to analyse from one to twenty-four samples within two working days.

2.5 UHPLC-MS/MS measurement

Liquid chromatography separation was achieved on an Acquity Ultra Performance Liquid Chromatography (UPLC) System (Waters, Milford, MA, USA) equipped with a Thermo Hypersil Gold column (100×2.1 mm, 1.9µm) (Thermo Fisher Scientific, San José, CA, USA) maintained at 40°C. Mobile phases consisted in 0.1% formic acid in water (MP A) and 0.1% formic acid in acetonitrile (MP B) for glucuronide fraction. For sulfate fraction analysis, mobile phases were 20 mM ammonium acetate in water (MP A') and 20 mM ammonium acetate in acetonitrile (MP B'). The flow rate was set at 0.4 mL min^{-1} for both analyses. The starting gradient was set at 90% of mobile phase A or A', for 1 min before performing a gradual increase of the organic phase (MP B or MP B') gradient to reach 20% at 3 min, 30% at 6.5 min and 100% at 7.5 min. Then the gradient was restored to its initial conditions within 0.5 min and remained as such for 2 min.

Detection was carried out using a XEVO TQ-S instrument (Waters, Milford, MA, USA) operating in the negative Electrospray Ionisation mode (ESI-). Capillary voltage was set at 2 kV, source temperature at 150°C, desolvatation temperature at 500°C, desolvatation gas (N_2) at 600 L h^{-1} and collision gas flow at 0.15 mL min^{-1}. The acquisition was performed using the Selected Reaction Monitoring (SRM). Diagnostic SRM transitions were first generated using waters Intellistart™ software and all the parameters were optimized individually for each diagnostic signal, as indicated in **Table 2**. Data acquisition and data processing were performed using MassLynx, version 4.1 software.

2.6 Quantification procedure

BPA-1G and BPA-1S quantification was achieved according to isotopic dilution method using ^{13}C-BPA-1G and BPA-d_6-1S, respectively (25 ng directly added to each homogenized sample). Area signals of BPA-1G (403>113), ^{13}C-BPA-1G (415>113), BPA-1S (307>227) and BPA-d_6-1S (313>233) were determined and both ratio, i.e. BPA-1G area/ ^{13}C-BPA-1G area and BPA-1S area/ BPA-d_6-1S area were calculated. For the other targeted molecules, i.e. BPA-2G, BPA-2S and BPS-1G, quantification was achieved using ^{13}C-BPA-1G and BPA-d_6-1S as surrogated internal standards. Therefore, area signals of BPA-2G (579>403) and BPS-1G (425>249) were determined and the corresponding ratio BPA-2G area/ ^{13}C-BPA-1G area and BPS-1G area/ ^{13}C-BPA-1G area were calculated. Finally, BPA-2S area signal (445>307) was measured and the ratio BPA-2S area/ BPA-d_6-1S area was calculated. In parallel, a calibration curve was performed in the range [LOQ-10 µg kg^{-1}] and the equation of linear regression was determined for each molecule of interest. The concentrations of metabolite compounds were determined regarding the calculated quantity of the molecule and its corresponding test sample weight.

2.7 Method performances assessment

Detection and quantification limits, specificity, linearity and efficiency of the extraction step were assessed before method implementation and food items subsequent characterisation. Limits of detection (LOD) and quantification (LOQ) were systematically determined in each analysed sample for BPA-1G and BPA-1S, on the basis of the observed signals of ^{13}C-BPA-1G and BPA-d_6-1S, from which the concentrations levels leading to an observed *S/N* ratio of *S/N*=3 (LOD), on the first SRM transition and *S/N*=3 (LOQ), on the second transition. This strategy is defined in the JRC guidance document [46], as "the concentration of an analyte in the extract of a sample which produces an instrumental response at two different ions to be monitored with a *S/N* ratio of 3:1 for the less intensive raw data signal".

For the other targeted molecules, i.e. BPA-2G, BPA-2S and BPS-1G, their corresponding LODs and LOQs were calculated on the basis of three different categories of fortified samples, in the range [0-0.5 µg kg^{-1}]. Therefore, three different LODs and LOQs were assessed for these three compounds of interest corresponding to the three investigated matrices, i.e. bovine liver, Pollock sample and porcine muscle. These three selected matrices were considered to be representative of all the samples of interest. LODs and LOQs were then

defined as the lowest concentration point of the calibration curve that gives an acceptable and consistent signal for the principal transition and the second one, respectively.

Specificity was assessed by checking the absence of interfering compounds responding to the diagnostic signals of the targeted molecules, in the range of their expected retention times. This parameter was also evaluated on the basis of the results obtained for the three representative matrices used in the framework of the validation process. Linearity was first evaluated for external calibration curves using standard solutions at six increasing concentration levels (namely 0, 0.5, 2.5, 5, 10 and 50 ng of the different analytes on-column). Secondly, the linearity for extracted calibration curves (liver, fish and meat) was evaluated within the 0-10 µg kg^{-1} concentration range. The quality of the linear regressions was assessed through their related coefficient of determination (R^2).

Finally, liquid/solid extraction and analytical strategy efficiency were checked using an ovine liver sample originating from an animal experiment.

3 Results and discussion

3.1 Analytical LC-MS/MS development

Chromatographic conditions were investigated with the objective of providing simultaneous efficient separation between all the targeted molecules combined with a good resolution of the different peaks. Preliminary experiments were carried out using water + 0.1% formic acid and acetonitrile + 0.1% formic acid as mobile phases on a Hypersil Gold column. Solvent gradient was then optimized within a 10 min run time, including a stabilization duration of 3 min. The flow rate and the column temperature were set to 0.4 mL min^{-1} and 40°C, respectively. While both efficient separation and resolution were obtained for the glucuronide compounds, these experimental chromatographic conditions were not adequate for the sulfate fraction. Peak tailing was indeed observed for compounds BPA-1S and BPA-d_6-1S. Additionally, BPA-2S was not detected with these initial conditions. These first results are illustrated in **Figure 2** on a salmon extract fortified with 5 ng of BPA-1S, BPA-2S (equivalent to 1 µg kg^{-1}) and 10 ng of BPA-d_6-1S (equivalent to 2 µg kg^{-1}). As the most acidic compound (BPA-2S) is not detected, its retention was probably very weak on reversed phase using acid modifier in the mobile phase. Furthermore, the mono-sulfated compounds would be stabilized under a neutral form to improve their peak shape. Therefore, the composition of mobile phases was modified

as follows: 0.1% of formic acid was substituted with 20 mM ammonium acetate for both mobile phases, i.e. water + 20 mM ammonium acetate (MP A') and acetonitrile + 20 mM ammonium acetate (MP B'), in accordance with our previous works [47]. Initial developed gradient was then implemented in order to compare both conditions. Peak shape and peak tailing were significantly improved for both BPA-1S and its corresponding internal standard using ammonium acetate as a modifier. Moreover, an efficient retention of BPA-2S was also observed with ammonium acetate modifier, allowing its analysis under these new chromatographic conditions.

The resulting chromatographic run was 10 min for each fraction of interest. Regarding glucuronide fraction, the first (more polar) compound (BPS-1G) eluted at 3.61 min in a first (glucuronide) fraction analysis and a last (less polar) one (BPA-1G) eluted at 6.77 min. on the same way, the first eluted compound for sulfate fraction was BPA-2S at 5.00 min and the second one (BPA-1S) at 7.36 min. These optimized chromatographic conditions were subsequently used in this study. In comparison with the previous published articles, the proposed chromatographic strategy allows the detection of a greater number of conjugated metabolites of both BPA and BPS, without any peak tailing or peak fronting observations.

