Running Title: PVPP reduces cross-reactivity of phenolic compounds during OTA ELISA

Polyvinylpyrrolidone reduces cross-reactions between antibodies and phenolic compounds in an enzyme-linked immunosorbent assay for the detection of ochratoxin A

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ABSTRACT

Ochratoxin A (OTA) is a fungal metabolite and putative carcinogen which can contaminate a variety of foods such as cereals, wine, and nuts. Commercial ELISA kits are known to give false-positive results for OTA concentrations when phenolic compounds are present. Pistachios represent a food matrix rich in phenolic compounds potentially contaminated with OTA, and were used to model OTA cross-reactivity. Polyvinylpolypyrrolidone (PVPP) was incorporated during extraction of OTA using a commercial ELISA protocol. HPLC methods were used to confirm that PVPP does not interact with OTA and levels of gallic acid and catechin remaining in pistachio extracts decreased with increasing PVPP application. Cross-reactivity of extracts also decreased with increasing PVPP application, and color loss was used as an indicator of anthocyanin removal. Incorporating PVPP into ELISA protocols allows for the continued use of rapid immunological methods in food matrices containing phenolic compounds.

Keywords: Ochratoxin A (OTA); ELISA; cross-reactivity; phenolic compounds; polyvinylpolypyrrolidone (PVPP)

1. Introduction

Ochratoxin A (OTA) is a chlorinated fungal secondary metabolite which is produced most frequently by *Aspergillus ochraceus*, *A. carbonarius*, *Penicillium verrucosum*, and *P. nordicum* (Serra, Braga, & Venancio, 2005). OTA is a putative carcinogen based on its ability to induce nephropathies in rats such as hyperplasia, degeneration, and karyomegaly of renal tubule epithelial cells (IARC, 1993; NTP, 1989). The diversity of OTA-producing fungi is paralleled in the range of agricultural food commodities which can be contaminated with OTA, which are most commonly cereals, nuts, and coffee (Zaied, Abid, Bouaziz, Chouchane, Jomaa, & Bacha, 2010; Coronel, Marin, Cano-Sancho, Ramos, & Sanchis, 2012; Amezcqueta et al., 2012). Robust analytical methods are required to quantify OTA concentrations in different food commodities in order to evaluate OTA risk assessment.

Analyzing food commodities using high performance liquid chromatography (HPLC) methods allows for a precise determination of OTA contamination. However, HPLC methods are lengthy and expensive as samples often need to undergo clean-up procedures before analysis (Zimmerli & Dick, 1995; Pittet, Tornare, Huggett, & Viani, 1996; Kieu & Ryu, 2014). Rapid methods, such as enzyme-linked immunosorbent assays (ELISA), offer a more cost-effective alternative to HPLC methods when analyzing a large amount of samples. ELISA kits for OTA quantification are easy to use, commercially available, and have been validated for use in multiple food matrices such as cereals, meats, and wine (Barna-Ventro et al., 1996; Visconti & Girolamo, 2006; Alcaide & Aguilar, 2008). ELISA kits showed comparable accuracy to HPLC methods when testing cereals that contained 0 – 80 ng/g, but the precision of ELISA methods decreased at lower OTA concentrations (Zheng, Hanneken, Houchins, King, Lee, & Richard, 2005; Flajs, Domijan, Ivic, Cvjetkovic, & Peraica, 2009). Moreover, ELISA methods are

susceptible to overestimation or false-positive results due to compounds which have structural similarities to the target analyte and which react with antibodies used in the assay.

Foods such as chocolate may be problematic during ELISA due to the presence of phenolic compounds as they can bind proteins or other compounds similar to target molecule or interrupt antigen-antibody reactions (Holzhauser, Stephan, & Vieths, 2002; Keck-Gassenmeier, Benet, Rosa, & Hischenhuber, 1999; Lee, Meldrum, Rivera, & Ryu, 2014; Tran, 2013). Phenolic compounds in wine are also known to interfere with OTA binding to antibodies (Visconti, Pascale, & Centornze, 1999). Analyzing pistachios for OTA using commercial ELISA kits resulted in significant overestimations of OTA content, which was not observed in kernels after the skins were removed (Lee et al., 2014). Pistachios contain a wide variety of phenolic compounds, which are located primarily in their skins (Tomiano, Martorana, Arcoraci, Monteleone, Giovinazzo, & Saija, 2010). Multiple methods have been proposed to reduce the interference effects of phenolic compounds including additional sample clean-up steps, sample dilution, or blocking agents (Alcaide & Aguilar, 2008; Steinitz, 2000; Flajs, Domijan, Ivic, Cvjetkovic, & Peraica, 2009; Lee, Wang, Allan, & Kennedy, 2004). Additional sample processing steps increase the cost and duration of analysis while diluting samples may decrease OTA concentrations below the detection limit of the ELISA kit. However, the utilization of extraction additive during extraction procedure can cause enhanced extraction efficiency and reduction of non-specific binding. Proteins, such as fish gelatin, skim milk power, bovine serum albumin (BSA), can be added to the extraction solvent to improve the extraction efficiency of the target antigens. A non-ionic detergent, such as Tween-20, is also used to help reduce non-specific binding.

