



Short Communication

GC–MS analysis of bisphenol A in human placental and fetal liver samples

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ABSTRACT

A method based on extraction with acetonitrile, followed by solid-phase extraction, derivatization with acetic anhydride, and isotope dilution gas chromatography–mass spectrometry (GC–MS) analysis was applied to determine levels of free and conjugated BPA in human tissues. β -Glucuronidase was used to de-conjugate the glucuronized BPA in the samples. The method was validated using various animal organ meat samples including pork liver and kidney, beef and calf liver, chicken liver and heart; recoveries were from 85% to 112% at two spiking levels. The average method limit of quantification (LOQ) was estimated at 0.77 ng/g for placenta samples and 1.2 ng/g for fetal liver samples based on 10 times the signal to noise ratio. BPA was detected in all animal tissue samples, with concentrations ranging from 1.8 ng/g in beef and calf livers to 17.1 ng/g in pork kidney. The method was used successfully to determine both free and conjugated BPA levels in human placental and fetal liver tissue samples. BPA was detected in 86% of the placental samples; concentrations of free BPA in the positive samples ranged from 0.60 ng/g to as high as 64 ng/g with an average of 9.5 ng/g and a median of 3.0 ng/g, and conjugated BPA was as high as 7.8 ng/g. BPA was also detected in most of the fetal liver samples (57%); concentrations of free BPA in the positive samples ranged from 1.3 to 27 ng/g with an average of 8.5 ng/g and a median of 3.2 ng/g. Conjugated BPA was also detected in most of the liver samples analysed for total BPA, ranging from 0.64 to 20 ng/g with an average of 3.9 ng/g and a median of 1.5 ng/g. This study, while primarily designed as a method validation, has demonstrated that BPA can be detected in human fetal liver samples as early as the third month of fetal life. Further work will be conducted to validate these preliminary findings.

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1. Introduction

Bisphenol A (BPA) is used as a monomer in the production of polycarbonate plastics and epoxy resins, and as an additive for the elimination of surplus hydrochloric acid in the production of PVC organosols. Polycarbonate is used in food storage containers such as water bottles and baby bottles, while epoxy resins and PVC organosols are used in the internal coating for food and beverage cans, and also in the internal coating on metal lids for foods in glass jars to protect the food from direct contact with metal. Residues of BPA in polycarbonate plastic containers and coatings can migrate into foods, especially at elevated temperatures, thus, humans are inevitably exposed to BPA through the primary source of exposure, diet, and other minor sources (environmental media, use of consumer products). There is considerable interest in individual body burdens of BPA as it is a known endocrine disruptor that mimics the actions of estrogen [1].

Due to the availability of data on BPA levels in food, exposure of the general population to BPA can be estimated. For example, exposure to BPA for the general population of Canada was estimated recently to be from 0.08 $\mu\text{g/kg}$ body weight/day to 4.30 $\mu\text{g/kg}$ -body weight/day. Estimates for the most highly exposed subpopulation (i.e. infants) range from an average of 0.50 $\mu\text{g/kg}$ body weight/day (maximum 4.30 $\mu\text{g/kg}$ body weight/day) for infants aged 0–1 month to an average of 0.27 $\mu\text{g/kg}$ body weight/day (maximum 1.75 $\mu\text{g/kg}$ body weight/day) for infants aged 12–18 months [2]. However, exposure of the human fetus to BPA during gestation is still relatively unknown. While several studies demonstrated transfer of BPA across placenta in the perfusion experiments using animal [3] or human placenta [4,5], only one study [6] measured BPA levels in human placental tissue samples from 37 pregnancies, with concentrations ranging from 1.0 to 104.9 ng/g. In a recent Korean study [7], BPA was assayed in maternal and umbilical cord blood samples from 300 full-term pregnancies, with concentrations as high as 66.68 and 8.86 ng/mL, respectively. However, the levels of BPA in human fetal organs were not measured in these studies. In addition, only free BPA was determined, which will result in an underestimation of the exposure for both mothers and fetuses. Glucuronidation is the major metabolic pathway of BPA; the majority

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of BPA is metabolized to BPA-glucuronide (BPA-glu) in the liver. Because the fetal liver is still in an immature state during gestation, its ability to glucuronidate is likely to be different from adult liver [8]. Thus, determination of both free BPA and BPA-glu in fetal liver and other organs is necessary to determine the level of exposure of the fetus to BPA as well as the degree of glucuronidation and elimination of BPA occurring during gestation.

