Agilent Application Solution

Analysis of bisphenol A leaching from baby feeding bottles

Application Note

Consumer Products

Author

Syed Salman Lateef
Agilent Technologies, Inc.
Bangalore, India

Abstract

Bisphenol A can leach out from the plastic surfaces of food containers and has been detected in various matrices such as plasma, urine and groundwater. Bipshenol A is an endocrine disruptor, which can mimic the body's own hormones such as estrogen and may lead to negative health effects. In this Application Note, we describe a method to quantify bisphenol A and a structurally similar analog bisphenol F extracted from baby feeding bottles. The method was developed on an Agilent 1260 Infinity LC system using an Agilent ZORBAX Eclipse Plus C18 column. Partial method validation was performed to demonstrate linearity, robustness and precision of area and retention time. The dilution series was generated automatically using an Agilent 7696A Sample Prep WorkBench, saving analyst time. The limit of quantitation (LOQ) for bisphenol A was found to be 1.06 ng/mL. During sample recovery studies, 80% recovery was obtained for bisphenol A. The method was transferred to an ultrahigh performance liquid chromatography (UHPLC) method using an Agilent 1290 Infinity LC System. The UHPLC method has the same experimental conditions but showed narrower and higher peaks, better resolution, and improved signal-to-noise response. Both methods can be applied for bisphenol A quantification in quality control of food containers such as baby feeding bottles.

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Introduction

Bisphenol A (BPA) is a monomer used to make polycarbonate plastic and epoxy resins. Traces of BPA can leach out of these polycarbonate plastic surfaces under various environmental conditions such as heat or pH changes and eventually are consumed by humans. BPA was detected in various matrices such as urine, groundwater and plasma. Based on the assumption that a threshold exists above which toxic effects are seen, the U.S. Environmental Protection Agency (US EPA) has established 50 µg/kg body weight/day as the reference dose (RfD) for BPA[1]. Baby bottles made of polycarbonate plastic are a potential risk to children. In this Application Note, we used a structurally similar commercially available compound bisphenol F, (BPF), (Figure 1) along with BPA to determine separation efficiency.

Ballesteros-Gomez *et.al.*, reviewed various analytical methods used to separate, identify and quantify BPA[2]. Also, the ASTM standard test method, D 7574-09, describes a SPE based offline method for extracting bisphenol A from environmental waters[3]. BPA is a fluorescent compound, and fluorescence detector (FLD) is sensitive to detect BPA concentration in baby bottles. In this Application Note, a method is described to simultaneously quantify BPA and BPF using a SPE based offline extraction procedure with (U)HPLC/FLD detection.

Experimental

Instruments and software

An Agilent 1260 Infinity Binary LC system consisting of the following modules was used:

- Agilent 1260 Infinity Binary Pump (G1312B)

- Agilent 1260 Infinity Autosampler and Thermostat (G1367E, G1330B)

- Agilent 1260 Infinity Thermostatted Column Compartment (TCC) (G1316A)

- Agilent 1260 Infinity Fluorescence Detector (G1312B) with 8 µL flow cell

The UHPLC analysis was developed and performed using an Agilent 1290 Infinity LC System consisting of the following modules:

- Agilent 1290 Infinity Binary Pump (G4220A)

- Agilent 1290 Infinity Autosampler and Thermostat (G4226A, G1330B)

- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

- Agilent 1260 Infinity Fluorescence Detector (G1312B) with 8 µL flow cell

Software:

- Agilent ChemStation B.04.02

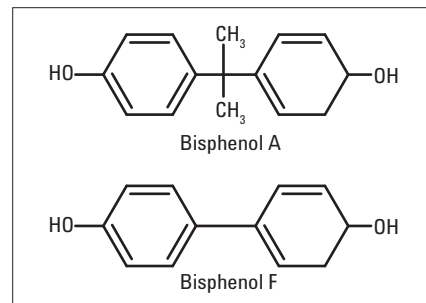Sample preparation:

- Agilent 7696A Sample Prep WorkBench

Figure 1
Molecular structures of bisphenol A and bisphenol F

Reagents and materials

All chemicals and solvents used were HPLC grade. Purified water was used from a Milli Q water purification system (Millipore Q-POD Element, USA). Acetonitrile and methanol super gradient were purchased from Lab-Scan (Bangkok, Thailand) and potassium phosphate monobasic was obtained from Fluka (Germany). Standards of bisphenol A and bisphenol F were purchased from Sigma-Aldrich (India). BPA-free baby bottles manufactured in USA and three different brands of polycarbonate baby bottles manufactured locally were purchased.