Then, the mass spectrometric parameters were optimized in the Selected Reaction Monitoring mode (SRM) for reaching high confidence level in terms of unambiguous identification of the molecules of interest at low concentrations according to 2002/657/EC decision requirements [48] (identification points >4) . Therefore, three or four diagnostic SRM transitions per targeted compound were monitored and optimized using Water's Intellistart system and then checked manually. Additionally, two SRM transitions were also monitored for each internal standard, namely ^{13}C-BPA-1G and BPA-d_6-1S. Final acquisition method was sequenced in two different time windows for glucuronide fraction (G-TW1 and G-TW2) and in a single time window for sulfate fraction (S-TW1) (**Table 2)**. Thanks to this analytical approach, selectivity, chromatographic efficiency and resolution were concluded compatible with expectations for food from animal origin characterization. While two diagnostic transitions were generally monitored in most of the published articles, the monitoring acquisition method was developed in order to strengthen the identification of the targeted molecules.

3.2 Standard Operating Procedure

The standard operating procedure (**Figure 1**) was developed and optimized for the direct determination of conjugated metabolites of bisphenol A and bisphenol S, in a wide range of food items from animal origin. Particular attention was given to the first extraction step efficiency. Therefore, different solid-liquid extractions have been evaluated on different foodstuffs. Water, water/acetonitrile (50:50, v/v), Folch [49] or Bligh and Dyer [50] procedures were compared on the basis on results obtained with liver, fish and muscle samples fortified with 5 ng of each compound of interest. Solid-liquid extraction carried out using water/acetonitrile mixture was preferred because acetonitrile allows efficient protein precipitation, enhancing thus the sample treatment facility. Indeed, no clogging was observed with subsequent SPE when using this strategy whereas a solid-liquid extraction driven using only water resulted in problematic SPEs. Additionally, no significant differences were observed between water/acetonitrile, Folch or Bligh and Dyer procedures, neither during sample treatment nor on the recovery yields. Therefore, two successive extractions using water/acetonitrile (50:50, v/v) were finally chosen for enhanced recovery yields/performances.

Afterwards, two consecutive orthogonal SPE purification stages were envisaged, *i.e.* a non-polar phase combined to a strong anion exchange sorbent. Regarding the non-polar SPE phase, several trials have been performed to optimize the washing step. Initially, various mixtures of water/methanol (from 100% water to 0%) were tested on reference standard solutions. A washing step using 5 mL water/methanol (90:10, v/v) was considered as suitable conditions. These experimental conditions were then implemented for the analysis of different matrices, such as liver, muscle and fish. For each tested matrix, both washing and elution fractions were collected and analyzed. No repeatable results were obtained insofar as variable recoveries of all targeted compound were observed in the washing fraction depending on the analyzed matrix. Actually, the eluting strength necessary for the elution of both glucuronide and sulfate compounds was observed as matrix-dependent. Therefore, cyclohexane was successfully tested in order to remove non polar interferences and then elution step was optimized as follows: sulfate compounds were eluted using acetonitrile while glucuronide molecules were secondly eluted using methanol. Because of lipid residual occasionally observed for fatty samples, a second SPE step using a strong anion exchange (SAX) phase was also included in the analytical workflow in order to improve the purification efficiency. Initially two different stationary phases have been investigated: the first one was a copolymeric strong anions exchanger bonded on a HR-X phase (HR-XA, Macherey Nagel)

and the second one was a strong anions exchanger on a functionalized silica (SAX, UCT). Regarding HR-XA cartridge, satisfactory recoveries were obtained for glucuronide compounds using an elution mixture of methanol + 2% formic acid (>90%). Nevertheless, sulfate compounds were not eluted in the corresponding fraction using methanol + ammonia, even with 15% ammonia, as described by Anizan et al. [47]. This kind of stationary phase presents too strong ionic affinities with BPA-1S, BPA-1S-d_6 and BPA-2S to allow their elution regarding the determination of conjugated metabolites of steroids in urine. Moreover, recovery yields obtained using SAX cartridge were excellent (>95%) for both glucuronide molecules eluted with methanol / formic acid (98:2, v/v) and sulfate compounds eluted with methanol / ammonium hydroxide 32% (85:15, v/v). Therefore, the additional step based on a SAX cartridge was chosen to achieve the purification before analysis. **Figure 3** illustrates an example of SRM chromatograms obtained for a salmon analysis, with the following analytical procedures: Solid-liquid extraction + filtration (A), Solid-liquid extraction + non polar SPE (B) and solid-liquid extraction + non polar SPE + SAX SPE (C). SRM chromatograms were slightly better using HR-X SPE in comparison to the single solid-liquid extraction: both intensities and S/N ratios were improved, especially regarding BPA-1S detection (307>227). Finally, the addition of SAX SPE purification improved significantly all results, especially in terms of S/N ratios. SRM chromatograms and its corresponding S/N calculation were investigated for the main diagnostic transition of BPA-1S (307>227) regarding the three different analytical strategies. S/N=8 was obtained for a single solid-liquid extraction, S/N=12 for solid-liquid extraction combined with non-polar SPE, and S/N=104 for the developed and optimized analytical method. This standard operating procedure is quite innovative in comparison with the analytical methods already published which focus on biological matrices, such as blood or urine. Indeed, the determination of conjugated metabolites of BPA and BPS in foodstuffs, at very low levels (from 0.02 to 0.50 µg/kg), require an efficient sample preparation which is fulfilled with the combination of both consecutive orthogonal SPE steps.

3.3 Validation results

3.3.1 Background contamination

The control of in-laboratory environmental contamination with BPA was considered as a major potential critical issue, as already mentioned in several articles [42, 51-55]. Despite their non-ubiquitous status, the potential background contamination of conjugated metabolites

of bisphenol A and bisphenol S was still necessary to be assessed. All materials and analytical measurement devices were tested. Moreover, one procedural blank sample was systematically included in each series of samples in the framework of the experimental plan implementation. The results demonstrated neither background contamination nor traces in more than thirty blank analyzed samples (<LOD) for the five metabolites of interest, allowing to conclude that no particular attention had to be paid to the background contamination for the determination of conjugated metabolites of both BPA and BPS, in the experimental conditions implemented in this analytical development.

3.3.2 Linearity – calibration curve

The linearity of the developed analytical method was determined on calibration curves made of standard solutions and extracted spiked foodstuffs (liver, muscle and fish). Eight concentration levels were used in the range of [0 – 10] µg kg^{-1}, with a majority of calibration points (n=6) included in the range 0 – 1 µg kg^{-1}. The intercept was not forced through the origin due to the possible presence of the targeted compounds in the non-fortified sample. Excellent linearity values were obtained for all the compounds of interest, as all resulting coefficients of determination (R^2) were found higher than 0.99 with residuals below 20% on the relative response factors (RRF). For each quantified analyte, both calibration curves (i.e., external and internal calibration) were found mimetic according to the Student statistical test, with equivalent slope. Therefore, sample quantification was carried out using the standard calibration curve.