Reducing the cross-reactivity requires an extraction additive which can facilitate the removal of phenolic compounds during OTA extraction. The insoluble phenol absorbent polyvinylpyrrolidone (PVP) has been used to reduce phenolic compound interference during enzyme activity assays (Loomis & Battaile, 1966). When phenolic compounds present in plants were interfering with ELISA tests screening for *Phytophthora* disease, PVP was used during extraction in phosphate-buffered saline to minimize interference (Olson, 1995). Polyvinylpolypyrrolidone (PVPP), an insoluble resin consisting of highly cross-linked PVP polymers has been used as a fining reagent to clarify wine and beer since 1961 (Dahlstrom & Sfat, 1972). Moreover, PVPP is often used to reduce interference of phenolic compounds in sample preparation (Charmont, Jamet, Pont-Lezica, & Canut, 2005). Multiple studies have investigated the effect of various fining reagents on the removal of OTA from wine, and PVPP did not significantly alter the OTA concentration during clarification (Fernandes, Ratola, Cerdeira, Alves, & Venacio, 2007; Castellari, Versari, Fabiani, Parpinello, & Galassi, 2001; Anh, Vural, & Bayram, 2001). The ability of PVPP to bind a wide range of phenolic compounds, remain insoluble in solution, and not interfere with OTA are all desirable characteristics for reducing cross-reactivity in commercial ELISA kits.

The aim of the current study was to determine the ability of PVPP to bind and remove phenolic compounds from a food matrix and reduce the cross-reactivity during OTA analysis using a commercial ELISA kit. PVPP binding characteristics were determined in model extraction solutions containing phenolic compounds and OTA. Pistachio skins were used as a model food matrix to evaluate the effect of PVPP application on removal of phenolic compounds, color loss, and cross-reactivity observed during ELISA.

2. Materials and Methods

2.1. Materials and Chemicals

Pistachios were purchased from a local supermarket chain (Moscow, ID, USA). Pistachio skins were collected by manually scraping kernels, ground into a fine powder using a coffee mill and stored at -20 °C until analysis. Purified standards of OTA, gallic acid, and catechin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Working standards were prepared in 50% methanol, stored at -20 °C in amber glass vials, and brought to room temperature before use. HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA), and acetic acid (99.5%), sodium acetate (> 99%) and phosphate buffered saline (PBS) tablets were obtained from Sigma-Aldrich (St. Louis, MO, USA). OchraTest WB immunoaffinity columns were purchased from VICAM (Watertown, MA, USA).

2.2. Model extraction

Solutions containing 5 mg/mL gallic acid were prepared in 0-70% methanol. Pre-weighed PVPP was added to give a final concentration of 10 mg/mL and the solutions were mixed using a wrist action shaker (Burrell Model 75, Pittsburgh, PA, USA) for 30 min. Two 1 mL aliquots of each sample were transferred to micro-centrifuge tubes before centrifugation ($9391 \times g$) at room temperature using an Eppendorf 5424 R centrifuge (Eppendorf, Hamburg, Germany). Supernatants were pooled and stored at 4 °C until analysis. For testing the effect of PVPP concentration, solutions containing 5 mg/mL of either gallic acid or catechin in 50% methanol (v/v) were spiked with OTA to give a final concentration of 10 ng/mL. Pre-weighed PVPP was added to give final concentrations of 10, 25, 50, 100, or 150 mg/mL. Samples were shaken and centrifuged as described above.

2.3. Pistachio skin extraction

Ground pistachio skins (0.5 g) were weighed and placed into 30 mL centrifuge tubes. PVPP in amounts of 50, 125, 250, 500, or 750 mg was added to each tube before the addition of 5 mL 50% methanol. Tubes were mixed using a wrist action shaker for 30 min before centrifugation ($12,062 \times g$) for 10 min at room temperature using a Sorvall RC-5 centrifuge (Thermo Scientific, Hudson, NH, USA). Supernatants were collected and stored at 4 °C until analysis. Supernatants were either analyzed directly using ELISA or HPLC for quantification of OTA or diluted in water for quantification of phenolic compounds.

