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Research Article

Effect of Various Treatment Methods on the Bisphenol A Concentration in Edible Mushroom Segments during Cultivation

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Abstract: The aim of study were to analyzed bisphenol A (BPA) concentrations in Polyvinyl Chloride (PVC) bottles used for cultivation of edible mushrooms, cultivation materials and fruiting bodies of *Pleurotus eryngii* with various treatments by HPLC-MS. BPA was detected in bottles, cultivation materials and fruiting bodies at levels greater than the limit of detection (0.000611 μg/g). BPA levels decreased from 19.851 to 6.230 μg/g following exposure to high temperature and pressure. In addition, the mean BPA levels increased in a pH-dependent manner to 11.37-30.80 μg/g. With the exception of those grown in new bottles, fruiting bodies contained BPA at levels not exceeding the recently established specific migration limits of 0.6 mg/kg for food established by the European Union. These data suggest that physical treatment methods could decrease BPA levels in new PVC bottles. Use of such treatments rendered fruiting bodies of *P. eryngii* safe for consumption.

Keywords: Biodegradation, bisphenol A, different treatment, HPLC-MS

INTRODUCTION

Bisphenol A (BPA; 2, 2-bis(4-hydroxyphenyl) propane) is an organic compound composed of two phenol rings connected by a methyl bridge, with two methyl functional groups attached to the bridge (Fig. 1a). BPA is widely used in polycarbonate plastics, epoxy, phenolic, polysulfone and polyetherimide resins, polyesters, polyacrylates and flame-retardant materials. It is also present in the lacquer lining of metal food and beverage cans, baby bottles and food packaging materials, among other items (Alexiadou *et al*., 2008; Takao *et al*., 2002). Numerous reports of BPA contamination in canned foods have been published (Kang and Kondo, 2003; Lin-Gibson *et al*., 2002). The main factors influencing the migration of BPA are heating time and temperature (Kang and Kondo, 2003; Munguía-López *et al*., 2005; Taylor *et al*., 2008). Moreover, due to increased use of products containing BPA, the likelihood of environmental contamination has increased. High levels of BPA have been identified in leachates of waste landfill (Nascimento Filho *et al*., 2003; Yamamoto and Yasuhara, 1999; Yamamoto *et al*., 2001). In addition, the leaching of BPA from plastic wastes into water has been reported. High BPA levels (9.8 and 139 μg/g) were detected in Polyvinyl Chloride (PVC) products, the manufacturing process of which involves use of BPA as a stabilizer. BPA can be biodegraded by microorganisms in the environment and metabolized by enzymes in plants, animals and mushrooms. Recently, interest in BPA has increased as *in vitro* experiments have shown that it is a potent estrogen mimic (Pulgar *et al*., 2000) and endocrine disruptor (Krishnan *et al*., 1993). Many methods for the determination of BPA levels in several matrices have been developed. Exposure to BPA is particularly important because of the increased susceptibility of the brain and other organs to estrogens during this time (Vandenberg *et al*., 2009). Furthermore, humans may be exposed to elevated levels of BPA due to a lack of metabolic enzymes capable of conjugating the compound (Taylor *et al*., 2008; Vandenberg *et al*., 2010).

In this study, BPA was extracted from mushroom and vegetable samples at Henan Institute of Science and Technology and analyzed by High-Performance Liquid Chromatography-mass Spectrometry (HPLC-MS). The BPA concentrations in PVC bottles, cultivation materials and fruiting bodies were also investigated. The findings were used to propose several methods of reducing BPA migration.

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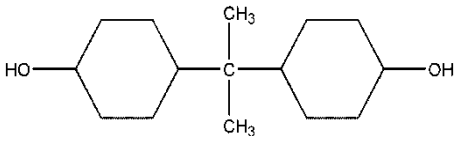
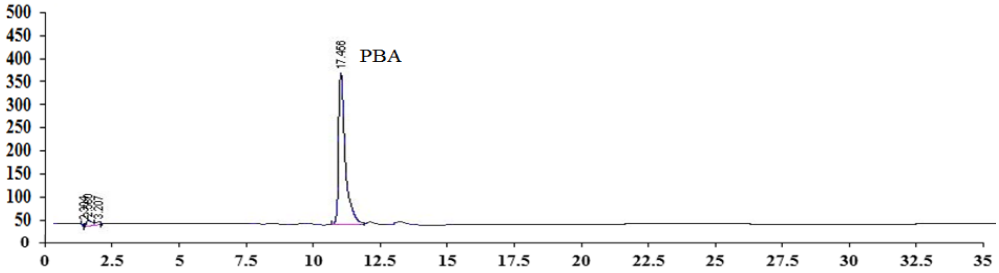


(a)



(b)

Fig. 1: The structure and hplc chromatogram for BPA; (a): Chemical structure of bisphenol A; (b): HPLC chromatogram of the standard compound for BPA

MATERIALS AND METHODS

Instrumentation and reagents: Bisphenol A (BPA) and chroman (minimum purity 99%) were purchased from Sigma-Aldrich (UK). Methanol, dichloromethane, acetone were purchased from Beijing Chemical Works. Deionized water was prepared by our lab apparatus. Glass Oasis™ HLB (5 mL/200 mg) cartridges were purchased from Waters (Milford, MA) and conditioned with 4 mL washes with methyl-tert-butyl-ether (MTBE), 3 mL of methanol and 5 mL of water.

