Proceedings of The National Conference
On Undergraduate Research (NCUR) 2018
University of Central Oklahoma
Edmond, Oklahoma
April 4-7, 2018

Determination of Ochratoxin A in Kentucky Black Walnuts

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Abstract

Previous research has shown that edible tree nuts are potential substrates for mycotoxins. Black walnuts (*Juglans nigra*) are frequently collected, dried, and consumed with minimal processing, especially in rural communities. This presents health risks if black walnuts contain high levels of mycotoxin contamination. This study aimed to determine the levels of one mycotoxin, ochratoxin A (OTA), in black walnuts from Kentucky. Black walnut samples were collected from three geographical locations near Lexington, Kentucky plus one commercially available sample (nut meat only) from another state. Analysis was performed using immunoaffinity column concentration and separation of the OTA followed by high-performance liquid chromatography with fluorescence detection (HPLC-FLD). Results from analysis of the commercial nuts and samples of the wild husks and nuts showed no quantifiable ochratoxin A present, even though analysis of OTA standards and of oat-containing cereals did test positive for OTA in expected levels.

Keywords: mycotoxin, nuts, HPLC-FLD

1. Introduction

Mycotoxin contamination of foods is an ongoing concern. Analysis of 489 breakfast cereals revealed the presence of ochratoxin A (OTA) in over 70% of oat-containing cereals.[1] Ochratoxin A is produced by several species of *Penicillium* and *Aspergillus* and, besides oats, has been found in other grains (wheat, barley, rye, corn, rice), grapes, coffee, and cocoa.[2] Ochratoxin A is the most common of the ochratoxins and a suspected human carcinogen. OTA is a renal carcinogen to particular animal species[3], reduces striatal dopamine in mouse brain cells[4], and acts as an immunosuppressant in animals.[5] Poultry fed with contaminated grains concentrated OTA in meat, resulting in depressed weight gain, reduced egg production and poor egg-shell quality.[6] Human health concerns relate to suspected higher incidences of urinary cancers, nephropathy, and neurotoxicity, although the results of animal studies have not been found easily transferrable to humans.[7]

In 2006 the European Food Safety Authority established the daily tolerable intake of OTA to be 5 μg/Kg for unprocessed cereals and 3 μg/Kg for processed cereals.[8] Roasted coffee beans were limited to 5 μg/Kg, and although tree nuts were not stated, dried fruit (currants, raisins, and sultanas) were limited to 10 μg/Kg. The US FDA has not yet established maximum limits for ochratoxin A.

In 2015 Tournas *et al.*[9] reported their analysis of 64 tree nut samples and 50 dried fruit samples purchased from local supermarkets in the Washington, DC area. Of these the highest mold counts were found in walnuts (100% of the samples) with a variety of *Penicillium* and *Aspergillus* species (including *Aspergillus niger*) detected. This study focused on fungi and not on the presence of mycotoxins. However, around the same time period Palumbo *et al.*[10] collected over 650 samples of dried fruits and nuts and found OTA highly concentrated in raisins and pistachios. The absence of OTA on walnuts is noteworthy.

Black walnuts (*Juglans nigra*) are prevalent in about two-thirds of the continental US.[11] They differ from the more common English walnut (*Juglans regia*), and besides being a small commercial crop, these nuts are also collected and eaten by many individuals for their unique and desirable taste for baking, ice cream, candies and other food preparations. The nuts have a fleshy outer husk around the hard shell that has to be removed before shelling. Because of the prevalence of black walnut trees in Kentucky[12], the popularity of individuals collecting the nuts and processing them in their homes (without any consideration for mold contamination), and the seemingly conflicting data regarding mycotoxins and walnuts, an investigation of the prevalence of ochratoxin A on black walnuts in the central Kentucky region was performed to assess what levels were present on both the outer husks as well as the nut meats themselves.

