EFFECT OF SOLVENT ON AFLATOXIN CONTENT IN EXTRACTS OF VIRGINIA TYPE PEANUT SKINS

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Abstract:

Peanut skins are currently a waste product that may be a potential source of flavonoid rich compounds. In order to make use of these compounds, an extract would need to be produced. One of the potential issues with a peanut skin extract could be that any aflatoxins in the whole skins may be concentrated by solvent extraction. A multistep peanut skin extraction (PSE) method (100% water) and a methanol-water method (30:70 and 80:20) were used to extract proanthocyanidins from natural peanut skins samples and from peanut skin samples treated with an aflatoxin standard to determine the recovery by each extraction procedure. Liquid Chromatography-Mass Spectrometry (LC-MS) detection was employed for the determination and quantitation of aflatoxins G2, G1, B2, and B1 in the extracts from each of the samples. No aflatoxins of interest were detected in the PSE, 30% methanol aqueous (aq.), and 80% methanol aq. extract of un-spiked peanut skin samples.

For aflatoxin spiked standard (G2, G1, B2, B1) samples, the PSE method (100% water) recovered the least aflatoxin with mean recoveries of 1-2%. The 30% methanol aq. procedure yielded mean recoveries of 17-36%. The 80% methanol aq. extraction method recovered the most aflatoxin, with mean recoveries of 35-63%. Statistical analysis revealed significant differences in aflatoxin recovery between the three extraction methods, indicating that both concentration and type of extraction solvent play significant roles in extraction of aflatoxin. This study provides evidence that if aflatoxins are present in the starting material for a flavonoid rich extract, the aflatoxins will carryover and concentrate in the resultant extract, especially if a high percentage of methanol is used as the major extraction solvent.

Keywords:

Phenolic compounds, peanut, aflatoxins, LC-MS

Introduction

The safety of the U.S. food supply is an important current issue. Millions of dollars are invested every year to ensure the safety of the U.S. food supply. Along with concerns about food pathogens, and allergens, mycotoxins including aflatoxins are also of high concern. Aflatoxins are naturally occurring mycotoxins that are frequently produced by *Aspergillus flavus* and *Aspergillus parasiticus* species of fungi. Although there are a range of different types of aflatoxins, the major aflatoxins of concern in food are designated B1, B2, G1, G2 (Sweeney and Dobson, 1998). The names of aflatoxins relate to their fluorescent color (B=blue and G=green). The order of their human toxicity is B1>G1>B2>G2 with B1 being at least two times more toxic than G1 (Hussein and Brasel, 2001). Contamination often occurs in nuts, peanuts, spices, corn, cottonseed, and other oil seeds (Stroka et al., 2000). Therefore the possibility exists that aflatoxins could be present in any manufactured good that contains the aforementioned ingredients in the product. Aflatoxins are quite stable compounds structurally due to ample conjugation. As such, aflatoxins are likely to remain present in a food product even after the processing or degradation of a contaminated food product because their decomposition temperatures range from 237°C to 306°C (Rustom, 1997). In the United States the level of aflatoxins in peanuts must be 15 ng/g or less (CFR, 2014); in the European Union the level is even stricter, at 2-4 ng/g (Blesa et al., 2003). Aflatoxins cost the southeastern U.S. peanut industry approximately \$ 25 million annually (Lamb and Sternitzke, 2001). Aflatoxin contamination is most likely to occur at or above 30°C, which promotes growth of the fungi that is known to produce the toxin (Scheidegger and Payne, 2005).

Aflatoxin detection can be difficult since the compounds need to be extracted from a complex food matrix. Aflatoxin concentration in a product is often not uniform. This requires that many samples be taken and tested for aflatoxin to ensure the safety of the lot. In one study the variation due to sampling in powdered ginger was found to be 87% (Whitaker et al., 2009). Aflatoxins are often extracted with chloroform, methanol, ethanol or their aqueous (aq.) mixtures when applicable (Do and Choi, 2007). Acetonitrile is another solvent that has been used to extract aflatoxins. Most often a

70% methanol-water mixture is used. Of concern, is that often similar or the same solvents are used to extract proanthocyanidins, therefore it is likely that these extracts could contain aflatoxins as well. Enzyme-linked immunosorbent assay (ELISA) kits are quite commonly used to detect aflatoxins because of their ease of use (Gilbert and Vargas, 2005). The use of Fourier Transform Infrared Resonance (FTIR) Spectroscopy to detect aflatoxins has been performed, but it has not gained widespread use (Mirghani et al., 2001). Spectroscopy such as FTIR would be highly useful since it is a non-destructive technique, and thus can be altered into an inline processing technique.