Collection of samples: All samples were collected from mushroom and vegetable base in Henan Institute of Science and Technology (May 25, 2014). The analyzed samples after different treatment were divided into three groups included cultivation bottles (PVC bottles) (E1-12), cultivation materials (C1-5) and fruiting bodies of *P. eryngii* (S1-4) (Fig. 2). All analyses were repeated three times for each sample.

Sample treatment: All necessary precautions were taken to avoid contamination with BPA during sample preparation. All glassware (glass bottles and glass pipettes) used in the extraction procedure was washed and baked for 8 h at 500°C (Sungur *et al*., 2014).

During sample treatment, all treatment steps divided into 3 steps: a partitioning step (Yi *et al*., 2010), solid-phase extraction step (Markham *et al*., 2010) and synthesis of derivative step (Xu *et al*., 2007).

A partitioning step was utilized prior to next step: 1 mg of the dry and clean sample of debris was mixed with 1 mL of acetonitrile in glass centrifuge tube and the sample was vortexed for 30 s. Then 3 mL of *n*-hexane were added and the samples were inverted by hand for 5 min, vortexed for 30 s and centrifuged at 5500 rpm, 4°C for 10 min. The *n*-hexane layer was discarded and the process was repeated with an additional 3 mL of *n*-hexane. After discarding the second *n*-hexane layer, as much of the aqueous layer as possible was transferred to a new centrifuge tube and evaporated down to approximately 1 mL under N_2, 37°C water bath.

Solid-phase extraction step: Samples were diluted with 9 mL of 1:8 methanol: water and vortexed for 1 min. The samples were loaded onto the solid-phase extract (SPE) column without vacuum and the sample vials were rinsed with 5 mL of water, which was also loaded without vacuum (Yi *et al*., 2010), then SEP rinsed with 3 mL of methanol: water (1:1/v:v) followed by 3 mL of methanol: ammonium hydroxide: water (5:1:44/v:v:v) and dried under N_2 for 50s with medium vacuum. A gas flow of approximately 8 L/min was used during the drying steps. Medium vacuum indicate a manifold reading of -25 kPa and -50 kPa, respectively. The final wash consisted of 3 mL of dichloromethane followed by 1 min drying time under N_2 with high vacuum (Markham *et al*., 2010). BPA was eluted into 5 mL reacti-vial from the column with 4 mL of MTBE without vacuum then derivatized (see procedure below) prior to analysis.

Synthesis of derivative step: Upon completion of sample extraction, this step has main mission to improved MS sensitivity and detection in Electrospray Ionization Positive (ESI^+) mode by synthesizing a BPA-Pyridine sulfonyl (BPA-PS) derivative Solution from solid-phase extraction step was evaporated to