3.3.3 Detection limits

The limit of detection (LOD) was estimated as the concentration from which a significant signal to noise ratio (*S/N=3*) was obtained. LODs were initially determined in three food matrices, namely liver, muscle and fish for the five targeted molecules. Regarding BPA-G and BPA-S, considered as the principal conjugated metabolites of BPA, LODs ranged from 0.02 to 0.10 µg kg^{-1} for the three investigated matrices. LODs obtained for BPA-2G and BPS-1G ranged from 0.02 µg kg^{-1} for BPA-2G in liver to 0.18 µg kg^{-1} for BPS-1G in fish sample. Detailed LOD results obtained for the five compounds of interest and the three investigated matrices are reported in **Table 3**. Additionally, LODs were systematically calculated for each analyzed sample for both BPA-1G and BPA-1S on the basis of the major transition of the corresponding internal standard, i.e.: 415>113 for ^{13}C-BPA-1G and 313>233 for BPA-d_6-1S.

Regarding, BPA-2S, observed LODs were significantly higher than the other targeted compounds, ranging from 0.10 µg kg^{-1} in liver sample to 0.50 µg kg^{-1} in bovine muscle. To our knowledge, no earlier studies have reported LODs of conjugated metabolites of both BPA and BPS, in foodstuffs. Nevertheless, the obtained LODs are consistent with BPA contamination levels reported in the framework of the second French total diet study [41].

3.4 Proof of the concept

The developed and validated analytical method was applied to various foodstuffs of animal origin.

Firstly, an incurred liver sample originating from an animal exposed to BPA on the basis of 50 µg kg^{-1} per day during 105 days was analyzed. The results show that BPA-1G, BPA-1S and BPA-2G were detected (>LOD) in this sample, unambiguously identified and quantified at a concentration of 3.81 µg kg^{-1}, 0.80 µg kg^{-1} and 0.09 µg kg^{-1}, respectively, as illustrated in **Figure 4**. Therefore, BPA-1G appears as the main conjugated metabolite of BPA in ovine liver representing 81% of the conjugated forms. BPA-1S was detected with a proportion of 17% and BPA-2G represented 2% of the conjugated forms. BPA-2S was not detected, (<LOD). This animal experiment results demonstrates that (i) BPA is extensively metabolized in the ovine liver, and, (ii) the efficiency of both extraction and purification efficiencies. Moreover, to the best of our knowledge it is the first time that BPA-2G has been identified in a liver sample originating from an animal exposed to BPA.

Secondly, fifty different foodstuffs of animal origin were selected as previously characterized for their contamination with free BPA [56]. The main objective was to investigate possible / potential presence of conjugated metabolites of BPA. As none of the investigated molecules (BPA-1G, BPA-2G, BPA-1S and BPA-2S) could be detected (<LOD) in any of the fifty selected foodstuffs samples, it means that BPA contamination was the result of direct contact with material containing BPA, and was not the result of living animal exposure to BPA which would have led to *in-vivo* metabolism and the subsequent presence of metabolites.

Therefore, the proof of the strategy efficiency was demonstrated insofar as the developed analytical method was successfully applied to a large variety of foodstuffs of animal origin and the observed results allows (i) to characterize BPA metabolism in ovine liver and (ii) determine the origin of BPA contamination.

4 Conclusion

Analytical method based on a first efficient solid/liquid extraction followed by the combination of two successive solid phase extraction (SPE) proved to be a suitable strategy for the direct detection and quantification of BPA-1G, BPA-2G, BPA-1S, BPA-2S and BPS-1G in a large scale of foodstuffs of animal origin. Conjugated bisphenol concentrations are usually deduced from "total" and "free" bisphenol concentrations with the major critical issue consideration of ubiquitous molecules. To the best of our knowledge, and according to the previous standard operating procedures already published, it is the first analytical methodology allowing the simultaneous determination of the four conjugated metabolites of BPA and mono glucuronide BPS. Targeted compounds were easily separated onto the UHPLC system and the Selected Reaction Monitoring mode of the triple quadrupole mass analyzer allowed the unambiguous detection and quantification of the five targeted molecules. Detection limits ranged from 0.02 µg kg^{-1} for BPA-1G in muscle or both BPA-1S and BPA-2G in liver, to 0.50 µg kg^{-1} for BPA-2S in muscle. Quantification was performed using both ^{13}C-BPA-1G and BPA-d_6-1S as internal standards; corresponding linearity was quite satisfying with all coefficients of determination above 0.99. While no targeted compound was detected in any of the analyzed samples (<LODs), The developed analytical method was successfully implemented to the determination of conjugated metabolites of BPA and BPS in a first heterogeneous set of 50 foodstuffs of animal origin, previously investigated regarding their BPA levels. The comparison between both results, i.e. free BPA concentrations and conjugated forms validated the *post mortem* origin of BPA in these samples. Additionally, an incurred liver sample originating from pregnant ewes administered subcutaneously with BPA (50 µg kg^{-1} per day) during 105 days, was analyzed using the developed analytical method. BPA-1G, BPA-1S and BPA-2G were detected (>LOD), unambiguously identified and quantified at a concentration of 3.81 µg kg^{-1}, 0.80 µg kg^{-1} and 0.09 µg kg^{-1}, respectively. These results confirm that BPA-1G is the main conjugated metabolite of BPA, since BPA-1S and BPA-2G were also observed.

Therefore, this analytical method could be used in the framework of investigations regarding both BPA and BPS conjugated metabolites, in food items but also in biological matrices.

REFERENCES

[1] S. Errico, M. Bianco, L. Mita, M. Migliaccio, S. Rossi, C. Nicolucci, C. Menale, M. Portaccio, P. Gallo, D.G. Mita, N. Diano, Migration of bisphenol A into canned tomatoes produced in Italy: dependence on temperature and storage conditions, Food chemistry, 160 (2014) 157-164.

[2] A. Goodson, H. Robin, W. Summerfield, I. Cooper, Migration of bisphenol A from can coatings--effects of damage, storage conditions and heating, Food additives and contaminants, 21 (2004) 1015-1026.

[3] E.M. Munguia-Lopez, S. Gerardo-Lugo, E. Peralta, S. Bolumen, H. Soto-Valdez, Migration of bisphenol A (BPA) from can coatings into a fatty-food simulant and tuna fish, Food additives and contaminants, 22 (2005) 892-898.

[4] M.I. Santillana, E. Ruiz, M.T. Nieto, J. Bustos, J. Maia, R. Sendon, J.J. Sanchez, Migration of bisphenol A from polycarbonate baby bottles purchased in the Spanish market by liquid chromatography and fluorescence detection, Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 28 (2011) 1610-1618.

[5] M.L. Spagnuolo, F. Marini, L.A. Sarabia, M.C. Ortiz, Migration test of Bisphenol A from polycarbonate cups using excitation-emission fluorescence data with parallel factor analysis, Talanta, 167 (2017) 367-378.

[6] Commission Regulation (EU) 2018/213 of 12 February 2018 on the use of bisphenol A in varnishes and coatings intended to come into contact with food and amending Regulation (EU) No 10/2011 as regards the use of that substance in plastic food contact materials, Off. J. Eur. L41/6 (2018).

[7] S.A. Baluka, W.K. Rumbeiha, Bisphenol A and food safety: Lessons from developed to developing countries, Food and Chemical Toxicology, 92 (2016) 58-63.

[8] S. Eladak, D. Moison, M.J. Guerquin, G. Matilionyte, K. Kilcoyne, T. N'Tumba-Byn, S. Messiaen, Y. Deceuninck, S. Pozzi-Gaudin, A. Benachi, G. Livera, J.P. Antignac, R. Mitchell, V. Rouiller-Fabre, R. Habert, Effects of environmental Bisphenol A exposures on germ cell development and Leydig cell function in the human fetal testis, PloS one, 13 (2018) e0191934.