Due to the ability of aflatoxins to fluoresce, Thin Layer Chromatography (TLC) and High Performance Chromatography (HPLC) with fluorescence detection is a common and inexpensive often-employed technique to detect aflatoxins. In addition, LC-MS has been used to detect aflatoxins in the positive mode since it gives increased ionization efficiency over negative mode (Blesa et al., 2003; Nonaka et al., 2009). LC-MS has been shown to be a specific and precise method for the determination and quantitation of both proanthocyanidins and aflatoxins. Common to both classes of compounds, a reversed phase column is often used (Ventura et al., 2004; Cavaliere et al., 2007; Bacaloni et al., 2008; Nonaka et al., 2009; Sarnoski et al., 2012). A gradient elution program is employed, with a choice of various effective solvents for the mobile phase. Cavaliere et al., (2007) used acetonitrile-water as mobile phase A and water as mobile phase B (both with added ammonium formate) to identify aflatoxins in maize extracts. The same solvents and concentrations were employed by Bacaloni et al., (2008) for the identification of aflatoxins in hazelnuts. A methanol-acetonitrile mobile phase A and aq. ammonium formate mobile phase B elution program were employed by Nonaka et al., (2009), also for the separation of aflatoxins. For the determination of proanthocyanidins in Virginia-Type peanut skins, Sarnoski et al., (2012) used an aq. ammonium acetate solution as solvent A and 100% methanol as solvent B. For its precision, selectivity, and reproducibility, LC-MS was the method of choice for the separation, identification, and quantitation of aflatoxins for this project.

Peanut skins are of particular interest to researchers as a potential source of a number of bioactive flavonoids, such as proanthocyanidins. Due to a growth in this field of research, the use of peanut skin extracts or components of peanut skin extracts as a beneficial additive in foods or in non-food materials is growing in likelihood and achievability. This presents a potential food safety issue, due to the possible concentration of harmful aflatoxins, primarily the most implicated aflatoxins of B1, B2, G1, and G2 in such extracts. Aflatoxins have been found to occur in peanut kernels and shells, but no researchers to date have analyzed the skins for aflatoxins. Therefore the objectives of this project were to determine if aflatoxins can exist in the skins of peanuts, and answer the question of whether the aflatoxins could be concentrated due to the extraction procedure used on the peanut skins. Emphasis was placed on the significance of the extraction solvent composition to the aflatoxin concentration of the final extract. The data obtained from the determinations made can assist in the search for a valuable and healthful use for peanut skins while maintaining food safety standards.

Materials and Methods

Extract source and standards

Peanut skins (Virginia variety) were obtained from a commercial source (Tidewater Blanching,

Suffolk, Va., U.S.A.). Upon receipt the peanut skins were frozen and stored at -20 °C. Unless otherwise noted, all chemicals were purchased from Fisher Scientific (Pittsburgh, Pa., U.S.A.). An aflatoxin mixed standard (1 µg/mL B1, 0.3 µg/mL B2, 1 µg/mL G1, 0.3 µg/mL G2) in methanol was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). The analytical concentration for each lot was specified on the certificate of analysis and differed slightly from lot to lot (see exact concentrations at footnote of Table 1).

Single solvent extraction

Peanut skins were extracted by placing 1 g of skins in 10 mL of 30% methanol aq. or 80% methanol aq. for 15 min, using a Fisher Scientific Ultrasonic Model FS20 (Pittsburgh, Pa., U.S.A) bath to assist extraction. The liquid portion was then 0.2 µm syringe filtered using Whatman PTFE Puradisc™ syringe filters (Whatman Inc., Piscataway, N.J., U.S.A.) and the volume was measured. A clean round bottom flask was pre-weighed and the liquid portion was transferred into the flask. The solvent was then evaporated under vacuum using a rotary evaporator (Buchi Rotavapor RII, Labor-technik AG, Switzerland) to dryness. The flask was then slowly oven dried at 50 °C, the weight was recorded when all solvent was lost, and constant weight was achieved. The yield was determined by the difference between the weights of the empty and extract containing flasks.

Table 1. Aflatoxin content in un-spiked and spiked samples (ng/g).