Chromatographic parameters

The chromatographic parameters for reverse phase liquid chromatography using Agilent 1260 and Agilent 1290 Infinity LC systems are shown in Table 1.

Parameters	Agilent 1260 Infinity LC system	Agilent 1290 Infinity LC system
Column:	Agilent ZORBAX Eclipse Plus C18 4.6×100 mm 5 µm (p/n 959996 902)	Agilent ZORBAX Eclipse Plus C18 4.6×100 mm 1.8 µm (p/n 959964 902)
TCC temperature:	40 °C	
FLD:	Ex: 230. Em: 316	
FLD acquisition rate, gain:	9.26 Hz, 15	
Sample thermostat:	4 °C	
Mobile phase A:	10 mM monobasic potassium phosphate in water	
Mobile phase B:	100% Acetonitrile	
Gradient:	Time (min)　　%B	
	0　　　　　　5	
	2　　　　　　5	
	2.1　　　　　35	
	12.5　　　　35	
	12.6　　　　70	
	17　　　　　70	
	18.1　　　　5	
	23　　　　　5	
Flow:	0.9 mL/min	
Injection volume:	20 µL. 5 sec needle wash at flush port for 5 sec, using mobile phase A	

Table 1
Chromatographic parameters used in the Agilent 1260 Infinity LC and Agilent 1290 Infinity LC systems.

Preparation of standards

BPA and BPF were accurately weighed out and dissolved in 100% methanol separately to obtain stock solutions of about 300 µg/mL each, which were stored at 4 °C when not in use. A 400 ng/mL solution of BPA and BPF was prepared freshly by diluting the stock solutions using the dilution buffer of 5% acetonitrile and 95% 10 mM monobasic potassium phosphate in water. Linearity levels shown in Table 2, were prepared by subsequent dilution of a 400 ng/mL solution. The Agilent 7696A Sample Prep WorkBench was used to make linearity levels, using serial dilutions. In the first sequence, 400 µL of dilution solution was added to all the vials. In the second sequence, 300 µL of 400 ng/mL solution was added to a Level 7 vial from the first sequence and vortexed for 15 seconds. Serial dilutions were carried out by taking 300 µL from the previous level and added to the next level vial. Note that instead of running two sequences, the steps can also be programmed into one method and running one sequence. The syringe parameters used in the setup of the Agilent 7696A Sample Prep WorkBench are given in Table 3. An Agilent Application Note[5], describes in detail the set up of 7696A Sample Prep WorkBench[4].

Sample preparation

BPA from polycarbonate baby bottles was extracted following the schematic shown in Figure 2. An SPE adapter (p/n 12131001) and 3 mm OD tubing (p/n 5062-2483) were used to load the sample onto an Agilent Bond Elut Plexa SPE column, 200 mg, 6 mL (p/n 12109206). An Agilent 20 port vacuum extraction manifold (p/n 12234104) was used for the setup of SPE. We followed the sample handling precautions as described in the ASTM method[3]. The reconstituted solution from the final step (Figure 2) was used directly for sample analysis.
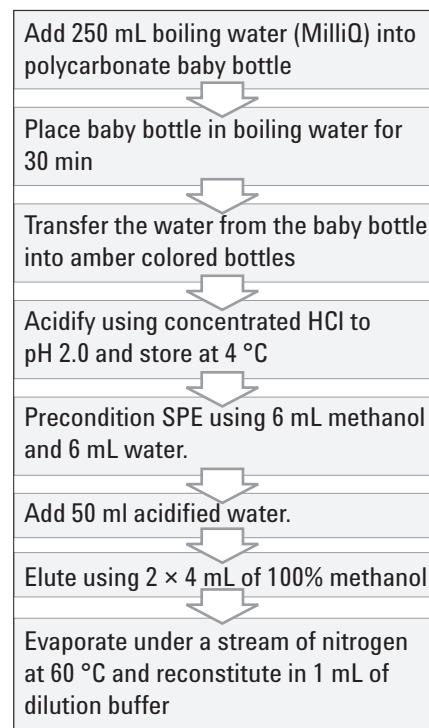
Add 250 mL boiling water (MilliQ) into polycarbonate baby bottle

Place baby bottle in boiling water for 30 min

Transfer the water from the baby bottle into amber colored bottles

Acidify using concentrated HCl to pH 2.0 and store at 4 °C

Precondition SPE using 6 mL methanol and 6 mL water.