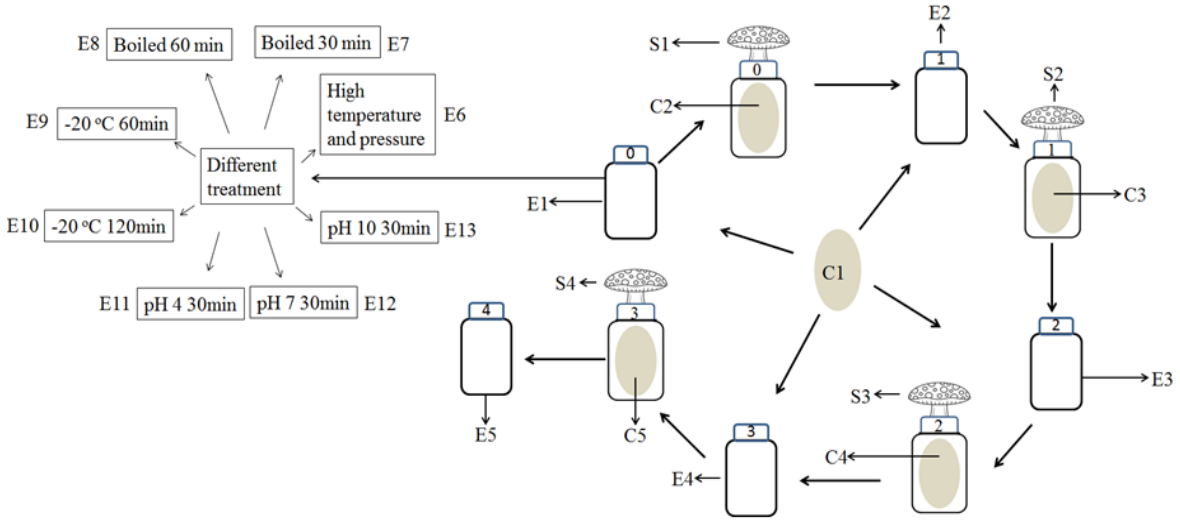


Fig. 2: Selection and treatment of samples
E1: new cultivation bottle; E2: bottle that used once; E3: bottle that used twice; E4: bottle that used thrice; E5: bottle that used quartic; E6: bottle in high temperature and pressure (1×10^5 Pa) for 30 min; E7: bottle in boiling water for 30 min; E8: bottle in boiling water for 60 min; E9: bottle in -20°C 60 min; E10: bottle in -20°C 120 min; E11: bottle in acid water (pH 4) for 30 min; E12: bottle in neutral water (pH 7) for 30 min; E13: bottle in alkaline water (pH10) for 30 min; C1: New cultivation materials; C2: cultivation materials from new bottle; C3: cultivation materials from bottle used once; C4: cultivation materials in bottle used twice; C5: cultivation materials in bottle used thrice; S1: fruiting bodies from new bottle; S2: fruiting bodies from bottle used once; S3: fruiting bodies in bottle used twice; S4: fruiting bodies in bottle used thrice

dryness under N_2, 37°C water baths. And then 95 µL of 1.13 mg/mL pyridine-3-sulfonyl chloride in acetone followed by 100 µL of 0.1 M sodium bicarbonate was added to the reacti-vial and vortexed for 30s. The vial was then placed in a 60°C and allowed to react for 7 min and immediately cooled on ice for 8 min. The solution was allowed to reach room temperature and extracted with 1 mL of *n*-hexane. The *n*-hexane solutions were saved and transferred to a new vial, evaporated to dryness under N_2, 37°C water bath and reconstituted with 1 mL of water: acetonitrile (1:1/ v:v).

The samples were vortexed for 30 s and passed through a membrane filter (0.45 µm, HLC-DISK 3, Beijing Chemical Co. Inc., Beijing). 1 µL was analyzed immediately by HPLC-MS (Braunrath *et al*., 2005). To evaluate the potential for BPA contaminations during the analysis, a large number of procedural water blanks were used in the study relative to the number of samples analyzed (8 water blanks and 21 samples).

Analytical conditions of BPA: Chromatographic separation of the 1 µL sample injection was achieved with a column Inertsil ODS-SP C_{18} chromatographic column (250 mm ×4.6 mm i.d., 5 µm; LC-20AT, Shimadzu, Japan). A linear gradient elution is eluents A (acetonitrile) and B (water with 0.1% acetic acid). The elution program was optimized and conducted as follows: a linear gradient of 0-70% B (0-5 min), 2% B (5-6 min), 3% B (6-7 min), 4% B (7-10 min), 3% B (10-15 min), 2% B (15-20 min), 1% B (20-25 min), 0% B (25-35 min). The solvent flow rate was 0.5 mL/min, argon was used as the collision gas and nitrogen was used as the nebulizer, desolation and cone gas. Triplicate injections were made for all sample extracts and standard solutions. The concentrations of BPA were calculated as by following Eq. (1):

$$\text{BPA concentration (\%)} = \text{BPA (µg)/Sample (mg)} \times 100\% \quad (1)$$

BPA (ng) was calculated by the BPA calibration curve, sample (mg) was 1 mg.

Method validation and application to samples: The BPA calibration curve of HPLC obtained by internal standard method (Each BPA standard would also contain a fixed concentration of internal standard (chroman)) with concentrations versus detector responses (peak areas). Relative Response Factor (RRF) was determined by calculating the ratio of the slope of BPA calibration curve to the slope of chroman calibration curve (Sungur *et al*., 2014). The Limit of Detection (LOD) was defined as (Hornung and Reed, 1990):

$$LOD = 3.3S_{y/x}/b \quad (2)$$

where, $S_{y/x}$ stands for the standard error of the predicted value for every x value in the regression of calibration curve ranging from peak areas with BPA of sample and b is the slope of the same curve.

