LABORATORY INFORMATION BULLETIN

Quantitative Determination of Bisphenol-A in Tuna using Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT

A sensitive, reliable, and convenient method was developed for the confirmation and quantification of bisphenol-A (BPA) using liquid chromatography tandem mass spectrometry. Studies have shown that traces of BPA can leach out of epoxy resin (i.e. used in food packaging materials like cans) under various environmental conditions (such as heat during processing and storage) and are eventually consumed by humans.

The Pacific Southwest Laboratory Food and Feed Laboratory (PSFFL) modified and optimized method parameters of previously published Laboratory Information Bulletins #4603[1] and #4495[2] to adequately detect and quantitate BPA in canned tuna samples. During the method development stage, it was concluded that a phenyl column and high pH mobile phase would assist in improving BPA chromatography. Additionally, the Center for Food Safety and Applied Nutrition (CFSAN) previously published an extraction and analytical method for BPA analysis in infant formula in 2010[3]. CFSAN further simplified the extraction method and expanded the analysis to various canned food products in 2011[4].

The proposed method, using the Irvine Rapid Analytical Method (IRAM) extraction procedure to analyze BPA in tuna samples, was validated at three fortification levels: 6 ng/g, 60 ng/g, and 120 ng/g. The calculated recoveries are within the range of 80-103%. Calculated RSD values ranged from 1-12%. The validated method demonstrates that it is reliable, accurate, and precise for the analysis of bisphenol-A in tuna.

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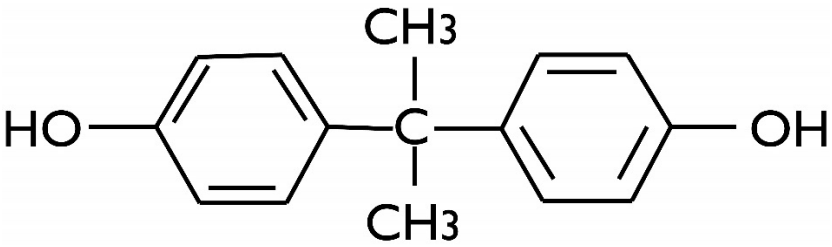
INTRODUCTION

Bisphenol-A (BPA) is a polyphenolic compound that is used in the manufacture of some food can liners and is a component of polycarbonate plastics (Scheme 1). The FDA has issued an assessment of any health hazards associated with BPA exposure, and has concluded that current regulatory levels are safe and there is no risk to the US consumer[5]. The FDA strives to have modern and accurate methods for the monitoring of target analytes should a surveillance program be requested. In this report, we describe a method developed to monitor BPA in canned tuna. PSFFL received canned tuna samples with a request to determine the level of BPA in the edible portion. This method was developed to analyze those samples and validated to allow for use on potential future samples.

Scheme 1. Chemical structure of bisphenol A.



The Center for Food Safety and Applied Nutrition (CFSAN) developed an extraction and analytical method for BPA analysis in infant formula in 2010[3]. In 2011, CFSAN simplified the extraction method and expanded the analysis to various canned food products[4]. The studies included FTIR analysis for the coating film of cans and a liquid chromatography mass spectrometry method for the brine and food contents.

Pacific Southwest Food and Feed Laboratory (PSFFL) used the Irvine Rapid Analytical Method (IRAM) extraction procedure to analyze BPA in tuna samples. The IRAM is an effective and reliable extraction method for the analysis of fatty food matrices[1,2]. The method was previously validated for fish but only included pesticides; thus, the extraction procedure developed by PSFFL extended the sample preparation specifically to BPA. While the analytical range of research methods seen in the literature[3,4] can detect at the high parts-per-trillion (ppt) level, for the routine regulatory analysis of BPA in tuna, the ability to detect and quantify low parts-per-billion (ppb) levels of BPA was determined to be satisfactory. This study was not designed to test the coating film of cans for BPA; it was conducted to analyze BPA in tuna using LC-MS/MS.

EXPERIMENT

Equipment and Materials

Note: Suppliers and part numbers provided denote products used in this method validation. Other suitable products may be available.

Equipment

a) 50 mL Corning® polypropylene centrifuge tubes
b) Thermo Scientific 0.45 µm Filter (Nylon, Catalog No. 44525 NN)
c) Kimble® KIMAX 15mL Conical Centrifuge Tube (Catalog No. 45164-15)
d) UTC QuEChERS Centrifuge Tube Extraction Product (Catalog No. ECQUUS150CT)
e) Homogenizer (SPEX Sample Prep Geno/Grinder 2010)
f) Fisherbrand™ Digital Vortex Mixer
g) Centrifuge (Eppendorf Centrifuge 5804/R)
h) VWR RapidVap® Vertex™ Dry Evaporator
i) Phenomenex KrudKatcher™ ULTRA HPLC In-Line Filter 0.5µm Depth Filter x 0.004in ID (Part No. AF0-8497)
j) Waters XBridge® Phenyl 3.5µm 3.0mm x 100mm column (Part No. 186003328)

Reagents and Standards

a) Acetone, HPLC Grade Fisher (Catalog No. A949-4)
b) Petroleum Ether, Optima™, Fisher (Catalog No. E120-4)
c) Acetonitrile, Optima™, Fisher (Catalog No. A955-4)
d) Water, Optima™, Fisher (Catalog No. W6-4)
e) Bisphenol-A (Sigma Aldrich, 239658)
f) Bisphenol-A-(*diphenyl*-$^{13}C_{12}$) (Cambridge Isotope Laboratories, CLM-4325-1.2)
g) BADGE*2H2O, {2,2-[bis-4-(2,3-dihydroxypropoxy)phenyl]propane} (Sigma Aldrich, 15137)

Standards Preparation

a) Internal Standard (ISTD): The isotope Bisphenol-A-(*diphenyl*-$^{13}C_{12}$), is received in acetonitrile at 100 µg/mL. Dilute 0.3125 mL of standard to 25 mL in acetonitrile to make a final concentration of 1250 ng/mL.
b) BPA stock standard: Prepare one stock standard by weighing BPA neat standard and dissolving it in acetone (BPA is also stable in water or acetonitrile). Final concentration of stock standard should be 1000 µg/mL. To create the ICV described in part (e) below, make a second stock solution from a separate neat standard, preferably from a different vendor.
c) BPA working standards (calibration curve): Using the BPA stock solution prepared in section (b) above, prepare stock solutions at two different concentrations to be used in Table 1. Prepare working standards by diluting an aliquot of BPA stock standard in acetonitrile to 300 ng/mL (used for standard 1-4) and 6000 ng/mL (used for standard 5-8).