2. Materials and Methods

2.1. Chemicals

Solid ochratoxin A (OTA) was purchased from Sigma-Aldrich (St. Louis, MO). OTA stock solution was prepared in toluene/glacial acetic acid (99:1 v/v) with a concentration of approximately 10 mg/L. OTA standard solution was prepared in toluene/glacial acetic acid (99:1 v/v) with a concentration of approximately 1 μg/mL. Stock and standard OTA solutions were stored at -2 ºC. OchraTest immunoaffinity columns (IAC) were purchased from VICAM (Watertown, MA). Phosphate-buffered saline (PBS) was prepared according to the method described by Sambrook *et al.*[13]

2.2. HPLC-FLD Analysis

Reverse-phase chromatography was performed using a Dionex UltiMate 3000 pump and UltiMate 3000 Fluorescence Detector with a Dionex Acclaim 120 C18 column (5 μm, 4.6 x 150 150 mm) with 20 μL sample loop. Isocratic elution consisted of 49.5% acetonitrile, 49.5% water, and 1% glacial acetic acid at a flow rate of 1.0 mL/min. OTA was detected at 333 and 460 nm wavelengths for excitation and emission, respectively. OTA fluorescence usually occurred at a retention time of about 9 minutes; however, the HPLC column was not thermostated, and fluctuations in the retention times of up to 1 minute occurred as a result of variable room temperatures from one day to another.

2.3. Calibration Standards

Five OTA calibration standards were prepared by diluting the OTA standard solution according to the AOAC Official Method 2001.01.[14] The concentrations of OTA in the calibration standards were 43.2, 28.8, 14.4, 4.32, and 1.44 ng/mL.

2.4. Recovery Experiments

Two sets of recovery experiments were performed. The first set consisted of spiking a testing material (either black walnut or green coffee bean sample) with an aliquot (1.0 mL) of either 43.2 ng/mL or 28.8 ng/mL OTA calibration standard. The OTA standard was added to the sample after it had been ground and mixed with the acetonitrile/water extraction solution, but before it had been shaken. The spiked sample then underwent the same preparation process and HPLC-FLD analysis as the other samples. For the second set of recovery experiments, an aliquot of OTA calibration standard (1.0 mL) was diluted with acetonitrile/water solution (50 mL, 80:20 v/v). An aliquot (10 mL) was diluted with PBS solution (50 mL). A portion (10 mL) of the diluted standard was filtered through the IAC, evaporated, and analyzed by HPLC-FLD identically to other samples.

2.5. Coffee And Oat Samples

Green coffee bean samples were purchased online and sourced from four countries: Brazil, Guatemala, Papau New Guinea, and Ethiopia. Raw oats and a processed toasted oats cereal were purchased from an Aldi supermarket in Lexington, KY. Both green coffee bean and oat-based cereal samples were stored in their original packaging at room temperature until analysis. Samples were ground, extracted, diluted, and filtered by the same method as previously described.

2.6. Black Walnut Sampling

Wild black walnut samples were collected from three geographical locations (Figure 1). Sampling locations included Asbury University campus (Wilmore, KY), Waveland State Historic Site (south Lexington, KY), and a private residence (north Lexington, KY). One commercial black walnut sample (from Stockton, MO) was obtained from a Sams Club in Nicholasville, KY. Raw nut samples were stored unhusked at -2 ^{o}C until analysis.

Figure 1. Black walnut sampling sites in Central KY: 1 (Asbury University Campus); 2 (Waveland Historic Site, Lexington, KY); 3 (Private Residence, Lexington, KY).

2.7. Black Walnut Sample Preparation

Samples were prepared and analyzed following the method used by Lee and Ryu[1]. Black walnut samples were analyzed in triplicate by analyzing three husk samples and three nut meat samples per sampling site. Samples were first dried in an oven at 110 ^{o}C for 48 hours. The husks of the dried samples were removed, and the nut shells were split with a hammer and the nut meats were removed. The husks and nut meats were separately ground to a fine powder using a Krups model F203 coffee grinder (Note: The commercial black walnut sample only included the nut meats). The grinder was thoroughly cleaned and dried in between samples. The material for analysis (husks or the meats, 12.5 g) was extracted with acetonitrile/water (80:20 v/v, 50 mL) by shaking at 100 RPM for 30 minutes with an Innova 2000 Platform Shaker (New Brunswick Scientific, Edison, NJ). The extract was filtered through qualitative grade 615 filter paper (Eaton-Dikeman, Beachwood, OH), and an aliquot of filtrate (10 mL) was diluted with PBS solution (40 mL). The diluted extract was filtered through a 0.45 µm nylon filter membrane to prevent blockage of the IAC. An aliquot (10 mL) of filtrate was filtered through an OchraTest IAC at 2-3 mL/min using a Visiprep SPE Vacuum Manifold (Sigma-Aldrich, St. Louis, MO). The IAC was then washed with PBS solution (10 mL) followed by water (10 mL). Methanol (3 mL) was used to elute OTA into a round-bottom flask, and the methanol was evaporated using a rotary evaporator. Any residue was re-dissolved in methanol/water (0.50 mL, 50:50 v/v) and was immediately analyzed by HPLC.