The precision of method was applied to sample E1 (duplicate) (Li *et al*., 2014; Zimmers *et al*., 2014). Analysis of five replicates during 1 day were conducted for the repeatability test (intra-day precision) with the first sample E1 and analysis of four replicates in 4 consecutive days was conducted for the reproducibility test (inter-day precision) with the second sample E1. To evaluate instrument sensitivity and stability over the time course of this study, it was assessed by analyzing the sample E1 at 0, 2, 4, 8, 12, 24 h and over two weeks. The accuracy of the method was determined as recovery studies. Using BPA as a target, the recovery of method was applied to sample E1 that were spiked with BPA standard solution at the high, intermediate and low levels using different glass bottle. The recovery (R%) was calculated by subtracting the concentration measured in the unspiked sample from that measured in the spiked sample and then dividing by the spiked concentration (Kuroda *et al*., 2003; Sungur *et al*., 2014; Zimmers *et al*., 2014):

$$R\% = (C_{\text{spiked sample}} - C_{\text{unspiked sample}})/C_{\text{spiked sample}} \times 100 \quad (3)$$

RESULTS AND DISCUSSION

Optimized sample processing: The *n*-hexane partitioning step described by Kang and Kondo (2003) was utilized to remove non-polar lipids from the samples. Additionally, the procedure developed by Markham *et al*. (2010) resulted in superior sample cleanup compared to other SPE methods tested. The ionization efficiency of negative ions was insufficient for analysis of BPA by HPLC-MS. By means of a derivatization reaction, we added basic pyridine-3-sulfonyl (PS) groups to BPA, which facilitated high efficiency analysis under ESI^+ conditions. Compared with the dansyl derivative, the PS derivative ensured a complete reaction with both hydroxyl groups of BPA. The BPA-PS derivative also ensured specificity, as a BPA-specific ion is formed as the major product ion in the collision-induced dissociation of BPA-PS (Xu and Spink, 2008). The BPA-PS derivative was detected with the MS operating in ESI^+ Multiple Reaction-Monitoring (MRM) mode. The transitions of the BPA-PS derivative were monitored at 511-354 *m/z*.

Quality assurance and quality control: HPLC-MS facilitates sensitive and selective determination of BPA. Precision was determined by analyzing two aliquots of sample E1. Intra-day analysis was performed by five replicate injections of the first sample E1. Inter-day analysis was conducted by four replicate injections of the second sample E1 on 4 consecutive days. Mean BPA concentrations in this sample were 19.75±0.071 μg/g for intra-day analysis and 19.90±0.05 μg/g for inter-day analysis, with an RSD <3.0%. To evaluate the sensitivity and stability of the HPLC-MS instrument, sample E1 was analyzed at 0, 2, 4, 8, 12 and 24 h; similar BPA concentrations were detected. The consistency of the results obtained over a 2-week period of sample processing and analysis supported this correlation. Sample E1 was spiked with high, intermediate and low levels of BPA and BPA recovery was evaluated. The native BPA signals from the original extract were subtracted from those of the spiked samples; the resulting data were used to determine BPA recovery. The recovery rate was 94.4%, with an RSD of <3.0%. This suggested that the extraction method and HPLC-MS analysis were suitable for determining BPA levels.

BPA concentrations in samples: Calibration standards were prepared from mixtures of equal concentrations of native BPA and chroman standards. The overall native/chroman Relative Response Factor (RRF) was 0.97. This was determined by calculating the ratio of the slope of the BPA calibration curve to the slope of the chroman calibration curve.

The curves had a slightly negative y-intercept, indicating that there was no background BPA contamination in the native or labeled internal standard solutions. Forcing the curves through zero had no effect on the calculated RRF (Yamamoto and Yasuhara, 1999). A procedural blank was processed with each batch of three samples. The free BPA concentration in the eight blanks analyzed during the study was 0.20±0.01 ng/mL. Prior to replacing the UV lamp and cartridges in the Milli-Q water purification system, the average BPA concentration in laboratory blanks was ~0.3 ng/mL. Zimmers *et al*. (2014) reported that the purity of the laboratory water supply could decrease the background BPA concentration. Therefore, the purity of the laboratory water supply is essential for maintaining a low background BPA concentration. Based on the blank results, the LOD was determined to be 0.22 ng/mL. Using this IUPAC criterion, there is a <1% probability of a false-positive result. Under the conditions described in above section, BPA was eluted at 11.25 min as a clear peak (Fig. 1b). The calibration curve was $Y = 9174X+12.101$, $R^2 = 0.998$ in a concentration range of 0.001-0.04 μg/μL.