For example, to make 25 mL of each working standard, dilute 7.5 µL of BPA stock standard up to the mark on a 25 mL volumetric flask with acetonitrile for the 300 ng/mL solution. For the 6000 ng/mL working standard, follow the same procedure to bring 150 µL of the stock solution up to the line in a 25 mL volumetric flask with acetonitrile. Use these two working standards to create the calibration curve by following the instructions in Table 1. The solvent is 1:1 HPLC grade water: acetonitrile.

d) BPA working standards (spiking solutions): Using the BPA stock solution, prepare three stock spiking solutions in acetonitrile at 150 ng/mL (low), 300 ng/mL (med), and 1500 ng/mL (high). For example, make up each spiking solution in separate 50 mL volumetric flasks by adding BPA stock solution at the following volumes and bringing it to the line with acetonitrile: 7.5 µL, 15 µL, and 75 µL.

e) Initial Calibration Verification (ICV): Make up a second working standard at 6000 ng/mL as described in part (c) above from the second stock solution described in part (b). From this second 6000 ng/mL working solution, prepare a working standard as described in Table 1, standard 6.

f) Continuing Calibration Verification (CCV): Prepare a second working standard as described in Table 1, standard 6.

Table 1. Standard preparation for calibration curve.

Level	Working Std used (ng/mL)	Amount of working standard added (µL)	Amount of IS added (µL)	Final Volume with Solvent (mL)	Final Concentration (ng/mL)
Standard 1	300	33.3	400	10.0	1
Standard 2	300	200	400	10.0	6
Standard 3	300	400	400	10.0	12
Standard 4	300	1000	400	10.0	30
Standard 5	6000	100	400	10.0	60
Standard 6	6000	200	400	10.0	120
Standard 7	6000	1000	400	10.0	600
Standard 8	6000	2000	400	10.0	1200
Internal Standard (IS): 1250 ng/mL					
Solvent: 1:1 HPLC grade Water: Acetonitrile					

Extraction Method

1. Weigh 5g of composited sample into a 50 mL centrifuge tube
2. Add 16 mL of Acetone
3. Vortex for 10 seconds
4. Add 4 mL of HPLC grade water
5. Vortex for 10 seconds
6. Shake the sample mixture using a Geo-Grinder at 500 strokes/min. for 2 min.
7. Centrifuge for 5 min. at 5000 rpm.

8. Pour supernatant (approximately 15 mL) into a 50 mL centrifuge tube containing 1 g PSA (primary secondary amine sorbent), 2 g C-18, 4 g Fructose, 4 g $MgSO_4$, 8 g NaCl. Shake and vortex the mixture for approximately 10 sec.
9. Add 1 mL of Petroleum Ether
10. Shake and vortex for approximately 10 sec.
11. Centrifuge the mixture for 5 min. at 5000 rpm.
12. Decant supernatant in to KIMAX 15mL Conical Centrifuge Tube. Concentrate the extractant down with Nitrogen gas to 0.5 mL using VWR Evaporator at 50°C for approximately 30 min.
13. Add ~4.5 mL 1:1 water and Acetonitrile, bringing the extract to 5.0 mL and vortex.
14. Filter extraction through a 75 mL reservoir fitted with a 0.45 µm Nylon syringe filter; elute with air pressure into 2 mL glass vial with PTFE lined cap.

Instrumentational Method

1. HPLC: Shimadzu Prominence Liquid Chromatograph (CBM-20A Controller, LC-20ADXR Pump, SIL-20ACXR Autosampler, CTO-20AC Column oven). See parameters in Tables 2 and 3.
   Mobile Phase A: Water + 5mM Ammonium Acetate (LC-MS Grade)
   Mobile Phase B: Acetonitrile (LC-MS Grade)
2. Mass spectrometer: AB Sciex QTrap 5500. See Tables 4 and 5 for MS/MS parameters.

Table 2. Shimadzu HPLC parameters used for the detection of BPA.

Equilibration Time (min):	0.1
Injection Volume (µL):	20
Total Flow (mL/min):	0.5
Rinsing Volume (µL):	500
Rinsing Speed (µL/sec):	25
Sampling Speed (µL/sec):	15
Cooler Temperature (°C):	15
Column Oven Temperature (°C):	45

Table 3. HPLC gradient for the detection of BPA.

Time	*Event*	*Parameter*
1.00	Pump B	20
2.00	Pump B	65
4.00	Pump B	75
5.00	Pump B	95
6.00	Pump B	95
6.10	Pump B	75
8.00	Pump B	65
9.00	Pump B	20
11.00		STOP

Table 4. Electrospray source conditions for the analysis of BPA.