2.4. HPLC analysis of OTA

For HPLC determination of OTA a previously validated method was used (Nguyen & Ryu, 2014). Briefly, 1.2 g pistachio skins and 12 mL acetonitrile/water (80:20, v/v) were mixed in a 30 mL centrifuge tube for 30 min before centrifugation at $9391 \times g$ as described above. Two milliliters of the supernatant were diluted with 8 mL PBS before filtering through an IAC at a flow rate of about 2-3 mL/min. The column was washed with 10 mL of PBS followed by 10 mL of water and OTA was eluted with 3 mL of methanol into a vial at a flow rate of 2-3 mL/min. The eluate was evaporated to dryness under a gentle stream of nitrogen at 50 °C. The residue was re-dissolved in 500 μ L 50% methanol (v/v) and 50 μ L of the aliquot was injected into HPLC (Agilent 1260 Infinity HPLC system, Palo Alto, CA, USA) equipped with a quaternary pump, an autosampler, a vacuum degasser, and a fluorescence detector. The chromatographic separation was performed on a reversed-phase Hypersil GOLD C₁₈ column (3 $\times$ 100 mm, particle size 1.9 μ m, Thermo Scientific, Hudson, NH, USA) with isocratic elution using 50% acetonitrile and

50% water containing 1% acetic acid at a flow rate of 0.4 mL/min. OTA was detected at 334 nm and 460 nm for excitation and emission, respectively.

2.5. OTA analysis using ELISA

A commercial OTA ELISA kit (Veratox®, Neogen Corporation, Lansing, MI, USA) was used to observe the cross-reactivity of pistachio skin extracts. Samples and standards were analyzed using the manufacturer's protocol, and the optical density was read at 620 nm using a microplate reader (Tecan Sunrise, Männedorf, Switzerland). Degree of interaction was calculated as the amount of OTA quantified using the ELISA protocol.

2.6. Analysis of phenolic compounds

The total phenolic compound contents of pistachio skins were determined by employing Folin-Ciocalteu colorimetric method (Arnous, Makris, & Kefalas, 2002). The total phenolic content was expressed as µg of gallic acid equivalents/g of fresh material, using calibration curves prepared with gallic acid as a standard.

All samples containing gallic acid or catechin were also analyzed for phenolic content by HPLC, in addition to the supernatants of all pistachio skin extracts. Gallic acid and catechin standards were prepared in water and used to generate standard curves for quantification. Phenolic compound content was also measured using an Agilent 1260 Infinity HPLC system (Palo Alto, CA, USA) equipped with a Luna C₁₈ column (4.6 × 250 mm, particle size 5 µm, Phenomenex, Torrance, CA, USA) and an UV-Vis detector. The mobile phase contained 1% acetic acid (solvent A) and acetonitrile (solvent B), with the linear gradient commencing at an

A/B ratio of 92/8 and finishing at 73/27 over 40 min at a flow rate of 0.8 mL/min. Detection was performed at 280 nm. Injection volume was 20 μ L for the analysis.

2.7. Color of pistachio skin extracts

To measure the effect of PVPP on clarifying pistachio skin extracts, a 1 mL aliquot of sample extract was loaded into a plastic dish and color was measured using a Minolta CR 400 colorimeter (Minolta, Osaka, Japan) mounted to a ring stand. Color values were recorded using the *Lab* color space, where *L* is the lightness dimension while *a* and *b* are color-opponent dimensions. To calculate the total color difference (ΔE), the following formula was used:

$$\Delta E = \sqrt{((L_{\text{sample}} - L_{\text{standard}})^2 + (a_{\text{sample}} - a_{\text{standard}})^2 + (b_{\text{sample}} - b_{\text{standard}})^2)}$$

2.8. Statistical analysis

The analytical results represent an average $\pm$ standard deviation of three determinations obtained for each parameter. Simple correlation coefficients and single factor ANOVA were used for determination of significant differences ($p < 0.05$). Statistical analysis was carried out using SPSS (IBM Corp., New York, NY, USA).