Add 50 ml acidified water.

Elute using 2 × 4 mL of 100% methanol

Evaporate under a stream of nitrogen at 60 °C and reconstitute in 1 mL of dilution buffer

Figure 2
Extraction of BPA from baby bottles and sample preparation using SPE.

Linearity levels	Bisphenol A (ng/mL)	Bisphenol F (ng/mL)
LOD	0.195105	0.195105
1	1.06224	1.06224
2	2.478559	2.478559
3	5.783305	5.783305
4	13.49438	13.49438
5	31.48688	31.48688
6	73.46939	73.46939
7	171.4286	171.4286

Table 2
Dilution table for bisphenol A and bisphenol F.

	Solvent prewash 1	Dispense wash	Dispense pumps	Dispense settings
Number of pumps or washes	1	1	3	
Wash volume (µL)	50	50	20	
Draw speed (µL/min)	1250	1250	1250	1250
Dispense speed (µL/min)	2500	2500	2500	2500
Needle dept off set (mm)	-2.0	-2.0	-2.0	-2.0
Viscosity delay (s)	0	0	0	0
Turret solvent	A			
Air gap (% syr. vol.)	0			0

Table 3
500 µL syringe parameters used for the Agilent 7696A Sample Prep WorkBench.

Procedure

The reconstituted extracts from the baby bottles were injected to measure the approximate concentration of BPA before establishing the linearity range. A 20 µL solution of mobile phase A was injected as blank, followed by each linearity level in six replicates. Area and retention time (RT) information for each level was used to calculate the relative standard deviation (RSD) values. The average area of each linearity level in the linearity range was plotted against the concentration to obtain a calibration curve. The limit of detection (LOD) and limit of quantitation (LOQ) for BPA and BPF were established from the lower linearity level injections.

To evaluate the robustness of the method, six critical method parameters were evaluated:

- Flow rate ±2%

- Column temperature ±2.5%

- Injector volume ±5%

- Excitation and emission wavelength ±3%

- Step gradient ±10%

- Buffer concentration ±10%

For each robustness parameter, a standard concentration of 30 ng/mL solution of BPA and BPF was injected in seven replicates.

To perform the recovery studies, we extracted samples from BPA-free baby bottles as described in Figure 2. To 50 mL of this sample, either a low or a higher quantity of BPA and BPF was spiked. Both spiked samples were subjected to SPE. The resulting concentrations of the samples were determined using the calibration curves. The theoretical concentrations were compared against the experimental values to obtain the recovery values.

Finally, three different brands of baby bottles were analyzed to determine the leaching concentrations of the two bisphenols using the standard HPLC method.

The method was then transferred to an Agilent 1290 Infinity LC system and run on a 1.8 µm column using the same experimental conditions to test resolution and sensitivity of the method. For this method, we also evaluated LOD, LOQ, linearity of each standard and precision of the method by area and RT RSD.

Results and Discussion

Separation and detection

The separation of BPA and BPF was tested on C18 columns using acidic and basic mobile phases during method development. Extracted water samples from baby bottles were also tested before finalizing the method.

An Agilent ZORBAX Eclipse Plus C18 column was used for further experiments. A low temperature (35 °C) of the TCC provided optimal separation of BPA from a closely eluting impurity, however 40 °C was found to be better when analyzing matrix samples. A linear gradient separated the two bisphenols, however a preliminary method robustness study showed large variation when the gradient was modified. A step gradient method was therefore adapted, which gave comparatively robust results. The ASTM method recommended storing bisphenols at low temperature, therefore the autosampler was maintained at 4 °C during the analysis. Figure 3 shows a chromatogram separating the two bisphenols using the final method.
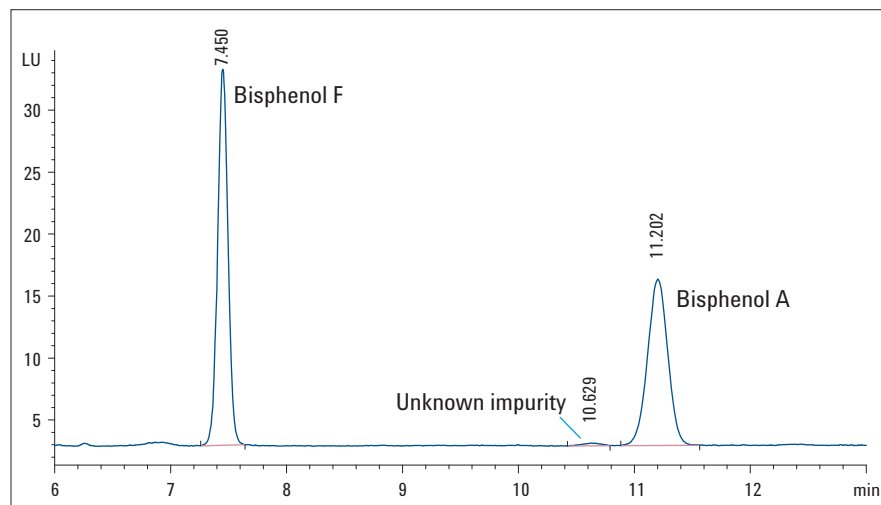