Parameter	*Value*
Curtain gas	28
Collision Gas	Medium
Ion Spray Voltage	-4000 V
Gas 1	70
Gas 2	65
Temperature	550ºC

Table 5. MRM transitions used for the identification of BPA. The quantitation ion is listed as transition 1

Transition	**Q1**	**Q3**	**DP**	**EP**	**CE**	**CXP**
BPA 1	227	212	-80	-10	-28	-20
BPA 2	227	133	-80	-10	-36	-7
$^{13}C_{12}$ BPA (BPA IS)	239.1	224	-80	-10	-28	-20

RESULTS AND DISCUSSION

The objective of this LIB was to develop a sensitive, reliable, and fit for use method for the extraction and quantitation of BPA using a LC-MS/MS. The experiment design and validation meet the requirements of the Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 2nd Ed, as a Level Two Validation[6]. The validation was performed over two days by two different analysts. The validation study included three tuna sources: canned, raw, and jarred tuna. Each matrix was fortified with BPA and the internal standard, Bisphenol-A-(*diphenyl*-$^{13}C_{12}$), at three levels in triplicate: 6 ng/g (low), 60 ng/g (medium), and 120 ng/g (high). Samples were fortified with BPA working standards made up at 150 µg/mL (low), 300 µg/mL (medium), and 1500 µg/mL (high). The acceptable limit of detection (LOD) and limit of quantitation (LOQ)were set at 2 ppb and 6 ppb, respectively.

A matrix blank, solvent blank, and double blank (solvent blank not fortified with internal standard) were injected and analyzed with each analytical batch of samples. Due to the ubiquity of BPA-laden plastics in the laboratory environment, the instrument was cleaned prior to the start of the validation study by running fresh acetonitrile through the LC-MS system. Additionally, all glassware was cleaned and rinsed with acetonitrile prior to use. No BPA was observed in solvent blank or double blank samples at least to the detection limit of BPA. This suggests that the instrument cleaning protocol was successful (Figure 1a and b). To semi-quantify any potential BPA contamination from labware or the instrument, solvent blanks were spiked with internal standard and analyzed without undergoing extraction (Figure 1c and d). Other solvent blanks spiked with internal standard underwent extraction and were analyzed (Figure 1e and f). In both cases, the samples were compared against a 1 ppb standard. Results from both types of blanks

were well below the LOD and could likely be attributed to ^{12}C contribution from the internal standard. Potential tuna sources were first analyzed for incurred residues to ensure minimal interference from incurred BPA. While all three tuna matrices showed the presence of some BPA, the level in each source was at or below the LOQ (Figure 1g and h). Several peaks at different retention times can also be observed in the tuna matrices; these are likely due to BPA analogs also present in can linings, but the peaks were not further investigated.

Chromatograms of BPA in solvent (Figures 2 & 3) and in canned tuna (Figure 4) demonstrate good signal-to-noise and baseline separation from potentially interfering peaks (discussed in greater detail below). The sharpness of the chromatographic peaks could be attributed to the pi-pi interaction between the phenyl column and BPA compound. Additionally, in comparison to commonly used acidic LC-MS conditions, a mobile phase buffered at pH 7 is beneficial for negative ionization.

Table 6. Summary spike recovery and RSD demonstration for BPA.

	Spiking Level	**No. of Recoveries**	**Ave. % Recovery**	**Average RSD (%)**
Canned	Low (6 ng/g)	7	89.6	11.9
	Medium (60 ng/g)	3	83.7	2.3
	High (120 ng/g)	3	80.4	3.5
Jar Tuna	Low (6 ng/g)	3	102.7	7.8
	Medium (60 ng/g)	3	86.4	3.0
	High (120 ng/g)	3	85.5	4.5
Raw Ahi Tuna	Low (6 ng/g)	3	86.4	9.9
	Medium (60 ng/g)	3	90.3	1.3
	High (120 ng/g)	3	96.7	10.1

Prior to calculating the recoveries for spiked samples, the response for a blank matrix analyzed the same day was subtracted from the response of the spiked sample. In practice, area ratios (peak area of the analyte/peak area of the internal standard) for matrix blanks were subtracted from the area ratios of spiked samples. The average percent recovery was calculated for BPA at each fortified level (Table 6) by comparing the blank-corrected response ratios of the spiked samples to the response ratio of the 60 ng/mL standard. Accuracy and precision were demonstrated for each tuna source. All spike recoveries were calculated to be within the acceptable range of 70-130%. The average percent recovery across the fortified three matrix sources was 89% (Table 6). Precision was determined by calculating the relative standard deviation (RSD). The calculated precision for each fortification level was within the acceptable range of less than 20% (Table 6). The calculated precision values varied across all three levels and various tuna sources; however,

the degree of dispersion of the measurements is relatively low. All recoveries from the study can be seen in Table 8.

The limit of detection (LOD) was demonstrated by spiking 6 ng/g of BPA in seven replicates of canned tuna. The LOD was determined using the method outlined in 40 CFR 136 appendix B[7] for method detection limits. Briefly, seven aliquots of canned tuna matrix were fortified at 6 ng/g and carried through the extraction and analysis procedures. After quantitation, the standard deviation of the seven replicates was determined and multiplied by 3.143 (giving the LOD at a 99% confidence level). The limit of quantitation (LOQ) was determined by multiplying the standard deviation of the peak area from six matrix blank measurements by 10. The LOD was calculated as 2.01 ng/g and the LOQ was calculated at 5.02 ng/g. These values meet the acceptance criteria of 2 ng/g (for LOD) and 6 ng/g (for LOQ).

The linear range of the method was determined by injecting eight standards in solvent ranging from 1 ng/mL – 1200 ng/mL. The correlation coefficient (r) was calculated by utilizing AB Sciex Analyst™ software and was determined to be 0.9989. The coefficient of determination (r^2) was determined by squaring the correlation coefficient. The calculated r^2 for the eight-point calibration was determined to be 0.9978 which is above the 0.995 requirement.