2.8. Software / Data Interpretation

Data from chromatographic analysis was interpreted with Dionex Chromeleon Chromatography Data System Software version 6.80 (Sr15, Build 4656) (Thermo Scientific, Waltham, MA).

3. Results and Discussion

3.1. Calibration Standards

A total of five OTA calibration standards were prepared and analyzed. The concentrations of OTA in the calibration standards were 43.2, 28.8, 14.4, 4.32, and 1.44 ng/mL. The area of the OTA signals for the five standards showed strong linearity with a coefficient of determination of 0.9999 (Figure 2). This suggests that the HPLC-FLD method is sensitive and effective at detecting OTA, even at low ppb levels.

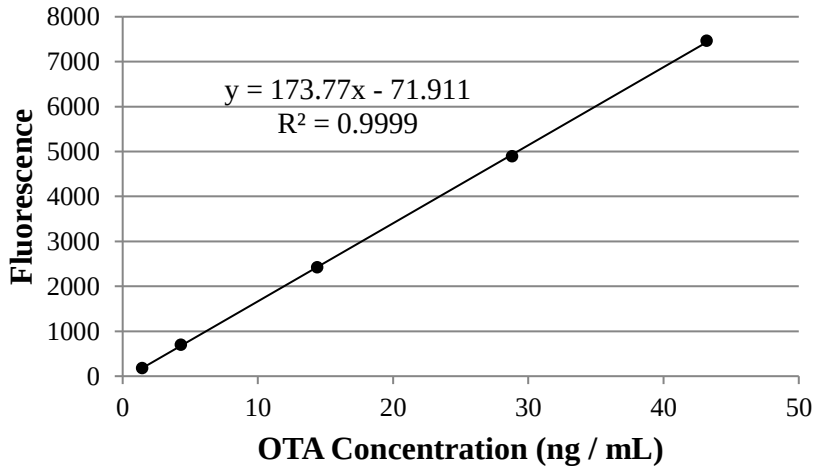

Figure 2. OTA standard curve

3.2. Recovery Experiments

Two separate sets of recovery experiments were used to assess the effectiveness and accuracy of the experimental method. Seven individual analyses were performed using the first recovery experiment method, in which a solid sample was spiked with OTA standard prior to stirring. Recovery values of the first set of recovery experiments ranged from 50% to 87% with median of 70%. Since these values were unexpectedly low, a second set of recovery experiments were performed to identify which part of the sample preparation process caused the low recovery. Five individual analyses were performed using the second recovery experiment method, in which an aliquot of OTA standard was diluted (in a manner identical to a sample) and filtered through the IAC. Recovery values of the second set of recovery experiments ranged from 85% to 100% with a median of 97%. These results suggest that matrix binding of OTA to the solid sample may be the cause of the low percent recoveries in the first set of recovery experiments, with filtration of solid material where some loss is occurring. Further study could be performed to determine if matrix binding of OTA to solid samples may cause the levels of OTA detected in food screenings to be below actual amounts. Furthermore, the results of the second set of recovery experiments suggest that the IAC yields accurate concentration and separation of OTA.

3.3. Coffee And Oat Analyses

As a means of testing the analysis method with foods that have previously been shown to be commonly contaminated with OTA, green coffee beans and oat cereal were tested.[1,2c] No quantifiable OTA was observed in any of the four green coffee bean samples that were tested, perhaps testifying to the improved processing methods being utilized to minimize OTA in coffee beans[2c]. However, the two types of oat cereal that were tested were both found to contain OTA (Figure 3). A one-time analysis of rolled-oats revealed an OTA concentration of 0.66 ng/g, and a one-time analysis of toasted oats breakfast cereal revealed an OTA concentration of 2.69 ng/g. This is slightly higher than the mean value for oat cereals (2.49 +/- 2.20 ng/g) found by Lee and Ryu in a comprehensive study of breakfast cereals across the United States.[1] Additionally, this concentration is close to the European Food Safety Authority's daily tolerable intake of OTA of 3 μg/Kg in processed cereals.[1]

