Advances in Environmental Research, *Vol. 4*, *No. 1* (*2015*) *39-48*
DOI: http://dx.doi.org/10.12989/aer.2015.4.1.039

Bisphenol A and the related alkylphenol contaminants in crustaceans and their potential bioeffects

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(*Received May 27*, *2014*, *Revised March 24*, *2015*, *Accepted April 03*, *2015*)

Abstract. Bisphenol A is widely used in plastic and other industrial consumer products. Release of bisphenol A and its analogues into the aquatic environment during manufacture, use and disposal has been a great scientific and public concern due to their toxicity and endocrine disrupting effects on aquatic wildlife and even human beings. More recent studies have shown that these alkylphenols may affect the molting processes and survival of crustacean species such as American lobster, crab and shrimp. In this study, we have developed gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometric (GC-MS) methods for the determination of bisphenol A and its analogues in shrimp *Macrobrachium rosenbergii*, blue crab *Callinectes sapidus* and American lobster *Homarus americanus* samples. Bisphenol A, 2,4-bis-(dimethylbenzyl)phenol and 4-cumylphenol were found in shrimp in the concentration ranges of 0.67–5.51, 0.36–1.61, and < LOD (the limit of detection)-1.96 ng/g (wet weight), and in crab of 0.10-0.44, 0.13-0.62, and 0.26-0.58 ng/g (wet weight), respectively. In lobster tissue samples, bisphenol A, 2-t-butyl-4-(dimethylbenzyl)phenol, 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol, 2,4-bis-(dimethybenzyl)phenol, 2,4-bis-(dimethylbenzyl)-6-t-butylphenol and 4-cumylphenol were determined at the concentration ranges of 4.48-7.01, 1.23-2.63, 2.71-9.10, 0.35-0.91, 0.64-3.25, and 0.44-1.00 ng/g (wet weight), respectively. At these concentration levels, BPA and its analogs may interfere the reproduction and development of crustaceans, such as larval survival, molting, metamorphosis and shell hardening.

Keywords: bisphenol A; alkylphenol; 4-cumylphenol; 2,4-bis-(dimethylbenzyl)phenol; 2-t-butyl-4-(dimethylbenzyl)phenol; gas chromatography-mass spectrometry; GC-FID; lobster, crab, shrimp

1. Introduction

Bisphenol A (BPA, 4,4'-isopropylidine diphenol) is a commercially important chemical with an estimated worldwide annual production of 5 million tons in 2010. Over 95% of all BPA produced is used as a monomer in the production of polycarbonate plastic and epoxy resins, which

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http://www.techno-press.org/?journal=aer&subpage=7 ISSN: 2234-1722 (Print), 2234-1730 (Online)

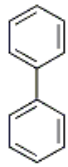
are found in numerous consumer products, including baby bottles, reusable water bottles, reusable food containers, coatings on metal lids, protective linings for canned foods and beverages, polyvinyl chloride stretch films, dental fillings, sealants, household electronic, papers and cardboards (WHO 2011, Shi 2012, Zhu and Zuo 2013, Michalowicz 2014, Zuo and Zhu 2014). The extensive manufacture and use of BPA has led to its ubiquitous occurrence in aquatic environments. Depending on location and sampling time, the concentrations of BPA in water samples varied widely. For example, in Germany, Fromme *et al*. (2002), Kuch and Ma (2001) examined BPA concentrations between 0.5 and 702 ng/L in river water; in the United States, Kolpin *et al*. (2002) found that BPA ranged from non-detectable to 12 μg/L. The BPA concentrations in sediments are often greater than those in water columns (Stuart *et al*. 2005, Wang *et al*. 2013, Zhu and Zuo 2013). Few studies have quantified BPA concentrations in animal tissues (Zuo and Zhu 2014).

