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Extraction of mycotoxin alternariol from red wine and from tomato juice with beta-cyclodextrin bead polymer

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ABSTRACT

Alternariol (AOH) is a mycotoxin which occurs in wine and tomato products as contaminant. Cyclodextrins (CDs) are ring-shaped glucose oligomers. CD polymers seem to be suitable for the removal of certain mycotoxins from aqueous solutions, including different beverages. In our recent study, insoluble β-CD bead polymer (BBP) almost completely removed AOH from aqueous solutions (pH 3.0–7.4). In this study, the time- and temperature-dependence of AOH-BBP interaction as well as the regenerability of the polymer after mycotoxin binding were examined. Furthermore, we tested the ability of BBP to extract AOH from spiked wine and tomato juice samples, during which the quality of beverages was also monitored. In addition, we describe here a novel albumin-based method for the extraction of AOH from tomato juice, used to analyze the rest of the mycotoxin in these samples. AOH-BBP interaction did not show temperature dependence (20–40 °C), while the incubation time markedly affected the mycotoxin extraction. After AOH binding, we successfully regenerated BBP with 50 *v*/v% ethanol-water mixture. Moreover, BBP strongly decreased the AOH content of both wine and tomato juice samples, suggesting the potential suitability of CD polymers as AOH binders in some beverages.

1. Introduction

Alternariol (AOH) is a dibenzo-α-pyrone mycotoxin (Fig. 1A) produced by *Alternaria* species. Its acute toxicity is considered to be low [1], however, the chronic exposure may cause mutagenic, carcinogenic, xenoestrogenic, and immunomodulatory effects [1–3]. AOH contamination has been reported in several commodities and processed products such as cereals [4], chestnuts, oilseeds [5], and fruits [6]. Furthermore, some milk thistle (*Silybum marianum*) based, hepatoprotective dietary supplements were found to contain high amounts of AOH (4560 μg/kg or 17.7 μmol/kg), which may partly compromise their beneficial therapeutic effects [4]. Tomato and grape are soft-skinned fruits which are particularly susceptible to *Alternaria* infection; therefore, their processed products (e.g., wine and tomato juice) are frequently contaminated with AOH [7,8]. AOH was detected in wines at 1.68–18 μg/L (0.007–0.07 μM) concentrations [9,10], while tomato products contained the mycotoxin between 6.1 and 25 μg/kg (0.024–0.1 μmol/kg) [6,10]. The average daily dietary exposure to AOH has been estimated between 1.9 and 39 ng/kg, which strongly exceeds the suggested threshold value (2.5 ng/kg/day) [11]. Vegetarians and infants, with the higher intake of cereal-based products, are likely exposed to higher amounts of AOH [4,5]. Due to its common incidence, AOH can be classified as an "emerging mycotoxin" [12]; however, there are no regulatory limits for AOH and other *Alternaria* mycotoxins in food and feed yet [13]. In addition, further analytical data are required for the proper risk assessment [5]. The emerging presence of mycotoxins in foodstuffs poses a serious threat to human health and makes the development of decontamination and/or detoxification methods particularly important. Decontamination strategies can be classified as physical, chemical, and microbiological approaches with varying degrees of effectiveness [14]. Traditional methods include heat treatment, irradiation, chemical detoxification, degradation by microorganisms, and adsorbents [15].

Cyclodextrins (CDs) are cyclic oligosaccharides, they have a lipophilic internal cavity and a hydrophilic outer surface. Therefore, CDs can form host-guest type inclusion complexes with lipophilic guest molecules [16]. The most commonly applied β-CDs are built up from seven glucose units. CDs are widely used by food, cosmetic, and pharmaceutical industries for solubilization or masking unpleasant odor/taste of certain components [17] as well as employed by analytical chemistry to enhance sample preparation, separation, and/or sensitivity of detection [16]. In previous studies, certain CDs successfully alleviated

Abbreviations: AOH, alternariol; BBP, β-cyclodextrin bead polymer; BSA, bovine serum albumin; CD, cyclodextrin.

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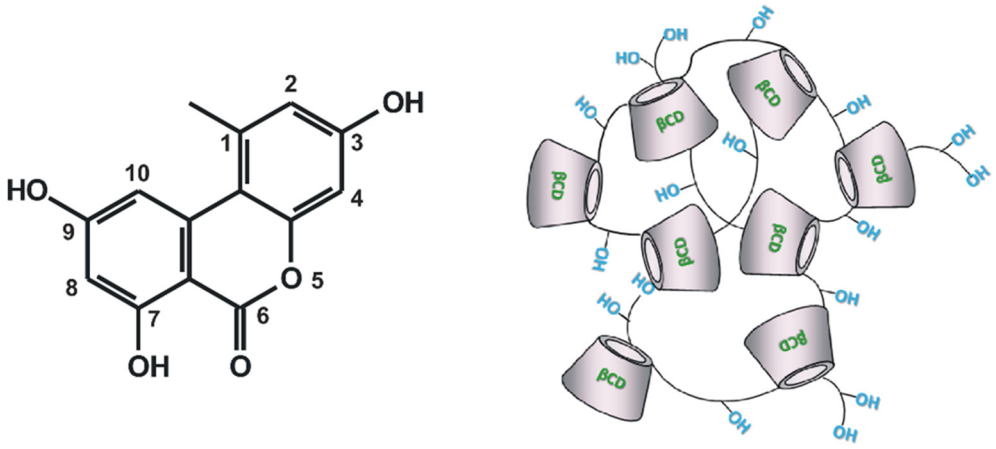

Fig. 1. Chemical structure of alternariol (AOH; A) and the schematic representation of epichlorohydrin cross-linked β-cyclodextrin bead polymer (BBP; B).

zearalenone-induced toxicity in cell experiments and in zebrafish embryos [18,19], due to the formation of stable mycotoxin-CD complexes. CD polymers are synthesized by cross-linking CD monomers with epichlorohydrin, polyurethane, or diisocyanate [20,21]. They have been extensively applied to remove contaminants from wastewater and freshwater [22,23] and to develop novel drug-delivery systems [24]. Furthermore, insoluble CD polymers have been successfully applied for the removal of mycotoxins from aqueous solutions [25,26], beer [25], wine [21], and apple juice [27]. Recent studies also revealed that masked (e.g., zearalenone-14-glucoside) and other modified (e.g., zearalenone-14-sulfate) mycotoxins can also be extracted by β-CD bead polymer [28,29].

