RESEARCH PAPER

# Determination of bisphenol A and related substitutes/analogues in human breast milk using gas chromatography-tandem mass spectrometry

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**Abstract** Bisphenol A (BPA) is an industrial chemical widely used in the production of polycarbonate and epoxy resins. Identified as an endocrine-disrupting chemical (EDC), BPA is a matter of existing or ongoing restrictive regulations and then is increasingly being replaced by other analogues used as BPA's substitutes. Human biomonitoring studies focusing on both BPA and emerging related analogues consequently appear as a requirement either for documenting the efficiency of regulatory actions toward BPA and for fuelling incoming risk assessment studies toward BPA's substitutes. In particular, the increasing concern about the late effects consecutive to early exposures naturally identify human breast milk as a target biological matrix of interest for priority exposure assessment focused on critical sub-populations such as pregnant women, fetuses, and/or newborns. In this context, an accurate and sensitive analytical method based on gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) was developed for the quantification of 18 "BPA-like" compounds in breast milk samples at trace levels (<0.05 µg kg$^{-1}$). The method includes a preliminary protein precipitation step followed by two successive solid-phase extraction (SPE) stages. Quantification of the targeted compounds was achieved according to the isotopic dilution method using $^{13}C_{12}$-BPA as internal standard. The method was validated according to current EU guidelines and criteria. Linearity ($R^2$) was better than 0.99 for each molecule within the concentration range 0–5 µg kg$^{-1}$. The detection and quantification limits ranged from 0.001 to 0.030 µg kg$^{-1}$ and from 0.002 to 0.050 µg kg$^{-1}$, respectively. The analytical method was successfully applied to the first set of human breast milk samples ($n=30$) originating from French women in the Region Pays-de-la-Loire. The measured levels of BPA were found in the <LOQ–1.16 µg kg$^{-1}$ range. BPS was detected in only one sample at 0.23 µg kg$^{-1}$, while the other targeted molecules were not detected. The proposed methodology then appeared suitable for the further monitoring of a potential decrease of BPA levels and an increase of other BPA analogue levels as reflective of the expected incoming trend in terms of human exposure.

**Keywords** Bisphenol A · BPA substitutes · Biomonitoring · Human exposure · Breast milk

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## Introduction

Bisphenol A (BPA, CAS Registry number 80-05-7) is an industrial chemical widely used in the production of polycarbonate and epoxy resins which have a large panel of applications including plastic food containers and epoxy food-can coatings. Currently, BPA is authorized as food contact materials (FCMs) within the EU (Commission Regulation No 10/

2011 [1]) in plastic materials and articles intended to come into contact with food. Therefore, foodstuffs and beverages may be in contact with BPA which can migrate from FCMs to foodstuffs [2, 3]. The European Food Safety Authority (EFSA) has set a regulatory specific migration limit at 600 µg kg$^{-1}$ or 100 µg dm$^{-2}$, as well as an acceptable daily intake of 0.005 mg kg$^{-1}$ body weight/day [1]. In January 2011, the prohibition of the use of BPA in the manufacture of polycarbonate infant-feeding bottles has been adopted within the EU [4], and on December 2012, a French law was also adopted for the suspension of the manufacture, import, export, and placing on the market of any food packaging containing BPA [5]. This prohibition will become effective on January 2015. These restrictive regulatory dispositions imply the substitution of BPA in food contact materials, which are considered as the main source of population exposure. Among the potential alternatives to BPA, other bisphenol-related analogues have been identified, some of them being already used for certain applications, e.g., in thermal paper. BPS and bisphenol F (BPF) are also used as monomer in the production of epoxy resins used as food contact material. While no legislation has been yet implemented regarding most of these BPA substitutes, a specific migration limit (SML) of 0.05 mg kg$^{-1}$ has been established for bisphenol S due to its chemical properties (more heat stable and photoresistant than BPA) [1]. In parallel, the toxicity and endocrine-disrupting activity of bisphenol S (BPS), bisphenol B (BPB), BPF, bisphenol AF (BPAF), or bisphenol E (BPE) have been studied and highlighted [6, 7].

In this context, the knowledge of both external and internal human exposure levels to BPA and other related analogues appears as a requirement either for documenting the efficiency of regulatory actions toward BPA and for fuelling incoming risk assessment studies toward BPA's substitutes. In particular, the increasing concern about the late effects consecutive to early exposures naturally identifies sensitive populations such as pregnant women and fetuses/newborns as a priority for biomonitoring studies. Breast milk then appears as a relevant biological material for conducting such studies, giving access both to an estimated internal exposure level of the mothers and their fetus and to an estimated food exposure level of the breastfed newborn. This last issue also refers to several studies having underlined the lack of metabolic enzymatic system able to conjugate BPA in newborns [8].

The determination of these targeted molecules in foodstuffs or biological matrices requires the development and the implementation of appropriate and consistent analytical methods. Few authors reported methodologies dedicated to the quantification of BPA-related compounds in biological human matrices. Data have been published regarding for instance the determination of bisphenol AF in both serum and urine of rats [9] or BPS, BPF, and BPB in human urine [10–13]. Several authors have also reported different analytical methods for the determination of BPA in breast milk [14–23]. Kuruto-Niwa et al. [14] and Migeot et al. [22] reported data for the determination of BPA in colostrum samples. Sun et al. [15] and Yi et al. [16] described two HPLC-FLD methods with detection limits of 0.11 and 1.8 ng mL$^{-1}$, respectively. Ye et al. reported an automated on-line column-switching LC-MS/MS method with a limit of detection (LOD) below 1 ng mL$^{-1}$ from a 100-µL test sample [17]. This analytical method has been widely used for the determination of BPA in different biological matrices in the framework of various cohort population studies. Samanidou et al. [20] and Otaka et al. [21] reported analytical methods based on MSPD and alkaline digestion, respectively, for the determination of BPA in breast milk samples. Cariot et al. also reported an on-line SPE-ultra high-performance liquid chromatography (UHPLC)-MS/MS method from a 500-µL test sample with a limit of quantification (LOQ) of 0.4 ng mL$^{-1}$ [18]. Zimmers et al. described an analytical method based on a solid-phase extraction step and LC-MS/MS for the determination of BPA in a breast milk sample with an associated LOD of 0.22 ng mL$^{-1}$ [19]. Rodríguez-Gómez reported two GC-MS/MS and UHPLC-MS/MS methods for the determination of endocrine-disrupting chemicals in human breast milk after stir-bar extraction, with a corresponding BPA LOD of 0.1 ng mL$^{-1}$ [23]. Now all these studies have considered a unique or limited number of targeted substances while a wide panel of BPA analogues/substitutes have already been identified by risk assessment agencies. Moreover, a relatively high percentage of reported undetected values at least in some of these studies may indicate a need for lower detection limits for better compatibility with the circulating concentration levels of BPA and analogues in humans. In this context, the aim of this study consisted in the development of an accurate and sensitive analytical method based on GC-MS/MS for the determination of BPA and a wide set of BPA analogues/substitutes in human breast milk, namely bisphenols A, B, C, E, F, M, P, S, Z, AP, AF, BP, Cl2, FL, and PH, DHDPE, biphenyl 4,4′-diol, and bis-2(hydroxyphenyl)methane. The method was then applied to a preliminary set of 30 breast milk samples collected from French mothers in the Region Pays-de-la-Loire (France).