More studies were performed to ensure that there was no coelution from interfering species. The authors considered two glycidyl derivatives of BPA: 2,2-[bis-4-(2,3-dihydroxypropoxy)phenyl] propane (BADGE* 2 H_2O) and 2-[4-(2,3-dihydroxypropoxy)phenyl]-2-[4'-hydroxyphenyl] propane (BAMGE* 2 H_2O), that can also leach out from epoxy can liners and have similar MRM transitions to BPA[8]. While there is no commercially available standard for BAMGE* 2 H_2O, there is a standard for BADGE* 2 H_2O. To determine if this method could effectively separate BPA and BADGE * 2 H_2O, both compounds were analyzed together. Analysis of standards in solvent, Figure 5, showed that the BADGE * 2 H_2O peak eluted (~3.27 min.) significantly earlier than BPA (~3.56 min). When both compounds were spiked into a tuna matrix and extracted, the BADGE * 2 H_2O peak was significantly smaller than the BPA peak despite being spiked at a higher level (Figure 6) suggesting the method may have better sensitivity for BPA than for BADGE * 2 H_2O. A second peak eluting between BADGE* 2 H_2O and BPA can be assumed to be BAMGE* 2 H_2O, based on discussions with technical experts[8]. However, without a standard for this compound, the authors cannot state conclusively that this peak is due to BAMGE* 2 H_2O. Overall, BADGE * 2 H_2O did not interfere with BPA detection or quantitation even at low levels.

CONCLUSIONS

PSFFL developed and validated a sensitive, reliable, and convenient extraction and instrumental procedure for the detection and quantitation of BPA in tuna using LC-MS/MS. The updated method is more convenient for implementation in a field regulatory environment. The calculated recoveries, accuracy, precision, LOQs, and LODs are acceptable for the validation study. A potential interference was chromatographically resolved. The results of this validation study demonstrated that this method is suitable for the identification and quantitation of BPA in tuna.

ACKNOWLEDGEMENTS

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Figure 1. Extracted ion chromatograms for different types of blank samples showing the transitions for the BPA quantifier ion (on the left) and the internal standard (on the right). (a,b) Double blank (solvent, not extracted), (c,d) solvent blank (solvent + internal standard, not extracted), (e,f) method blank (solvent + internal standard, carried through extraction procedure), (g,h) matrix blank (canned tuna + internal standard, carried through extraction procedure). Vertical axes were normalized to the internal standard peak.

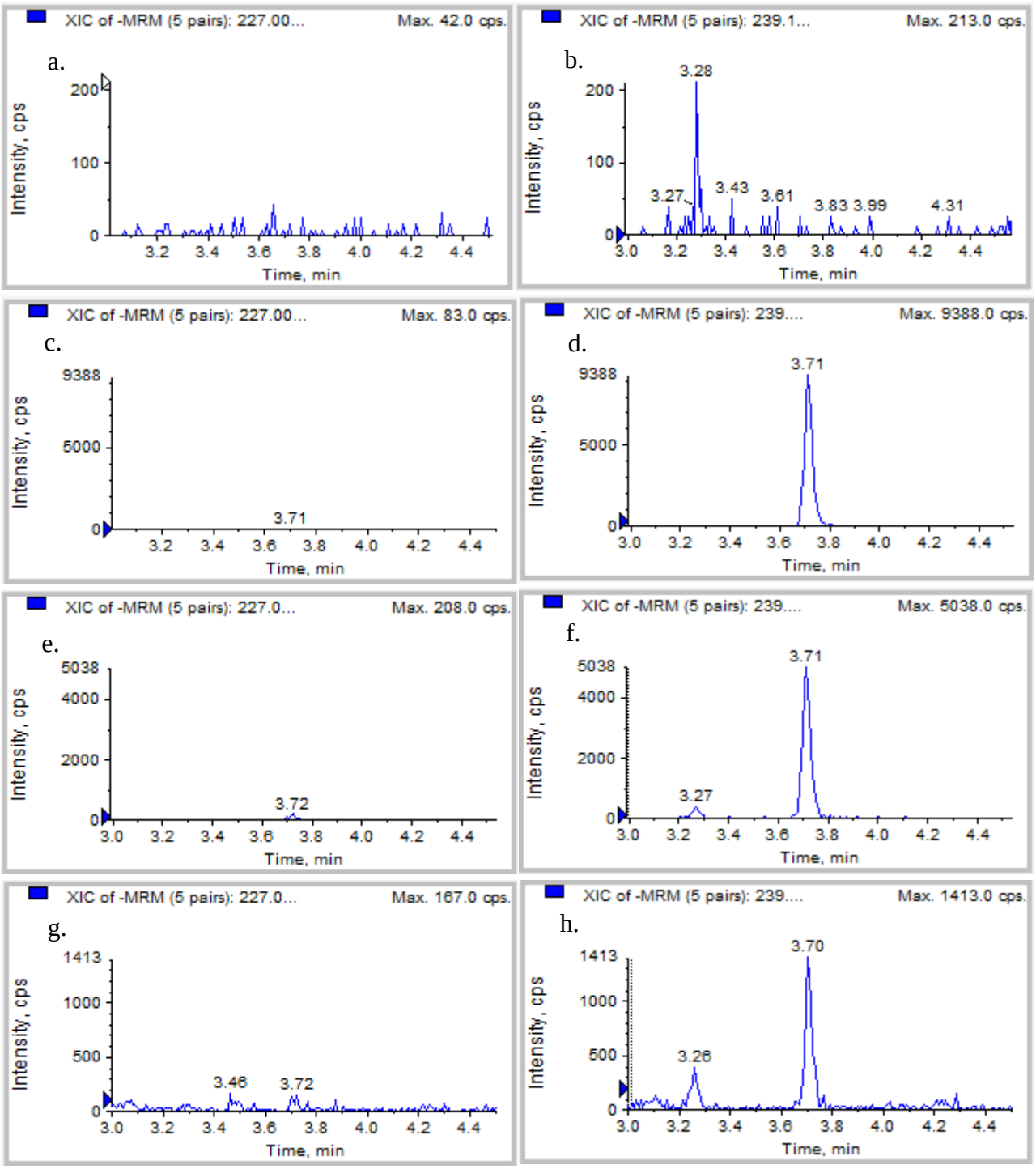


Figure 2. Extracted ion chromatograms of BPA quantifier (a) and confirmation ion (b) from a standard prepared at 6 ng/mL. The internal standard is shown in (c).