3. Results and discussion

3.1. Effect of methanol concentration on PVPP binding efficiency

Previous studies showed that PVPP binding of phenolic compounds was optimal when the methanol concentration was below 10% (Loomis & Battaile, 1966; Andersen & Sowers, 1968; Magalhaes et al., 2010). The first goal of this work was to verify if PVPP could remove phenolic compounds in 50% methanol used by the commercial ELISA protocol during the

extraction of OTA. Treating 5 mg/mL gallic acid solutions with 10 mg/mL PVPP removed 46%, 41%, and 34% of the gallic acid in 10%, 30%, and 50% methanol, respectively (data not shown). This negative trend has been reported for flavonoids, as increasing the methanol concentration from 25% to 70% decreased the fraction of quercetin bound by PVPP from 85% to 50%, respectively (Doner, Becard, & Irwin, 1993). While phenolic compound removal would be more efficient at lower methanol concentrations, OTA is not as soluble in water as in methanol (el Khoury & Atoui, 2010). To evaluate the recovery, OTA was incubated at four levels of 2, 5, 10, and 20 ng/g into 50% methanol containing PVPP. The mean recoveries of OTA were 98%, 99%, 100%, and 101% at four levels of 2, 5, 10, and 20 ng/g, respectively. This data indicated PVPP in 50% methanol did not affect OTA at different levels. Due to the detection range of commercial ELISA kits used in this research was 2 – 25 ng/g, a middle range concentration of OTA (10 ng/g) was chosen for further study. The methanol concentration remained at 50% for all subsequent experiments so that the only deviation from the manufacturer's protocol was PVPP incorporation.

3.2. Selective removal of phenolic compounds by PVPP

Gallic acid and catechin were chosen as they are the two of the most abundant phenolic compounds in pistachio skins (Seeram, Zhang, Henning, Lee, Niu, Lin, & Heber, 2006; Tomaino, Martorana, Arcoraci, Monteleone, Giovinazzo, & Saija, 2010). In addition, previous work demonstrated that gallic acid and catechin exhibited the highest degree of interference in two commercial ELISA kits for OTA detection (Lee et al., 2014). Following the PVPP treatment, OTA recovery and remaining individual phenolic compounds were measured by HPLC. Model extraction using 5 mg/mL of either gallic acid or catechin with 10 ng/g OTA were used to

observe any PVPP interference with OTA quantitation while binding phenolic compounds. The chemical structures of compounds used in this study are presented in Figure 1. Increasing the concentration of PVPP displayed a dose-dependent decrease in the amount of phenolic compounds in solution without affecting the OTA concentration (Fig. 2). PVPP treatment significantly ($p < 0.05$) decreased the amount of gallic acid and catechin remaining. It appeared that during model extractions OTA remains in solution while phenolic compounds are interacting with PVPP, which may be attributed to the methanol concentration used during extraction. When wine was spiked with OTA and diluted in water before treatment using 10 mg/mL PVPP, 16% of the spiked OTA was lost (Bazin, Faucet-Marquis, Monje, El Khoury, Marty, & Pfohl-Leskowicz, 2013). The minimal interaction of PVPP with OTA concentrations in wine observed in previous studies (Fernandes et al., 2007; Castellari et al., 2001; Anh et al., 2001) could have been due to the use of low concentrations of PVPP used for wine clarification (1.5 µg/mL to 1 mg/mL). Minimal amounts of fining reagents were used to reduce the loss of phenolic compounds which provide beneficial flavors.

PVPP was able to bind and remove catechin more effectively than gallic acid (Fig 2). This observation is in accordance with previous studies using PVPP binding assays (Magalhaes et al., 2010; Duran-Lara et al., 2015). The increased number of hydroxyl groups and additional benzene ring present on the catechin molecule compared to gallic acid may explain the difference in PVPP binding affinity. The main non-covalent interactions between a phenolic solute and PVPP are hydrophobic effects between the benzene ring of phenols and methylene groups of the polymer and hydrogen bonding between phenolic hydroxyl groups and the carbonyl group in the pyrrolidone ring (Molyneux & Vekavakayanondha, 1986). After testing various flavonoids in PVP binding assays, a trend emerged where PVP binding efficiency

increased with the number of hydroxyl groups present on the molecule (Doner et al., 1993). Increasing the number of phenol groups also increases the PVP binding efficiency during dialysis (Molyneux & Frank, 1961). By calculating interaction energies, the lower gap energy between PVPP and catechin compared to gallic acid was used to explain the difference in observed binding affinities by PVPP (Duran-Lara et al., 2015).