## Material and methods

### Standards and reagents

Bisphenol A [2,2-bis(4-hydroxyphenyl)propane, BPA, CAS number 80-05-7] and $^{13}C_{12}$-BPA used as internal standard were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Bisphenol B [2,2-bis(4-hydroxyphenyl)butane, BPB, CAS number 77-40-7], bisphenol AP [1,1-bis(4-hydroxyphenyl)-1-phenyl-ethane,

BPAP, CAS number 1571-75-1], bisphenol AF [2,2-bis(4-hydroxyphenyl)hexafluoropropane, BPAF, CAS number 1478-61-1], bisphenol BP [bis-(4-hydroxyphenyl)diphenylmethane, BPBP, CAS number 1844-01-5], bisphenol C [2,2-bis(3-methyl-4-hydroxyphenyl)propane, BPC, CAS number 79-97-0], bisphenol Cl2 [bis(4-hydroxyphenyl)-2,2-dichlorethylene, BPCl2, CAS number 14868-03-2], bisphenol E [1,1-bis(4-hydroxyphenyl)ethane, BPE, CAS number 2081-08-5], bisphenol PH [5,5′-(1-methylethyliden)-bis[1,1′-(bisphenyl)-2-ol]propane, BPPH, CAS number 24038-68-4], bisphenol S [bis(4-hydroxyphenyl)sulfone, BPS, CAS number 80-09-1], bisphenol F [bis(4-hydroxydiphenyl)methane, BPF, CAS number 1333-16-0], DHDPE [4,4′-dihydroxydiphenyl ether, CAS number 1965-09-9], bisphenol FL [9,9′-bis(4-hydroxyphenyl)fluorene, BPFL, CAS number 3236-71-3], bisphenol Z [1,1-bis(4-hydroxyphenyl)-cyclohexane, BPZ, CAS number 843-55-0], biphenyl-4,4′-diol [BP4,4′, CAS number 92-88-6], bisphenol M [1,3-bis(2-(4-hydroxyphenyl)-2-propyl)benzene, BPM, CAS number 13595-25-0], bisphenol P [1,4-bis(2-(4-hydroxyphenyl)-2-propyl)benzene, BPP, CAS number 2167-51-3], bis-2(hydroxyphenyl)methane [BIS2, CAS number 2467-09-9], and biphenyl-2,2′-diol [BP2,2′, CAS number 1806-29-7] used as external standard were purchased from Sigma-Aldrich Inc. (St. Louis, USA). The chemical structures and monoisotopic masses of these molecules are shown in Table 1. Stock standard solutions of both $^{13}C_{12}$-BPA and native bisphenols were prepared in acetonitrile at a concentration of 5 and 100 ng $μL^{-1}$, respectively. Working solutions were obtained by an appropriate dilution in acetonitrile. All standard solutions were stored at 4 °C, in the dark.

Acetonitrile and methanol (HPLC gradient-grade quality), acetone, cyclohexane, and formic acid were obtained from Promochem (Wesel, Germany). Deionized water was purchased from Panreac (Castellar del Vallès, Spain). β-Glucuronidase/aryl-sulfatase solution was obtained from Merck (Darmstadt, Germany). Solid-phase extraction columns, i.e., CHROMABOND HR-X and AFFINIMIP BPA, were obtained respectively from MACHEREY-NAGEL (Hoerdt, France) and POLYINTELL (Val de Reuil, France). The derivatization reagent (N-methyl-N(trimethylsilyl)-trifluoroacetamide—MSTFA) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

## Quality assurance/quality control procedures

As for other ubiquitary substances, the measurement of BPA is imposing a significant challenge in terms of characterization, control, and management of residual background contamination, i.e., external to the sample to be analyzed and present in procedural blank samples, as already discussed by Deceuninck et al. [24] and Ye et al. [25]. In particular, all

solvents and consumables used have been previously tested for all the targeted monitored compounds, as well as containers used for breast milk sample collection. Moreover, glassware used in the extraction procedure was previously heated for 12 h at 500 °C. Six procedural blank samples were systematically included in each series of analysis, and the corresponding BPA's signal average and relative standard deviation (RSD) were calculated. Then, a maximal acceptable cutoff value was determined for blank samples. The corresponding background contamination level was then systematically subtracted from the primary BPA concentration determined in the analyzed samples. A quality control chart was also implemented in order to control the background contamination during the time.

## Sample preparation

An analytical method developed for the determination of BPA in a large set of food items has been already described by Deceuninck et al. [24], which represented the first basis for the present method development dedicated to biological matrices. Nevertheless, some adaptations of this initial protocol have been implemented in order (1) to extend the range of targeted substances and to include the other BPA's analogues and (2) to take into account the specificities associated with the breast milk matrix (Fig. 1).

Briefly, 3 g of breast milk was weighted in a glass tube, and 30 μL of the surrogate $^{13}C_{12}$-BPA solution at 0.5 ng $μL^{-1}$ was added (internal standard for quantification according to the isotopic dilution method). An enzymatic hydrolysis was carried out at 50 °C, during 4 h, by adding 20 μL of a β-glucuronidase/aryl-sulfatase mixture, extracted from *Helix pomatia*, in order to finally quantify the total forms of the monitored compounds (i.e., free+conjugated phase II metabolites). Afterwards, a double protein precipitation was performed by adding 5 and 3 mL of acetone. After evaporation of the organic layer, two successive solid-phase extraction steps were performed. The first SPE was carried out using porous adsorptive resin based on polystyrene-divinylbenzene stationary phase cartridge (HR-X) previously activated successively with 20 mL methanol and 10 mL water. After loading the sample, the column was washed successively with 4 mL water, 8 mL water/methanol (90:10, v/v), and 4 mL water/methanol (40:60, v/v), before eluting the targeted molecules with 14 mL acetonitrile. The second specific SPE was based on a molecularly imprinted polymers (MIP) stationary phase conditioned successively with 10 mL methanol/formic acid (100:2, v/v), 4 mL acetonitrile, and 4 mL water. After loading the extract, the column was rinsed with 5 mL water, 3 mL water/acetonitrile (60:40, v/v), and 2.5 mL acetonitrile. The compounds of interest were eluted using 10 mL methanol. The extracts were evaporated to dryness under a gentle nitrogen stream (45 °C), reconstituted in 100 μL of acetonitrile, and