The presence of BPA in the environment has caused considerable concerns due to its toxicity and endocrine disrupting property. Exposures to BPA and other alkylphenols can result in developmental and reproductive changes in aquatic organisms (Laufer *et al*. 2012a, b, 2013, Segner *et al*. 2003, Zhu and Zuo 2013), laboratory animals (Adewale *et al*. 2009) and humans (Watkins *et al*. 2014). However, the biological effect of BPA at low concentrations is currently still a subject of scientific debate (Goodman *et al*. 2006, Zhu and Zuo 2013). It has been argued that BPA's binding affinity and transcriptional activity for estrogen receptors (ER) is over 1000-fold lower than that of 17β-estradiol (E2) (Gould *et al*. 1998, Kuiper *et al*. 1998). Nonetheless, *in vivo* studies indicated that BPA still possessed active estrogenic effects at a concentration as low as 1 nM. Recent studies have shown that some degradation products and metabolites of BPA have much higher estrogenicity or toxicity than BPA. For instance, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), a metabolite of bisphenol A, has shown an estrogenic activity approximately 1000-fold higher than BPA (Okuda *et al*. 2010, Yoshihara *et al*. 2004); 4-cumylphenol (4-CP), a BPA analog in plastics, resins and the degradation products, has exhibited an estrogenic activity over 12-times higher than BPA (Terasaki *et al*. 2005). Thus, to better understand the estrogenic and toxic effects of BPA in aquatic organisms, it is critical to identify and quantify not only BPA but also its analogs, metabolites and related degradation products of polycarbonate plastic and epoxy resins in various biological and environmental matrices. Both gas chromatography (GC) and high-performance liquid chromatography (HPLC), especially combined with mass spectrometry (MS), are suitable techniques for the determination of BPA and other endocrine disrupting compounds in aquatic and biological samples (Shi 2012, Stuart *et al*. 2005, Chen *et al*. 2013, Zuo *et al*. 2007, 2013, Zuo 2014). In this study, fast and accurate GC with flame ionization detection (FID) and GC-MS techniques have been developed and applied for the simultaneous identification and quantitation of bisphenol A and five its analog and degradation products contamination in American lobsters, crabs and shrimps. The chemical structures of BPA and its analogs studied are presented in Fig. 1.

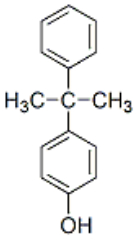
2. Material and methods

2.1 Chemicals and crustacean samples

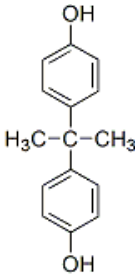
Bisphenol A, 2-t-butyl-4-(dimethylbenzyl)phenol, 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol, 2,4-bis-(dimethybenzyl)phenol, 2,4-bis-(dimethylbenzyl)-6-t-butylphenol and 4-cumylphenol



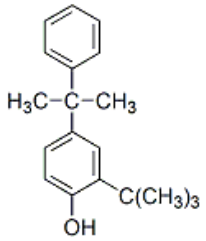
(a) IS: Biphenyl



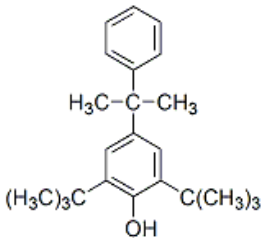
(b) 1: 4-cumylphenol



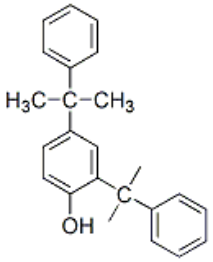
(c) 2: Bisphenol A



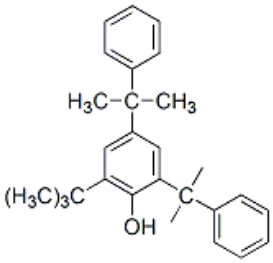
(d) 3: 2-t-butyl-4-(dimethylbenzyl)phenol