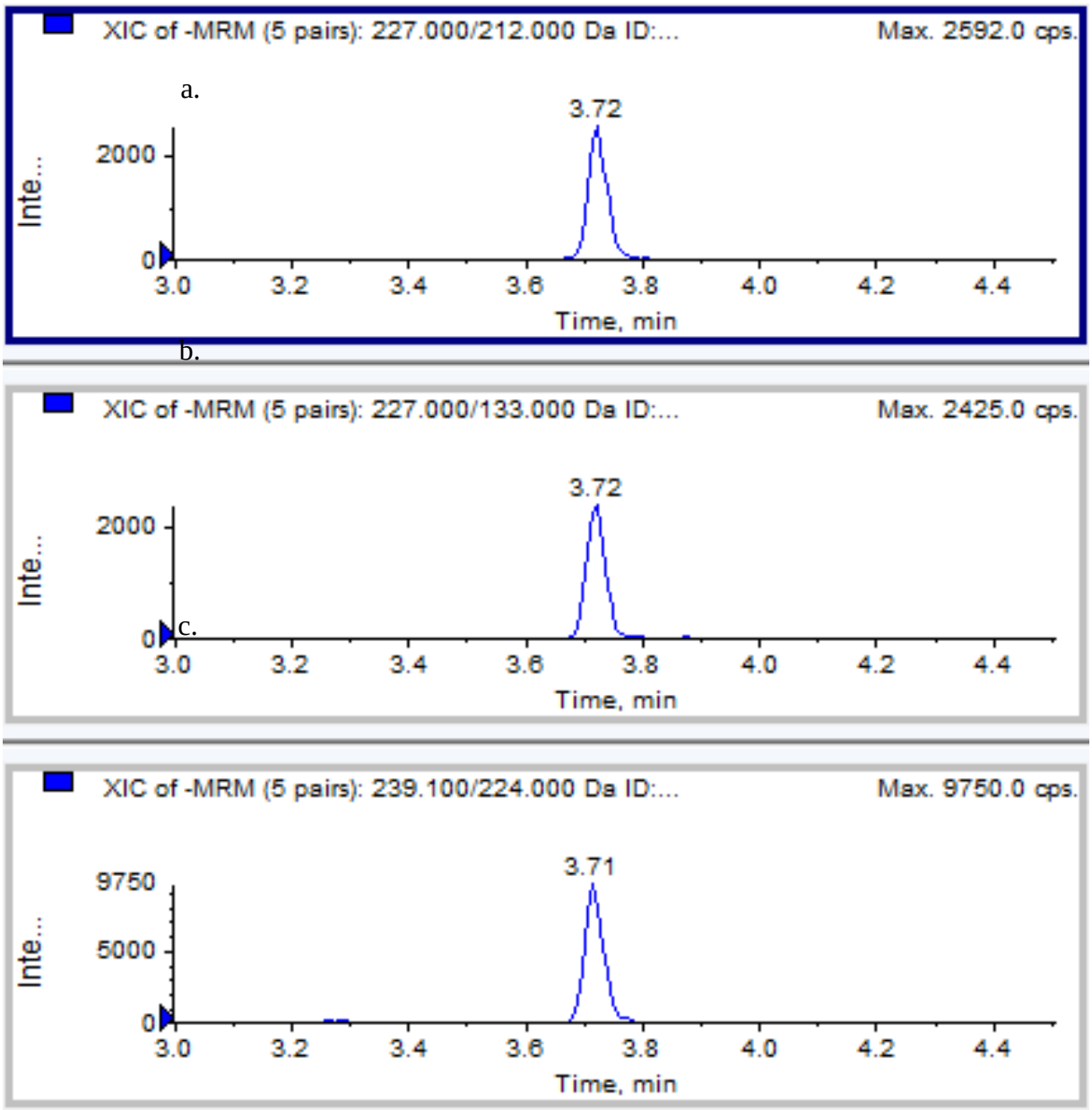


Figure 3. Extracted ion chromatograms of BPA quantifier (a) and confirmation ion (b) from a standard prepared at 120 ng/mL. The internal standard is shown in (c).

