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LPS-004

Determination of Bisphenol A in Liquid Infant Formula

by Solid Phase Extraction with Acetic Anhydride Derivatization and Gas Chromatography–Mass Spectrometry

Bureau of Chemical Safety
Food Directorate
Health Products and Food Branch

A WHO Collaborating Centre for Food Contamination Monitoring
World Health Organization

Canada

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1.0 Principle and Scope

This method is for determination of bisphenol-A in liquid infant formulae at levels from 0.3 to 25 ppb.

Labelled BPA-d16 is used as internal standard. The milk proteins are denatured and separated using acetonitrile. Fats, carbohydrates and some of the added fatty acids are removed by SPE.
BPA is derivatized to the di-ester using acetic anhydride in basic aqueous solution. The derivative of BPA is extracted using a non-polar solvent, and measured by GC/MS in SIM mode using 4 ions (one target and 3 qualifiers). Calibration standards are prepared the same way as samples (from the derivation step).

2.0 Definitions

BPA: Bisphenol A

BPA-d16 : Isotopic labelled Bisphenol A-d16

ACN: Acetonitrile

3.0 Performance Characteristics

Intra-assay

BPA levels (ng/g)	Recovery (%)	RSD %
2.5 (n=8)	94	2.8
20.0 (n=5)	94	3.9
8.0 (n=8)	85	2.7

Inter-assay validation data

		BPA		
	n	ng/g	Recovery (%)	% RSD
High concentration reference material	6	10.4	----------	2.8
Low concentration reference material	6	0.54	----------	5.0
Method blanks	6	0.13	----------	----------
Spiked blanks	6	----------	100	1.4
Duplicates	5			1.9

4.0 Equipment and Supplies

4.1 Equipment

4.1.1 Agilent 6890 gas chromatograph with 5975 mass selective detector

4.1.2 Oven

4.1.3 Balances

4.1.4 50-position stirring plate for 22-mL vials (Barnstead # PS80037A)

4.1.5 Ultrasonic bath

4.1.6 pH meter and calibration solutions

4.1.7 N_2 evaporator 12 or 24 positions

4.1.8 Vortexes, simple and multi-tubes

4.1.9 Speedvac evaporator system

4.2 Chemicals

4.2.1 Acetonitrile (HPLC grade) from J.T. Baker (Phillipsburg, N.J.).

4.2.2 Methanol (HPLC grade) from J.T. Baker (Phillipsburg, N.J.).

4.2.3 Toluene (glass distilled) from Sigma-Aldrich (Oakville, ON).

4.2.4 Potassium carbonate (ACS grade) from Sigma-Aldrich (Oakville, ON).

4.2.5 Bisphenol A (99%) from Sigma-Aldrich (Oakville, ON).

4.2.6 Bisphenol A-d16 (98%) from Sigma-Aldrich (Oakville, ON).

4.2.7 Isooctane (pesticide-residue grade) from Sigma-Aldrich (Oakville, ON).

4.2.8 MTBE (methyl t-butyl ether, 99.9%) from Sigma-Aldrich (Oakville, ON).

4.2.9 K_2HPO_4 (ACS) from Sigma-Aldrich (Oakville, ON).

4.2.10 Na_2SO_4 (anhydrous, ACS grade) from Sigma-Aldrich (Oakville, ON).

4.2.11 1-Pentanol (99%), dodecane (99%) from Sigma-Aldrich (Oakville, ON).

4.2.12 Acetic anhydride (ACS grade) from Fisher (Ottawa, ON).

4.2.13 H_3PO_4 (85% HPLC grade) from Fisher (Ottawa, ON).

4.2.14 Deionized water from a milli-Q system.

4.3 Materials

4.3.1 15 mL polypropylene centrifuge tubes

4.3.2 Volumetric flasks, 10, 25, 50, 100 mL

4.3.3 Disposable glass tubes, 13 x 100 mm, 16 x 100 mm, 20 x 150 mm

4.3.4 2 mL autosampler amber vials

4.3.5 Stirring bars, 12.5 x 5 mm

4.3.6 22 mL vials with Teflon-coated septum caps

4.3.7 Eppendorf pipettes, 100 – 1000 μL, 10 – 100 μL, 20 – 250 μL

4.3.8 pH indicating papers, 1 – 14

4.3.9 C18 SPE cartridges, 500 mg/6 cc from Varian (Mississauga, ON.)

4.3.10 80 mL reservoirs for SPE with adaptors

4.3.11 Vacuum manifold for SPE 20 positions

4.3.12 10 – 50 mL bottle-top dispenser

4.3.13 70 mL glass tubes with Teflon-coated septum and screw caps

4.3.14 Empty glass column with frit.

5.0 Health and Safety

6.0 Procedures

6.1 Preparation of Standard Solutions

6.1.1 BPA stock Solutions (400 ppm): Prepare in 25 mL volumetric flask, using acetonitrile as solvent; store at 4°C.

6.1.2 BPA Intermediate and spiking solution (10.0 ppm): In a 25 mL volumetric flask, pipette the necessary volume of stock solution of BPA to get a concentration of 10.0 μg/mL. Fill up with acetonitrile.