Unlike the methods used in animal toxicology experiments, where the doses of BPA are at much higher levels (ppm and above), the background BPA levels in human tissues are much lower (ppb and sub-ppb) and the amount of tissue available for analysis is very small. Thus, this requires the analytical method to be very sensitive. The method also needs to be as selective as possible, due to the complex nature of the tissue samples, in order to minimize interferences, and should be able to measure both free and conjugated BPA. Although methods for determination of BPA in foods are widely available and well validated, information on methods for measuring BPA in human tissues is very limited and conjugated BPA was not always measured [6,9]. In this study, the method based on solvent and solid phase extraction followed by derivatization and GC–MS analysis developed previously [10] was adapted and validated for determination of BPA in various tissue samples. This method was used to analyze human placental and fetal liver tissue samples for both free and conjugated BPA, generating information for the first time on human fetal exposure to BPA during early to mid-gestation and transfer of BPA from human placenta to fetuses.

2. Experimental

2.1. Sample collection and storage

Various animal organ meat samples including pork liver, pork kidney, calf liver, chicken liver, and chicken heart were used for method validation. These samples were purchased from local grocery stores in Ottawa. Human placental ($n=21$, 12.3–20 weeks fetal age (wFA)) and fetal liver ($n=21$, 11.3–22 wFA) samples were obtained after elective pregnancy termination during 1998–2006 in the Greater Montreal area of Quebec with informed written consent. The tissues were placed in sterile vials and flash-frozen in a dry ice/acetone bath, initially stored at McGill University (-80°C), and then transferred in dry ice to Health Canada for processing. Ethics approval for the collection and use of the human placental and fetal tissues was obtained from McGill University and Health Canada. All samples were stored at -80°C prior to analysis.

2.2. Materials and reagents

BPA (99%), β -glucuronidase (from *Escherichia coli*), toluene (anhydrous, 99.8%), isooctane (ACS grade), K_2CO_3 (ACS grade), MTBE (methyl *tert*-butyl ether, HPLC grade), Na_2SO_4 (anhydrous, ACS grade), 1-pentanol (99%), and dodecane (99%) were purchased from Sigma–Aldrich (Oakville, ON). BPA-d14 (99%) was obtained from Polymer Source Inc. (Montreal, PQ). Acetonitrile (HPLC grade) and acetic anhydride (ACS grade) were purchased from Fisher Scientific (Fairlawn, NJ). Methanol (HPLC grade) and sodium acetate (anhydrous, ACS grade) were obtained from EMD Chemical Inc. (Gibbstown, NJ). Deionized (DI) water was obtained from a Barnstead nanopure ultrapure water system (Dubuque, IA).

Individual standard solutions of BPA (3.162 ng/ μL) and BPA-d14 (3.171 ng/ μL) were made by dissolving each compound in acetonitrile and stored at 4°C . Calibration standard solutions were prepared by adding 15 μL of BPA-d14 internal standard solution (i.e., 47.565 ng BPA-d14) and various amounts (2, 10, 20, 30, 40 μL) of BPA standard solution (i.e., 6.324, 31.62, 63.24, 94.86, 126.48 ng BPA respectively) to 22 mL vials containing 12 mL of 1.0 M K_2CO_3

solution, and by going through the same derivatization procedure as for the samples. Since the final volume of extract is 250 μL (30 μL keeper solution + 220 μL toluene), concentrations of BPA in the final extracts ranged from 25 to 506 ng/mL with BPA-d14 at 190 ng/mL. All glassware were pre-cleaned with water followed by methanol, and conditioned in an oven overnight at 200°C to eliminate environmental BPA.

2.3. Sample extraction and derivatization

About 1 g of sample was initially homogenized in 2 mL of acetate buffer solution (1 M, pH 6.4) using an IKA homogenizer (Wilmington, NC) for 15 s. Then each sample was spiked with 15 μL of BPA-d14 internal standard solution, and the sample was vortexed and left for 18 h. To measure the concentration of total BPA (free BPA and BPA-glu), separate samples were also spiked with 2000 IU of β -glucuronidase, and incubated at 37°C for 18 h. A 4 mL aliquot of acetonitrile was added to each sample. The samples were placed on a shaker for 2 h to ensure thorough extraction, and then centrifuged for 15 min at 4000 rpm.