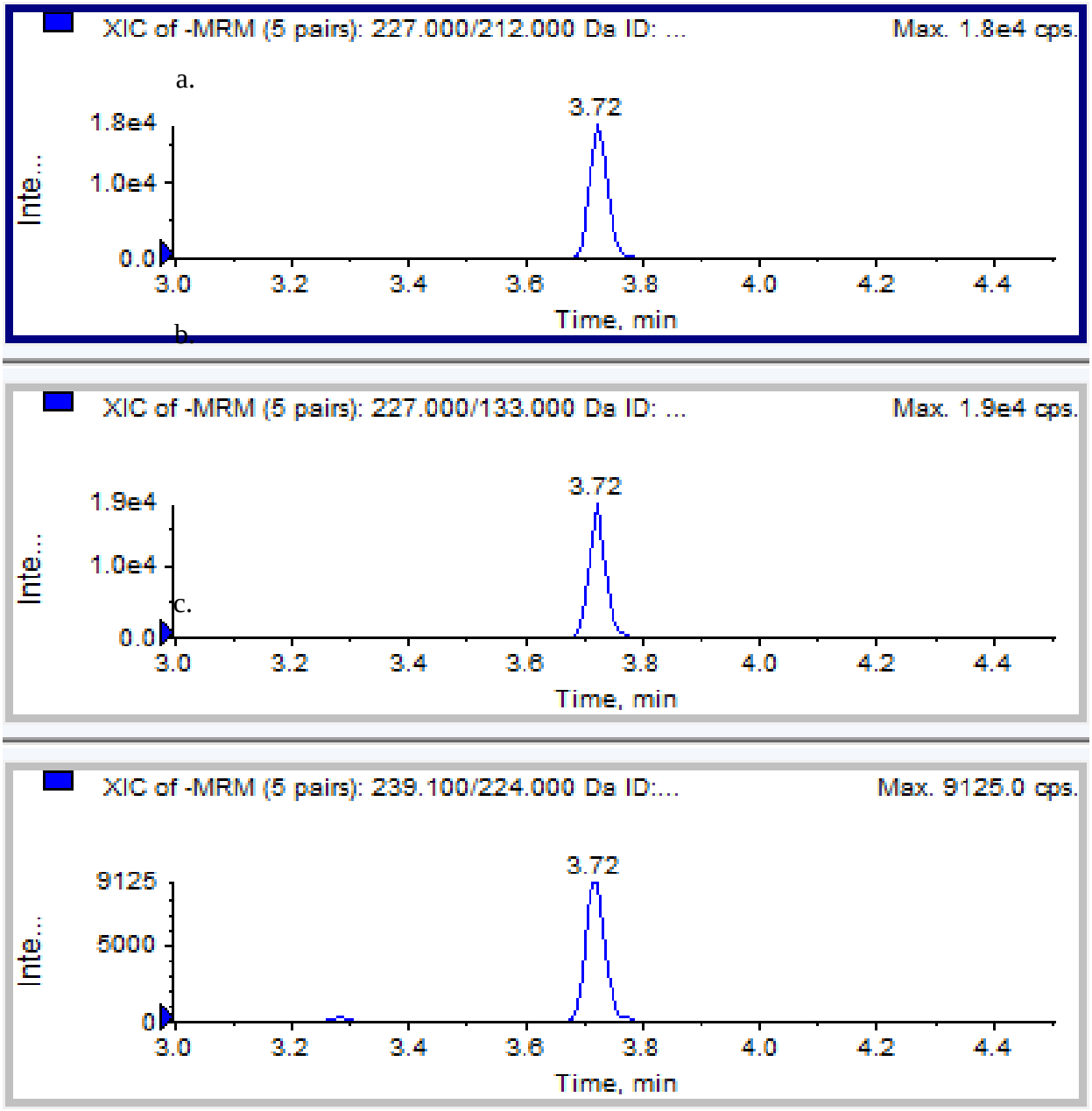


Figure 4. Extracted ion chromatograms of BPA quantifier (a) and confirmation ion (b) from a sample of tuna spiked with 50 ng/mL of BPA. The internal standard is shown in (c).

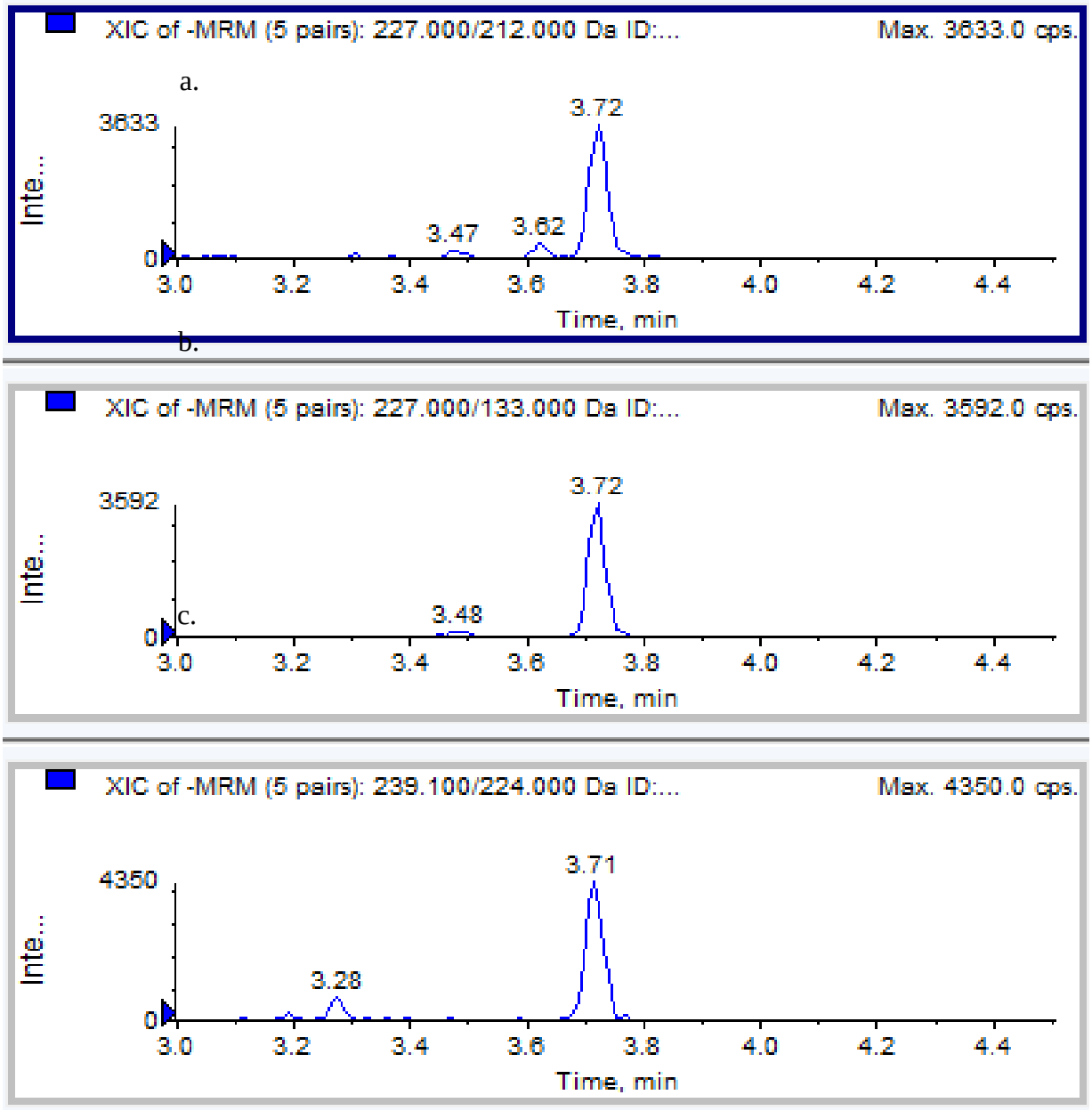
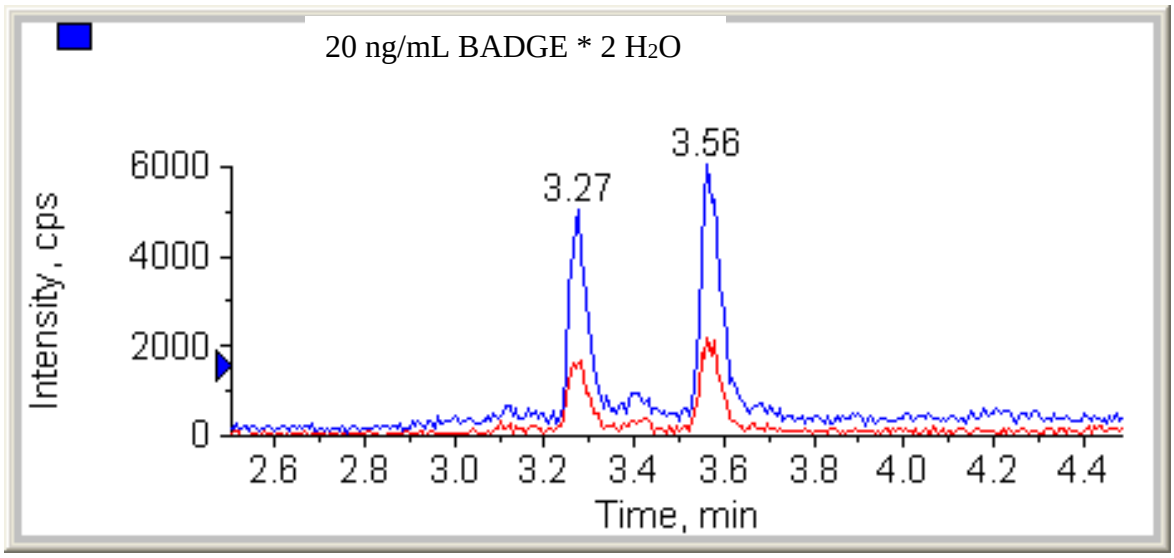