[9] C. Desdoits-Lethimonier, L. Lesne, P. Gaudriault, D. Zalko, J.P. Antignac, Y. Deceuninck, C. Platel, N. Dejucq-Rainsford, S. Mazaud-Guittot, B. Jegou, Parallel assessment of the effects of bisphenol A and several of its analogs on the adult human testis, Hum Reprod, 32 (2017) 1465-1473.

[10] X.H. Xu, J. Zhang, Y.M. Wang, Y.P. Ye, Q.Q. Luo, Perinatal exposure to bisphenol-A impairs learning-memory by concomitant down-regulation of N-methyl-D-aspartate receptors of hippocampus in male offspring mice, Hormones and behavior, 58 (2010) 326-333.

[11] X. Xu, Y. Ye, T. Li, L. Chen, D. Tian, Q. Luo, M. Lu, Bisphenol-A rapidly promotes dynamic changes in hippocampal dendritic morphology through estrogen receptor-mediated pathway by concomitant phosphorylation of NMDA receptor subunit NR2B, Toxicology and applied pharmacology, 249 (2010) 188-196.

[12] R. Moral, R. Wang, I.H. Russo, C.A. Lamartiniere, J. Pereira, J. Russo, Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature, The Journal of endocrinology, 196 (2008) 101-112.

[13] M. Ben Maamar, L. Lesne, C. Desdoits-Lethimonier, I. Coiffec, J. Lassurguere, V. Lavoue, Y. Deceuninck, J.P. Antignac, B. Le Bizec, E. Perdu, D. Zalko, C. Pineau, C. Chevrier, N. Dejucq-Rainsford, S. Mazaud-Guittot, B. Jegou, An investigation of the endocrine-disruptive effects of bisphenol a in human and rat fetal testes, PloS one, 10 (2015) e0117226.

[14] E.F. Efsa Panel on Food Contact Materials, A. Processing, Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs, EFSA Journal, 13 (2015) 3978-n/a.

[15] M.T. Do, V.C. Chang, M.A. Mendez, M. de Groh, Urinary bisphenol A and obesity in adults: results from the Canadian Health Measures Survey, Health Promot Chronic Dis Prev Can, 37 (2017) 403-412.

[16] D.G. Skledar, J. Schmidt, A. Fic, I. Klopcic, J. Trontelj, M.S. Dolenc, M. Finel, L.P. Masic, Influence of metabolism on endocrine activities of bisphenol S, Chemosphere, 157 (2016) 152-159.

[17] V. Le Fol, S. Ait-Aissa, M. Sonavane, J.M. Porcher, P. Balaguer, J.P. Cravedi, D. Zalko, F. Brion, In vitro and in vivo estrogenic activity of BPA, BPF and BPS in zebrafish-specific assays, Ecotoxicology and environmental safety, 142 (2017) 150-156.

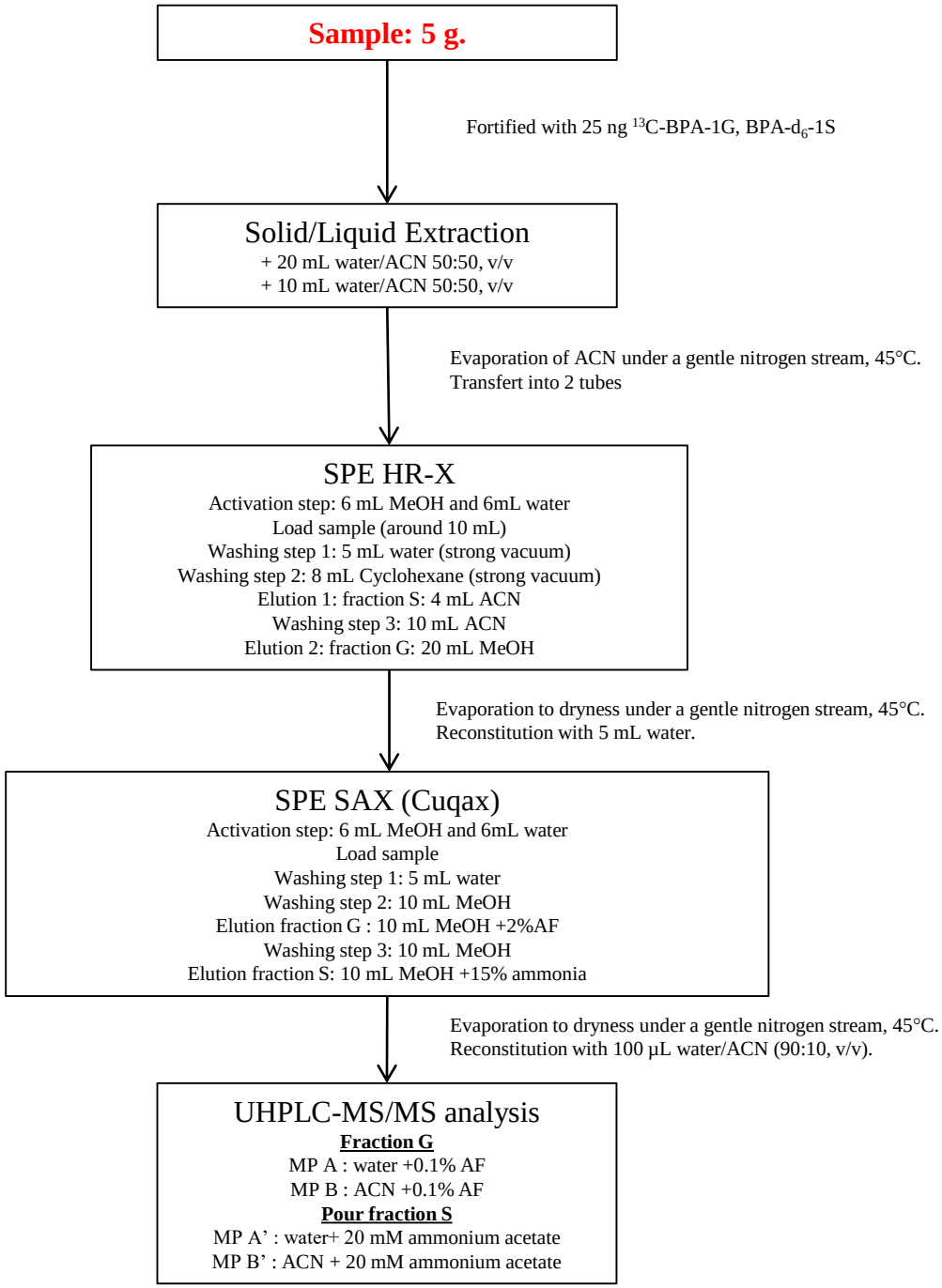
[18] L. Ivry Del Moral, L. Le Corre, H. Poirier, I. Niot, T. Truntzer, J.F. Merlin, P. Rouimi, P. Besnard, R. Rahmani, M.C. Chagnon, Obesogen effects after perinatal exposure of 4,4'-sulfonyldiphenol (Bisphenol S) in C57BL/6 mice, Toxicology, 357-358 (2016) 11-20.