Sample supernatants were diluted with 40 mL of water and loaded onto the Varian C18 Bond Elut cartridges (500 mg, 6 cc) pre-conditioned with 12 mL of methanol and 12 mL of water. The cartridges were washed with 6 mL of water and 12 mL of 30% methanol in water, and eluted with 6 mL of 50% acetonitrile in water. The eluate was collected in a glass tube and concentrated to about 3 mL under a nitrogen stream.

The concentrated extract was derivatized to the di-ester using acetic anhydride in a K_2CO_3 solution followed the same procedure developed previously [10]. Briefly, the concentrated aqueous extract was transferred to a 22-mL glass vial, and 10 mL of 1.0 M K_2CO_3 solution and 400 μL of acetic anhydride were added to each vial. All sample vials were placed in a Barnstead stirring block (Dubuque, IA) and stirred for 5 min at low speed. Another 400 μL of acetic anhydride was added, and the solution was kept stirring for 10 min. Next, 5 mL of isooctane and 400 μL of acetic anhydride were added to each vial and the solution was kept stirring for 10 min. One mL of 3.0 M K_2CO_3 solution was added to each sample to ensure that the pH was above 10, and then stirred for another 10 min. The stirring was then stopped, and the two phases were allowed to separate. The isooctane phase was transferred through an anhydrous Na_2SO_4 column into a glass tube. The aqueous phase was re-extracted with 5 mL of MTBE; the MTBE phase was passed through the Na_2SO_4 column and combined with the isooctane extract. The dry organic extract was transferred through a disposable Pasteur pipette packed with Na_2SO_4 into a disposable glass tube, and 30 μL of keeper solution (a mixture of 1-pentanol and dodecane, 50/50, v/v) was added. The sample extract was evaporated to almost dryness under a nitrogen stream, reconstituted in 220 μL of toluene, and transferred to a gas chromatography (GC) vial for analysis.

2.4. GC/MS analysis

An Agilent 6890 gas chromatograph (GC) coupled to a 5973 mass selective detector (MSD) was used for the analysis. The chromatographic separation was achieved on a DB-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) using helium as the carrier gas (1.2 mL/min, constant pressure). The injector temperature was 280°C . One μL of the extract was injected in splitless mode. The following oven temperature program was used: initial temperature 100°C , held for 1.0 min, ramped at $20^{\circ}\text{C}/\text{min}$ to 225°C , held for 5.0 min, then ramped at $35^{\circ}\text{C}/\text{min}$ to 300°C and held for 3.0 min. A solvent delay time of 8 min was used to protect the ion multiplier of the MS instrument from saturation. The MS was operated with electron impact ionization in selected ion monitoring (SIM) mode.