In our previous investigation, the interactions of AOH with CDs and CD polymers have been examined in different buffers (pH 3.0–10.0) [26]. Interestingly, both soluble and insoluble β-CD polymers proved to be more effective binders of AOH compared to β-CD monomers, suggesting the cooperativity of CD rings in polymers [26]. These studies also demonstrated that the insoluble (water-swellable) β-CD bead polymer (BBP; Fig. 1B) can almost completely remove AOH from aqueous solutions between pH 3.0 and 7.4 (while BBP was poorly effective at pH 10.0). Furthermore, in our recent work, AOH-BBP interaction has been quantitatively characterized employing the Langmuir (42 mg AOH is bound by 1 g of BBP) and Freundlich ($K_F = 5.5$ (mg/g) × (L/mg)$^{1/n}$) models [26]. These data suggest the potential utilization of BBP for the removal of AOH from solutions. Therefore, in the current study, we aimed to further characterize the AOH-BBP interaction, including the time- and temperature-dependence as well as the regenerability of the polymer after mycotoxin binding. In addition, the extraction of AOH by BBP from beverages (spiked red wine and tomato juice samples) has been investigated. In our preliminary studies, liquid-liquid and solid-phase extraction methods failed to effectively extract AOH from tomato juice, therefore, we developed and optimized a novel sample preparation method based on the high-affinity interaction of the mycotoxin with bovine serum albumin (BSA). Finally, we examined the effects of BBP on the color intensity and total polyphenol concentration in red wine and tomato juice, to monitor the potential BBP-induced quality changes of these beverages.

2. Materials and methods

2.1. Reagents

Alternariol (AOH) was purchased from Cfm Oskar Tropitzsch GmbH (Marktredwitz, Germany). Insoluble β-CD bead polymer (BBP; cross-linked with epichlorohydrin, β-CD content: 50 *m*/*m*%) was provided by CycloLab Cyclodextrin Research and Development Laboratory Ltd. (Budapest, Hungary). HPLC grade acetonitrile and ethanol (96 *v*/*v*%) were obtained from VWR (Budapest, Hungary). Dichloromethane was purchased from Reanal (Budapest, Hungary). Bovine serum albumin (BSA), gallic acid, and Folin-Ciocalteu reagent were obtained from Merck (Darmstadt, Germany). Stock solution of AOH (5000 μM) was prepared in dimethyl sulfoxide (Fluka, NJ, USA) and stored protected from light at −20 °C.

2.2. Effect of incubation time on the removal of AOH from sodium tartrate buffer (pH 3.0) by BBP

To test the effect of incubation time on the binding ability of BBP, AOH solution (2 μM, 1.5 mL) was incubated with 5 mg BBP in a thermomixer, in sodium tartrate buffer (50 mM, pH 3.0) for 0, 2.5, 5, 10, 30, and 60 min (1000 rpm, 25 °C). After incubation, beads were sedimented by pulse centrifugation (4000 *g*, 3 s, room temperature). Then 250 μL acetonitrile was added to a 500-μL aliquot of the supernatant, after which AOH was quantified by HPLC-FLD (see in Section 2.7).

2.3. Effect of temperature on the removal of AOH from sodium tartrate buffer (pH 3.0) by BBP

To test the temperature-dependence of AOH-BBP interaction, AOH solution (2 μM, 1.5 mL) was incubated with 5 mg BBP in a thermomixer, in sodium tartrate buffer (50 mM, pH 3.0) for 40 min (1000 rpm) at 20, 25, 30, 35, and 40 °C. After incubation, sample preparation and analyses were identical as described in Section 2.2.

2.4. Testing the regenerability and reusability of BBP after AOH binding

The regenerability of BBP as AOH binder was also investigated, using the previously described protocol regarding zearalenone-BBP interaction [25], with minor modifications (see consecutive steps in Fig. 2): (1) AOH (2.0 μM, 1.5 mL) was incubated with BBP (5 mg) in sodium tartrate buffer (50 mM, pH 3.0) in a thermomixer (40 min, 1000 rpm, 25 °C). (2) The polymer was sedimented by pulse centrifugation (4000 *g*, 3 s, room temperature), then the supernatant was completely removed. (3) To elute the bound mycotoxin from BBP, beads were washed twice with 1.5 mL ethanol-water mixture (50:50 *v*/*v*%) for 20 min (1000 rpm, 25 °C). (4) After centrifugation (4000 *g*, 3 s, room temperature), ethanol-water mixtures were removed and combined. (5) Finally, BBP was conditioned by 1.5 mL sodium tartrate buffer (pH 3.0) for 15 s, and the supernatant was removed after centrifugation (4000 *g*, 3 s, room temperature). Subsequently, the process was repeated two times. After 1.5-fold dilution of samples with acetonitrile, AOH was quantified by HPLC-FLD (see in Section 2.7).