**Table 1**  Chemical structures and monoisotopic masses of the 18 targeted bisphenol compounds

| Molecule | Abbreviation | CAS Number | Structure | Monoisotopic mass (amu) |
|---|---|---|---|---|
| **Bisphenol A** *2,2-Bis(4-hydroxyphenyl)propane* | BPA | 80-05-7 |  | 228.2863 |
| **Bisphenol B** *2,2-Bis(4-hydroxyphenyl)butane* | BPB | 77-40-7 |  | 242.3188 |
| **Bisphenol AP** *1,1-Bis(4-hydroxyphenyl)-1-phenyl-ethane* | BPAP | 1571-75-1 |  | 290.3629 |
| **Bisphenol AF** *2,2-Bis(4-hydroxyphenyl)hexafluoropropane* | BPAF | 1478-61-1 |  | 336.2343 |
| **Bisphenol BP** *Bis-(4-hydroxyphenyl)diphenylmethane* | BPBP | 1844-01-5 |  | 352.4341 |
| **Bisphenol C** *2,2-Bis(3-methyl-4-hydroxyphenyl)propane* | BPC | 79-97-0 |  | 256.3458 |
| **Bisphenol Cl2** *Bis(4-hydroxyphenyl)-2,2-dichlorethylene* | BPCl2 | 14868-03-2 |  | 281.1384 |
| **Bisphenol E** *1,1-Bis(4-hydroxyphenyl)ethane* | BPE | 2081-08-5 |  | 214.2648 |
| **Bisphenol PH** *5,5'-(1-Methylethyliden)-bis[1,1'-(bisphenyl)-2-ol]propane* | BPPH | 24038-68-4 |  | 380.4880 |
| **Bisphenol S** *Bis(4-hydroxyphenyl)sulfone* | BPS | 80-09-1 |  | 250.2714 |
| **Bisphenol F** *Bis(4-hydroxydiphenyl)methane* | BPF | 1333-16-0 |  | 200.2332 |
| **DHDPE** *4,4'-Dihydroxydiphenyl ether* | DHDPE | 1965-09-9 |  | 202.2060 |
| **Bisphenol FL** *9,9'-Bis(4-hydroxyphenyl)fluorene* | BPFL | 3236-71-3 |  | 350.4181 |
| **Bisphenol Z** *1,1-Bis(4-hydroxyphenyl)-cyclohexane* | BPZ | 843-55-0 |  | 268.3568 |
| **Biphenyl-4,4'-diol** | BP4,4' | 92-88-6 |  | 186.2066 |
| **Bisphenol M** *1,3-Bis(2-(4-hydroxyphenyl)-2-propyl)benzene* | BPM | 13595-25-0 |  | 346.4709 |
| **Bisphenol P** *1,4-Bis(2-(4-hydroxyphenyl)-2-propyl)benzene* | BPP | 2167-51-3 |  | 346.4709 |
| **Bis-2(hydroxyphenyl)methane** | BIS2 | 2467-09-9 |  | 200.2332 |
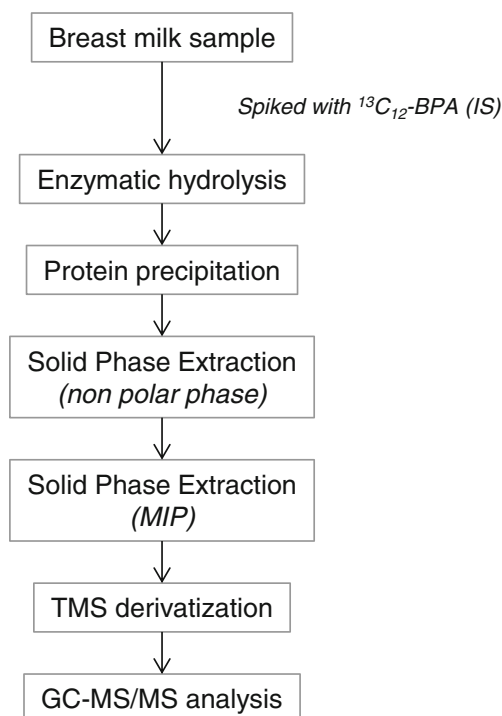
**Fig. 1** Standard operating procedure for the determination of BPA and substitutes/analogues in human breast milk samples

transferred into an injection vial. Finally, the derivatization step for GC-MS/MS analysis was achieved by adding 20 μL of N-methyl-N(trimethylsilyl)-trifluoroacetamide (MSTFA) reagent into the vial and heated for about 30 min at 45 °C.

## GC-MS/MS measurement

An Agilent 7890 gas chromatograph coupled to a triple quadrupole mass analyzer Agilent 7000 (Agilent Technologies, Santa Clara, USA) was used for both identification and quantification of the targeted analytes. The chromatographic separation was achieved using an Optima$^®$-17-MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (MACHEREY-NAGEL, Hoerdt, France). The temperature program was set as follows: 120 °C (2 min), 16 °C min$^{-1}$ until 300 °C (12 min), and 5 °C min$^{-1}$ until 320 °C (5 min). Injector and transfer line temperatures were set at 280 and 320 °C, respectively. Source temperature was set at 250 °C. Electron energy was set at 70 eV. Helium (Alpha 2 purity grade) was used as carrier gas at 1 mL min$^{-1}$. Argon was used as collision gas at 1.5 mL min$^{-1}$. Injections were performed using a 4-mm-i.d. glass liner containing glass wool, operating in the pulsed splitless mode set as follows: initial temperature 300 °C, initial pressure 40 psi, purge flow 60 mL min$^{-1}$, and purge time 1.5 min. The injection volume was 2 μL. Two transitions per molecule were monitored for both bisphenols (BP$_X$) and the corresponding internal standard: $^{13}C_{12}$-BPA (see Table 2). BPx quantification was performed on the most intense

diagnostic signal ("SRM transition 1") and the corresponding signal of $^{13}C_{12}$-BPA (369.2>197.2). The second recorded diagnostic signal (noted as "SRM transition 2") was used for identification purpose. Additionally, GC-MS-MS stability was checked throughout the series of injections according to SRM transition 1 of the most intense diagnostic signal of biphenyl-2,2′-diol (330.2>315.2). The dwell time was set at 20 ms allowing from 15 to 20 points per peak. The MS1 quadrupole DC (direct current) and MS2 quadrupole DC were set respectively at 12.2 and 1.2 V. Data acquisition and data processing were performed using the version B04.00 Mass Hunter constructor software (Agilent Technologies, Santa Clara, USA).

## Validation procedure and method performances

A full validation procedure of the developed method was performed, based on the current European Commission requirements (2002/657/EC decision). Background contamination, repeatability, linearity, within reproducibility, ruggedness, as well as both detection and quantification limits were evaluated. Specificity was evaluated by checking the absence of interfering compounds appearing on the diagnostic signals of the targeted substances, in the range of their expected retention time in breast milk samples. Ruggedness was determined with regard to various factors of variability such as fat milk content, levels of supplementation, operators, as well as different reference standards, solvents, and material supplies. Linearity was first evaluated for external calibration curves using standard solutions at eight increasing concentration levels (namely 0, 0.03, 0.15, 0.3, 0.75, 1.5, 3, and 15 ng of the different analytes on-column). The obtained data permitted to confirm the suitability (mimetic properties) of the surrogate $^{13}C_{12}$-BPA internal standards used for quantifying the different targeted molecules, then to determine the relative response factors (RRF) further used in this quantification process. Secondly, the linearity for extracted calibration curves (i.e., on real breast milk spiked samples) was evaluated within the 0–5 μg kg$^{-1}$ concentration range. The quality of the obtained linear regressions was assessed through their related coefficient of determination ($R^2$). Limits of detection (LOD) and quantification (LOQ) were determined on the basis of fortified samples (0.01 and 0.05 μg kg$^{-1}$), from which the concentration levels leading to observed S/N ratios of S/N=3 (LOD) and S/N=9 (LOQ) were estimated, respectively.