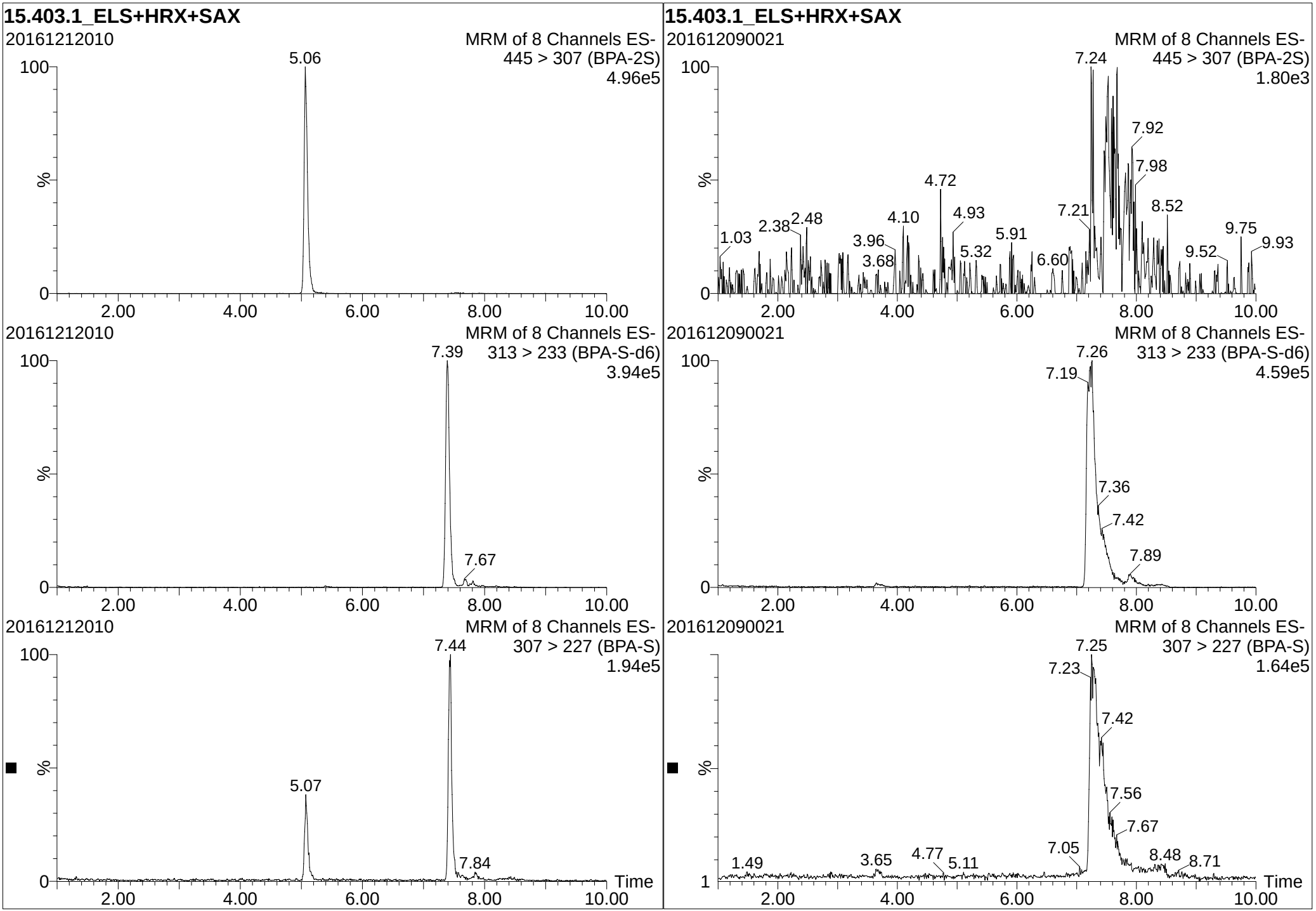
[19] N. Ahsan, H. Ullah, W. Ullah, S. Jahan, Comparative effects of Bisphenol S and Bisphenol A on the development of female reproductive system in rats; a neonatal exposure study, Chemosphere, 197 (2018) 336-343.
[20] S. Kolla, M. Morcos, B. Martin, L.N. Vandenberg, Low dose bisphenol S or ethinyl estradiol exposures during the perinatal period alter female mouse mammary gland development, Reprod Toxicol, 78 (2018) 50-59.
[21] J.C. Park, M.C. Lee, D.S. Yoon, J. Han, M. Kim, U.K. Hwang, J.H. Jung, J.S. Lee, Effects of bisphenol A and its analogs bisphenol F and S on life parameters, antioxidant system, and response of defensome in the marine rotifer Brachionus koreanus, Aquatic toxicology, 199 (2018) 21-29.
[22] Y. Chen, J. Fang, L. Ren, R. Fan, J. Zhang, G. Liu, L. Zhou, D. Chen, Y. Yu, S. Lu, Urinary bisphenol analogues and triclosan in children from south China and implications for human exposure, Environmental pollution, 238 (2018) 299-305.
[23] G. Russo, F. Barbato, E. Cardone, M. Fattore, S. Albrizio, L. Grumetto, Bisphenol A and Bisphenol S release in milk under household conditions from baby bottles marketed in Italy, J Environ Sci Health B, 53 (2018) 116-120.
[24] L.H. Wu, X.M. Zhang, F. Wang, C.J. Gao, D. Chen, J.R. Palumbo, Y. Guo, E.Y. Zeng, Occurrence of bisphenol S in the environment and implications for human exposure: A short review, The Science of the total environment, 615 (2018) 87-98.
[25] D. Chen, K. Kannan, H. Tan, Z. Zheng, Y.L. Feng, Y. Wu, M. Widelka, Bisphenol Analogues Other Than BPA: Environmental Occurrence, Human Exposure, and Toxicity-A Review, Environmental science & technology, 50 (2016) 5438-5453.
[26] W. Volkel, T. Colnot, G.A. Csanady, J.G. Filser, W. Dekant, Metabolism and kinetics of bisphenol a in humans at low doses following oral administration, Chemical research in toxicology, 15 (2002) 1281-1287.
[27] W. Dekant, W. Volkel, Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures, Toxicology and applied pharmacology, 228 (2008) 114-134.
[28] W. Volkel, N. Bittner, W. Dekant, Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry, Drug metabolism and disposition: the biological fate of chemicals, 33 (2005) 1748-1757.
[29] V. Le Fol, S. Ait-Aissa, N. Cabaton, L. Dolo, M. Grimaldi, P. Balaguer, E. Perdu, L. Debrauwer, F. Brion, D. Zalko, Cell-specific biotransformation of benzophenone-2 and bisphenol-s in zebrafish and human in vitro models used for toxicity and estrogenicity screening, Environmental science & technology, 49 (2015) 3860-3868.
[30] N. Cabaton, D. Zalko, E. Rathahao, C. Canlet, G. Delous, M.C. Chagnon, J.P. Cravedi, E. Perdu, Biotransformation of bisphenol F by human and rat liver subcellular fractions, Toxicology in vitro : an international journal published in association with BIBRA, 22 (2008) 1697-1704.
[31] N. Cabaton, M.C. Chagnon, J.C. Lhuguenot, J.P. Cravedi, D. Zalko, Disposition and metabolic profiling of bisphenol F in pregnant and nonpregnant rats, Journal of agricultural and food chemistry, 54 (2006) 10307-10314.
[32] G. Provencher, R. Berube, P. Dumas, J.F. Bienvenu, E. Gaudreau, P. Belanger, P. Ayotte, Determination of bisphenol A, triclosan and their metabolites in human urine using isotope-dilution liquid chromatography-tandem mass spectrometry, Journal of chromatography. A, 1348 (2014) 97-104.
[33] R.R. Gerona, T.J. Woodruff, C.A. Dickenson, J. Pan, J.M. Schwartz, S. Sen, M.W. Friesen, V.Y. Fujimoto, P.A. Hunt, Bisphenol-A (BPA), BPA glucuronide, and BPA sulfate in midgestation umbilical cord serum in a northern and central California population, Environmental science & technology, 47 (2013) 12477-12485.
[34] C. Liao, K. Kannan, Determination of free and conjugated forms of bisphenol A in human urine and serum by liquid chromatography-tandem mass spectrometry, Environmental science & technology, 46 (2012) 5003-5009.
[35] F. Grandin, N. Picard-Hagen, V. Gayrard, S. Puel, C. Viguie, P.L. Toutain, L. Debrauwer, M.Z. Lacroix, Development of an on-line solid phase extraction ultra-high-performance liquid chromatography technique coupled to tandem mass spectrometry for quantification of bisphenol S and bisphenol S glucuronide: Applicability to toxicokinetic investigations, Journal of chromatography. A, 1526 (2017) 39-46.
[36] Z.Z. Hauck, K. Huang, G. Li, R.B. van Breemen, Determination of bisphenol A-glucuronide in human urine using ultrahigh-pressure liquid chromatography/tandem mass spectrometry, Rapid communications in mass spectrometry : RCM, 30 (2016) 400-406.