There were also noticeable differences in BPA concentration range among samples. BPA concentration differed significantly according to the treatment applied to the bottles (Fig. 3 and 6a). The peak corresponding to BPA was found in the HPLC chromatograms of samples E1-E13. The chromatographic peaks in samples subjected to different treatments with identical relative retention times were defined as the common peaks. The peaks that were too close to the solvent peak (retention time 5 min) were excluded from the list of common peaks. Thirteen peaks were determined to be common peaks and numbered P1 to P13 (Fig. 3). The peak corresponding to BPA was P7. The area sum of all common peaks accounted for >90% of the total area of all peaks in the chromatograms. The point of the

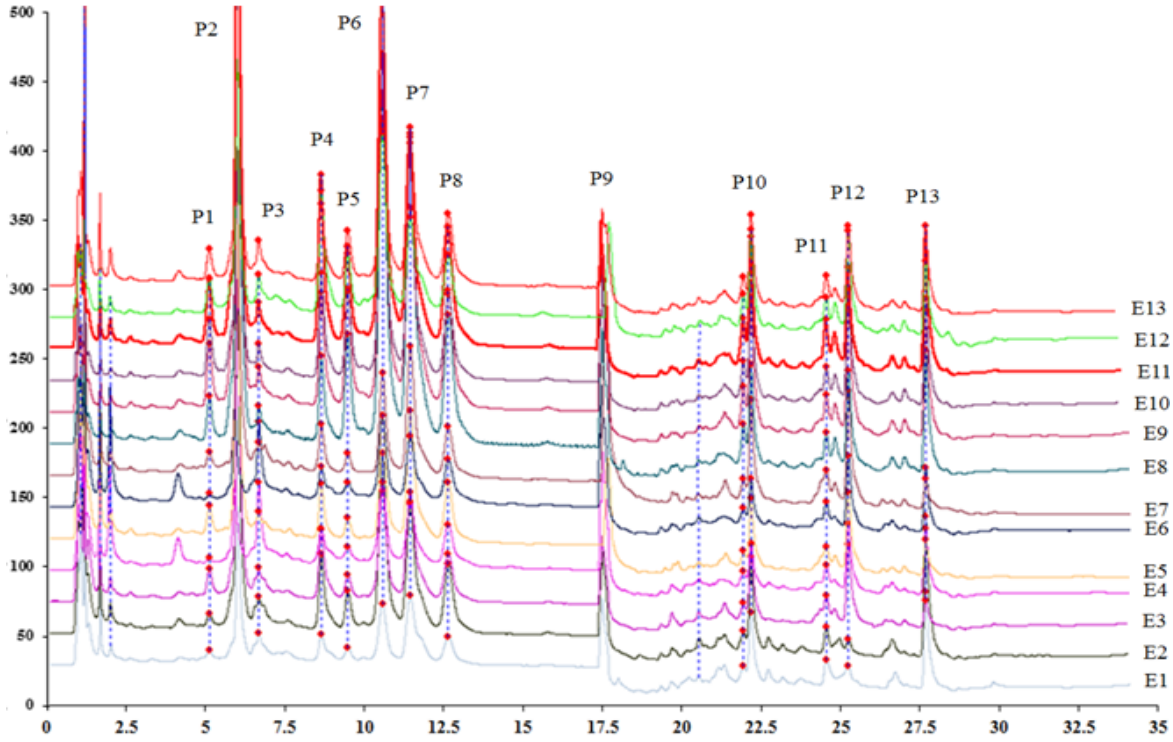


Fig. 3: Overlaid HPLC chromatograms of extracts of samples E1–E13

superposition of the relative retention time was good, no point was out with the curve and the peaks matched well. These were thus taken as characteristic peaks for samples E1-E13. The highest mean concentration of BPA (19.85 μg/g) was found in new bottles (E1) and the lowest (1.92 μg/g) in bottles used quartic (E5). Thus, BPA levels gradually decreased with increasing frequency of bottle use. BPA levels in bottles subjected to treatment with low temperature (-20°C) (E9 and E10, respectively) and alkaline water (E13) were higher than those in bottles subjected to high temperature and pressure (E6), acid water and neutral water (E11 and E12, respectively). The BPA concentration increased initially and then decreased in a time-dependent manner in bottles subjected to treatment with boiling water (E7 and E8). Regarding the physical methods, high temperature and pressure resulted in the greatest reduction in BPA concentrations (to 6.232 μg/g). The BPA concentration in the bottles subjected to treatment with high temperature and pressure for 30 min was significantly lower than that following treatment with boiling water for 30 and 60 min (15.14 and 14.12 μg/g, respectively). Therefore, the temperature of sterilization as much as possible was considered other safety factor.

Cultivation materials are necessary for the growth of edible mushrooms. Fresh cultivation materials (C1) contained lower BPA concentrations (0.563 μg/g) than other samples (Fig. 4 and 6b). However, cultivation materials (C2) after cultivation in new bottles had a higher BPA concentration (3.552 μg/g) and those of cultivation materials from bottles used once, twice and thrice (C3-5) were 2.27, 1.77 and 1.17 μg/g, respectively. Therefore, the BPA concentrations of cultivation materials from bottles decreased in a usage frequency-dependent manner. Thus, BPA was transferred from bottles to the cultivation materials during cultivation.