## Breast milk samples

Breast milk samples (n=30) used for both development/ validation and generation of preliminary exposure data were collected in the frame of a French regional research project globally aiming to investigate the link between perinatal nutrition and metabolic programming. One aspect of this project

**Table 2** MS/MS parameters for each molecule of interest

| Molecule | SRM transition 1 (T1) | Collision energy T1 (eV) | SRM transition 2 (T2) | Collision energy T2 (eV) | Retention time (min) |
|---|---|---|---|---|---|
| $^{13}C_{12}$-Bisphenol A (internal standard) | 369.2>197.2 | 20 | 384.2>369.2 | 12 | 11.12 |
| Biphenyl-2,2′-diol (external standard) | 330.2>315.2 | 12 | | | 7.79 |
| Bisphenol A | 357.2>191.2 | 20 | 372.2>357.2 | 12 | 11.12 |
| Bisphenol B | 386.2>357.2 | 10 | 371.3>221.3 | 12 | 11.53 |
| Bisphenol AP | 434.2>419.3 | 10 | 419.3>341.2 | 20 | 14.09 |
| Bisphenol AF | 480.3>411.2 | 15 | 465.3>315.1 | 12 | 9.06 |
| Bisphenol BP | 496.4>419.2 | 12 | 419.2>253.2 | 20 | 17.68 |
| Bisphenol C | 385.3>205.2 | 20 | 400.4>385.3 | 12 | 11.41 |
| Bisphenol C2 | 426.2>261.1 | 25 | 424.2>409.1 | 15 | 12.59 |
| Bisphenol E | 343.2>193.2 | 12 | 358.3>343.2 | 15 | 11.05 |
| Bisphenol PH | 509.4>267.2 | 25 | 524.4>509.4 | 15 | 17.92 |
| Bisphenol S | 394.2>229.2 | 20 | 394.2>379.2 | 12 | 14.53 |
| Bisphenol F | 344.2>179.2 | 15 | 329.3>179.2 | 12 | 10.92 |
| DHDPE | 346.2>181.2 | 15 | 331.2>181.2 | 10 | 10.78 |
| Bisphenol FL | 494.4>329.2 | 25 | 494.4>313.1 | 25 | 21.32 |
| Bisphenol Z | 412.3>369.2 | 12 | 412.3>203.2 | 25 | 13.39 |
| Biphenyl-4,4′-diol | 330.2>315.2 | 12 | 330.2>299.2 | 25 | 10.92 |
| Bisphenol M | 490.4>475.4 | 15 | 475.4>445.3 | 30 | 14.83 |
| Bisphenol P | 490.4>475.4 | 15 | 475.4>445.3 | 30 | 16.83 |
| Bis-2(hydroxyphenyl)methane | 344.2>329.2 | 10 | 344.2>241.2 | 12 | 9.41 |

was indeed dedicated to the characterization of breast milk with regard to the presence (quantitative levels and qualitative profiles) of environmental chemical contaminants as a possible contributor to the studied biological endpoints (metabolic syndrome, prematurity, etc.) in addition to other genetic or nutritional factors. These breast milk samples were originating from the Nantes Human Milk Biobank (LACTATHEQUE DC-2009-0982), declared and approved by the local ethics committee on 24th June 2010. For each donation, mothers were asked to complete a medical questionnaire integrating biobank consent. All the milk samples were only mature milk when lactation is well established. All samples were stored at −20 °C until analysis.

## Results and discussion

### GC-MS/MS measurement

Chromatographic conditions were optimized in order to obtain an efficient separation between all the targeted molecules. The final acquisition method was then sequenced in eight different time windows as illustrated in Fig. 2 presenting the total ion chromatogram (TIC) obtained for a breast milk sample spiked with each targeted molecule at 1 µg kg$^{-1}$ (ppb). The covered range of compounds lead to a significant chromatographic

distance between the first and the last eluted molecule, i.e., biphenyl-2,2′-diol and bisphenol FL, observed at a retention time ($t_R$) of 7.79 and 21.32 min, respectively. A good chromatographic resolution ($R>1$) was obtained for the main molecules of interest except for two of them, namely bisphenol F and biphenyl-4,4′-diol both observed at $t_R=10.92$ min. Nevertheless, these two compounds could be unambiguously identified according to their respective specific diagnostic signals (344.2>179.2 for BPF and 330.2>315.2 for biphenyl-4, 4′-diol). All optimized SRM are reported in Table 2. On the same way, two transitions were also monitored for both internal ($^{13}C_{12}$-BPA) and recovery (biphenyl-2,2′-diol) standards used respectively for quantification and recovery determination purposes. While significantly different response factors were observed between the targeted compounds (lower sensitivity especially for bisphenol FL and bisphenol C), the unambiguous detection and identification of all molecules was achieved at trace level (<1 µg L$^{-1}$).

### Sample preparation

The standard operating procedure previously developed and validated by Deceuninck et al. [24] for the determination of BPA in foodstuffs was adapted and optimized for the determination of the other targeted BPA analogues in human breast milk samples. Moreover, contrary to the previous procedure, this analytical method was developed
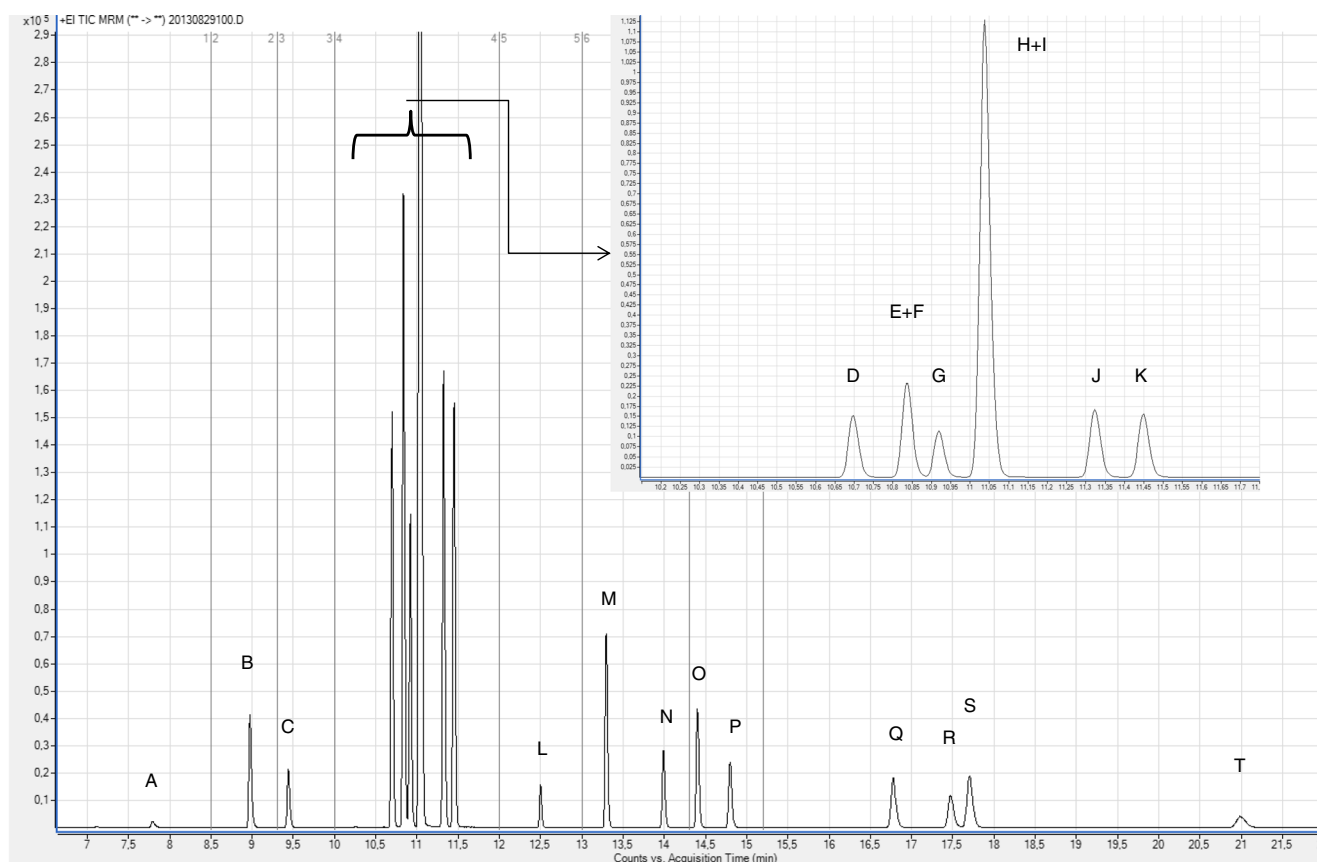
**Fig. 2** GC-MS/MS total ion chromatogram of human breast milk fortified with 1 μg kg$^{-1}$ of each molecule: *A* biphenyl 2,2′-diol, *B* bis(2-hydroxyphenyl)methane, *C* bisphenol AF, *D* DHDPE, *E* bisphenol F, *F* biphenyl 4,4′-diol, *G* bisphenol E, *H* $^{13}C_{12}$-bisphenol A, *I* bisphenol A, *J* bisphenol C, *K* bisphenol B, *L* bisphenol Cl2, *M* bisphenol Z, *N* bisphenol S, *O* bisphenol AP, *P* bisphenol M, *Q* bisphenol P, *R* bisphenol BP, *S* bisphenol PH, *T* bisphenol FL