Figure 3
Separation of 30 ng/mL solution of bisphenol F and bisphenol A using an Agilent ZORBAX Eclipse Plus C18 column. The chromatogram was collected using FLD settings of excitation at 230 and emission at 316 nm.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The analyte concentration that provides a signal-to-noise ratio (S/N) of > 3 was considered as LOD and the analyte concentration with S/N ratio > 10 was considered as LOQ. A peak-to-peak method was used to calculate noise. Figure 4 shows a chromatogram of BPA at the LOQ level overlaid with a blank (mobile phase) injection. For BPA, the LOD was 0.19 ng/mL with S/N = 4.3 and LOQ was 1.06 ng/mL with S/N = 15.1.

Linearity

Calibration curves with linearity range (see Table 2) were prepared using an Agilent 7696A Sample Prep WorkBench. The WorkBench automates the sample handling, providing consistent results. Different sets of linearity ranges can be prepared by simply rerunning the program. The linearity levels were established starting from the LOQ level of BPA. LOD and LOQ values, along with the linearity results are included in Table 4. LOD and LOQ values can be further decreased by increasing the injection volume but it was not necessary for this application since the values obtained from baby bottles were found to be within the linearity range.

Precision of retention time (RT) and area

The area precision was measured as RSD(%) across the linearity levels. The maximum RSD value of 5.6% and 7.2% for level 1 (L1) were obtained for BPA and BPF respectively. Similarly, RT precision calculations showed a maximum RSD value of only 0.14% and 0.11% for the BPA and BPF. Graphical representation of area RSD values are shown in Figure 5.

SI no.	Name	LOD ng/mL	S/N	LOQ ng/mL	S/N	Linearity range	R^2 value	No. of levels
1	Bisphenol F	0.19	5.1	0.46	12.4	1.06 –171.43	0.99999	7
2	Bisphenol A	0.19	4.3	1.06	15.1	1.06–171.43	0.99998	7

Table 4
LOD, LOQ and linearity for BPA and BPF. Samples were prepared using an Agilent 7696A Sample Prep WorkBench. BPA levels found in polycarbonate baby bottles were within the linearity range.
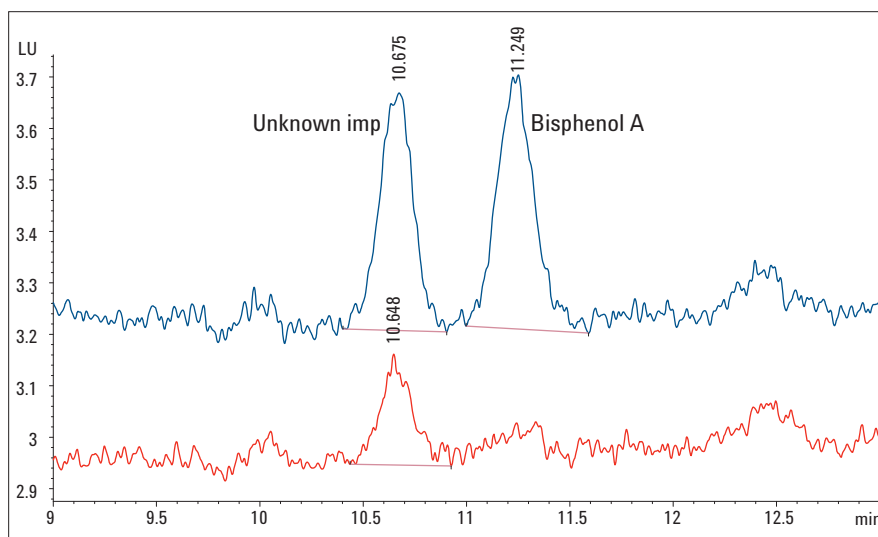