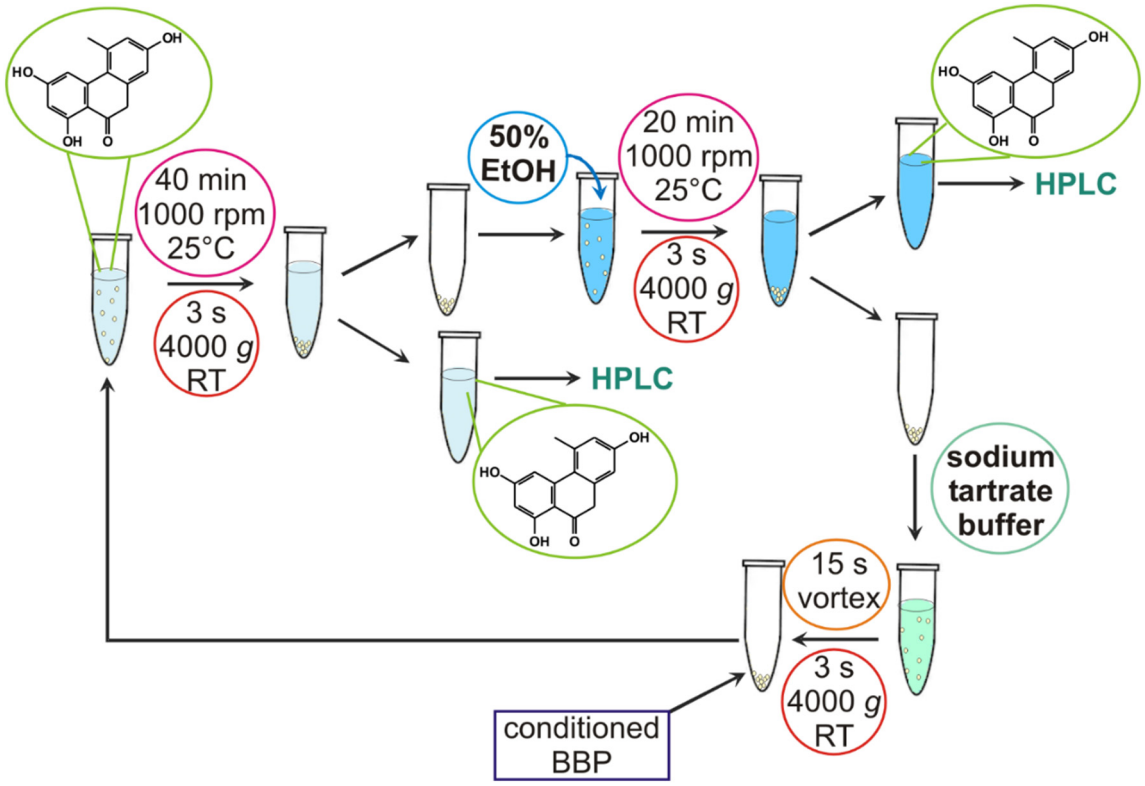

Fig. 2. Schematic representation of the extraction of AOH from sodium tartrate buffer (50 mM, pH 3.0) by BBP and the regeneration of the polymer with ethanol-water mixture (RT, room temperature; EtOH, ethanol). Magenta color represents incubation conditions in the thermomixer and red depicts centrifugation steps.

2.5. Removal of AOH from spiked red wine samples by BBP

Commercially available red wine (Csányi Winery: Cabernet Sauvignon 2016, Villány, Hungary) was spiked with AOH (final concentration: 2.0 μM). Spiked wine fractions (1.5 mL each) were incubated with increasing amounts of BBP (0, 1, 2.5, 5, 10, and 25 mg) in a thermomixer (1000 rpm, 40 min, 25 °C). After pulse centrifugation (4000 *g*, 3 s, room temperature), a 1000-μL aliquot of the supernatant was carefully removed.

After the treatment with BBP, the rest of the AOH was extracted from wine samples employing dispersive liquid-liquid extraction, based on the previously reported protocol [30], with minor modifications. Sodium chloride (0.05 g), acetonitrile (188 μL), and dichloromethane (2.0 mL) were added to the previously removed 1000 μL fraction of the supernatant (see above). Following 1 min vigorous vortexing, the cloudy mixture was centrifuged for 5 min (5000 *g*, room temperature). Thereafter, dichloromethane (lower phase) was carefully removed. Then the extraction with dichloromethane (2.0 mL) was repeated one more time. The organic solvent phases from the two extraction steps were combined, after which the residual water was removed by anhydrous sodium sulfate. After centrifugation (1 min, 5000 *g*, room temperature), a 3.5-mL fraction of the liquid was removed, and completely evaporated (Vacuum Pump, Büchi V-850 Vacuum Controller; Flawil, Switzerland) with a rotary evaporator (Büchi Rotavapor R-3; Flawil, Switzerland) at 40 °C. The dry residue was dissolved in 500 μL HPLC eluent (acetonitrile and pH 3.0 orthophosphoric acid, 40:60 *v/v%*), then AOH was quantified by HPLC-FLD (see in Section 2.7).

2.6. Removal of AOH from spiked tomato samples by BBP and by bovine serum albumin

Commercially available tomato juice (Solevita, manufactured in Hungary) was spiked with AOH (2.0 μM). Spiked samples (1.5 mL each) were incubated with BBP (0, 2.5, 5, 10, and 25 mg), using the same conditions described for wine samples (see in Section 2.5). After incubation, samples were centrifuged (14,000 *g*, 3 min, room temperature), then a 1000-μL aliquot of the supernatant was carefully removed.

After the treatment with BBP, the remaining AOH was quantified following an extraction procedure from tomato juice samples, based on its high-affinity interaction with BSA [31]. Incubation with BBP and extraction steps with BSA are demonstrated in Fig. 3. During the development of the extraction procedure with BSA, we tested the optimal environmental conditions in the pH range of 3 to 8. These preliminary studies suggested the highest recoveries of AOH between pH 7–8. Therefore, to produce appropriate conditions for AOH-BSA complex formation, the previously removed 1000 μL fraction of the supernatant (see above) was tuned approximately to pH 7 with 4 μL of 12 M sodium hydroxide, after which 1000 μL of 100 μM BSA solution (dissolved in phosphate buffered saline, pH 7.4) was added. Then ultrafiltration of these samples was carried out (7500 *g*, 10 min, 25 °C) employing Pall Microsep™ Advance centrifugal devices (30 kDa molecular weight cut-off; VWR, Budapest, Hungary) as described previously [31,32]. Since high BSA concentration was applied, it entraps practically the total amount of the mycotoxin in the retentate. Retentate was collected and diluted with twofold volume of acetonitrile, to precipitate albumin (which consequently liberates AOH from its BSA complex). After centrifugation (10 min, 14,000 *g*, 3 °C), AOH content of the supernatant was quantified by HPLC-FLD (see in Section 2.7).