in order to analyze either free bisphenols (active form) or total (after enzymatic deconjugation of glucuronide and sulfate phase II metabolites) bisphenol concentrations. This last option is achieved by using β-glucuronidase and aryl-sulfatase from *H. pomatia* (37 °C overnight). These experimental conditions allow the hydrolysis of more than 98 % of both monoglucuronide-BPA and monosulfate-BPA (data not shown). Furthermore, an additional protein precipitation step was used to enhance the efficiency of the analytical method, i.e., sample treatment facility. Finally, additional minor changes have been made to the initially developed protocol, especially regarding both solid-phase extractions insofar as the volumes of cleaning and elution have been optimized in the field of the determination of the 18 bisphenols of interest. Regarding MIP SPE, cross-reactivity was observed for all tested bisphenol compounds insofar as the column affinity is based on the shape of the bisphenol analogue and also with strong binding site interaction inside the cavity for phenolic moieties. Figure 2 illustrates the resulting total ion chromatogram obtained for the 18 monitored bisphenols in typical human breast milk samples from a reduced sample volume (3 mL).

## Background contamination

Six procedural blank samples were systematically included in each batch of analyses. The corresponding measured level (average) and associated variability (RSD) of such external contamination were then reported in a control chart. The different contamination sources that have been identified and described in a previous paper [24] were a matter of reinforced attention in the present study. Finally, an expected residual background contamination has been observed for BPA; therefore, a maximal acceptable cutoff value in the blank sample was fixed to the equivalent of 0.10 μg kg$^{-1}$. A background determination in the blank above this concentration automatically generated the rejection of the corresponding series of samples. No similar external contamination has been detected for the 17 other compounds at a concentration level higher than 0.01 μg kg$^{-1}$. As an illustrating example, typical diagnostic chromatograms of bisphenol B from a pool of three human breast milk samples fortified with 0, 0.01, 0.05, 0.10, and 0.25 μg kg$^{-1}$ are shown in Fig. 3. The most sensitive signal of BPB (386.2>357.2) is reported

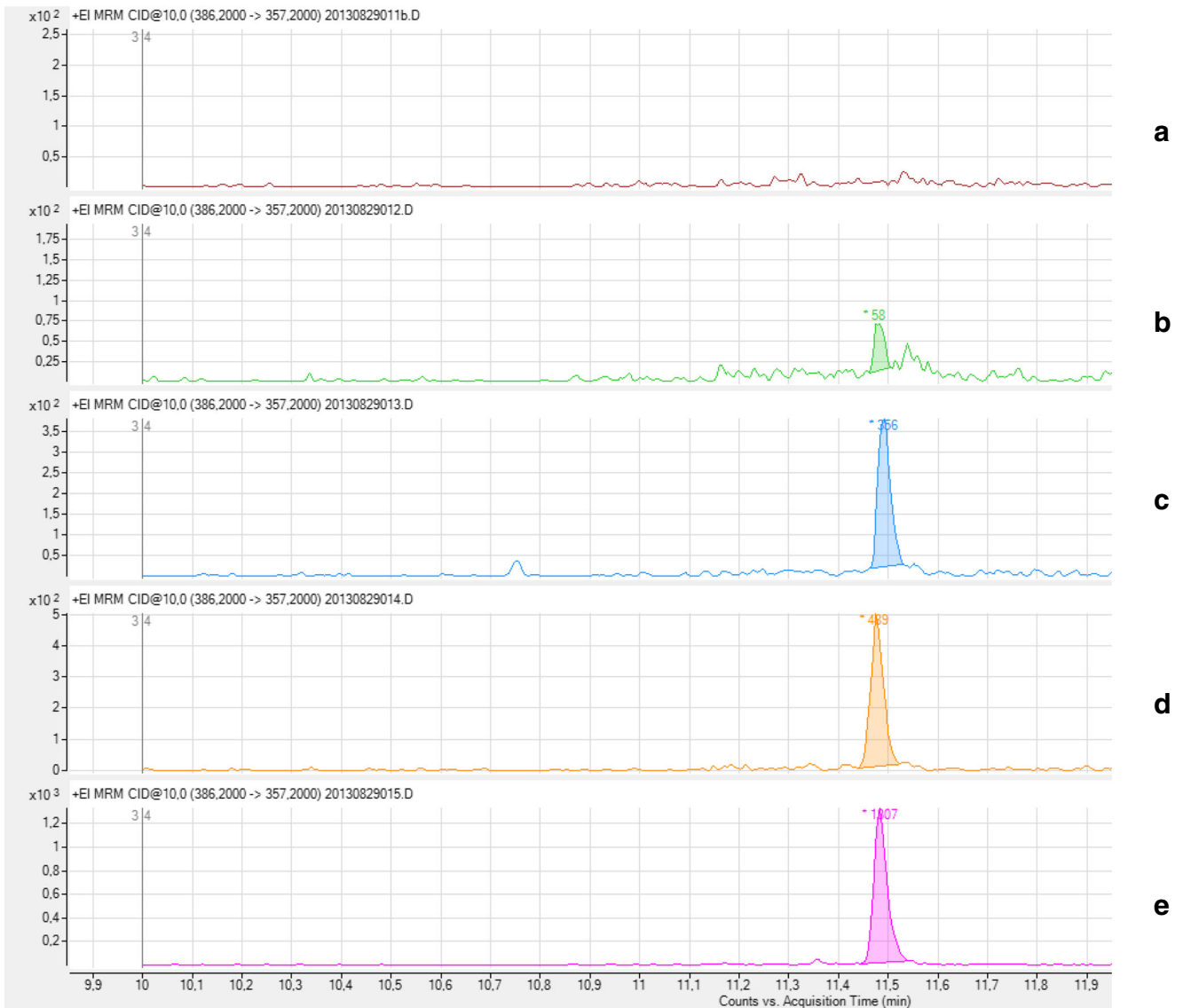**Fig. 3** Pool of three breast milk samples not fortified (**a**) and fortified with 0.01 µg kg$^{-1}$ (**b**), 0.05 µg kg$^{-1}$ (**c**), 0.10 µg kg$^{-1}$ (**d**), and 0.25 µg kg$^{-1}$ (**e**)

for each spiked sample in a fixed *y*-axis. No signal was observed for the non-fortified sample, indicating the absence of procedural contamination. While a *S/N*<3 is observed at 0.01 µg kg$^{-1}$ spiked sample with BPB (close to the LOD), significant signals (*S/N*>3) were detected for the corresponding fortifications at 0.05, 0.10, and 0.25 µg kg$^{-1}$. In conclusion, the cutoff value was only used for BPA determination, the lowest reported point for the 16 other compounds being the LOD/LOQ levels.