[37] M.Z. Lacroix, S. Puel, S.H. Collet, T. Corbel, N. Picard-Hagen, P.L. Toutain, C. Viguie, V. Gayrard, Simultaneous quantification of bisphenol A and its glucuronide metabolite (BPA-G) in plasma and urine: applicability to toxicokinetic investigations, Talanta, 85 (2011) 2053-2059.
[38] C. Kubwabo, I. Kosarac, K. Lalonde, W.G. Foster, Quantitative determination of free and total bisphenol A in human urine using labeled BPA glucuronide and isotope dilution mass spectrometry, Analytical and bioanalytical chemistry, 406 (2014) 4381-4392.
[39] I. Kosarac, C. Kubwabo, K. Lalonde, W. Foster, A novel method for the quantitative determination of free and conjugated bisphenol A in human maternal and umbilical cord blood serum using a two-step solid phase extraction and gas chromatography/tandem mass spectrometry, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 898 (2012) 90-94.
[40] ANSES, Opinion and expert appraisal report of the French Agency for Food, Environmental and Occupational Health & Safety on the assessment of the risks of bisphenol A (BPA) for human health. Maisons Alfort, Volume 1 (2013).
[41] N. Bemrah, J. Jean, G. Riviere, M. Sanaa, S. Leconte, M. Bachelot, Y. Deceuninck, B.L. Bizec, X. Dauchy, A.C. Roudot, V. Camel, K. Grob, C. Feidt, N. Picard-Hagen, P.M. Badot, F. Foures, J.C. Leblanc, Assessment of dietary exposure to bisphenol A in the French population with a special focus on risk characterisation for pregnant French women, Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 72 (2014) 90-97.
[42] Y. Deceuninck, E. Bichon, S. Durand, N. Bemrah, Z. Zendong, M.L. Morvan, P. Marchand, G. Dervilly-Pinel, J.P. Antignac, J.C. Leblanc, B. Le Bizec, Development and validation of a specific and sensitive gas chromatography tandem mass spectrometry method for the determination of bisphenol A residues in a large set of food items, Journal of chromatography. A, 1362 (2014) 241-249.
[43] ANSES, Second French Total Diet Study (TDS2) – Volume 1: Inorganic contaminants, minerals, persistent organic pollutants, mycotoxins and phyto-oestrogens. Expert appraisal report. Scientific Editions. Maisons-Alfort, ANSES: 305., (2011).

[44] V. Sirot, J.L. Volatier, G. Calamassi-Tran, C. Dubuisson, C. Menard, A. Dufour, J.C. Leblanc, Core food of the French food supply: second Total Diet Study, Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 26 (2009) 623-639.
[45] D. Guignard, V. Gayrard, M.Z. Lacroix, S. Puel, N. Picard-Hagen, C. Viguie, Evidence for bisphenol A-induced disruption of maternal thyroid homeostasis in the pregnant ewe at low level representative of human exposure, Chemosphere, 182 (2017) 458-467.
[46] E.C. Joint Research Centre, Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food, Publications Office of the European Union, (2016).
[47] S. Anizan, D. Di Nardo, E. Bichon, F. Monteau, N. Cesbron, J.P. Antignac, B. Le Bizec, Targeted phase II metabolites profiling as new screening strategy to investigate natural steroid abuse in animal breeding, Analytica chimica acta, 700 (2011) 105-113.
[48] Commission Decision of 12 August 2002 (2002/657/EC) implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.
[49] F.N. Lebaron, J. Folch, The effect of pH and salt concentration on aqueous extraction of brain proteins and lipoproteins, Journal of neurochemistry, 4 (1959) 1-8.
[50] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can J Biochem Physiol, 37 (1959) 911-917.
[51] R. Carabias-Martinez, E. Rodriguez-Gonzalo, P. Revilla-Ruiz, J. Hernandez-Mendez, Pressurized liquid extraction in the analysis of food and biological samples, Journal of chromatography. A, 1089 (2005) 1-17.
[52] B. Shao, H. Han, X. Tu, L. Huang, Analysis of alkylphenol and bisphenol A in eggs and milk by matrix solid phase dispersion extraction and liquid chromatography with tandem mass spectrometry, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 850 (2007) 412-416.
[53] Y. Watabe, T. Kondo, M. Morita, N. Tanaka, J. Haginaka, K. Hosoya, Determination of bisphenol A in environmental water at ultra-low level by high-performance liquid chromatography with an effective on-line pretreatment device, Journal of chromatography. A, 1032 (2004) 45-49.
[54] K. Inoue, K. Kato, Y. Yoshimura, T. Makino, H. Nakazawa, Determination of bisphenol A in human serum by high-performance liquid chromatography with multi-electrode electrochemical detection, Journal of chromatography. B, Biomedical sciences and applications, 749 (2000) 17-23.
[55] W. Volkel, M. Kiranoglu, H. Fromme, Determination of free and total bisphenol A in human urine to assess daily uptake as a basis for a valid risk assessment, Toxicology letters, 179 (2008) 155-162.

627 [56] S. Gorecki, N. Bemrah, A.C. Roudot, E. Marchioni, B. Le Bizec, F. Faivre, M. Kadawathagedara,
628 J. Botton, G. Riviere, E.m.-c.c.s. group, Human health risks related to the consumption of foodstuffs of
629 animal origin contaminated by bisphenol A, Food and chemical toxicology : an international journal
630 published for the British Industrial Biological Research Association, 110 (2017) 333-339.