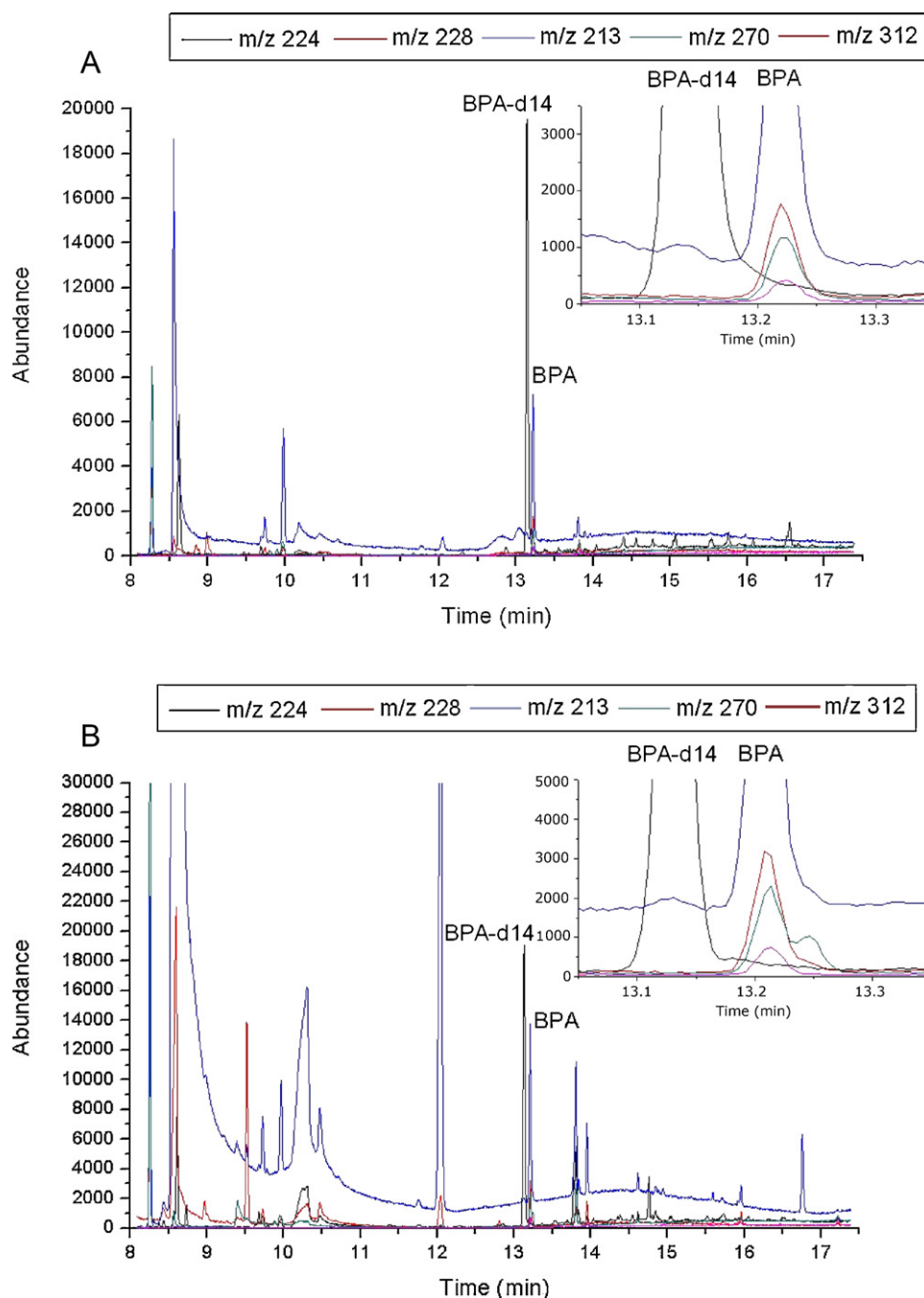


Fig. 1. Typical GC/MS chromatograms of chicken liver (A) and pork kidney (B). BPA-d14 was used as an internal standard.

The GC-MSD interface and MSD source temperatures were 280 and 230 °C, respectively. The ions of m/z 213, 228, 270 and 312 were selected for derivatized BPA, and the ion of m/z 224 was selected for BPA-d14. Dwell time was 35 ms for each ion.

Identification and confirmation of BPA in the samples were based on the retention time and the peak area ratios of the confirmation ions (m/z 228, 270 and 312) to the quantification ion (m/z 213). The BPA concentrations in samples were calculated according to the isotope dilution method; calibration curve was made by plotting the peak area ratios of native BPA (m/z 213) to BPA-d14 (m/z 224) versus the mass ratios of native BPA to BPA-d14. Two method blanks were analysed in each batch of analysis, and the results of all samples were corrected for the trace levels of BPA in blanks (about 0.5 ng). This method was used to analyse the proficiency test sam-

ples from FAPAS in 2009 and 2010, and the z-scores of our results were as low as 0.2.

3. Results and discussion

3.1. Method performance

Due to the limited amount of human placental and fetal liver tissue samples available (from less than 1 g to as low as 0.1 g), it was impossible to validate the method using the human tissue samples. Thus, animal organ meats purchased from local grocery stores were used instead. Chicken liver was used initially, but a background BPA level of 8.1 ng/g was detected. In order to check the BPA levels in other organ meats and to test the robustness of the method, other