On the basis of *n*=12 measurements from blank samples, the mean residual background contamination level for BPA has been calculated at 0.05 µg kg$^{-1}$±0.01 µg kg$^{-1}$. If 3 mL of human breast milk sample aliquots constituted the maximum available volume in the present case, it is noteworthy that a

larger volume would permit to minimize again the eventual influence of this residual background.

Analytical performances

*Stability of analytical standards*

The stability of standard solutions was identified as a possible factor that could influence the results. Therefore, the stability of the 20 targeted analytical standards (both internal and external standards included) was checked by preparing and testing the stock solutions at the beginning and at the end of the work. The resulting variations of stability for all the compounds of interest were determined below 5 %.

## Linearity

The linearity of the developed method was determined for both the external standard and extracted spiked milk sample (pool of five individual samples) calibration curves and on the basis of eight concentration levels in the range 0–5 µg kg$^{-1}$, with the majority of values ($n=6$) in the range 0–1 µg kg$^{-1}$. The intercept was not forced through the origin due to the possible presence of the targeted molecule in the non-fortified sample (i.e., residual background contamination for BPA or presence in the initial pool of breast milk samples for BPA and other analogues). An excellent linearity was observed as the resulting coefficients of determination ($R^2$) were found higher than 0.998 with residuals below 20 % on the relative response factors (RRF) as indicated in Table 3. For each quantified analyte, namely BPA and BPS, both the calibration curves (i.e., external and internal calibration) were found mimetic, with equivalent slope. Therefore, sample quantification was carried out using the standard calibration curve. Within-laboratory reproducibility was also determined for BPA at two levels of concentrations (+0.10 and + 1.0 µg kg$^{-1}$). The corresponding RSD ranged from 13 to 20 % for the fortification levels of 1.0 and 0.10 µg kg$^{-1}$, respectively.

## Detection and quantification limits

The limit of detection (LOD) was classically estimated as the concentration from which a significant signal to noise ratio ($S/N=3$) is obtained. It has been determined for the 17 BPA analogue molecules from a pool of six individual breast milk samples. Resulting LODs ranged from 0.001 µg kg$^{-1}$ for bisphenol AF, bisphenol Cl$_2$, and bisphenol S to 0.030 µg kg$^{-1}$ for both DHDPE and bis-2(hydroxyphenyl)methane. The limit of quantification (LOQ) was calculated on the basis of ten times the ratio $S/N$, resulting in calculated values ranging from 0.003 to 0.100 µg kg$^{-1}$ as reported in Table 3. The obtained detection and quantification limits were very satisfactory for all the targeted molecules, taking into account both LOD and LOQ already reported in literature.

## Recovery

The recovery of the method was assessed by adding known amounts of each targeted molecule in the range 0.1–10 ng in human breast milk samples. Recoveries were determined by analyzing the fortified samples, and the concentration of each compound of interest

**Table 3** Validation parameters: linearity (comparison of both single isotopic dilution and standard addition method), detection, and quantification limits

| Molecule | Abbreviation | Linearity (calibration curve) | | | Linearity (spiked sample method) | | | Detection limit (LOD) (µg kg$^{-1}$) | Quantification limit (LOQ) (µg kg$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | | $R^2$ | Slope ($a$), µg kg$^{-1}$ | Intercept ($b$) | $R^2$ | Slope ($a$) µg kg$^{-1}$ | Intercept ($b$) | | |
| Bisphenol A | BPA | 0.9987 | 0.2179 | 0.0060 | 0.9956 | 0.2346 | 0.0568 | <0.0030 | <0.010 |
| Bisphenol B | BPB | 0.9992 | 0.0274 | 0.0001 | 0.9995 | 0.0227 | 0.0004 | 0.006 | 0.020 |
| Bisphenol AP | BPAP | 0.9994 | 0.0319 | 0.0004 | 0.9999 | 0.0233 | 0.0000 | 0.002 | 0.007 |
| Bisphenol AF | BPAF | 0.9994 | 0.0731 | 0.0013 | 0.9979 | 0.0413 | 0.0000 | 0.001 | 0.003 |
| Bisphenol BP | BPBP | 0.9996 | 0.0209 | 0.0000 | 0.9984 | 0.0093 | 0.0000 | 0.002 | 0.006 |
| Bisphenol C | BPC | 0.9991 | 0.2260 | 0.0021 | 0.9991 | 0.2065 | 0.0000 | 0.003 | 0.009 |
| Bisphenol Cl2 | BPC2 | 0.9994 | 0.0169 | 0.0000 | 0.9977 | 0.0140 | 0.0000 | 0.001 | 0.003 |
| Bisphenol E | BPE | 0.9991 | 0.1591 | 0.0020 | 0.9994 | 0.1904 | 0.0077 | 0.006 | 0.018 |
| Bisphenol PH | BPPH | 0.9997 | 0.0282 | 0.0000 | 0.9966 | 0.0082 | 0.0000 | 0.003 | 0.009 |
| Bisphenol S | BPS | 0.9993 | 0.0319 | 0.0000 | 0.9997 | 0.0295 | 0.0000 | 0.001 | 0.003 |
| Bisphenol F | BPF | 0.9985 | 0.0805 | 0.0021 | 0.9996 | 0.1064 | 0.0000 | 0.006 | 0.018 |
| DHDPE | DHDPE | 0.9989 | 0.2249 | 0.0051 | 0.9826 | 0.1001 | 0.0000 | 0.030 | 0.100 |
| Bisphenol FL | BPFL | 0.9998 | 0.0081 | 0.0000 | 0.9993 | 0.0040 | 0.0000 | 0.004 | 0.012 |
| Bisphenol Z | BPZ | 0.9998 | 0.0951 | 0.0001 | 0.9995 | 0.0758 | 0.0000 | 0.002 | 0.006 |
| Biphenyl-4,4′-diol | BP4,4′ | 0.9982 | 0.1964 | 0.0060 | 0.9981 | 0.2039 | 0.0000 | 0.005 | 0.015 |
| Bisphenol M | BPM | 0.9994 | 0.0504 | 0.0000 | 0.9980 | 0.0234 | 0.0000 | 0.002 | 0.010 |
| Bisphenol P | BPP | 0.9995 | 0.0287 | 0.0000 | 0.9974 | 0.0103 | 0.0001 | 0.004 | 0.012 |
| Bis-2(hydroxyphenyl)methane | BIS2 | 0.9984 | 0.0455 | 0.0017 | 0.9959 | 0.0153 | 0.0000 | 0.030 | 0.100 |