2.7. HPLC analyses

AOH was quantified employing a Jasco HPLC system (Tokyo, Japan), which includes a binary pump (PU-4180), an autosampler (AS-4050), and a fluorescent detector (FP-920). Chromatographic data were evaluated employing ChromNAV2 software (Jasco, Tokyo, Japan). Limit of

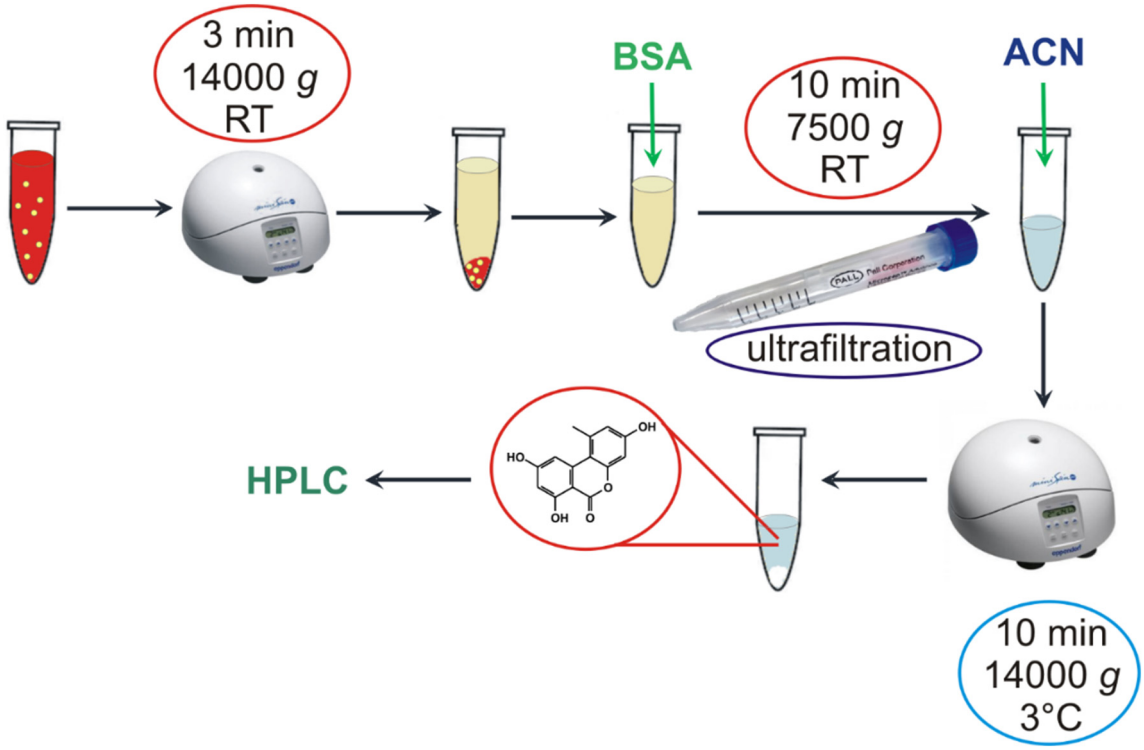

Fig. 3. Schematic representation of the extraction processes of AOH from tomato juice (after the treatment with BBP), using BSA as affinity protein (ACN, acetonitrile; RT, room temperature).

detection (LOD) and limit of quantification (LOQ) values were determined as the lowest concentrations where the signal-to-noise ratios were 3 and 10, respectively.

AOH concentrations in aqueous buffers and in extracts from wine samples were determined using the previously described HPLC-FLD method, without modification [26]. LOD and LOQ values were 25 nM (6.5 μg/L) and 50 nM (12.9 μg/L), respectively. The method showed good linearity ($R^2 = 0.999$) in the 0.1–2.5 μM concentration range. The intra-day repeatability was tested as well, showing 5.4% as the coefficient of variation ($n = 5$).

In the extracts from tomato juice samples, AOH was co-eluted with other constituents; therefore, the following HPLC-FLD method was employed in these experiments. Samples (injected volume: 20 μL) were driven through a guard column (Phenomenex C18, 4.0 × 3.0 mm; Phenomenex, Torrance, CA, USA) linked to an analytical column (Kinetex XB-C18, 250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA) with 1.0 mL/min flow rate at room temperature. The isocratic elution applied sodium phosphate buffer (10 mM, pH 5.0) and acetonitrile (70:30 *v/v%*). AOH was detected at 455 nm ($\lambda_{ex} = 345$ nm). LOD and LOQ values were 100 nM (25.8 μg/L) and 200 nM (51.6 μg/L), respectively. The method showed good linearity ($R^2 = 0.994$) in the 0.2–2.5 μM concentration range. The intra-day repeatability was tested as well, showing 3.7% as the coefficient of variation ($n = 5$).

2.8. Testing the effect of BBP on the quality of red wine

Wine samples were incubated with BBP (0.0, 1.0, 2.5, 5.0, 10.0, and 25.0 mg/1.5 mL) using the same conditions as in Section 2.5. The color intensity and the total polyphenol content were examined with UV–Vis spectroscopy, employing a Jasco V-730 spectrophotometer (Jasco, Tokyo, Japan). Wine color intensity (WCI) was determined after five-fold dilution of 400 μL supernatant with distilled water [33]:

$$WCI = A_{420} + A_{520} + A_{620} \quad (1)$$

where A_{420}, A_{520}, and A_{620} are the absorbance values of samples at 420, 520, and 620 nm, respectively.

Investigation of total polyphenol content was performed with Folin-Ciocalteu reagent, using the previously reported method [34], with minor modifications. A 100-μL aliquot of the supernatant was diluted five-fold with distilled water. Then a 20-μL volume of these diluted samples was mixed with 100 μL Folin-Ciocalteu reagent, 300 μL sodium carbonate solution (20 *m/m%*), and 1580 μL distilled water. Samples were incubated for 30 min at room temperature in the dark, after which their absorbance was measured at 760 nm. Total polyphenol content of samples was expressed in gallic acid equivalents (GAE), using a calibration curve of gallic acid standards incubated in the same way.