Sample: 5 g.
Fortified with 25 ng ^{13}C-BPA-1G, BPA-d_6-1S
Solid/Liquid Extraction
+ 20 mL water/ACN 50:50, v/v
+ 10 mL water/ACN 50:50, v/v
Evaporation of ACN under a gentle nitrogen stream, 45°C.
Transfert into 2 tubes
SPE HR-X
Activation step: 6 mL MeOH and 6mL water
Load sample (around 10 mL)
Washing step 1: 5 mL water (strong vacuum)
Washing step 2: 8 mL Cyclohexane (strong vacuum)
Elution 1: fraction S: 4 mL ACN
Washing step 3: 10 mL ACN
Elution 2: fraction G: 20 mL MeOH
Evaporation to dryness under a gentle nitrogen stream, 45°C.
Reconstitution with 5 mL water.
SPE SAX (Cuqax)
Activation step: 6 mL MeOH and 6mL water
Load sample
Washing step 1: 5 mL water
Washing step 2: 10 mL MeOH
Elution fraction G : 10 mL MeOH +2%AF
Washing step 3: 10 mL MeOH
Elution fraction S: 10 mL MeOH +15% ammonia
Evaporation to dryness under a gentle nitrogen stream, 45°C.
Reconstitution with 100 µL water/ACN (90:10, v/v).
UHPLC-MS/MS analysis
Fraction G
MP A : water +0.1% AF
MP B : ACN +0.1% AF
Pour fraction S
MP A' : water+ 20 mM ammonium acetate
MP B' : ACN + 20 mM ammonium acetate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4.96e5
5.06
MRM of 8 Channels ES-
313 > 233 (BPA-S-d6)
3.94e5
7.39
7.67
MRM of 8 Channels ES-
307 > 227 (BPA-S)
1.94e5
7.44
5.07
7.84
Time
201612090021
MRM of 8 Channels ES-
445 > 307 (BPA-2S)
1.80e3
7.24
7.92
7.98
4.72
8.52
7.21
4.10
4.93
2.48
2.38
5.91
9.75
1.03
9.93
3.96
9.52
5.32
3.68
6.60
MRM of 8 Channels ES-
313 > 233 (BPA-S-d6)
4.59e5
7.26
7.19
7.36
7.42
7.89
MRM of 8 Channels ES-
307 > 227 (BPA-S)
1.64e5
7.25
7.23
7.42
7.56
7.67
7.05
1.49
3.65
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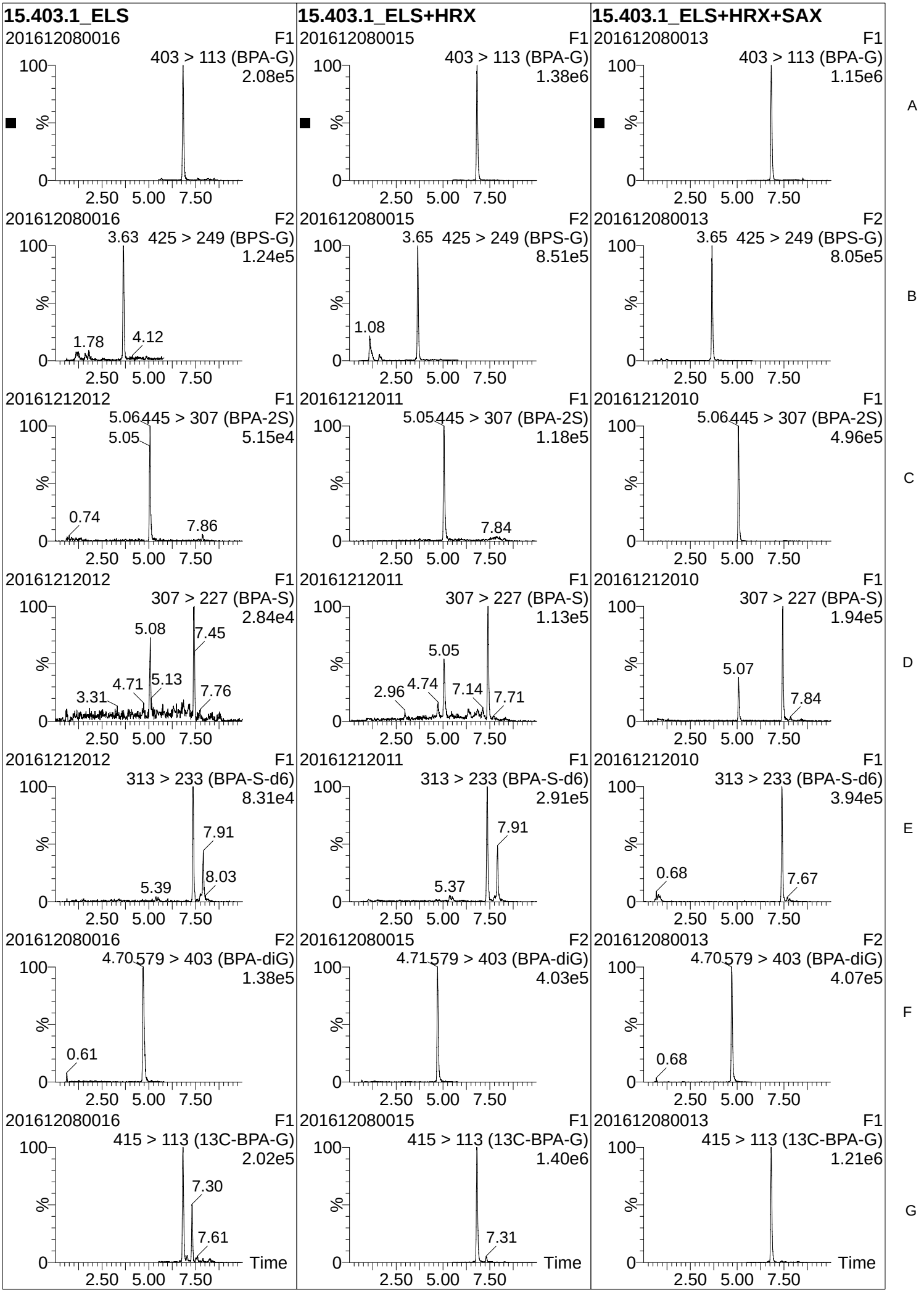
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1.78
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8.51e5
1.08
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B
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7.45
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7.14
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20161212010 F1
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1.94e5
5.07
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2.91e5
7.91
5.37
20161212010 F1
313 > 233 (BPA-S-d6)
3.94e5
0.68
7.67
E
201612080016 F2
4.70 579 > 403 (BPA-diG)
1.38e5
0.61
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201612080013 F2
4.70 579 > 403 (BPA-diG)
4.07e5
0.68
F
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7.61
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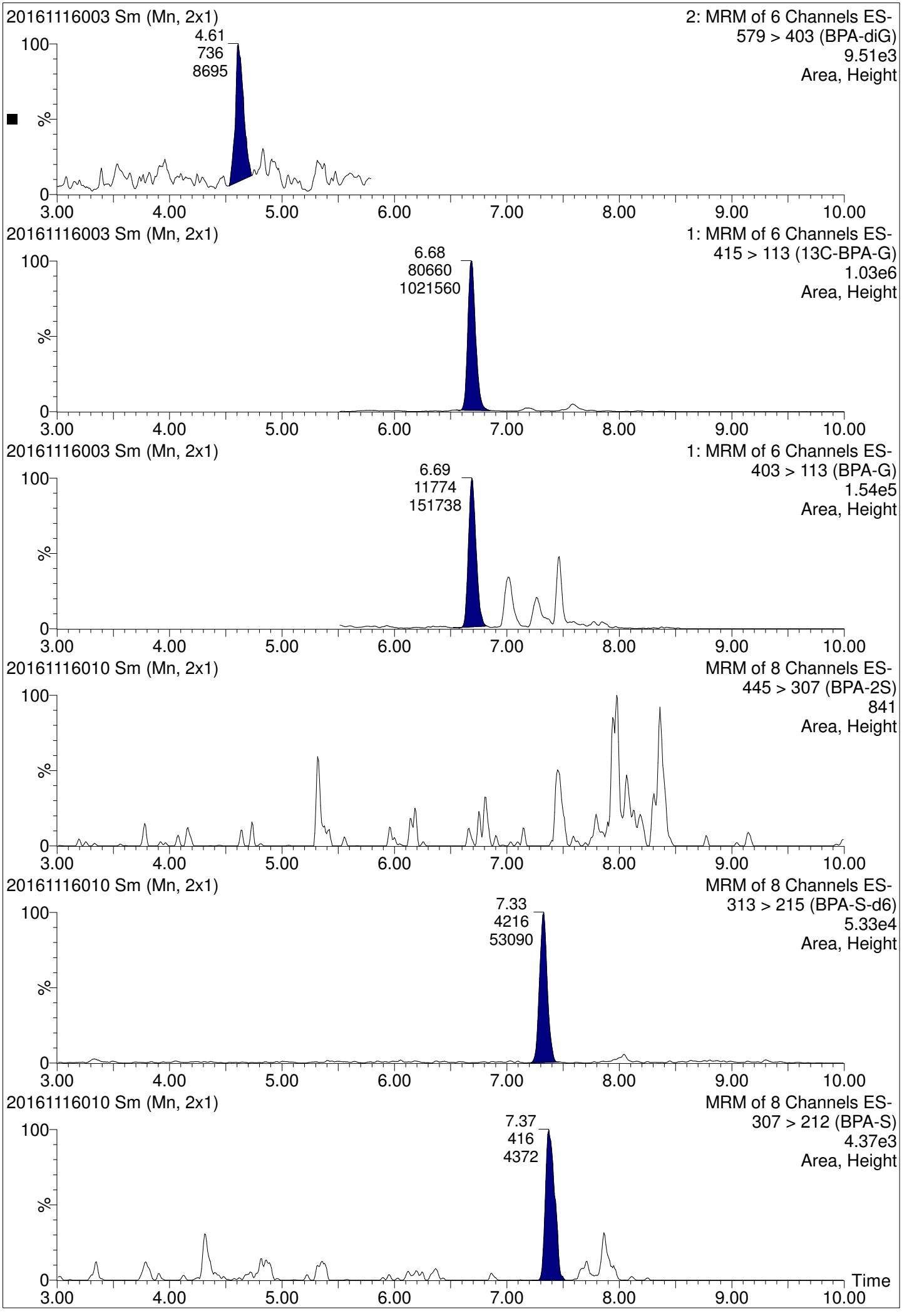
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579 > 403 (BPA-diG)
9.51e3
Area, Height
4.61
736
8695
100
%
0
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
20161116003 Sm (Mn, 2x1)
1: MRM of 6 Channels ES-
415 > 113 (13C-BPA-G)
1.03e6
Area, Height
6.68
80660
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20161116003 Sm (Mn, 2x1)
1: MRM of 6 Channels ES-
403 > 113 (BPA-G)
1.54e5
Area, Height
6.69
11774
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20161116010 Sm (Mn, 2x1)
MRM of 8 Channels ES-
445 > 307 (BPA-2S)
841
Area, Height
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MRM of 8 Channels ES-
313 > 215 (BPA-S-d6)
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Area, Height
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4216
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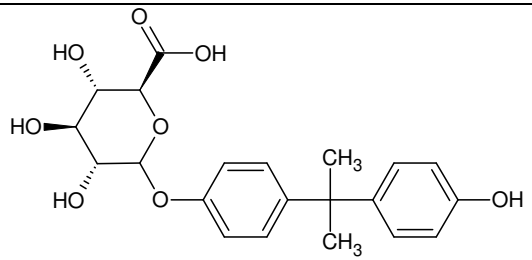
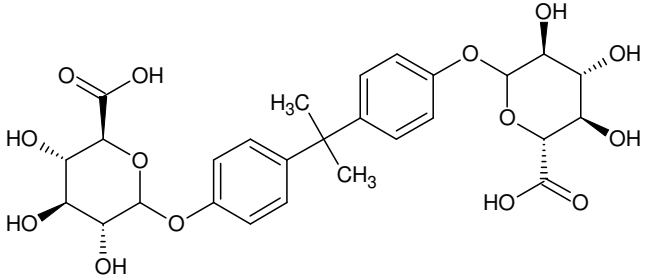
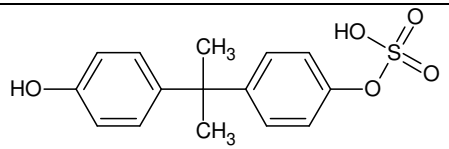
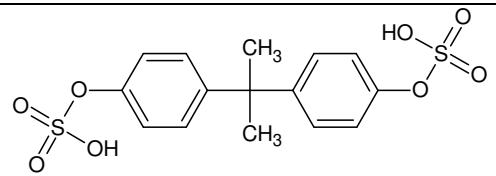
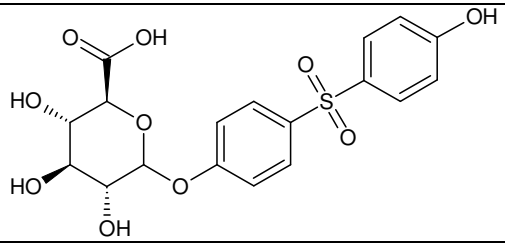
20161116010 Sm (Mn, 2x1)
MRM of 8 Channels ES-
307 > 212 (BPA-S)
4.37e3
Area, Height
7.37
416
4372
Time