**Table 4** Recovery determination of targeted compounds in human breast milk

| Molecule/abbreviation | Spiked (ng) | Recovery % |
|---|---|---|
| Bisphenol A | 0.1 | 97 |
| | 1 | 94 |
| | 10 | 105 |
| Bisphenol B | 0.1 | 96 |
| | 1 | 99 |
| | 10 | 102 |
| Bisphenol AP | 0.1 | 100 |
| | 1 | 90 |
| | 10 | 92 |
| Bisphenol AF | 0.1 | 100 |
| | 1 | 96 |
| | 10 | 90 |
| Bisphenol BP | 0.1 | 108 |
| | 1 | 109 |
| | 10 | 99 |
| Bisphenol C | 0.1 | 92 |
| | 1 | 94 |
| | 10 | 97 |
| Bisphenol Cl2 | 0.1 | 102 |
| | 1 | 101 |
| | 10 | 93 |
| Bisphenol E | 0.1 | 96 |
| | 1 | 94 |
| | 10 | 102 |
| Bisphenol PH | 0.1 | 94 |
| | 1 | 93 |
| | 10 | 102 |
| Bisphenol S | 0.1 | 100 |
| | 1 | 99 |
| | 10 | 93 |
| Bisphenol F | 0.1 | 103 |
| | 1 | 109 |
| | 10 | 104 |
| DHDPE | 0.1 | 104 |
| | 1 | 92 |
| | 10 | 100 |
| Bisphenol FL | 0.1 | 103 |
| | 1 | 100 |
| | 10 | 96 |
| Bisphenol Z | 0.1 | 100 |
| | 1 | 97 |
| | 10 | 103 |
| Biphenyl-4,4′-diol | 0.1 | 109 |
| | 1 | 103 |
| | 10 | 104 |
| Bisphenol M | 0.1 | 96 |
| | 1 | 96 |
| | 10 | 94 |

**Table 4** (continued)

| Molecule/abbreviation | Spiked (ng) | Recovery % |
|---|---|---|
| Bisphenol P | 0.1 | 97 |
| | 1 | 92 |
| | 10 | 99 |
| Bis-2(hydroxyphenyl)methane | 0.1 | 108 |
| | 1 | 103 |
| | 10 | 109 |

was assessed by interpolation in the calibration curve within the linear dynamic range and compared with the amount previously added. The recoveries obtained with this method ranged from 90 to 109 % (Table 4) and were highly satisfactory with results close to 100 % for each molecule.

**Table 5** Determination of BPA concentrations in human breast milk samples

| Sample | [BPA] µg kg$^{-1}$ |
|---|---|
| 1 | 0.62 |
| 2 | 0.17 |
| 3 | 0.09 |
| 4 | <LOQ |
| 5 | 0.17 |
| 6 | 0.04 |
| 7 | <LOQ |
| 8 | 0.03 |
| 9 | 0.20 |
| 10 | 0.08 |
| 11 | 0.87 |
| 12 | 0.10 |
| 13 | 0.07 |
| 14 | 0.13 |
| 15 | 0.14 |
| 16 | 0.08 |
| 17 | 0.19 |
| 18 | 0.04 |
| 19 | 0.08 |
| 20 | 0.13 |
| 21 | 0.07 |
| 22 | 0.12 |
| 23 | 1.16 |
| 24 | 0.80 |
| 25 | 0.13 |
| 26 | <LOQ |
| 27 | 0.03 |
| 28 | 1.09 |
| 29 | <LOQ |
| 30 | 0.25 |

Measurement of BPA and analogues in the first set of French human breast milk samples

The developed method was applied to the analysis of 30 human breast milk samples originating from French mothers in Region Pays-de-la-Loire. The 18 targeted compounds were successfully monitored, but BPA was almost the only one identified and quantified in 90 % of these samples. The observed levels of BPA (total forms, i.e., free+deconjugated glucuronides and sulfates forms) were in the range <LOQ–1.16 µg kg$^{-1}$ with mean and median values of 0.23 and 0.11 µg kg$^{-1}$, respectively (Table 5). These concentrations are consistent with those

already reported in literature [20, 23]. A typical SRM diagnostic ion chromatogram obtained for a sample quantified at 0.87 µg kg$^{-1}$ is illustrated in Fig. 4. Both diagnostic signals (357.2>191.2 and 372.2> 357.2), used respectively for quantification and identification purposes, as well as the corresponding signal of internal standard (369.2>197.2) are clearly observed at the expected retention time, illustrating the developed method performances in terms of specificity and sensitivity.

Interestingly, BPS was quantified in only one analyzed breast milk sample as illustrated in Fig. 5, at a concentration of 0.23 µg kg$^{-1}$. Three specific diagnostic