2.9. Testing the effect of BBP on the quality of tomato juice

Effects of BBP (0.0, 2.5, 5.0, 10.0, and 25.0 mg/1.5 mL) on the color and polyphenol content of tomato juice were also tested, using the same conditions as in Section 2.6. After incubation and centrifugation, a 200-μL aliquot of the supernatant was diluted ten-fold with distilled water, after which the color quality (CQ) was evaluated based on the absorbance measured at 550 and 650 nm [35]:

$$CQ = A_{650}/A_{550} \quad (2)$$

where A_{650} and A_{550} are absorbance values at 650 and 550 nm, respectively. The polyphenol content of tomato juice after its incubation with BBP was tested as described in Section 2.8.

2.10. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM) derived from at least three independent measurements. Statistical significance was established ($p < 0.05$ and $p < 0.01$) employing IBM SPSS Statistics software (IBM Corporation, New York, NY, USA), based on one-way ANOVA and Tukey's post-hoc test.

3. Results

3.1. Effects of incubation time and temperature on AOH-BBP interaction

First, the time- and temperature-dependence of AOH-BBP interaction were tested. The incubation time strongly influenced the removal of AOH by the polymer from aqueous buffer (pH 3.0). As Fig. 4A demonstrates, the concentration of the mycotoxin was reduced by 42, 63, and 85% after 2.5, 10, and 60 min incubation, respectively. However, after 30 min, BBP did not induce further relevant decrease in AOH concentration. Furthermore, AOH-BBP interaction did not show significant temperature-dependence in the 20–40 °C range (Fig. 4B).

3.2. Regenerability of BBP as AOH binder

After the removal of AOH by BBP from sodium tartrate buffer (pH 3.0), we tried to displace the bound toxin from the polymer by washing it twice with 50 *v/v%* ethanol-water mixture (see experimental details in Section 2.4 and in Fig. 2). Then the binding procedure was repeated twice. Results are summarized in Table 1, where line "A" shows the concentration of AOH in the buffer after incubation with BBP, while line "B" represents the amount of the mycotoxin in the combined (first and second washing steps) ethanol-water mixtures after elution. Both "A" and "B" were expressed as % of the initial amount of AOH in the buffer. BBP was successfully regenerated with 50 *v/v%* ethanol, and proved to be similarly effective AOH binder during its second and third application as first time. In addition, we were able to regain the bound mycotoxin from the polymer.

3.3. Removal of AOH from spiked red wine samples by BBP

To test the removal of AOH by BBP from red wine, samples were spiked with 2 μM AOH then incubated with increasing concentrations of BBP. Before spiking, AOH content of the wine was tested, it did not contain detectable amount of the mycotoxin (LOD = 25 nM or 6.5 μg/L). After the incubation of spiked wine with BBP, AOH content of samples were extracted and quantified as described in Sections 2.5 and 2.7, respectively. The recovery of the liquid-liquid extraction method used was 70.8 ± 1.8% in the 0.25–2 μM concentration range. As Fig. 5A demonstrates, BBP decreased the AOH content of wine in a concentration-dependent fashion. Even 2.5 mg/1.5 mL concentration of BBP reduced significantly the AOH content, and 25 mg/1.5 mL polymer removed approximately 80% of the mycotoxin.

3.4. Effects of BBP on the color and total polyphenol content of wine

Since BBP is not a selective binder of AOH, it likely interacts with other wine components, including anthocyanins and other polyphenols. These interactions may affect the quality of the wine. Therefore, the effects of BBP on the color and total polyphenol content of red wine were also examined (see details in Section 2.8). In the controls (without BBP), the WCI value and the total polyphenol content of the wine were 1.32 ± 0.01 and 1.60 ± 0.06 g/L GAE, respectively. Both the color intensity and the polyphenol content of wine samples were gradually decreased after the incubation with increasing amounts of BBP (Fig. 5B). However, BBP induced a considerably lower relative decrease in WCI value and polyphenol level compared to the AOH content of spiked samples. For example, 25 mg/1.5 mL BBP caused 25 and 39% decrease in color and polyphenol concentration, respectively; while induced 80% reduction in AOH content (Fig. 5).

3.5. Extraction of AOH from spiked tomato juice samples with bovine serum albumin

Since in our preliminary experiments liquid-liquid and solid-phase extractions did not prove to be appropriate in tomato juice (and affinity

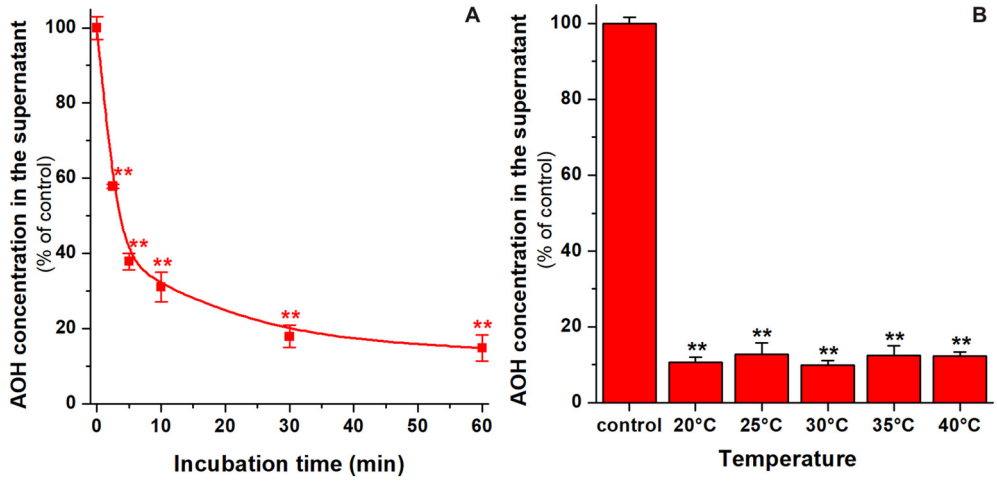

Fig. 4. Time- (A) and temperature-dependence (B) of AOH removal from sodium tartrate buffer (50 mM, pH 3.0). Extraction was performed in a thermomixer (1000 rpm), in the presence of 2 μM mycotoxin and 5 mg/1.5 mL BBP ($n = 3$; $^{**}p < 0.01$). Time-dependence was tested at 25 °C, while temperature-dependence was examined after 40 min incubation of samples with BBP. The control means the concentration of AOH in the solution at room temperature, without the incubation with BBP.