(e) 4: 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol



(f) 5: 2,4-bis-(dimethylbenzyl)phenol



(g) 6: 2,4-bis-(dimenthylbenzyl)-6-t-butyphenol

Fig. 1 The chemical structures of BPA and its analogs studied

standards were all purchased from Sigma-Aldrich (St. Louis, MO) or provided by Dr. H. Laufer at the University of Connecticut. Biphenyl (internal standard) was purchased from Acros Organics (Morris Plains, NJ). Acetonitrile, acetone and hexane, all of HPLC grade, were obtained from Pharmco products (Brookfield, CT). Except where noted, all reagents were of analytical grade and all aqueous solutions were prepared by using doubly-distilled and deionized water. Mature male and female lobsters, ranging in weight between 300 and 450 g (shorts) were collected from Long Island Sound and from Vineyard Sound, Massachusetts (Fig. 2). Long Island Sound is an estuary of the Atlantic Ocean, located in the United States between Connecticut to the north and Long Island, New York to the south. The mouth of the Connecticut River at Old Saybrook, CT, empties into the sound. Vineyard Sound is the stretch of the Atlantic Ocean which separates the Elizabeth Islands and the southwestern part of Cape Cod from the island of Martha's Vineyard, offshore from the state of Massachusetts in the USA. To the west, it joins Rhode Island Sound and on its eastern end it connects to Nantucket Sound. Muscle tissue samples were taken and preserved by the Marine Biological Laboratory, Woods Hole, MA. The muscle samples were frozen at -20°C.

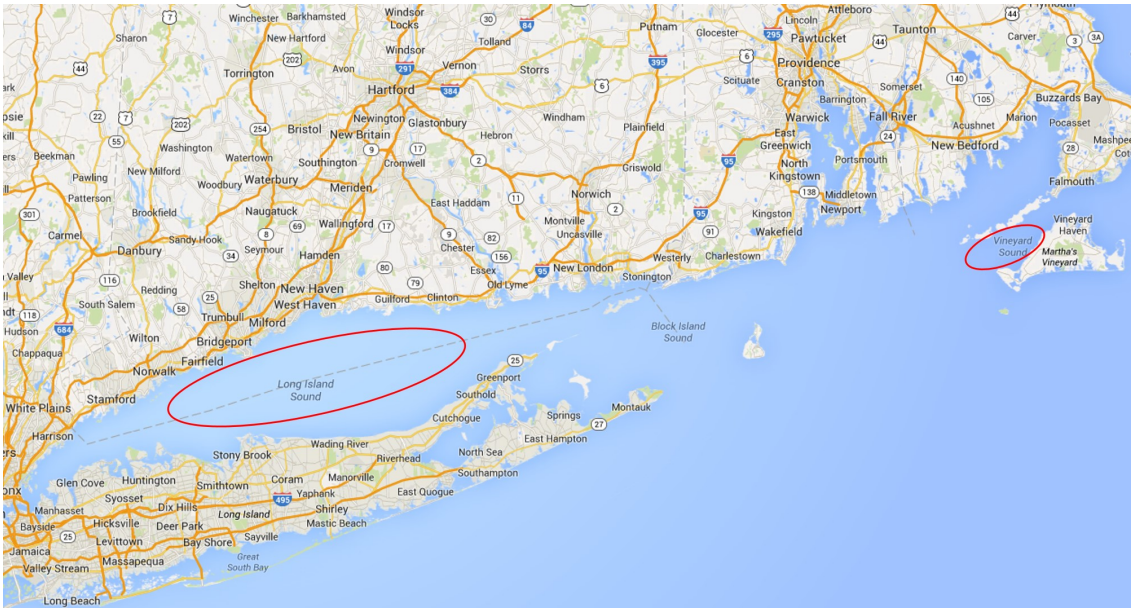


Fig. 2 Location of lobster sampling sites around Long Island Sound, CT and Vineyard Sound, MA, in the United States

The blue crab samples were collected from Narragansett Bay, Rhode Island and donated by Davis Seafood Market in Fall River, Massachusetts, U.S.A. Shrimp samples were purchased from local supermarkets in New Bedford and North Dartmouth, MA and stored at 4°C until used in this study.

2.2 Preparation of standard solutions and samples

The standard stock solutions of analytes (1.000 mg/mL) were prepared by dissolving the standard chemicals in 10 mL of methanol. Internal standard stock solution (1.030 mg/mL) was prepared by dissolving 10.30 mg of biphenyl in 10.00 mL methanol. All these solutions were stored in the dark at 4°C. The lower concentrations of working solutions were freshly prepared by dilution of these stock solutions.