Figure 5. 20 ng/mL and 100 ng/mL standards of BADGE * 2 H_2O showing the increase in the 3.27 min peak and stability of the 3.56 min peak suggesting that the BPA is incurred and not related to BADGE * 2 H_2O.



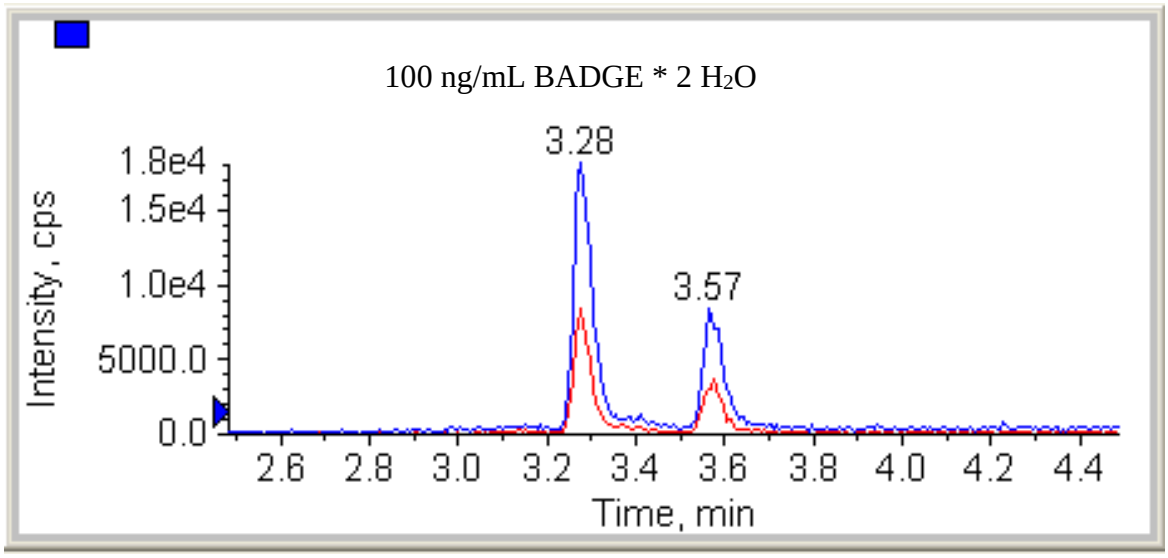
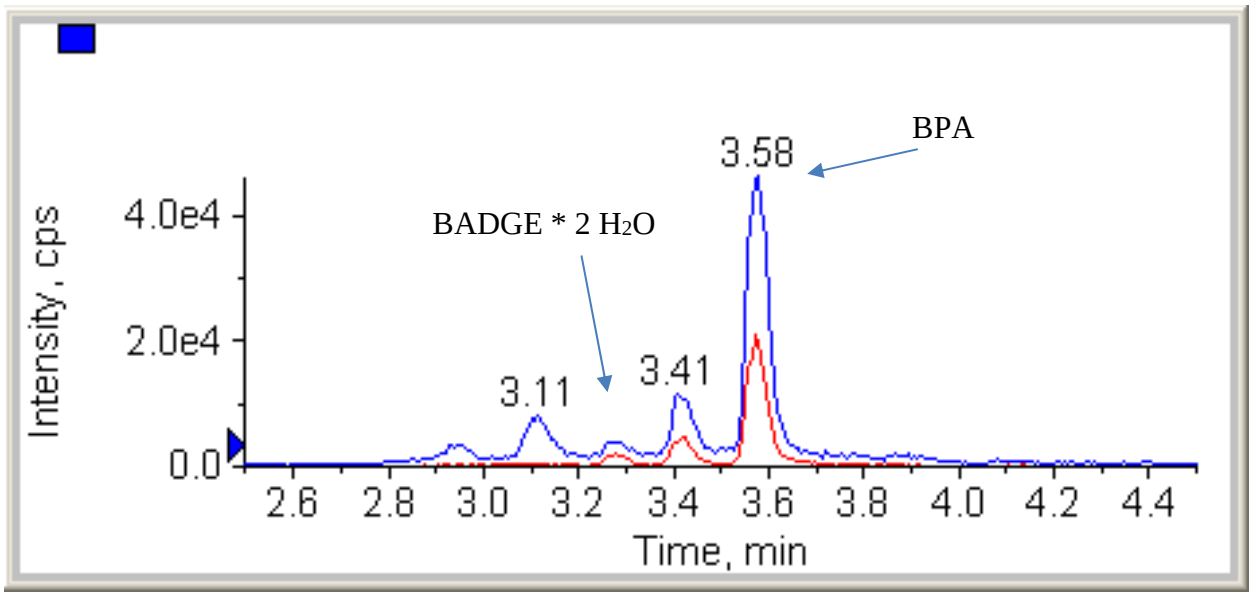