Table 1
Testing the regenerability and the reusability of BBP as AOH binder. Removal of AOH by BBP from sodium tartrate buffer (pH 3.0, 25 °C, 40 min), and the elution of the mycotoxin from the polymer by 50 *v/v%* ethanol-water mixture. "A" and "B" were expressed as % of the initial amount of AOH in the buffer ($n = 3$).

Number of applications	Procedure performed	AOH (% ± SEM) in the buffer (A) or in the eluent (B)	Σ (% ± SEM)
1st application of the polymer	A: After extraction with BBP	15.8 ± 1.2 (buffer)	100.9 ± 5.0
	B: After two washing steps of BBP with 50 *v/v%* ethanol	85.1 ± 3.8 (eluent)	
2nd application of the polymer	A: After extraction with BBP	14.5 ± 2.1 (buffer)	95.7 ± 6.2
	B: After two washing steps of BBP with 50 *v/v%* ethanol	81.2 ± 4.1 (eluent)	
3rd application of the polymer	A: After extraction with BBP	14.6 ± 0.5 (buffer)	–

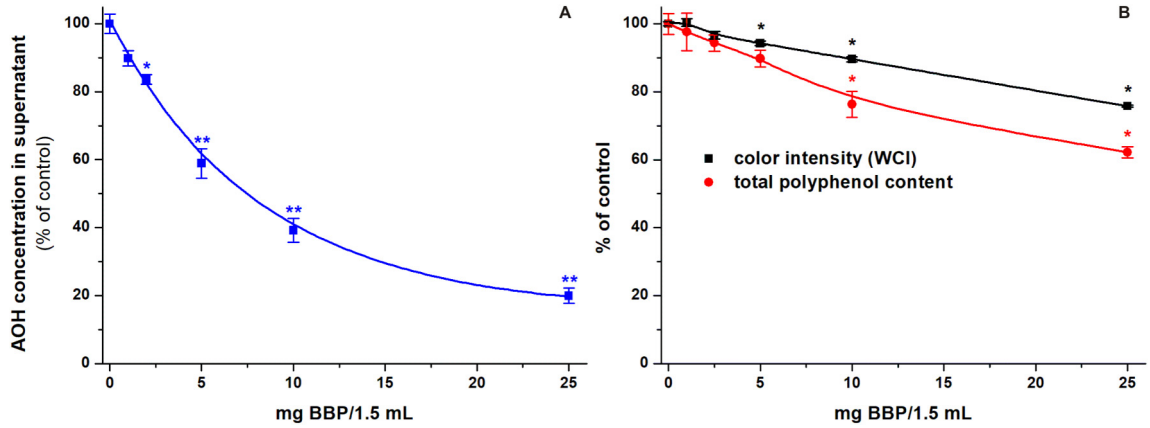

Fig. 5. (A) Extraction of AOH (2 μM) from spiked red wine samples by increasing concentrations of BBP (0.0, 1.0, 2.5, 5.0, 10.0, and 25.0 mg/1.5 mL). (B) Changes in the color and total polyphenol content of red wine after its incubation with BBP (0.0, 1.0, 2.5, 5.0, 10.0, and 25.0 mg/1.5 mL). Incubations were performed in a thermomixer (40 min, 1000 rpm, 25 °C; $n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$).

columns for AOH are not commercially available), we developed a BSA-based method (see details in Section 2.6 and in Fig. 3) to extract the remaining fraction of AOH after the treatment with BBP. Because the interaction of AOH with albumin is not selective, high albumin excess (50 μM BSA vs. 2 μM mycotoxin) was applied. The further increase in BSA concentration did not improve the recovery in tomato juice (data not shown). The extraction was similarly effective from both aqueous buffer (potassium phosphate, pH 7) and tomato juice: In the 0.5–2.5 μM concentration range, the recovery of the extraction was 59.4 ± 1.6% and 55.7 ± 2.4% in potassium phosphate buffer and in spiked tomato juice samples, respectively.

3.6. Removal of AOH from spiked tomato juice samples by BBP

To investigate the extraction of AOH from tomato juice by BBP, samples were spiked with 2 μM AOH and incubated in the presence of increasing BBP concentrations. Before spiking, AOH content of tomato juice was tested, it did not contain detectable amount of the mycotoxin (LOD = 100 nM or 25.8 μg/L). After the incubation with BBP and the centrifugation of these samples, the remaining AOH was extracted from the supernatant using BSA as affinity protein (see in Fig. 3 and Section 3.5), then the mycotoxin was quantified by HPLC-FLD (see details in Section 2.7). Our results demonstrate that BBP decreased the concentration of AOH in tomato juice in a concentration dependent fashion (Fig. 6A). BBP induced 19% decrease in AOH content at 2.5 mg/1.5 mL concentration. The highest applied amount of BBP (25 mg/1.5 mL) caused approximately 50% reduction in AOH content; however, it only slightly exceeded the effect of 10 mg/1.5 mL polymer (Fig. 6A).

3.7. Effects of BBP on the quality of tomato juice

Since BBP may interact with certain components in tomato juice, we tested its effects on the color quality and polyphenol content. In the controls (without BBP), the CQ value and the total polyphenol content were 0.734 ± 0.002 and 0.24 ± 0.03 g/L GAE, respectively. As Fig. 6B demonstrates, BBP did not affect the color quality of tomato juice, even at the highest concentration applied. However, the total polyphenol content was reduced in a concentration-dependent fashion. The lowest (2.5 mg/1.5 mL) and highest (25 mg/1.5 mL) concentrations of the polymer induced 27 and 46% decrease in the polyphenol content of tomato juice, respectively. It is close to the relative decrease in the AOH content caused by BBP in this beverage (Fig. 6).