Two grams of crustacean muscle samples were rinsed with 4% NaCl and weighed. 2 mL of acetonitrile was added to denature the proteins. Then the muscle tissue samples were homogenized with 4 mL of 4% NaCl and transferred to a 30 mL tube. 4 mL of hexane were added to extract alkylphenol analytes, the whole solution were vortexed for 2 min and ultrasonicated at room temperature for 20 min in an ultrasonic bath (VWR Signature Ultrasonic Cleaner Model 75D, 90W) with Digital Timer, Heat, and Power (VWR, S. Plainfield, NJ, USA). Then the samples were centrifuged for 5 min, and the hexane phase was pipetted out and filtered by folded filter paper (12.5 cm Whatman #5). After that, 4 mL of hexane was added to each vial for another ultrasonic extraction, centrifugation and filtration. The extraction process was repeated for 3 times (n = 3), and the combined 12 mL hexane solution was evaporated to dryness at ambient temperature under nitrogen. The residue in the tube was reconstituted with 380 μl of methanol and 20 μl of internal standard (IS) (1.03 μg/mL of biphenyl) to make a final volume of 400 μl for GC analysis.

2.3 GC analysis

GC-FID analysis was carried out on a Shimadzu GC-17A gas chromatograph equipped with a FID, a Shimadzu AOG-20i GC auto-injector, and a Gateway E-4200 computer that utilizes CLASS-VIP Chromatography Data System Version 4.2 (Shimadzu Scientific Instruments, Columbia, MD, USA). Samples were separated on a 30 m × 0.32 mm id, 1.00 μm film EC^{TM}-5 capillary column (Alltech, Deerfield, IL, USA). The stationary phase was composed of 5% phenyl-and 95% dimethyl-polysiloxane mixture. Helium was employed as a carrier gas with a linear velocity of 27 cm/s. Nitrogen make-up gas, hydrogen and compressed air were used for the FID. A split/splitless injector was used in a splitless mode. The injector volume was 2.0 μl. The operating conditions for GC began with an initial temperature of 40°C for 2 min, followed by a 15°C /min ramp to 260°C, then a 13 min hold at 265°C, for a total run time of 30 min.

A Thermo Scientific Focus^{TM} GC Gas Chromatograph coupled with a Thermo Scientific Xcalibur Ion Trap and DSQ (ITQ700) series mass selective detector and a Thermo Scientific AI 3000/AS 3000 series automatic sampling system was employed for GC-MS analyses. Samples were separated on a Thermo TR-5ms SQC 30 m × 0.25 mm id × 0.25 μm capillary column. The column temperature was initially held at 40°C for 2 min, then the temperature was raised to 265°C at a rate of 15°C per min and held for 13 min. The total run time was 30 min. Ultra high purity helium with an inline Alltech trap was used as carrier gas. The carrier gas was set at a constant flow of 1.5 mL/min. Injector temperature was maintained at 250°C, and the injection volume was 1.0 μl in the splitless mode. The temperature of transfer line was held at 265°C. Mass spectra were scanned from m/z 50-450. Electron impact ionization energy was 70 ev.

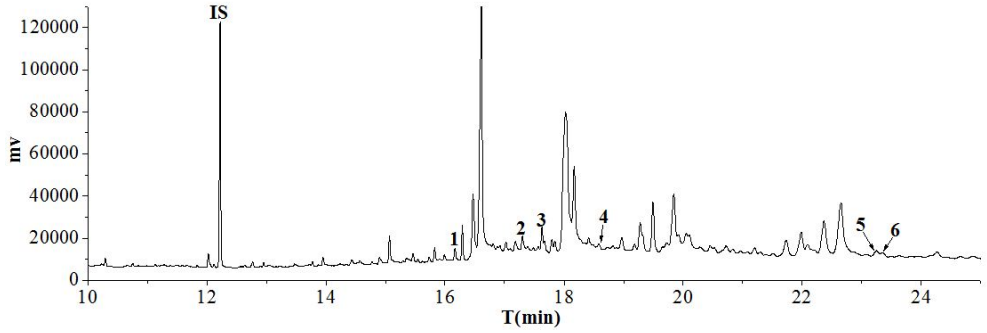
Identification of the peaks of alkylphenol analytes was achieved by two ways. One was based on the GC retention time together with co-injection of standards with the samples. The other identification method, more convincing, was through the characteristic ions from the mass spectrum. Quantification of these alkylphenol analytes was conducted by the standard calibration curve method. Calibration curves were constructed by linear regression of the ratio of peak areas of the standards and the internal standard versus the standard concentration. All the calibration standards and crustacean samples were run in triplicate.