Un-Spiked Samples (ng/g)				
Solvent	Aflatoxins			
	G2	G1	B2	B1
PSE (100% H ₂ O)	n.d.	n.d.	n.d.	n.d.
Methanol 30%	n.d.	n.d.	n.d.	n.d.
Methanol 80%	n.d.	n.d.	n.d.	n.d.
Spiked Samples (ng/g)				
Solvent	Aflatoxins			
	G2	G1	B2	B1
PSE (100% H ₂ O)	7.05 ± 0.15	14.75 ± 0.95	3.70 ± 0.40	9.20 ± 0.70
Methanol 30%	107.7 ± 3.95	209.9 ± 1.50	51.95 ± 2.45	162.8 ± 5.50
Methanol 80%	185.2 ± 4.90	471.8 ± 2.10	99.20 ± 6.10	410.1 ± 7.90

n.d. signifies not detected

Analytical standard concentrations for the lot were (0.961 µg/mL B1, 0.280 µg/mL B2, 0.903 µg/mL G1, 0.296 µg/mL G2) in methanol.

Multistep peanut skin extraction (PSE) procedure

The method used for extraction of proanthocyanidins has been previously described (Masquelier, 1987). Briefly, peanut skins (100 g) were extracted with boiling deionized water (99.7 °C) at a ratio of 1 g to 10 mL. Filtration was performed to remove any remaining solid particles. Sodium chloride was added to the filtrate to the point of saturation (approximately 20% w/w). Filtration was repeated to remove precipitates. The phenolic compounds were then extracted in a separatory funnel using ethyl acetate and dried with sodium sulfate. The ethyl acetate extract was then concentrated via a rotary evaporator to about 1/5 the initial volume. Finally, the proanthocyanidins were precipitated with three equal volumes of chloroform and filtered. After air drying, the proanthocyanidin precipitate was a tan powder. Yields of approximately 1-1.5 g of powder were obtained from 100 g of peanut skins.

Determination of Aflatoxin Recovery

Aflatoxin recovery was determined by adding 1 mL of the aflatoxin mixed standard to 1 g of peanut skins in a glass tube for the PSE procedure, 30% methanol aq., and 80% methanol aq. extractions. The aflatoxin mixed standards (all from the same lot) were added to triplicate portions of dry peanut skins, and homogenized manually with a glass rod. The skins were then allowed to dry overnight at ambient temperature in a fume hood. Peanut skins were then extracted according to the 30% methanol aq., 80% methanol aq., or PSE procedure. One-way ANOVA was used to test for difference between treatment groups at the $\alpha = 0.05$ level.

MS Identification

The ESI-LC-MS system consisted of an Agilent (Palo Alto, Ca., U.S.A.) 1100 series HPLC coupled to a ThermoFinnigan (San Jose, Ca., U.S.A.) LCQ quadrupole ion trap mass spectrometer with electrospray ionization (ESI). LC separation of the extracts was accomplished with a Waters (Milford, Ma., U.S.A.) XTerra MS C18 (15 cm x 2.1 mm i.d., 3.5 μ m) column with guard column. A gradient method, consisting of solvent A (0.2% acetic acid in water) and solvent B (0.2% acetic acid in methanol), was applied at a flow rate of 0.15 mL/min as follows: 5-50% B from 0 to 15

min, 50-95% B from 15 to 45 min, and held at 95% B from 45 to 60 min, followed by column equilibration. MS data was acquired under positive mode, using Xcalibur (Thermo Fisher Scientific, Waltham, Ma., U.S.A.) and analyzed with the same software. Comparison of product ion spectra in samples and standards was used to quantify aflatoxins. The optimized electrospray/ion optics parameters were as follows: spray voltage 3.3 kV; sheath gas (nitrogen), capillary voltage 12.5 V; capillary temperature 250°C; tube lens offset voltage 0 V.

Results and Discussion

Aflatoxin content was quantitated by (+) ESI-MS/MS integration of aflatoxin product ion peaks (Figure 1). In un-spiked peanut skin extracts the product ion abundances were below the acceptable range for detection/quantification or the presence of the aflatoxins was absent in these samples. For the spiked samples aflatoxin product ions were in range for quantification, and matched the expected fragmentation pattern for identification. Aflatoxins B1, B2, G1, and G2 give characteristic $[M+H]^+$ fragment ions at m/z 313, 315, 329, and 331 respectively, and have differentiating MS/MS spectra (Blesa et al., 2003). To determine the recovery using the different extraction procedures, peanut skin samples were spiked at ratio of (1 mL/g of mixed standard). The PSE procedure which uses boiling water as the main solvent for extraction, exhibited the least amount of recovery of aflatoxin into the final extract (Table 1). Overall recoveries of the aflatoxins for the PSE method were around 1% (Table 2). The 30% methanol aq. procedure extracted more aflatoxins, and recoveries were around 25% using this extraction solvent. Finally, the 80% methanol aq. extract recovered the greatest amount of aflatoxins, with recoveries being in the 50% range. Overall, when the methanol content used for extraction increased, so did the efficiency of the recovery for aflatoxin. According to ANOVA results every aflatoxin (B1, B2, G1, G2) by extraction method was statistically different (Table 2). This study provides evidence that concentration of aflatoxin as a result of producing a phenolic extract is occurring. When the amount of aflatoxins in the extract was calculated for all treatments except for the water based extraction values were found to be concentrated over their spike levels in the skins.