3.3. Color loss of pistachio skin extracts as affected by PVPP concentration.

The concentration of phenolic compounds in pistachio skins influence the degree of red coloration (Bellomo & Fallico, 2007). Therefore, the color of extract can be used as an indicator to measure the efficacy of PVPP in removing phenolic compounds that may interfere with anti-OTA antibodies in the commercial ELISA kits. Since the pistachio skins produced extracts with a dark red hue which faded with increasing PVPP application, color loss was measured as an indicator of PVPP effectiveness in removing anthocyanins. Color values of the extracts were recorded in the *Lab* color space and are presented in Table 1. All color dimensions were significantly affected by PVPP application ($p < 0.01$). The total color loss, ΔE , was linearly correlated to PVPP treatment ($R^2 = 0.90$). PVPP treatment clarified the extracts since the lightness dimension, L , increased while both a and b color dimensions approached zero with increasing PVPP treatment. Positive a and b color dimensions produce red, and a zero value translates to a neutral grey color in the *Lab* color space.

3.4. Effect of phenolic compound removal on ELISA cross-reactivity

Previous work demonstrated that phenolic compounds in pistachio skins can cross-react with antibodies in commercially available ELISA kits for OTA detection (Lee et al., 2014). The

pistachio skins contained 38919 ± 1428 $\mu\text{g/g}$ total phenolic compounds as determined by the Folin-Ciocalteu method.

Pistachio skin extracts without addition of PVPP displayed a degree of interaction of approximately 16.74 ng/g. The degree of interaction represents the amount of OTA quantified by the ELISA kit, and was used to measure cross-reactivity as HPLC analysis verified no detectable OTA present in pistachio skins (data not shown). Increasing the PVPP concentration decreased the amount of phenolic compounds remaining, in addition to decreasing the cross-reactivity of the supernatants (Fig. 3). Concomitant to the model extract solutions, PVPP was able to bind and remove catechin more effectively than gallic acid. The gallic acid concentration of the supernatants was loosely correlated ($R^2 = 0.78$) to the resulting cross-reactivity, while the concentration of catechin was not ($R^2 = 0.32$). Gallic acid exhibited a significantly greater degree of interference in ELISA kits compared to catechin (Lee et al., 2014). The degree of interaction was not significantly different between 50 and 100 mg/mL PVPP. At 150 mg/mL, PVPP was able to reduce the degree of interaction below the 2 ng/g OTA detection limit in two out of three replications and completely bound all gallic acid and catechin present in the pistachio skins.

4. Conclusions

Overall, the simple and convenient addition of PVPP during the extraction step was able to significantly remove phenolic compounds from pistachio skins. PVPP demonstrated no interaction with OTA during model extraction in 50% methanol. After separating phenolic compounds from a model food matrix which had been bound to PVPP using centrifugation, the resulting supernatants showed a dose-dependent decrease in cross-reactivity. Incorporating PVPP

during OTA extraction using commercial ELISA kits is a potential method to reduce the overestimation of OTA content in foods containing phenolic compounds.

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Figure Legend

Fig. 1. Chemical structure of ochratoxin A (OTA), gallic acid, catechin, and polyvinylpolypyrrolidone (PVPP)

Fig. 2. Effect of polyvinylpolypyrrolidone (PVPP) incorporation on the amount of gallic acid (A) and catechin (B) remaining in model extraction solvents spiked with 10 ng/g ochratoxin A (OTA).

Fig. 3. Effect of polyvinylpolypyrrolidone (PVPP) incorporation on the amount of gallic acid and catechin remaining in pistachio skin extracts, and the resulting ochratoxin A (OTA) cross-reactivity observed during ELISA.

Table 1. Color properties of pistachio skin extracts as affected by polyvinylpolypyrrolidone (PVPP) concentration.

Color Properties*	PVPP used during extraction (mg/ml)				
	0	10	25	50	100
<i>L</i>	25.1 ± 1.36	30.33 ± 1.66	29.35 ± 1.12	30.75 ± 0.47	33.47 ± 0.35
<i>a</i>	2.08 ± 0.64	1.27 ± 0.62	0.24 ± 0.49	-0.22 ± 0.18	-0.68 ± 0.03
<i>b</i>	3.86 ± 0.74	3.11 ± 1.07	1.33 ± 1.23	0.71 ± 0.22	-0.54 ± 0.47
<i>ΔE</i>	1.53 ± 0.62	4.61 ± 1.21	5.51 ± 0.58	6.89 ± 0.25	9.83 ± 0.36

**L*: Degree of whiteness (white +100 → 0 black); *a*: Degree of redness (red +100 → -80 green); *b*: Degree of yellowness (yellow +70 → -80 blue); *ΔE*: Total color difference =

$$\sqrt{((L_{\text{sample}} - L_{\text{standard}})^2 + (a_{\text{sample}} - a_{\text{standard}})^2 + (b_{\text{sample}} - b_{\text{standard}})^2)}$$

Figure 1.

OTA

Gallic acid

Catechin

PVPP

Figure 2.

Figure 3.