6.1.3 BPA spiking solution (1 ppm): Pipet 5.0 mL of 10 ppm solution in a 50 mL volumetric flask. Fill in with acetonitrile.

6.1.4 BPA-d16 internal standard solution (1.0 ppm): In a 25 mL volumetric flask, pipette the necessary volume of stock solution of d16-BPA to get a concentration of 1.0 μg/mL. Fill up to volume with acetonitrile.

6.1.5 Derivatized BPA calibration standards

Prepare these only prior to derivatization of the sample extracts.

Add standards to a set of 22 ml vial containing 12 ml of 1.0 M K_2CO_3 solution.

Proceed to the derivatization step. Concentrations (in ng/mL) below refer to a 150 μL final volume.

ng total	ng/mL injected	μL of 1.0 ppm BPA-d16	μL of 1.0 ppm BPA std.	μL of 0.1 ppm BPA std.
0	0	0	-------	0
1.5	10	30	----------	15
3	20	30	----------	30
9	60	30	---------	90
24	160	30	---------	240
72	480	30	72	------

6.1.6 Phosphate pH 7.0, 0.1M buffer: Weigh 28.6 g of Na_2HPO_4 in a 2 L Erlenmeyer flask. Add a stirring bar and approximately 1950 ml H_2O. Dissolve and add concentrated H_3PO_4 to pH 7.0 ± 0.1. Complete to 2.0 L and store at 4 ^oC; prepare weekly.

6.1.7 50% ACN / H_2O solution: In a glass bottle, mix 250 mL acetonitrile and 250 mL H_2O.

6.1.8 30% methanol / H_2O solution: In a glass bottle, mix 150 ml methanol and 350 ml H_2O.

6.1.9 1.0 M K_2CO_3 solution: In a 500 mL volumetric flask, dissolve 69g of anhydrous K_2CO_3; complete to volume with water.

6.1.10 1-pentanol/dodecane keeper solution: In a 15 mL stoppered glass tube, mix 4 mL 1-pentanol and 4ml of dodecane.

6.2 Sample Extraction

6.2.1 Before starting, condition the following glassware in an oven at 260 °C for at least 2 hours: a set of 70 mL glass tubes, a set of 16 x 150 mm and 2 set of 16 x 100mm disposable glass tubes in order to eliminate environmental BPA that may be present.

6.2.2 Every extraction batch contained the following control samples: (1) one method blank (6 mL of water), (2) one method blank spiked with BPA at 20 ng g^{-1}, (3) one or two in-house reference materials, and (4) one unknown sample spiked with BPA at 20 ng g^{-1}.

6.2.3 In a 15 mL PP centrifuge tube, weigh about 6.0 g of liquid formulae milk. For concentrated formulae, use 3.0g and add 3.0 ml H_2O.

6.2.4 To each sample (exception for the blank), add 30 μL of d16-BPA 1.0 ppm internal standard solution and mix.

6.2.5 Add 6.0 mL of ACN

6.2.6 Shake for 30 seconds and vortex for 30 seconds

6.2.7 Centrifuge at 4000 rpm 4 ^{o}C for 12 minutes

6.2.8 Decant the liquid in a 70 mL glass tube

6.2.9 Add 55 ml pH 7.0 buffer to each tube; cap and mix.

Purification by solid phase extraction –SPE –
C18 BONDELUT 500 mg 6cc (Varian #1210-2052)

6.2.10 Condition with 2 reservoir volumes (13 mL) of methanol and 2 volumes H_2O.

6.2.11 Identify each cartridge with the sample #

6.2.12 Use 80 ml reservoir fitted with an adaptor.

6.2.13 If filtration is needed before, only use a Fiberglass cartridge filter of 2 um (never nylon or organic membrane which all absorb the analytes of interest), inserted between the reservoir and the adaptor.

6.2.14 Pour the extract into the reservoir and allow for gravity absorption (no vacuum unless flow has completely stopped).

6.2.15 Rinse with 1 volume (6.5 ml) of H_2O and discard

6.2.16 Rinse with 2 volumes (13 ml) of 30% methanol / H_2O and discard.

6.2.17 Identify a set of 16x100mm glass tubes with sample #. Place the tubes in the rack and the rack in the vacuum manifold to collect the eluate.

6.2.18 Elute the C18 with 6.5 ml (1 volume) of 50 % ACN. At the end, apply a light vacuum to collect the last drops.

6.2.19 Mix the eluate using a vortexer.

6.2.20 Concentrate to about 3 ml using N_2 evaporator.

6.3 Derivatization

6.3.1 Transfer the concentrated aqueous extract to a 22 mL vial; add a small stirring bar.

6.3.2 If this has not yet been done before extraction, add 30 µL of 1.0 ppm internal standard BPA-d16 (in acetonitrile) to each sample, exception for the reagents blank.