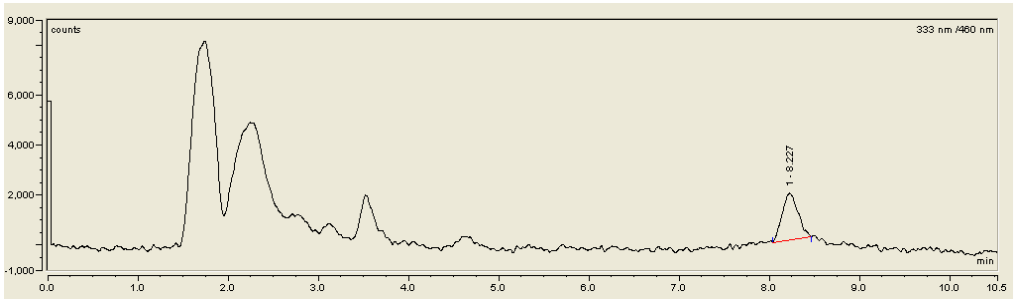

Figure 3. HPLC chromatogram of an oat-based breakfast cereal correlating to an OTA concentration of 2.69 ng/g. Note that the retention time of OTA in this analysis was closer to 8 minutes due to fluctuations in room temperature.

3.4. Black Walnut Analysis

A total of eighteen black walnut samples were analyzed. Three husk and three nut meat samples from Asbury University's campus, three husk and three nut meat samples from Waveland State Historic Site, three husk samples from a private residence in Lexington, KY, and three nut meat samples from the commercial sample were analyzed. No quantifiable OTA was found in any of the black walnuts tested (Figure 4).

The absence of OTA in the black walnuts may have been the result of a number of possibilities. For example, black walnuts may not be commonly infected with OTA-producing fungi. Alternatively, the fungi that produce OTA may not be as common in central Kentucky as other areas of the United States in which previous studies with other walnut varieties have been conducted. The absence of OTA in black walnuts suggests that black walnuts do not present a risk of OTA contamination upon consumption, even when the walnuts are collected and dried without any commercial processing conditions, although a larger sampling is needed to know this with higher certainty. This may be especially noteworthy for rural areas where black walnuts are frequently consumed in this manner.

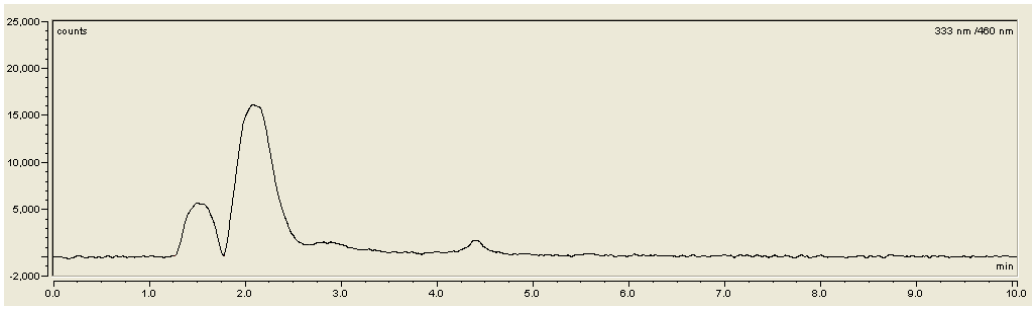

Figure 4. HPLC chromatogram of a black walnut husk sample with no OTA present.

4. Conclusions

In summary, black walnuts collected in Kentucky were tested for ochratoxin A (OTA) using immunoaffinty column concentration followed by HPLC-FLD analysis. No quantifiable OTA was found in any of the black walnuts tested. Other food samples (green coffee and oats) commonly infected with OTA were analyzed, and while OTA was not found in any green coffee, it was found in two types of oat cereal. Recovery experiments were performed to assess the reliability of the method, and although OTA recovery was high with OTA standards by themselves, levels of OTA in spiked food samples were not, suggesting that matrix binding may inhibit the levels of OTA detected during analysis. Further study could be performed to determine if matrix binding impacts food screening for OTA and other mycotoxins. The lack of OTA in black walnuts suggests that they do not present a risk of OTA contamination, although our recovery experiment results may indicate that small levels of OTA could be present but not detected.

5. Acknowledgements

This work was supported by an Asbury University Faculty Research Grant for purchase of the Visiprep sampler. We thank Asbury University's Department of Science and Health for purchase of the fluorescence detector and for support to present this research at the National Conference on Undergraduate Research (NCUR) 2018. We thank Dr. Marvin Ruffner and Professor Ann Witherington (Asbury University) for providing black walnut samples.

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