Figure 4
A 20 µL injection of LOQ level, 1.06 ng/mL (21 pg on column) solution, of bisphenol A overlaid with blank injection. S/N ratio obtained at this concentration was 15.
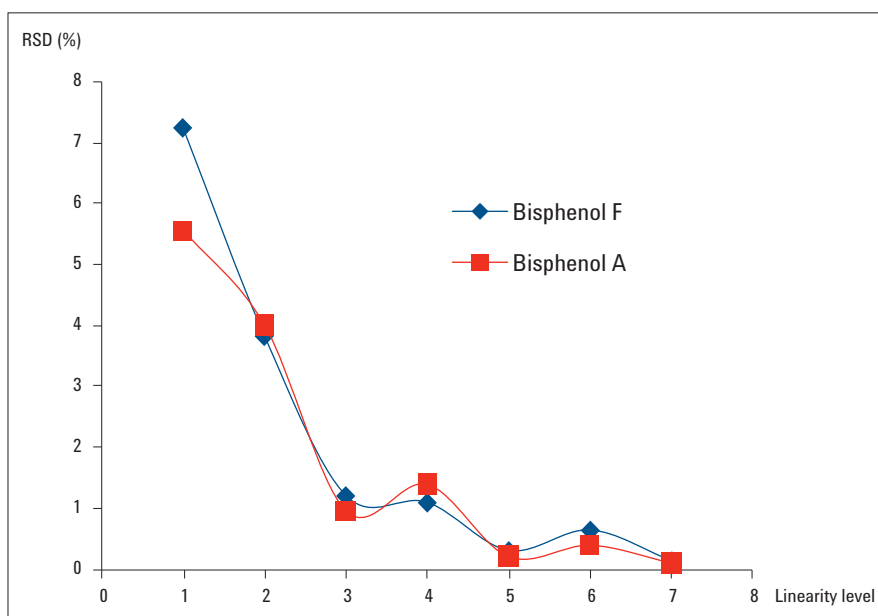

Figure 5
Area precision measured as RSD(%) for six replicates at each concentration level for BPA and BPF.

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Robustness

To test the robustness of the method, a standard solution containing 30 ng/mL of BPA and BPF was used. Six critical method parameters (flow rate, column temperature, injector volume, excitation and emission wavelength, step gradient and buffer concentration) were varied separately and data were collected for seven replicate injections. The compound peak areas from the last six replicates were compared for analysis. The allowed deviation for the area and retention time was set to ± 5% and ± 3% respectively.

The results of the robustness tests are summarized in Table 5. The red numbers indicate where the result exceeded the allowed deviation. A flow rate change of +2% resulted in a decreased area of the two bisphenols. Specifically for bisphenol A, the peak area was found to have the negative deviation caused by 2.5% change in column temperature. The resolution of BPA compared to the impurity (see RT 10.6, Figure 3) showed poor results when the temperature was increased and improved results when the temperature was decreased to 35 °C. A temperature of 40 °C was found to be better for sample analysis. Robustness results show the importance of maintaining the column temperature during the analysis. Setting the FLD excitation at 230 nm and emission at 316 nm was found to be ideal, as it displays the maximum peak area. The emission setting of 316 nm is robust since a variation of 3 nm does not deviate the area percentage from the allowed limit. The excitation wavelength however, needs to be controlled. A change in buffer concentration is also critical as a deviation of 10% decreases the peak area for both BPA and BPF. Robustness results indicate that the method is reliable for normal usage and to a great extent the performance remains unaffected by deliberately changing method parameters. However, some parameters are critical and must be carefully controlled.

Recovery from sample matrix

BPA-free baby bottles were used as blank matrix. The recoveries of the BPA and BPF were tested by spiking experiments in duplicates. A low standard spike contained BPA (30 ng) and BPF (30 ng) each spiked into 50 mL water extracts of BPA-free bottles. Another high standard spike consisted of BPA (50 ng), BPF (50 ng) each spiked into a 50 mL water extracts from BPA-free bottles. The analytes were extracted from the water sample as described above. Using the aqueous linearity curve (see section Linearity), the area was converted to concentration values. The low and high concentration values were compared against the theoretical value. The recovery experiment results are shown in Table 6. BPA shows a recovery value of 80% at the high concentration value. The value of 80% is higher than the value reported in the ASTM method where an average single laboratory results shows a recovery of 70%.