Edible mushrooms can be consumed raw and therefore their BPA concentration may directly impact health. Fruiting bodies (S1) grown in new bottles had the highest BPA concentration (0.686 μg/g). The BPA content of fruiting bodies cultivated in bottles used once and twice (C2-3) did not differ significantly (0.341 and 0.304 μg/g, respectively) (Fig. 5 and 6c). The BPA content of fruiting bodies from bottles used thrice (C4) was the lowest (0.232 μg/g). This suggests that fruiting bodies cultivated in used bottles had lower BPA levels than those cultivated in new bottles.

The previous study focused on the determination of free BPA levels (Matthews *et al*., 2001). Although others exist, the oral route is the major means of human exposure to BPA (Rubin, 2011; Völkel *et al*., 2002). BPA is an endocrine disruptor that interferes with the production, secretion, transport, action, function and elimination of natural hormones (Krishnan *et al*., 1993; Sun *et al*., 2004). BPA can imitate human hormones in a way that could be hazardous to health (Braekevelt *et al*., 2011). An earlier study of BPA metabolism

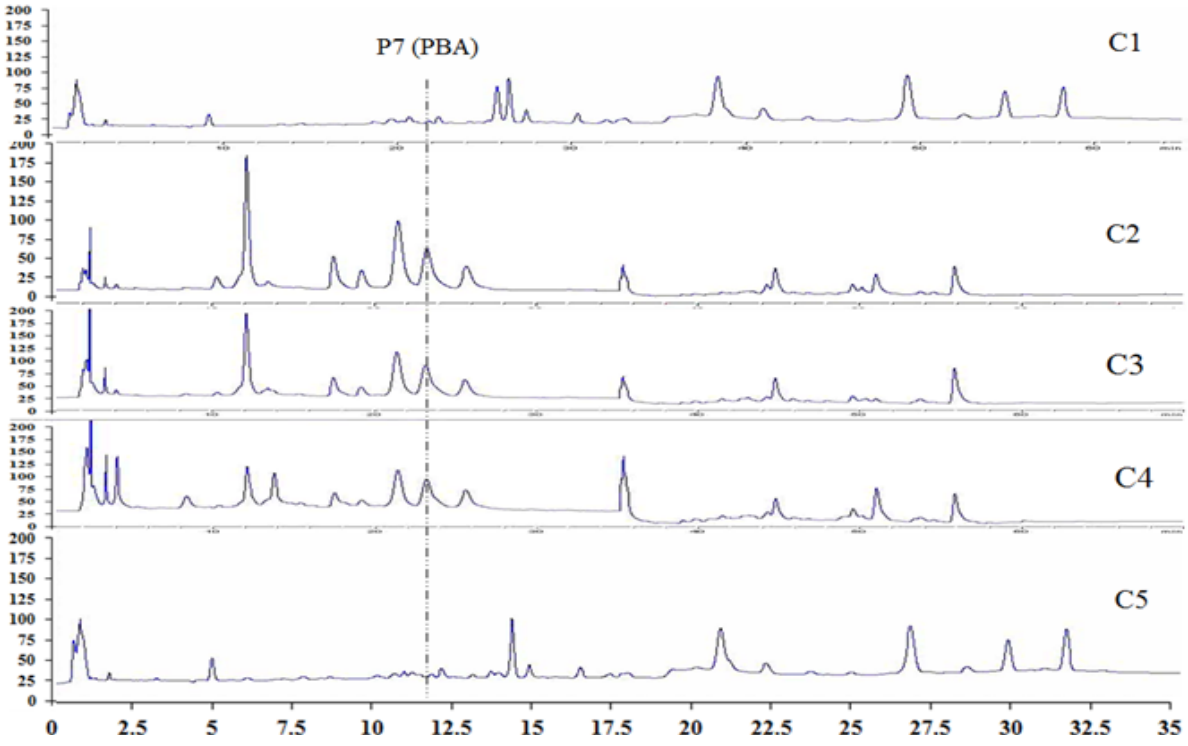


Fig. 4: HPLC chromatograms of extracts of samples C1–C5

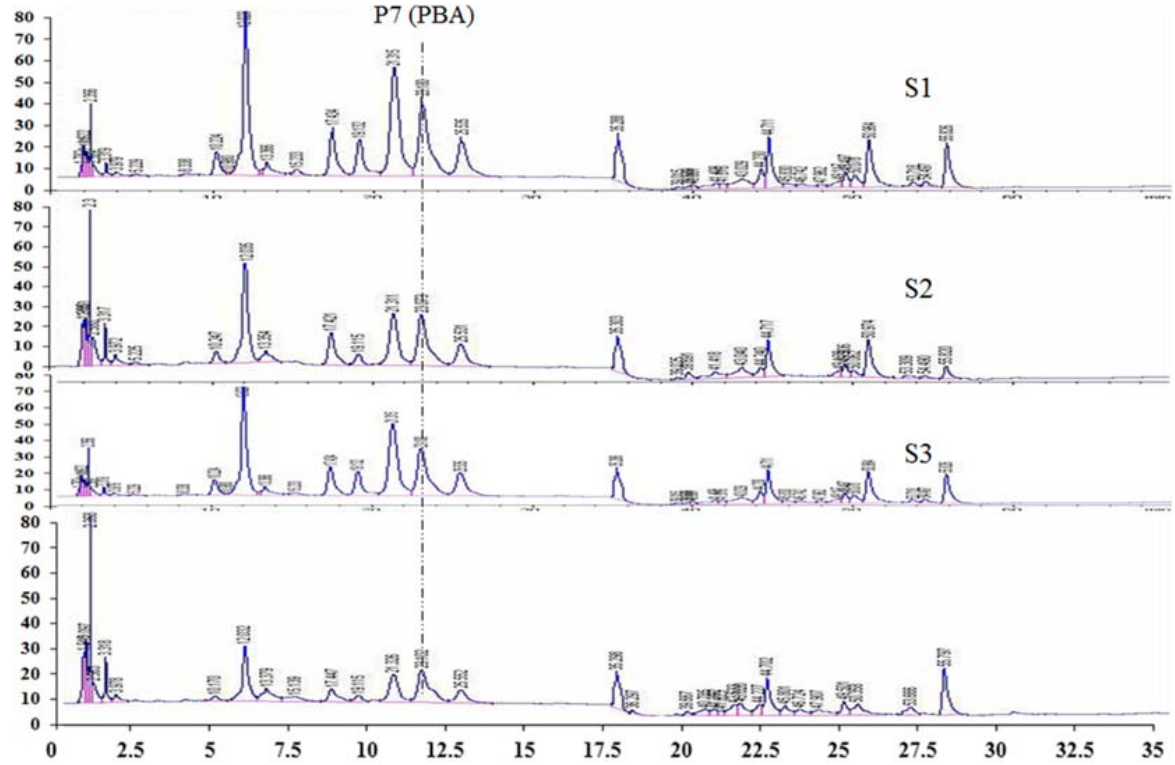


Fig. 5: HPLC chromatograms of extracts of samples S1–S4

concluded that BPA is rapidly metabolized to conjugates and excreted after ingestion (Völkel *et al*., 2002). However, there is mounting evidence that humans are internally exposed to unconjugated BPA.