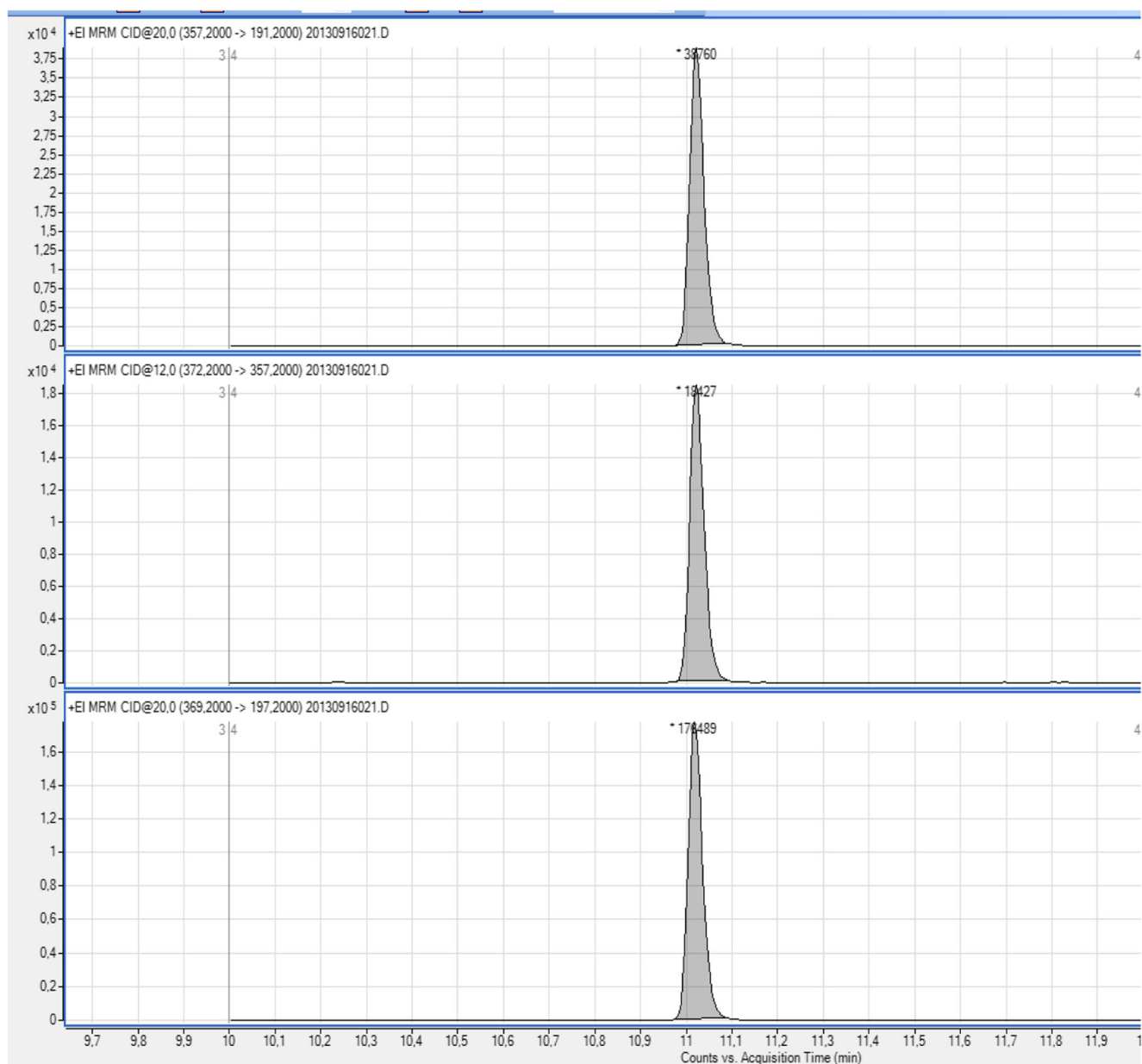**Fig. 4** GC-MS/MS chromatograms (SRM mode) of a human breast milk sample. [BPA] estimated to 0.87 µg kg$^{-1}$: two diagnostic transitions for BPA (357.2>191.2: *top* and 372.2>357.2: *middle*) and one diagnostic transition for $^{13}$C$_{12}$-BPA (369.2>197.2: *bottom*) used as internal standard
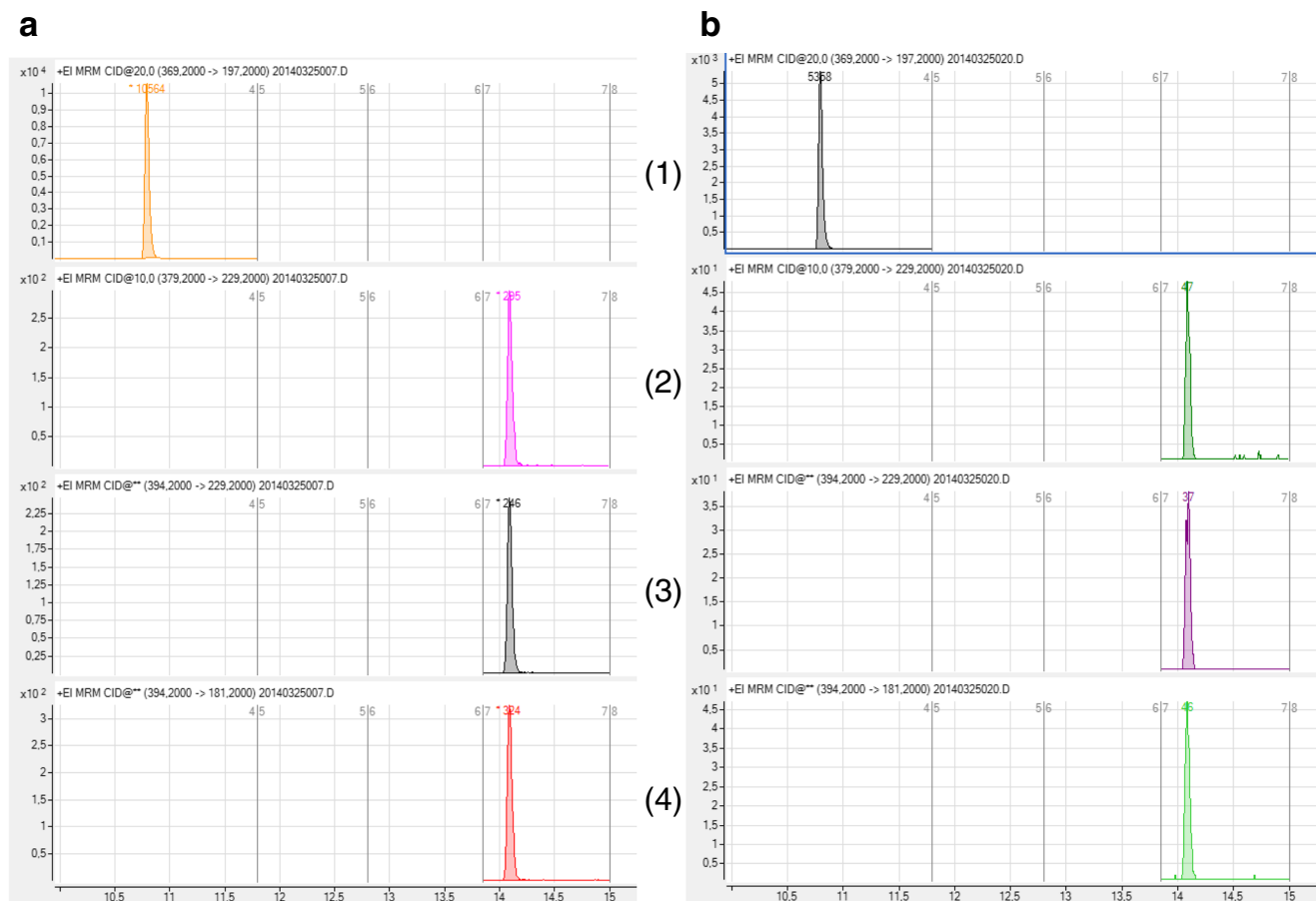
**a**



**b**



Fig. 5 GC-MS/MS chromatograms (SRM mode) of both BPS standard solution (**a**) and a human breast milk sample in which [BPS] has been estimated at 0.23 μg kg$^{-1}$ (**b**): one diagnostic transition for $^{13}$C$_{12}$-BPA

(369.2>197.2: *1*) used as internal standard and three diagnostic transitions for BPS: 379.2>229.2 (*1*), 394.2>229.2 (*3*), and 394.2> 191.2 (*4*)

signals were recorded for this compound (379.2>229.2, 394.2>229.2, and 394.2>181.2) allowing an unambiguous identification of the molecule.

## Conclusions

The accurate and sensitive determination of BPA and other BPA substitutes/analogues is a crucial need for both biomonitoring and research studies aiming to investigate the possible link between such internal exposure and human health. The quantification of these molecules in human breast milk samples especially appears relevant in that context with regard to the criticality of sensitive populations such as pregnant women, fetuses, and newborns. The developed method combined both an optimized selective sample preparation and a specific GC-MS/MS measurement in the SRM acquisition mode. Validation parameters, such as linearity, recovery, and sensitivity performance limits, were evaluated for all

targeted molecules. Linearity was satisfying, with a coefficient of determination above 0.9982 and results below 20 % on the relative response factors (RRF). Accurate quantification was demonstrated down to the concentration of 0.05 μg kg$^{-1}$ for each molecule using $^{13}$C$_{12}$-BPA as internal standard. The analytical method was successfully implemented to the determination of BPA and related analogues in the first set of human breast milk samples originating from French mothers in Region Pays-de-la-Loire. The observed levels of BPA ranged from <LOQ to 1.16 μg kg$^{-1}$, while the other molecules were found below their corresponding LOQ. However, BPS was detected in only one sample, at 0.23 μg kg$^{-1}$. These BPA concentrations are consistent with those already reported in literature since, to our knowledge, no BPS concentrations have been reported yet. The proposed methodology then appeared suitable for further monitoring of a potential decrease of BPA levels and an increase of other BPA analogue levels as reflective of the expected incoming trend in terms of human exposure.

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