Compound name	Recovery low conc. (%)	Recovery high conc. (%)
Bisphenol F	70.2	75.9
	70.1	74.1
Bisphenol A	76.9	79.6
	75.1	81.1

Table 6
Recovery results from spiking experiments performed in duplicates.

Parameters	Changes		BPF % area	% RT	Resolution of BPA with unknown	BPA % area	% RT
Flow: 0.9 mL/min ± 2%	High:	0.92 mL/min	-4.6	-1.2	1.9	-5.1	-1.2
	Low:	0.88 mL/min	0.1	1.9	1.9	-1.9	2.2
TCC: 40 °C ± 2.5%	High:	41 °C	-4.2	-0.4	1.7	-5.0	-0.6
	Low:	39 °C	-3.1	0.9	2.1	-10.0	1.3
Injector: 20 µL ± 5%	High:	21 µL	2.6	0.2	1.9	0.0	0.2
	Low:	19 µL	-7.6	0.1	1.9	-9.8	0.1
Wavelength: 230–316 ±3 nm	233–316		-2.2	0.0	1.9	-5.7	0.0
	227–316		-7.0	0.2	1.9	-4.6	0.2
	230–319		-3.2	0.1	1.9	-4.5	0.1
	230–313		-3.5	0.1	1.9	-3.0	0.1
Step gradient starting point: 2 min ±10%	High:	2.2 min	-3.8	2.9	1.9	-4.1	2.0
	Low:	1.8 min	-3.3	-2.4	1.9	-3.8	-1.5
Buffer concentration: 10 mM ±10%	High:	11 mM	-4.2	0.2	1.9	-6.0	0.2
	Low:	9 mM	-5.9	0.1	1.8	-9.8	0.1

Table 5
Robustness test method results compared to the standard method at concentration of 30 ng/mL. The red values in the table indicate that the deviations exceeding the allowed limits of 5% for area and 3% for retention time.

Sample analysis

The content of BPA and BPF in baby bottles was determined using the extraction procedure and the developed chromatographic method. Baby bottles labeled as brand 1, brand 2, and brand 3, were analyzed in duplicates. The results of the analysis were compared against the calibration curve prepared prior to sample analysis. Blank water samples subjected to SPE did not show BPA, suggesting that no BPA leached out of plastics used in the experiment[3]. Different amounts of BPA were detected in the three brands of baby bottles (see Figure 6A). The BPA emission spectra from the standard was overlaid with the spectra from the sample. A good overlap was observed confirming the presence of BPA (see Figure 6B). Different brand analysis showed a high concentration of 4 ng/mL while a low value of 0.5 ng/mL (see Table 7). These values are consistent with those observed earlier by Sun *et al.*, who showed a value of 0.6 ng/mL[5]. If a baby of 10 kg were to drink 250 mL from brand 2 baby bottle, the baby would consume 0.1 µg/kg/day of BPA. This value is less than the reference dose of 50 µg/kg/day established by the EPA but is of concern according to some other studies[6]. The results also show that BPF was not detected in any bottle.

Compound name	BPF (ng/mL)	BPA (ng/mL)
Brand 1	0	0.76
	0	0.52
Brand 2	0	4.26
	0	4.46
Brand 3	0	2.08
	0	2.58