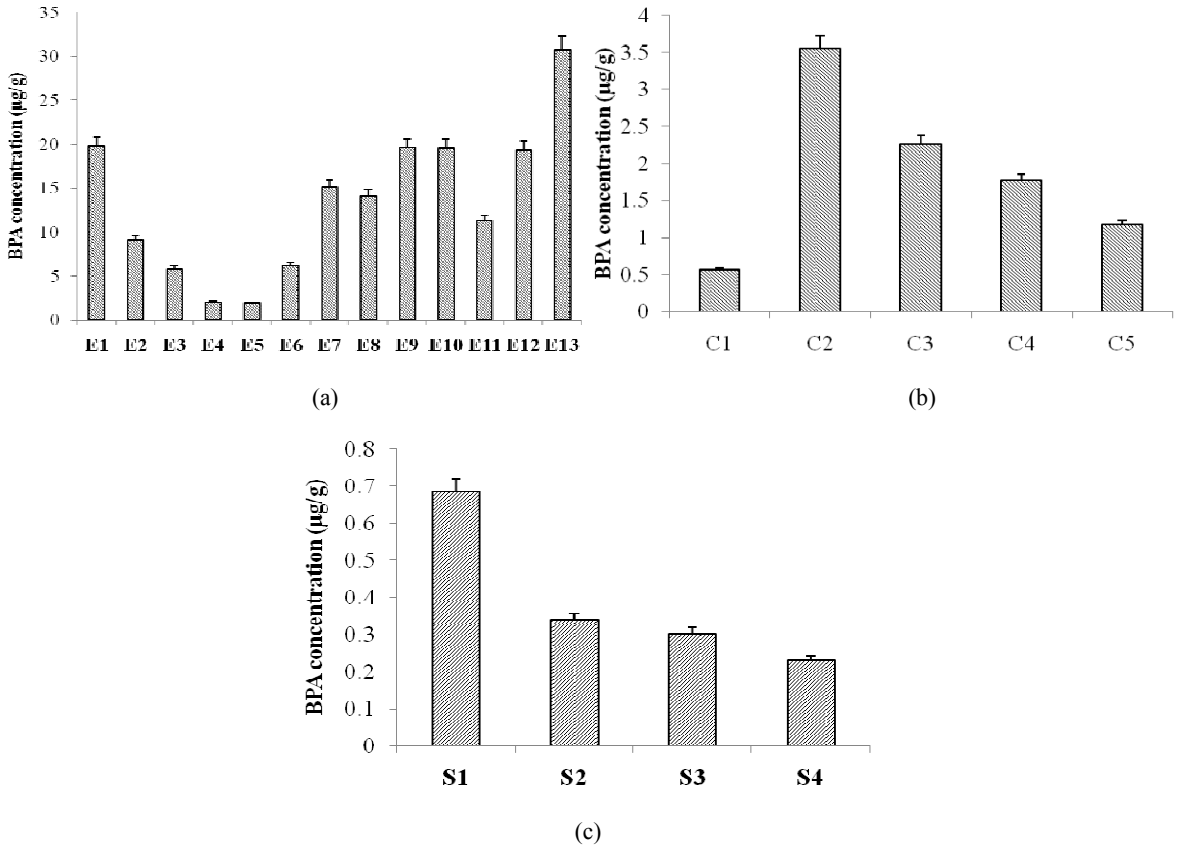


Fig. 6: The concentrations of BPA in different proessed samples (E1-E13, C1-C5 and S1-S4); (a): BPA concentrations in sample with cultivation bottles (E1-E13); (b): BPA concentrations in sample with cultivation materials (C1-C5); (c): BPA concentrations in sample with fruiting bodies (S1-S4)

As reviewed by Vandenberg *et al*. (2009, 2010), free BPA has been detected in urine, blood/serum, amniotic fluid, placental tissue and breast milk.

The effects of bottle usage frequency and applied treatments on BPA levels were investigated. Treatment at high temperature and pressure reduced BPA levels by up to two-thirds. This suggested that the reduction in BPA level was dependent on the state of BPA in the polymer, such as the degree of polymerization of the resin, or whether it was used as a primary material or an additive. Two possible structural relationships with the polymer were inferred:

- A low degree of polymerization of the resin with BPA resulted in breakage of chemical bonds by alkaline water, resulting in the release of free BPA.
- A portion of BPA decomposed rapidly due to gasification under high temperature and pressure.

BPA levels in cultivation materials increased and then decreased with increasing bottle usage frequency; new cultivation materials had the lowest BPA concentration (0.56 μg/g) (Fig. 6). Possible reasons for this phenomenon are as follows:

- The water mixed with cultivation materials contained BPA
- During long-term storage of cultivation materials, BPA in the packaging slowly penetrated cultivation materials
- During sterilization of cultivation materials before inoculation with *P. eryngii*, BPA was transferred from the bottles to cultivation materials, leading to increased BPA levels.

The BPA in fruiting bodies of *P. eryngii* originated mainly from cultivation materials and water vapor in the air and fruiting bodies cultivated in new bottles had higher BPA contents (0.586 μg/g) than those in used bottles. The BPA concentration in fruiting bodies decreased with increasing bottle usage frequency; i.e., 0.341, 0.304 and 0.232 μg/g BPA in bottles used once, twice and thrice, respectively. The BPA concentration in fruiting bodies was significantly lower than those in bottles and cultivation materials. This suggests that *P. eryngii* might degrade BPA. BPA biodegradation by fungi is mediated mainly by lignin-degrading enzymes such as manganese peroxidase (MnP) and lactase. MnP is a heme peroxidase that oxidizes phenolic compounds

in the presence of Mn (II) and H_2O_2 (Braunrath *et al*., 2005; Trasande *et al*., 2012). Laccase is a multi-copper oxidase and catalyzes one-electron oxidation of phenolic compounds by reducing oxygen to water (Kang *et al*., 2006). With the exception of those in new cultivation materials and fruiting bodies from bottles used once, twice and thrice, BPA concentrations were greater than the European Union migration limit of 0.6 mg BPA/kg for food (Kuroda *et al*., 2003). Treatment of bottles with high temperature and pressure, acid water and boiling water resulted in significant reductions in BPA levels. In contrast, BPA levels were not significantly affected by cold storage (-20°C). These treatments are used to reduce BPA levels in other plastic products.

CONCLUSION

Therefore, the BPA levels of fruiting bodies (*P. eryngii*) produced in bottles used once, twice and thrice from mushroom and vegetable do not pose a risk to human health. During actual production, we suggest the following methods of reducing BPA content: New and empty cultivation bottles should be subjected to high-temperature sterilization in the first and second maintenance of a neutral pH during sterilization of cultivation materials. Under alkaline conditions, total BPA content of cultivation materials might be increased due to breaking of chemical bonds used to polymerize the resin with BPA.

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