The blank control test was necessary for the whole experimental design to exclude the interferences from sampling to detecting process. For the blank, 2 g of the clean sea sands were extracted 3 times with n-hexane, followed exactly with the sample preparation procedures. There were no interested ananlytes detected in the procedural blank.

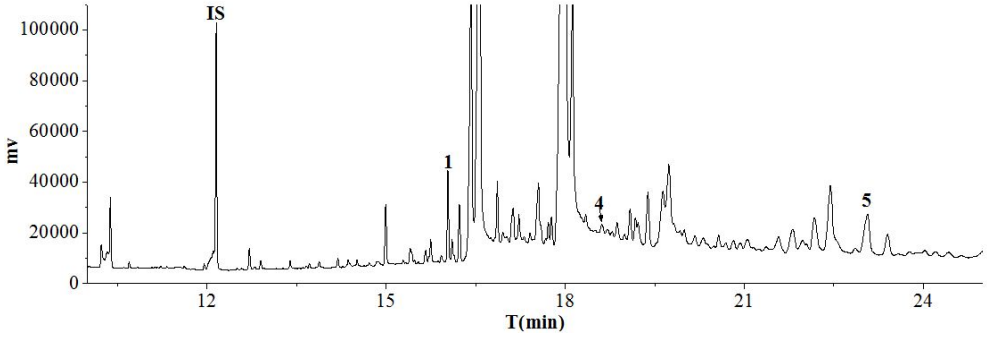
The percent recovery of alkylphenols through the liquid-liquid extraction and GC analysis was conducted by spiking 100 μL of 100 μg/L standard solution to the tissue samples (muscle), and then extracted by hexane, following the exact same sample preparation procedures. The concentration of the normal samples and the concentration of the samples with spiked standards were analyzed by GC-FID, and the recoveries for the amount of spiked standards were calculated.

3. Results and discussion

Fig. 3 showed typical gas chromatograms for a lobster (A) and shrimp muscle extract (B), respectively. Bisphenol A (MW: 228.29) and five of its analogs, 4-cumylphenol (MW: 212.29), 2-t-butyl-4-(dimethylbenzyl)phenol (MW:268.39), 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol (MW:324.50), 2,4-bis-(dimethybenzyl)phenol (MW: 330.46), and 2,4-bis-(dimethylbenzyl)-6-t-butylphenol (MW: 386.57) were identified in lobster muscle extracts; BPA, 4-cumylphenol



(a)



(b)

Fig. 3 (a) A typical gas chromatogram of an American lobster muscle sample; and (b) A typical gas chromatogram of a shrimp muscle sample: (1) 4-cumylphenol; (2) 2-t-butyl-4-(dimethylbenzyl)phenol; (3) 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol; (4) bisphenol A; (5) 2,4-bis-(dimethybenzyl)phenol; and (6) 2,4-bis-(dimethylbenzyl)-6-t-butylphenol

Table 1 Characteristic ions for alkylphenols studied

Compound	Characteristic ions (m/z)
4-cumylphenol	197, 212
Bisphenol A	213,228
2-t-butyl-4-(dimethylbenzyl)phenol	253, 268
2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol	309, 324
2,4-bis-(dimethybenzyl)phenol	237, 315, 330
2,4-bis-(dimethylbenzyl)-6-t-butylphenol	293, 371, 386

and 2,4-bis-(dimethybenzyl)phenol were detected in shrimp and crab samples. These alkylphenol analytes, except BPA, were eluted out in order of their molecular weight from the nonpolar poly(dimethylsiloxane) column. The identification of each akylphenols was confirmed by GC-MS. The characteristic ions for each alkylphenols were summarized in Table 1.