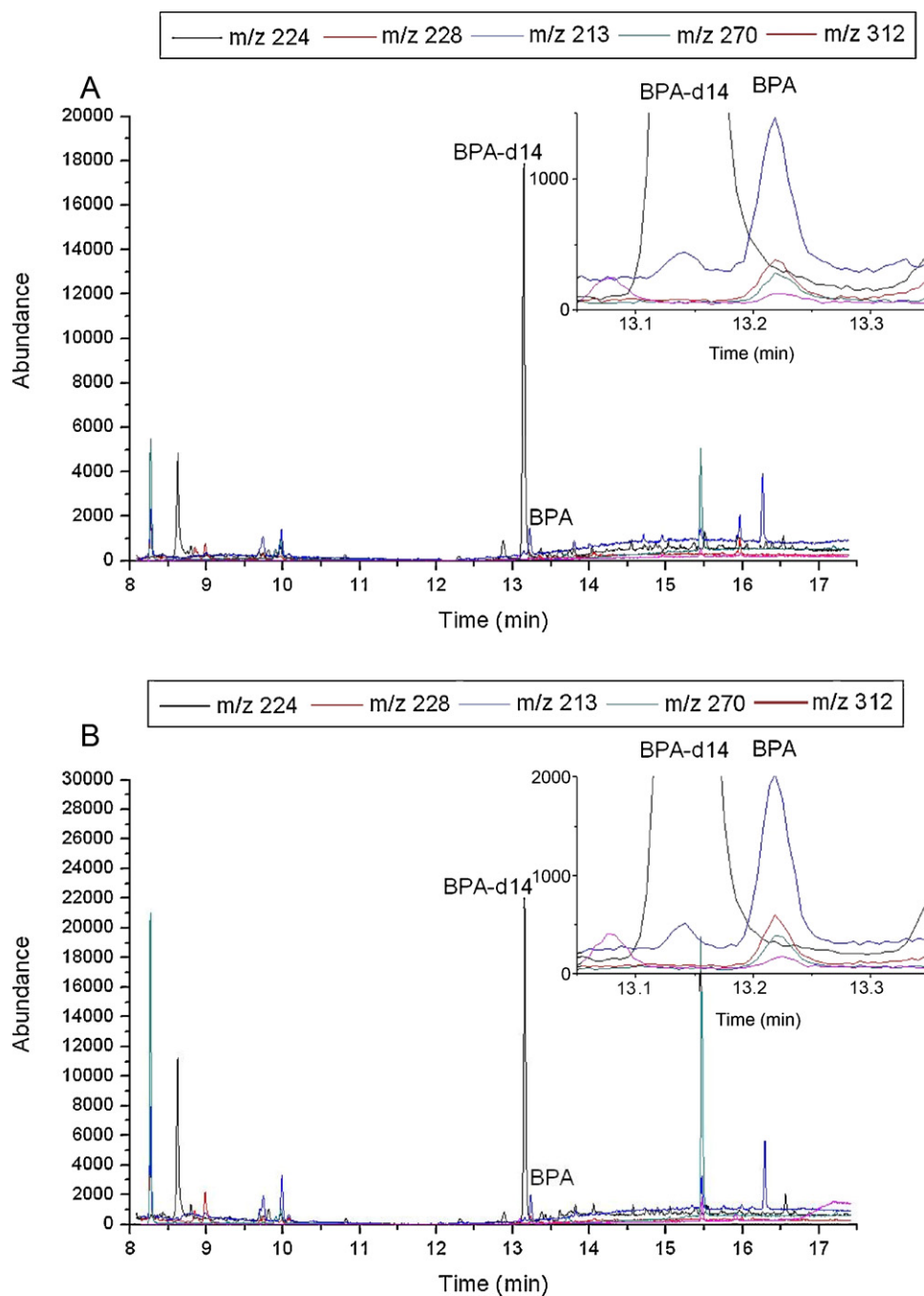


Fig. 2. Typical GC/MS chromatograms of fetal liver (A) and placenta (B). BPA-d14 was used as an internal standard.

organ meats including chicken heart, pork kidney and liver, beef and calf liver were also analysed for BPA to validate the method. BPA was detected in all organ meat samples, with levels from as low as 1.8 ng/g in beef and calf livers to as high as 17 ng/g in pork kidney (Table 1). The typical GC–MS ion chromatograms of the extracts from chicken liver and pork kidney are shown in Fig. 1. The sources of BPA in these organ meat samples are not known. Packaging is unlikely to be the source since they were all packaged the same way and the cling film is not known to contain BPA. But this does raise awareness that BPA is present in foods other than canned foods and that, for more accurate exposure assessment, organ meats and other non-canned foods should also be analysed.

Linearity of the instrument and the method was demonstrated using five standard solutions with BPA concentrations in the final

Table 1

Background BPA levels (ng/g) in animal organ meat samples and recoveries.