Table 7
Concentration of BPA and BPF extracted in 250 mL water from different brands of baby bottles.
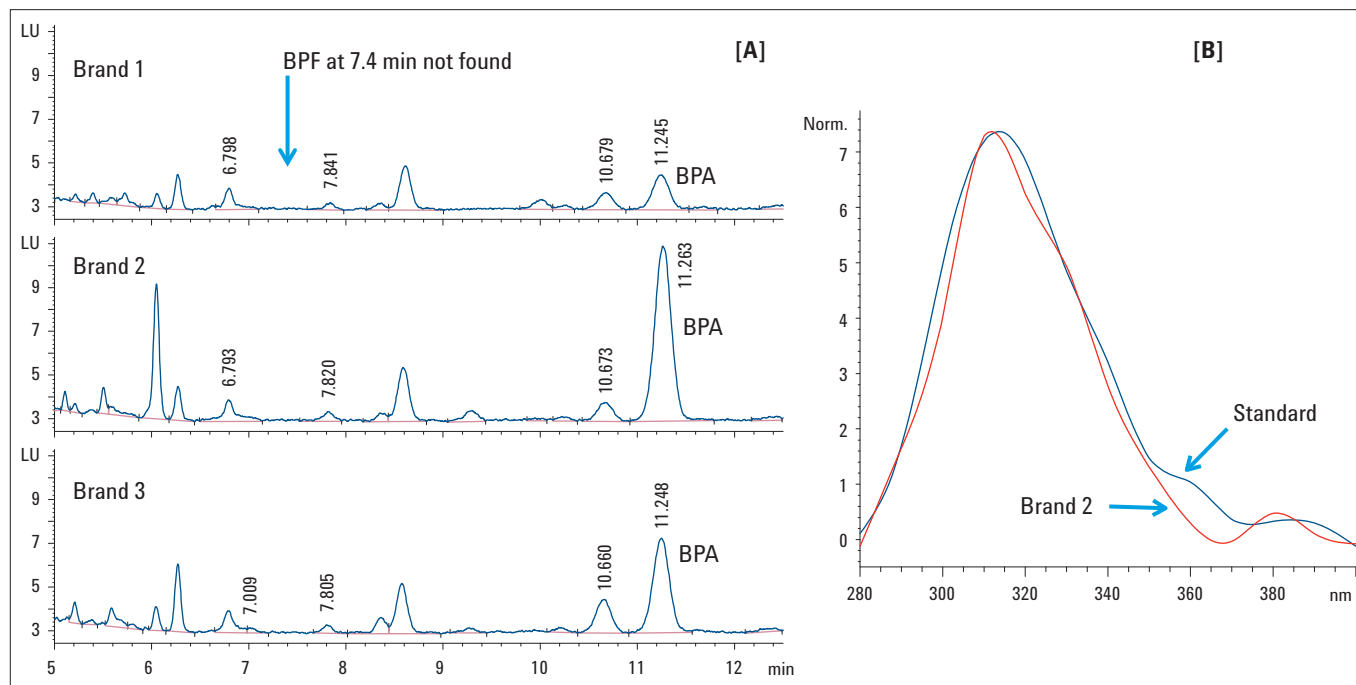

Figure 6
[A] The overlay of chromatogram from three different baby bottles analyzed for BPA and BPF. [B] The overlay of the emission spectrum of BPA from standard and that obtained from brand 2 sample.

UHPLC method

The HPLC method was transferred to an UHPLC method on an Agilent 1290 Infinity LC system keeping the same run time as shown in Figure 7. The transfer was performed to study the effect on resolution and sensitivity. The UHPLC method used the same mobile phase, gradient, and detector settings. The column dimensions were kept the same but the particle size was reduced from 5 µm to 1.8 µm for the UHPLC method. The peaks in the UHPLC method elute about 1.2 minutes earlier, which is due to the lower delay volume in an Agilent 1290 Infinity LC system. The UHPLC method also showed narrower peaks and better resolution compared to the HPLC method. The peak properties such as peak area, peak height, peak width, resolution and S/N at the lowest linearity level L1 and the highest linearity level L7 are compared in Table 8. The results show that resolution of BPA increased from 1.9 in the HPLC method to 2.5 in the UHPLC method. The S/N ratio almost doubled thereby adding sensitivity and allowing the possibility to redefine the LOQ and LOD levels.
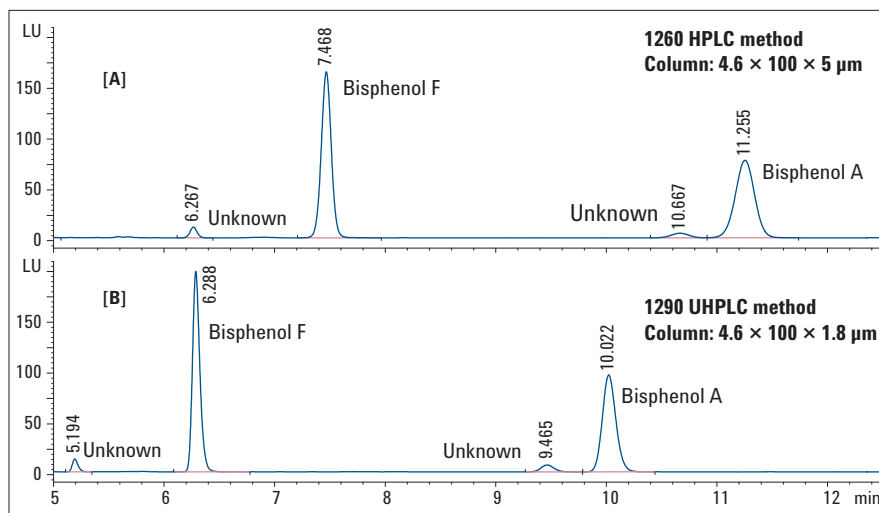

Figure 7
An overlaid chromatogram of the HPLC method [A] and UHPLC method [B] separating the level 7 standards for BPA and BPF on an Agilent ZORBAX Eclipse Plus C18 4.6×100 column. A 5 µm particle size was used for HPLC method while 1.8 µm was used for UHPLC method.