4. Discussion

In this study, the interaction of AOH with BBP was tested in aqueous buffer as well as in spiked wine and tomato juice samples. In previous experiments, BBP proved to be an effective binder of AOH under acidic and slightly alkaline conditions (pH 3.0–7.4), while it was less effective under strongly alkaline circumstances (pH 10.0) [26]. AOH is a common contaminant in wines and tomato products [4,9], the pH values of these beverages are around 3.1–4.4 [36,37]. Therefore, we performed our

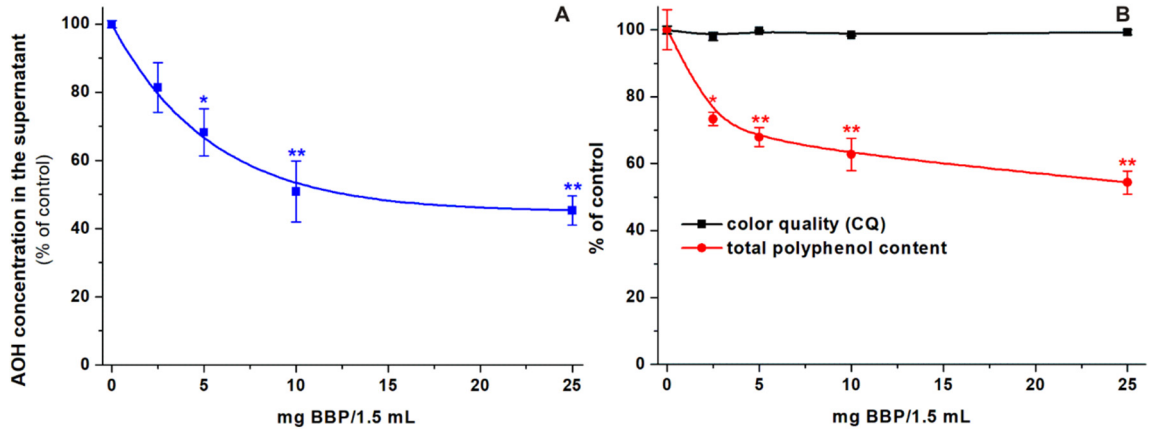

Fig. 6. (A) Extraction of AOH (2 μM) from spiked tomato juice samples by increasing concentrations of BBP (0.0, 2.5, 5.0, 10.0, and 25.0 mg/1.5 mL). (B) Changes in color and total polyphenol content of tomato juice after its incubation with BBP (0.0, 2.5, 5.0, 10.0, and 25.0 mg/1.5 mL). Incubations were performed in a thermomixer (40 min, 1000 rpm, 25 °C; $n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$).

investigations at pH 3.0 regarding the time- and temperature-dependence of AOH-BBP interaction and the regenerability of the polymer after AOH binding. Similarly to the environmental pH, the incubation time is also an important parameter of AOH-BBP interaction, at least 30 min incubation seems to be reasonable to achieve close to maximal removal of the mycotoxin (Fig. 4A). However, we did not observe relevant temperature-dependence (20–40 °C) (Fig. 4B). In previous experiments, zearalenone and some of its reduced/conjugated metabolites [25,29] were extracted by BBP from aqueous solutions with comparable efficacy as AOH in the current study. Similarly to AOH-BBP, zearalenone-BBP interaction did not show relevant temperature-dependence; nevertheless, BBP removed close to maximal amount of zearalenone after 10 min incubation [25].

The reusability of BBP as AOH binder is an important issue if we consider the cost-efficiency aspect of its potential application. Since high concentrations of organic solvents can displace guest molecules from the apolar cavity of CDs [25,38], CDs can be regenerated with relatively concentrated ethanol-water or methanol-water mixtures without the damage of their structure or their ability to interact again with guest molecules [23,39]. In this study, BBP was successfully regenerated after AOH extraction by 50 *v/v%* ethanol-water mixture, showing the same AOH binding ability during the first, second, and third applications (Table 1). These results are in agreement with our previous studies with zearalenone-BBP interaction [25], and with other studies employing ethanol-water mixture to regenerate hexamethylene diisocyanate- and epichlorohydrin-crosslinked β- and γ-CD polymers [39,40]. Some reports also demonstrated the regenerability and reusability of CD polymers in three to four cycles [38,39,42]. Moreover, other studies examined the limits of regenerability, suggesting that CD polymers can be applied even 20–25 times without the relevant decrease in their binding ability [23,43–45]. In addition, the bound fraction of the mycotoxin was completely eluted from BBP by two consecutive washing steps (Table 1). This observation demonstrates that CD technology may be suitable for the solid-phase extraction of AOH for analytical purposes, as it has been described regarding mycotoxin patulin [27,41].