Sample	BPA concentration (ng/g) ^a	Recovery (%) ^b	
		10 ng/g	20 ng/g
Chicken liver	8.1 ± 0.29 (4)	85.2 ± 1.9	87.7 ± 2.0
Chicken heart	8.3 ± 1.3 (4)	95.3 ± 12.4	101.5 ± 5.4
Pork kidney	17.1 ± 1.0 (3)	64.3 ± 3.1	99.4 ± 4.1
Pork liver	10.5 ± 0.5 (4)	98.7 ± 5.5	92.7 ± 0.4
Beef liver	1.8 ± 0.19 (4)	107.4 ± 3.5	100.7 ± 2.8
Calf liver	1.8 ± 0.21 (3)	112.5 ± 8.2	107.7 ± 4.9

^a Numbers in brackets are the number of replicates.

^b Recoveries were measured at two spiking levels in triplicates, $n = 3$.

Table 2
Concentrations (ng/g) of free and conjugated BPA in human placental and fetal liver samples.

Placenta				Liver			
Sample ID	Fetal age (week)	Free BPA	BPA-glu	ID	Fetal age (week)	Free BPA	BPA-glu
P01	18	5.5	– ^a	L01	18.5	ND	0.73
P02	19	1.8	0.99	L02	13.8	ND	–
P03	20	7.4	–	L03	12	1.3	–
P04	13.5	4.8	1.5	L04	18	3.6	4.2
P05	12.3	2.0	3.5	L05 ^b	16	2.4	0.64
P06	18	1.2	7.8	L06 ^b	16	1.3	1.9
P07	14.7	1.6	–	L07	13.8	ND	–
P08	15	7.9	–	L08	15.3	2.8	20
P09	13.8	3.4	7.1	L09	19	2.1	1.6
P10	18	ND	ND ^c	L10	11.3	27	–
P11	17	4.0	–	L11	16.5	2.7	1.4
P12	18	0.60	–	L12	22	ND	–
P13	14.5	0.84	1.0	L13	18	ND	ND
P14	17	ND	ND	L14	15.5	ND	ND
P15	13	1.2	6.8	L15	17	ND	0.77
P16	12.8	1.9	–	L16	13.5	ND	–
P17	12.5	64	–	L17	13.5	21	–
P18	12.6	39	–	L18	15	ND	–
P19	13.3	ND	–	L19	14.7	7.6	–
P20	15.8	2.7	–	L20	12	5.8	–
P21	16.5	21	–	L21	13	24	–

^a The total BPA concentration were not measured due to the limited amount of sample.^b Samples from a twin pregnancy.^c Not detected, <LOQ

extracts from 25 ng/mL to 506 ng/mL. Linearity with an r^2 value >0.999 was observed for BPA's calibration curve plotted with the peak areas normalized to BPA-d14 versus mass ratios of BPA normalized to BPA-d14. Due to the variations in the amount of samples for analysis for both placenta and fetal liver, the method limit of quantification (LOQ), defined as the 10 times the signal to noise ratio, was estimated for each one of the placenta and fetal liver samples according to the amount of BPA-d14 spiked to the samples. The LOQ for placenta samples ranged from 0.21 to 2.4 ng/g with an average of 0.77 ng/g, while the LOQ for fetal liver samples ranged from 0.25 to 4.5 ng/g with an average of 1.2 ng/g.

The method extraction recoveries for BPA from six different animal tissue matrices were determined by spiking each sample in triplicates with BPA at two different levels, 10 ng/g and 20 ng/g. As shown in Table 1, recoveries were better than 85%, except for pork kidney which spiked at 10 ng/g, with relative standard deviations (RSD) from 0.4 to 13%.

3.2. BPA in human placental and fetal liver tissues

As mentioned previously, the amount of human placental and fetal liver tissue samples was limited. Thus, although all placental and fetal liver samples were analysed for free BPA, only nine of the 21 placental samples and 10 of the 21 fetal liver samples were also analysed for total BPA in order to determine levels of conjugated BPA. The typical GC–MS ion chromatograms of the placental and fetal liver extracts are shown in Fig. 2. Interferences were not observed around either the BPA or the BPA-d14 peaks, which confirms that this method works very well with human tissue samples. Table 2 shows the concentrations of free and conjugated BPA in the

placenta and fetal liver samples together with the information on fetal ages, and the average, median, standard deviations, and the range of the concentrations of both the free BPA and the conjugated BPA in the placental and fetal liver samples are shown in Table 3.