Mixtures of the six alkylphenol standards in the concentration range of 0.00-50.0 μg/L were prepared for calibration curves. The linearity and detection limits for the six alkylphenols were

investigated. A good linearity was obtained for each of the analytes tested with the coefficients of determination for these studied alkylphenols ranged between 0.994 and 0.999, indicating that the analytical method developed was very stable and had high reproducibility. The detection limits measured at 3 times of the signal to noise (S/N) ratio were 0.728 μg/L for bisphenol A, and between 0.010 to 0.091 μg/L for other alkylphenols tested, respectively. The corresponding limits of quantification, at 10 times of the S/N ratio were 2.43 μg/L for BPA and between 0.303 and 0.033 μg/L for the other alkylphenols, respectively. The recovery rate for the alkylphenols tested except BPA ranged from 82.5 to 98.6%. Although percentage recoveries for bisphenol A were low due to its high polarity, reproducibility between multiple extractions was high with a relative standard deviation of less than 4.8%. No ananlytes were detected in the procedural blank.

Inter-day precision of the developed method was evaluated by performing six injections of standard solutions and shrimp samples each day on five different days within a week period. Inter-day precision (RSD) on the basis of the retention time and peak area was better than 0.3 and 1.6%, respectively. Intra-day repeatability of the method was performed by three analysts (five determinations by each analyst) using the described method and the same instruments. The results showed no significant differences: RSD% = 2.0.

The internal standard calibration method was used for the quantification of BPA and other alkylphenol compounds. As summarized in Table 2, the contents of BPA and its analogs measured in 10 American lobster muscle samples were 4.48-7.01 ng/g of bisphenol A, 1.23-2.63 ng/g of 2-t-butyl-4-(dimethylbenzyl)phenol, 2.71-9.10 ng/g of 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol, 0.35-0.91 ng/g of 2,4-bis-(dimethybenzyl)phenol, 0.64-3.25 ng/g of 2,4-bis-(dimethylbenzyl)-6-t-butylphenol and 0.44-1.00 ng/g of 4-cumylphenol. For 8 shrimp muscles, bisphenol A, 2,4-bis-(dimethylbenzyl)phenol and 4-cumylphenol were found in the concentration ranges of 0.67–5.51, 0.36-1.61, and 0.00-1.96 ng/g (wet weight), respectively, and for 8 crab samples the corresponding concentration ranges were of 0.10-0.44, 0.13-0.62, and 0.26-0.58 ng/g (wet weight), respectively.

The concentration level of the BPA and other alkylhenols (APs) in crab and shrimp samples measured in this study is lower than that found in American lobster. The blue crab and fresh water shrimp have a much shorter lifetime, that means a less total exposure to the environmental pollutants, than the lobster, which may explain the lower APs concentrations in the blue crab and shrimp tissues. The concentration of BPA in crabs and shrimps detected here is also much lower than those in canned shrimp and fish food from Japan (Yoshida *et al*., 2001) at the level ranging from 17 to 602 ng/g. However, The BPA concentrations in crabs and shrimps found in this study are comparable to the concentrations detected in marketable fishes in Netherland (0.24-2.6 ng/g), Beijing, China (0.33-7.8 ng/g), Sweden (0.24-4.7 ng/g) and Italy (0.1-4.9 ng/g) (Belfroid *et al*. 2002, Shao *et al*. 2005, WSP 2007, Mita *et al*. 2011), and also in the same range as in some aquatic

Table 2 Concentrations of BPA and other alkylphenols in lobster, crab and shrimp muscle samples

Samples compounds	Lobster muscle	Crab muscle	Shrimp muscle
4-cumylphenol (ng/g)	0.44-1.00	0.26-0.58	< LOD-1.96
bisphenol A (ng/g)	4.48-7.01	0.10-0.44	0.67-5.51
2-t-butyl-4-(dimethylbenzyl)phenol (ng/g)	1.23-2.63	< LOD	< LOD
2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol (ng/g)	2.71-9.10	< LOD	< LOD
2,4-bis-(dimethybenzyl)phenol (ng/g)	0.35-0.91	0.13-0.62	0.36-1.61
2,4-bis-(dimethylbenzyl)-6-t-butylphenol (ng/g)	0.64-3.25	< LOD	< LOD

environmental samples, for example, around 1.5 to 3.5 ng/g in the sediment samples collected near wastewater outfalls in Boston Harbor's Deer Island (Stuart *et al*. 2005). These results have in some degree indicated that BPA may not significantly bio-accumulated in blue crab and shrimp muscle tissues. But moderate bioaccumulation of BPA and its analogs was observed in American lobster muscle tissues.