The detection of mycotoxins in commodities and beverages can be performed by various analytical procedures [30,46–48]. The development of selective and sensitive analytical methods requires appropriate sample clean-up procedures. Dispersive liquid-liquid microextraction (DLLME) is based on the formation of a stable emulsion between the aqueous sample, the extraction solvent, and the dispersive solvent [30]. It is a relatively novel technique applied in mycotoxin analyses in complex liquid matrices (including beverages) [30,48]. In a previous study, DLLME was applied for the extraction of ochratoxin A form spiked wine samples [30]. We successfully employed this method (with minor modifications) for the extraction of AOH from red wine (see in Section 2.5). However, the same method did not work regarding spiked tomato juice samples, and we failed to reproduce the liquid-liquid extraction method reported by Rodríguez-Carrasco and coworkers (this procedure was used for the analysis of *Alternaria* toxins in fresh tomatoes and tomato-based products) [48]. Nevertheless, in the latter study, mycotoxins were quantified by mass spectrometry, which has higher selectivity and sensitivity compared to FLD, and the differences in the quality of tomato products may also affect the extraction. We tried to extract AOH from tomato juice by solid-phase extraction employing SEP-PAK C18 cartridges (Waters, Milford, MA, USA), but it was also unsuccessful. Immunoaffinity-based clean-up of mycotoxins is a reliable method [49,50]; however, based on our knowledge, there is no marketed immunoaffinity clean-up column for the selective extraction of AOH. Therefore, we tried to solve this problem employing a non-selective mycotoxin binding protein. Human serum albumin can form highly stable complexes with certain mycotoxins, including ochratoxin A and AOH where the association constants (K_a) are approximately 10^7 L/mol [51] and 10^5 L/mol [26], respectively. Since albumin bound to magnetic beads [52] or immobilized on agarose solid phase extraction column [49] were successfully applied for the extraction and clean-up of ochratoxin A from aqueous solution and spiked wine, albumin seems to be a cheap alternative of antibodies for affinity-based extraction. Bovine albumin is widely available, cheaper, and forms more stable (approximately two-fold) complexes with AOH ($K_a = 8.1 \times 10^5$ L/mol) than human and porcine albumins [31]; therefore, we selected BSA for the extraction of AOH from tomato juice. In agreement with our expectations, BSA proved to be a suitable protein to extract AOH from aqueous solution and from tomato juice.

The development of decontamination methods has been in the focus of interest, due to the widespread occurrence of mycotoxins in animal feed, food, and beverages. The thermal degradation of *Alternaria* toxins in cereal-based products was tested; however, the limited success of this strategy can be explained by the high thermal stability of AOH [53]. Approximately 44% decrease in the AOH content of tomato juice was observed as a result of its heating to 110 °C for 90 min [54]. Adsorbents are also widely used for decontamination purposes. Removal of ochratoxin A (6.2 nM) from spiked wine samples was tested employing different adsorbents (e.g., egg albumin, chitin, bentonite, and chitosan) [55]. Among these adsorbents, chitosan (5 mg/mL) was the most effective (67% of ochratoxin A was removed). β-CD polyurethane polymer (2 mg/mL) extracted approximately 88–95% of ochratoxin A (6.2–24.8 nM) from spiked wine samples [21]. In the current study, BBP was a less effective binder of AOH in spiked tomato juice samples (approximately 50% of the mycotoxin was removed with 16.7 mg/mL polymer) than in red wine (Figs. 5 and 6). The lower binding ability in tomato juice (vs. wine) may be partly explained by the presence of fibers.

Furthermore, similarly to adsorbents, BBP does not bind selectively mycotoxins. Therefore, the polymer interacts with certain apolar constituents in beverages, such as some polyphenols. Interestingly, BBP decreased the color of red wine, while the color intensity of tomato juice was not affected. It may be explained by the fact that the red color of wine is mainly provided by malvidin-3-monoglucoside [33], which can interact with the CD cavity as an anthocyanin [56]. In contrast, tomato carotenoids (such as lycopene which is mainly responsible for the color of tomato) form poorly stable complexes with CDs [57,58]. Furthermore, BBP significantly decreased the polyphenol content in both red wine and tomato juice (Figs. 5 and 6). In wine, the relative decrease in AOH concentration was much higher than the reduction in the polyphenol content (Fig. 5). However, in tomato juice, the extraction of the relative AOH and polyphenol contents were close (Fig. 6). It may be explained by the higher polyphenol content of red wine vs. tomato juice and/or the different polyphenol composition in the two beverages. The latter may be also partly responsible for the better extraction of AOH from wine. Similarly to our previous study, we determined the K_a values of the formed complexes related to the molar monomer (β-CD) content of BBP [26]. These association constants (5.3×10^2 L/mol in wine and 3.5×10^2 L/mol in tomato juice) were approximately ten-fold lower compared to data determined in aqueous buffer (50 mM sodium phosphate, pH 3.0; $K_a = 4.7 \times 10^3$ L/mol) [26], suggesting again the interactions of BBP with other components of beverages.

Finally, it is important to discuss the potential contamination of beverages by BBP (e.g., degradation or byproducts), because it raises a concern regarding its safe application by food industry. During the synthesis of cross-linked CD polymers, the polymerization reaction is followed by neutralization, multiple exhaustive dialysis, and membrane filtration steps. Therefore, there is a very low probability that inorganic salts as well as hydrolysis byproducts used and formed upon the chemical modification remain in the bead polymers [59,60]. After analytical release, cross-linked CD polymers can be applied by both food and pharmaceutical industries. Therefore, besides their utilization for food processing, β-CD bead polymers can be used as surgical wound healing agents [59,60]. This latter application obviously requires very high purity.

5. Conclusions

In summary, we demonstrated the potential applicability of BBP for AOH extraction in aqueous buffer, red wine, and tomato juice. As disadvantages, we can consider the application of BBP as mycotoxin binder only if the stability of mycotoxin-BBP complexes are appropriately high (it gives better selectivity), and some changes in the quality of treated beverages are possible. As advantages, the water-insoluble (but water-swellable) BBP can be easily removed from solutions by filtration or sedimentation (with the bound mycotoxin), and it is recyclable due to the simple regenerability of the polymer. Since chemical modification of CDs can strongly influence their binding ability, it is reasonable to hypothesize that the affinity and/or selectivity of BBP toward AOH (or other mycotoxins) can be improved. Considering the above-listed observations, BBP seems to be worthy for further evaluation as a mycotoxin binder to test its suitability for food industry and/or analytical chemistry. In addition, herein, we also described a novel albumin-based extraction protocol for AOH, which is suitable to extract the mycotoxin from aqueous solutions, including tomato juice.

CRediT authorship contribution statement

Eszter Fliszár-Nyúl: Methodology, Investigation, Formal analysis, Writing – original draft. Ákos Szabó: Investigation, Formal analysis. Lajos Szente: Conceptualization, Resources. Miklós Poór: Conceptualization, Funding acquisition, Methodology, Writing – original draft. All authors have read, edited, and approved the final version of the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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