6.3.3 Add 10 mL of 1.0M K_2CO_3 solution

6.3.4 Put all the samples vials on the 50-positions- stirring plate and start stirring at low speed

6.3.5 Prepare also a set of calibration standards -see section 6.1.5

6.3.6 Add 200 µL of acetic anhydride to each vial.

6.3.7 After 5 minutes, repeat addition of 200 µl acetic anhydride and keep stirring for 10 more minutes.

6.3.8 Add 5.0 ml of isooctane to the vial

6.3.9 Using a pH indicating strip and Pasteur pipette, check a few samples for pH. It must be above 10. If needed, add 0.5 mL of concentrated (3M) K_2CO_3 solution.

6.3.10 Add 100 µl more acetic anhydride and stir for 10 minutes more.

6.3.11 Stop stirring and allow the 2 phases to separate (10 minutes or more if needed).

6.3.12 If there is emulsion, split the sample into 2 vials, dilute with H_2O, add some more isooctane and re-extract.

For organic phase:

6.3.13 Fill up to ½ a small glass column with anhydrous Na_2SO_4 and place over a 20x150 mm glass tube

6.3.14 Use a Pasteur pipette to transfer the isooctane phase to the Na_2SO_4 column.

6.3.15 Re-extract the aqueous solution (the 22 ml vial) using 5.0 ml MTBE (methyl t-butyll ether).

6.3.16 Stir at HIGH speed for at least 10 minutes.

6.3.17 Stop stirring and allow the phases to separate.

6.3.18 Transfer the MTBE organic phase to the Na_2SO_4 column.

6.3.19 Transfer the dry organic extract to a 13x100 mm tube. It may be necessary to add Na_2SO_4 to the large tube and vortex if water seems to be present (cloudiness)

6.3.20 To the 13x100mm tube, add 30 µl of 1-pentanol/dodecane solution as a keeper

6.3.21 Rinse the Na_2SO_4 column and tube using 1 mL isooctane and 1 mL MTBE and combine to the 13x100 mm tube.

6.3.22 Evaporate the solvent using the speedvac, at 35 °C for about 30 minutes. There should only be a small drop of the keeper left at the bottom.

6.3.23 If there is presence of water residue, add 1 ml of acetone and re-evaporate.

6.3.24 Reconstitute with 120 µl of toluene.

6.3.25 Vortex 30 seconds

6.3.26 Ultrasonic bath for 5 minutes

6.3.27 Transfer to a GC autosampler vial containing an insert for analysis (use an eppendorf pipette, instead of a Pasteur pipette).

6.4 Instrumental Conditions

6.4.1 Flow rate of the helium carrier gas: 1.1 mL min^{-1}.

6.4.2 Injector temperature: 280°C.

6.4.3 Sample injection volume: 1.0 µL, in splitless mode.

6.4.4 GC column: ZB-5ms capillary column (5% diphenyl-95% dimethyl-silicone, 30 m x 0.25 mm x 0.25 µm).

6.4.5 GC oven temperature program: 100°C for 1 minute, raised to 225°C for 5 minutes at 20°C min^{-1}, then raised to 325°C at 35°C min^{-1}, and held for 1 minute.

6.4.6 MSD was operated with electron impact ionization in selected ion monitoring (SIM) mode. A standard spectra autotune is used. EM offset at + 300 eV.

6.4.7 The following ions were selected for BPA: 213, 228, 270, 312, and for BPA-d16: 224.

6.4.8 Dwell time was 35 ms for each ion.

6.4.9 GC-MSD interface temperatures: 280 °C.

6.4.10 MSD source temperatures: 230°C.

Under these conditions, the retention time of BPA should be about 13.4 minutes.

6.5 Data analysis, Calculations

6.5.1 Enter the multiplier in the sequence table BEFORE data acquisition.

Multilplier = Final volume (mL) / sample weight

For a 6.0 g sample, M=0.025

Use CHEMSTATION built-in data analysis program to calibrate in Internal standard mode, with ion m/z 228 as the target. Enter calibration standard concentration in ng/mL in order to get concentrations results in ng/g of sample. Calibration curve using 1/C2 weighing without forcing to zero (blank has a positive signal).

6.5.2 Quantitate each sample using "Calculate report" and "generate detailed report".

7.0 Quality Control and Quality Assurance

7.1 Confirmation of BPA identity was based on the retention time and the ion ratios.

7.2 Every extraction batch contained the following control samples: (1) one method blank (6 mL of water), (2) one method blank spiked with BPA at 20 ng g^{-1}, (3) one or two in-house reference materials, and (4) one unknown sample spiked with BPA at 20 ng g^{-1}.

7.3 Linearity: The R^2 value for the calibration curve with peak areas normalized to internal standard versus concentrations should be better than 0.99. At least 4 out of 5 calibration standards must be used. Back-calculated values of each calibration std ±15 % of their nominal value (±20 % for lowest calibration standard).

7.4 Method quantification limit was established at 0.5 ng g^{-1}, this was equivalent to the lowest calibration standard of 10 ng mL^{-1} for a 3 g sample

8.0 Associated Documents