Figure 6. Chromatogram of BADGE * 2 H_2O (spiked at 20 ng/g) and BPA (spiked at 6 ng/g) extracted from tuna. Chromatogram of BPA spiked in tuna (at 6 ng/g) with no BADGE * 2 H_2O present is shown in the bottom chromatogram.



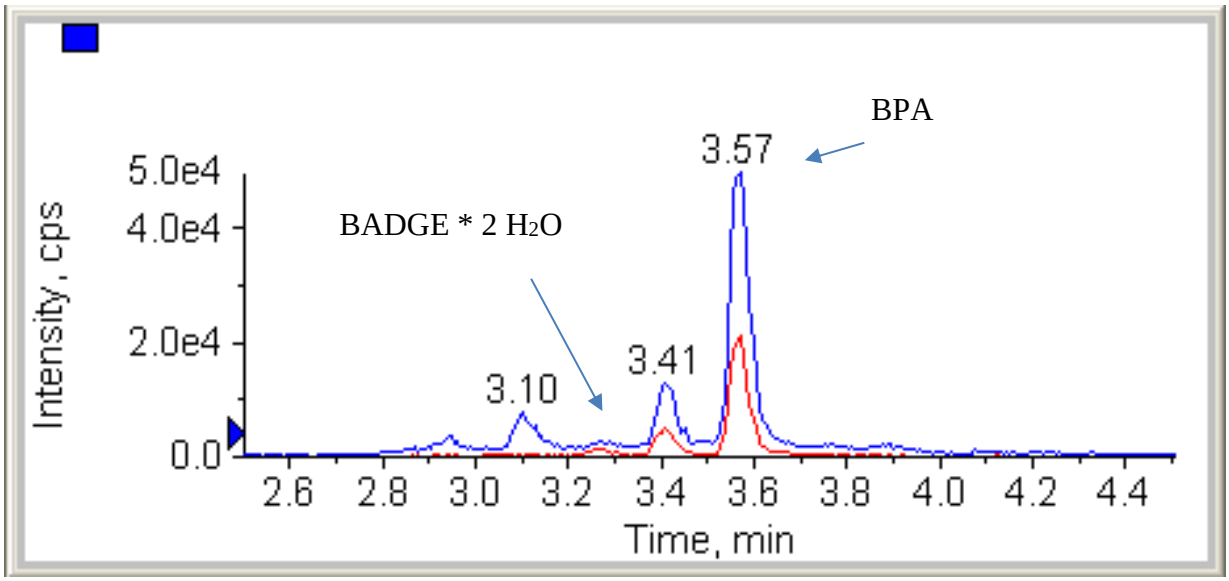


Table 8. Detailed summary of spike recovery demonstrations of three levels in three tuna sources.

	Sample Name	Final Conc. (ng/g)	% Recovery
Can Tuna	Spiked Low 6 ng/g	6.02	100.4
	Spiked Low 6 ng/g	6.10	101.7
	Spiked Low 6 ng/g	5.21	86.98
	Spiked Low 6 ng/g	5.81	96.86
	Spiked Low 6 ng/g	4.43	73.84
	Spiked Low 6 ng/g	4.76	79.44
	Spiked Low 6 ng/g	5.27	87.89
	Spiked Medium 60 ng/g	51.5	85.80
	Spiked Medium 60 ng/g	49.2	82.07
	Spiked Medium 60 ng/g	50.0	83.32
	Spiked High 120 ng/g	95.9	79.95
	Spiked High 120 ng/g	93.5	77.90
	Spiked High 120 ng/g	100.1	83.40
Jar Tuna	Spiked Low 6 ng/g	5.86	97.63
	Spiked Low 6 ng/g	5.91	98.50
	Spiked Low 6 ng/g	6.72	111.9
	Spiked Medium 60 ng/g	50.1	83.44
	Spiked Medium 60 ng/g	52.3	87.22
	Spiked Medium 60 ng/g	53.0	88.40
	Spiked High 120 ng/g	99.5	82.90
	Spiked High 120 ng/g	100.3	83.62
	Spiked High 120 ng/g	107.8	89.85
Raw Tuna	Spiked Low 6 ng/g	4.59	76.49
	Spiked Low 6 ng/g	5.52	92.00
	Spiked Low 6 ng/g	5.44	90.61
	Spiked Medium 60 ng/g	54.7	91.21
	Spiked Medium 60 ng/g	54.4	90.61
	Spiked Medium 60 ng/g	53.4	89.02
	Spiked High 120 ng/g	128.7	107.5
	Spiked High 120 ng/g	112.8	94.06
	Spiked High 120 ng/g	106.1	88.49