Compound name (Level)	HPLC method					UHPLC method				
	Peak area	Peak width at half height	Peak height	Resolution	S/N	Peak area	Peak width at half height	Peak height	Resolution	S/N
BPF (L7)	1037.0	0.10	163.7	-	3683.0	930.5	0.07	199.6	-	6784.8
BPA (L7)	934.1	0.19	76.3	1.9	1715.7	825.8	0.13	96.4	2.5	3276.7
BPF (L1)	7.5	0.10	1.1	-	27.4	7.2	0.07	1.4	-	42.6
BPA (L1)	8.1	0.20	0.6	1.8	15.1	9.4	0.14	1.0	2.5	31.6

Table 8
Comparison of peak area, peak width at half height, peak height, resolution and S/N ratio between HPLC and UHPLC method from first level and last linearity level. The UHPLC method provides better sensitivity and resolution compared to the HPLC method.

The calibration for BPA and BPF, when using the same calibration levels (see Table 2) was found to be linear - R^2: 0.99991 for BPF and R^2: 0.99993 for BPA. RSD(%) deviation on area and RT was calculated for all concentration levels. The results show that RSD(%) on area deviation was comparatively lower in the UHPLC method. As shown in Figure 8, a value of 3.0% was found for level 1 for BPA. The maximum RSD of RT for both BPA and BPF was less than 0.1%.

Conclusion

Bisphenol A and bisphenol F were separated and quantified using an Agilent 1260 Infinity LC system and an Agilent ZORBAX Eclipse Plus C18 column. Calibration standards were prepared using the Agilent 7696A Sample Prep WorkBench. A method was developed and partially validated. This method quantifies bisphenol A and bisphenol F from various baby bottles with 80% recovery values. The method can be applied to determine BPA and BPF levels for quality control of baby bottles. A method transfer to an Agilent 1290 Infinity LC system was effectively carried out by keeping the same detector and method conditions. Both HPLC and UHPLC methods were linear and give precise results. The UHPLC method however showed better resolution, S/N ratio, narrower peak width and increased peak height compared to the HPLC method.
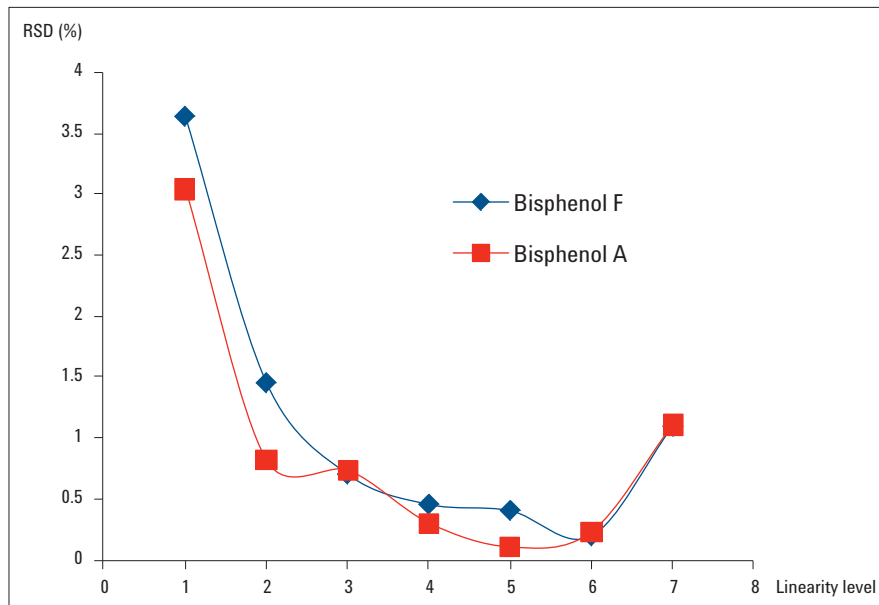

Figure 8
Area precision measured as RSD (%) for BPF and BPA with UHPLC. Six replicates at each concentration level were measured.

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