Figure 1. Typical chromatograms obtained from peanut skins samples spiked with aflatoxins. Quantitation was performed by integration of each aflatoxins major product ion.

Table 2. Average Aflatoxin Recoveries (%) $\pm$ standard error (SE).

Control Samples (%) – aflatoxins not detected				
Solvent	Spiked Samples (% Recovery)			
	Aflatoxins			
	G2	G1	B2	B1
PSE (100% H ₂ O)	2.38 ^a $\pm$ 0.05	1.63 ^a $\pm$ 0.10	1.33 ^a $\pm$ 0.15	0.96 ^a $\pm$ 0.05
Methanol 30%	36.36 ^b $\pm$ 1.35	23.24 ^b $\pm$ 2.35	18.55 ^b $\pm$ 0.85	16.94 ^b $\pm$ 1.60
Methanol 80%	62.56 ^c $\pm$ 8.40	52.25 ^c $\pm$ 4.65	35.42 ^c $\pm$ 2.15	42.68 ^c $\pm$ 0.80

Mean values with the same letter in a column indicate no significant difference.

Extract Production

Methanol is an extraction solvent often used to extract phenolic compounds. When aq. methanol, ethanol, and acetone extracts of peanut skins were compared, the methanolic extracts were found to have the highest phenolic contents (Nepote et al., 2000). Consequently, 80% (v/v) methanol aq. extracts have been used to extract aflatoxins (Shannon and Shotwell, 1979) and more recently by Nonaka et al., (2009). Decreasing the amount of organic solvent for extraction generally results in

lower aflatoxin extraction efficiencies (Bradburn et al., 1990). Although methanol extraction is quite efficient at extracting phenolic compounds, there may be some unintended food safety consequences of using methanol to extract phenolics, namely that aflatoxins will be extracted and concentrated during the process. This experiment generally illustrates that if a higher concentration of organic solvent is used, then there would likely be a greater concentration of aflatoxin in the resultant extract. Therefore a lower concentration of or-

ganic solvent would not be recommended for extracting phenolic compounds, but it should limit the extraction of mycotoxins in peanut skins.

Recovery and Extraction Efficiency

The performance of each of the extraction methods can be evaluated based upon their aflatoxin recovery capabilities. No aflatoxins could be detected in un-spiked samples because the aflatoxins were absent from the samples, or the aflatoxins were present at a level below the detection threshold. The lack of aflatoxins in the un-spiked samples was most likely due to the lack of the presence of *Aspergillus* species fungi on the skins, which are the microorganisms commonly implicated as the cause of aflatoxin presence (Passone et al., 2007). The boiling water (PSE) extraction performed on the spiked samples had the lowest recovery (Table 2). When using a methanol concentration of 80% and employing the same ultrasonic procedure used in the 30% methanol aq. procedure aflatoxin recoveries averaged around 50% (Table 2). Aflatoxin G2 consistently showed the highest recovery irrespective of extraction method. Interestingly, the methanol aq. extractions were particularly efficient for the extraction of B1 and G1 (Table 2), which has been identified as more carcinogenic and toxic than aflatoxins B2 and G2 (Van Egmond et al., 1995; Jaimez et al., 2000).

Overall, the resulting aflatoxin recoveries for this project were lower than expected based upon comparison with other studies. Nonaka et al., (2009) reported recoveries ranging from 83%-109%, and Bacaloni et al., (2008) reported recoveries ranging from 60% -94%. However these studies examined aflatoxins in the peanut kernel and other food samples, and the extraction process was different than described in this study. The recoveries in this study make evident the effects of solvent type (and composition) on the four aflatoxins as a group and between specific aflatoxins (i.e. B1 vs B2). Sample type also seems to play a significant role in recovery efficiency, due to the effects of matrix differences. In a comparative study by McDaniel et al., (2011) aflatoxin recoveries were found to be dependent not only upon the type of cleanup method applied, but also upon the sample type. As reported by McDaniel et al., (2011) samples of both corn and dried distillers grains (DDG) were spiked and subjected to the same extraction and clean up (SPE) method. In the case of aflatoxins B1 and B2, a significant recovery difference was found in extraction recoveries ($104.5\% \pm 7.78$ from corn vs. $74\% \pm 10.07$ from DDG for B1 and $67\% \pm 9.9$ from