Molecule	Structure	Molecular formula	Molecular Weight (amu)
Bisphenol A ß-D-Glucuronide		$C_{21}H_{24}O_8$	404.41
Bisphenol A bis-ß-D-Glucuronide		$C_{27}H_{32}O_{14}$	580.53
Bisphenol A monosulfate		$C_{15}H_{16}O_5S$	308.35
Bisphenol A disulfate		$C_{15}H_{16}O_8S_2$	388.41
Bisphenol S ß-D-Glucuronide		$C_{18}H_{18}O_{10}S$	426.39

Molecules	abbreviation	SRM transitions (m/z)	Cone voltage (V)	Collision energy (eV)	Retention time (min)	Fraction and time window (TW)
BPA monoglucuronide	BPA-1G	403>227	52	20	6.77	G- TW2
		403>175	52	15		
		403>113	52	15		
		403>85	52	18		
^{13}C-BPA monoglucuronide	^{13}C-BPA-1G	415>175	52	20	6.77	G- TW2
		415>113	52	15		
BPA diglucuronide	BPA-2G	579>403	40	20	4.68	G- TW1
		579>175	40	20		
		579>113	40	20		
BPS monoglucuronide	BPS-1G	425>249	40	25	3.61	G- TW1
		425>175	40	15		
		425>113	40	15		
BPA monosulfate	BPA-1S	307>227	40	25	7.36	S- TW1
		307>212	40	30		
		307>133	40	40		
BPA monosulfate-d_6	BPA-d_6-1S	313>233	40	25	7.32	S- TW1
		313>138	40	40		
		313>215	40	30		
BPA disulfate	BPA-2S	445>307	40	20	5.00	S- TW1
		387>307	40	20		
		307>227	40	25		

	Limits of detection - LOD (µg kg^{-1})				
	BPA-1G	**BPA-1S**	**BPS-1G**	**BPA-2G**	**BPA-2S**
Liver	0.04	0.02	0.10	0.02	0.10
Muscle	0.02	0.09	0.04	0.12	0.50
Fish	0.10	0.10	0.18	0.05	0.25