BPA is slightly to moderately toxic to fish (Alexander *et al*. 1988). But the acute toxicity of 4-cumylphenol (4-CP) to fish and invertebrates is over 18 and 4 folds higher than BPA, respectively. Although a standard acute toxicity test for fish and invertebrates has not been reported for 2,4-bis-(dimethylbenzyl)phenol (2,4-DCP) and other BPA analogs tested here, a 96-hour LC_{50} for fish and 48-hour EC_{50} value for invertebrates estimated by Ecological Structure Activity Relationships (ECOSAR version 1.00) indicates that 2,4-DCP is over 45 and 17 times more toxic than 4-CP to fish and invertebrates, respectively (U.S. EPA 2009). Thus the occurrence of 4-cumylphenol, 2,4-bis-(dimethybenzyl)phenol and other BPA analogs in crustacean muscle could be a more serious concern due to their high toxicity and estrogenicity to aquatic organisms.

At the concentration levels detected in this study, BPA, 4-CP, 2,4-DCP and other BPA analogs all may negatively affect the development and reproduction of aquatic organisms, including crustaceans. The bioassay study by Biggers and Laufer (2004) indicated that BPA, 4-CP and 2,4-DCP had juvenile hormone activity in the marine polychaete worm *Capeitella capitata*. The reproduction, development and metamorphosis of crustaceans are mostly regulated by the crustacean juvenile hormone, methyl farnesoate (MF). BPA's juvenile hormone activity was 20 times higher than MF. Laufer *et al*. (2013) tested the effects of BPA and 2,4-DCP on lobster larvae. Their results shown that the alkylphenols were toxic to second and third stage larvae, and fourth stage post-larvae when they were fed with 5 or 10 ng alkylphenol per day in their diet, with 13-21% surviving to 25 days compared to 69% of controls. The compounds significantly delayed each molt by 2-3.5 days, and the surviving treated larvae were predominantly larval-juvenile intermediates at metamorphosis (62%). BPA and 2,4-DCP may also disrupt shell hardening through interfering with tyrosine cross-linking due to the similarity of their chemical structure to tyrosine derivatives. Laufer *et al*. (2012b) have shown that both BPA and 2,4-DCP competed strongly with tyrosine during the hardening of new cuticle following lobster molting and weakened the shell of lobsters, which increased the shell's susceptibility to microorganisms. In the light of this study and potential estrogenic and toxic effects of BPA, particularly its analogues, metabolites and other degradation products, an extensive survey on not only BPA but also its analogues and their degradation products is urgently needed for the environmental risk assessment on crustaceans and other aquatic organisms and regulations of these plasticizers.

4. Conclusions

A gas chromatography combined with flame ionization detector and mass spectrometric detector has been developed for the simultaneously identification and quantitation of bisphenol A (BPA) and its analog, metabolite and degradation products in this study. Bisphenol A has been detected in all crustacean muscle samples. Five analogs of BPA were found in lobster muscle samples, two in crab and two in shrimp samples at a concentration range up to 23.5 ng/g (wet weight). At these concentration levels, BPA and its analogs can adversely affect the development and reproduction of American lobsters, crabs and shrimps. Further study on the metabolites of BPA and its analogs and their bio-effects are needed.

Acknowledgments

The authors would like to thank Drs. E. Ojadi, C. Neto, D. Ryan, J. Stuart, H. Laufer for their discussion and contributions. The research described in this paper was financially supported by the US Natural Science Foundation under Grant OCE 0752033, The US Geological Survey (USGS and Water Resources Research Center at the University of Massachusetts Amherst.

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