corn vs. $40\% \pm 5.86$ from DDG for B2) was found in the McDaniel et al., (2011) study. Significant differences were also found in HPLC column elution capabilities ($120\% \pm 21.78$ from corn vs. $75\% \pm 23.97$ from DDG for B1 and $74\% \pm 9.07$ from corn vs. $40\% \pm 5.86$ from DDG for B2), through the analysis of spiked extracts of these grains (McDaniel et al., 2011). The differences in recoveries were attributed to a varying ability for some aflatoxins to bind to and to be released from both SPE columns and HPLC columns (McDaniel et al., 2011).

Various sample preparation procedures have been used for extraction of aflatoxins, and the specific procedure used usually depends upon the type of food sample used. Differing sample preparation procedures also seem to affect measurable recoveries. Recovery yields would have likely differed if the samples had been finely ground before extraction. Instead, the peanut skin samples used for this project were used in their natural form. It has been shown for hazelnuts that an increase in ultrasonication time (up to 30 minutes) is required for obtaining absolute recoveries when samples are higher in proteins or carbohydrates (Bacaloni et al., 2008). Given that peanut skins can average 49.2% carbohydrates (Hoffpauir, 1953) and hazelnuts average 6% carbohydrates (Bacaloni et al., 2008), peanut skin recoveries using the methanol method may have been greater if ultrasonication times had been extended to 30 minutes or more. However, in order to keep the procedure fairly rapid a 15 minute sonication time was chosen for this study.

The PSE procedure using boiling water as an extraction solvent did not employ an ultrasonication step. As such, the minimal aflatoxin recovery could potentially be attributed to solvent type, due to the lack of ultrasonication, or to a combination of the two variables. While methanol has an average polarity index of approximately 5.1, water has an average polarity index of 9. Aflatoxins solubility is reported to be 3.150 g/L in water (DHHS, 2011). Solubility of aflatoxins has been reported to be better in organic solvents like methanol, acetone, and chloroform (Garcia et al., 1994). As such, one would expect that water (PSE procedure) would be less likely to extract aflatoxins than the methanol solutions. However, the dielectric constant of water decreases from approximately 88 to 56 going from 0°C to 100°C (Malmbert and Maryott, 1956), and the average dielectric constant of methanol is approximately 32.7 (University of Washington, 2014). As such,

the polarity of boiling water should be more closely comparable to the polarities of the two aq. methanol solvents, therefore poor recovery of aflatoxins cannot be attributed to the use of boiling water alone, but may also relate to the multiple steps of the purification procedure. In a separate study proanthocyanidin contents were found to be higher using the PSE method, as opposed to water-methanol, or water-acetone extracts of peanut skins (Sarnoski et al., 2012). Thus the PSE procedure provides the dual advantage of efficiently extracting proanthocyanidins, and posing the least amount of risk of extracting aflatoxins.

In order to explain the low aflatoxin recoveries using the boiling water method as opposed to the methanol aq. methods, the effects of using different extraction procedures must be considered. The water extraction employed a multi-step procedure with a final precipitation step, in which chloroform is added to the extract. Aflatoxins are notably soluble in chloroform (DHHS, 2011). As such, this final precipitation step may provide a secondary manner in which remaining aflatoxins in the extract can be separated from the resulting precipitate. Also important to note, the water extraction did not employ an ultrasonication step, which has been shown to increase efficiency of aflatoxin extraction from foods such as hazelnuts, peanuts, cashews, and pistachios (Bacaloni et al., 2008) and may lower the recovery further in this instance.

Conclusions

A statistically significant difference in recovery efficiencies were found among each of the extraction procedures used in this project. In order to minimize the recovery of aflatoxins while extracting plant flavonoids, it is necessary to consider a number of factors. In the interest of public health and safety, a delicate balance must be maintained between solvent flavonoid extraction potential and solvent aflatoxin recovery efficiency. This project provides evidence that solvents with potentially similar aflatoxin recovery capacities may result in very different recovery efficiencies due to procedural differences. This research demonstrates that a 100% boiling water, multi-step extraction method for proanthocyanidins exhibits a lower recovery rate for aflatoxins compared with methanol aq. extractions and is therefore potentially the safest method explored within the scope of this project for extracting flavonoids from peanut skins.

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