BPA was detected in 86% of the early to mid-gestation placental samples (12.3–20 wFA), concentrations of free BPA in the positive samples range from 0.60 ng/g to as high as 64 ng/g, with an average of 9.5 ng/g and a median of 3.0 ng/g. These results are within the range of BPA concentrations (1.0–104.9 ng/g, median = 12.7 ng/g) reported for human term placental samples by Schönfelder et al. [6], suggesting that exposure of the feto-placental compartment to BPA is similar from early gestation to term. Among the nine placental samples analysed for total BPA, conjugated BPA was detected in seven samples at concentrations ranging from 0.99 to 7.8 ng/g (12.3–19 wFA). The ratios of conjugated BPA concentrations over the free BPA concentrations for the positive placental samples analysed for both free and total BPA varied from 0.32 to 6.6 with an average of 2.7. Significant trend in the placental BPA levels relative to fetal age (12.3–20 wFA) was not observed. There were too few BPA-glu data for correlation analysis.

BPA was also detected in most of the fetal liver samples (57%, 11.3–19 wFA), concentrations of free BPA in the positive samples range from 1.3 ng/g to 27 ng/g, with an average of 8.5 ng/g and a median of 3.2 ng/g. Concentrations of free and conjugated BPA in the liver samples from twin pregnancy also agreed well (free BPA: 2.4 vs. 1.3 ng/g; BPA-glu: 0.64 vs. 1.9 ng/g), which indicates the relative even distribution of the BPA transferred from placenta to the fetuses. This clearly demonstrates, for the first time, that human fetus exposed to BPA from as early as the first trimester due to the transfer of BPA from placenta to fetal liver. Conjugated BPA was also

Table 3
The mean, median, standard deviation (SD), and the range of BPA results in human placental and fetal liver samples.

Sample		Number of samples	Number of positive samples	BPA concentration (ng/g)				
				Min	Max	Mean	Median	SD
Placenta	Free BPA	21	18	0.60	64	9.5	3.0	16
	BPA-glu	9	7	0.99	7.8	4.1	3.5	3.1
Fetal liver	Free BPA	21	12	1.3	27	8.5	3.2	9.7
	BPA-glu	10	8	0.64	20	3.9	1.5	6.6

detected in most of the liver samples analysed for total BPA, ranging from 0.64 ng/g to 20 ng/g, with an average of 3.9 ng/g and a median of 1.5 ng/g (15.3–19 wFA). The level of conjugated BPA in one liver sample (20 ng/g) was seven times that of free BPA (2.8 ng/g) in the same sample, while the ratios of conjugated BPA concentrations over the free BPA concentrations for the other liver samples varied from 0.27 to 1.48. A significant decline in liver BPA concentrations as a function of fetal age ($p \leq 0.03$, 11–22 wFA) was observed, but there were too few BPA-glu data for correlation analysis.

The reasons for the wide variability in the ratios of conjugated over free BPA in both the placental and the fetal liver tissues are not known. It could be due to the differences in the ages of the fetuses since the ability of the fetal liver to glucuronidate BPA by UDP-glucosyltransferase may vary considerably during gestation. In addition, since the free BPA in mothers can pass through the placenta to the fetal circulation, it is also possible that maternal conjugated BPA can transfer through the placenta to the fetal compartment. The significant age-related decline in liver BPA levels may be indicative of an increasing ability of the hepatocytes to metabolize and excrete BPA.

This study, while primarily designed as a method validation, has demonstrated that BPA can be detected in human fetal liver samples as early as the third month of fetal life. The conjugated BPA found in the fetal tissues could be due to glucuronidation of BPA by the fetal hepatocyte and/or to direct transfer from the maternal circulation. Because the fetus is more sensitive to estrogenic chemicals than adults, BPA accumulation in fetal tissues may result in increased risk for abnormal organ development. Thus, a more comprehensive study using human fetal liver samples should now be conducted, not only to determine the ability of fetal hepatocytes to glucuronidate BPA as a function of fetal age but also to investigate

if there are effects of BPA at the concentrations measured in this study on fetal hepatocyte differentiation and function.

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