



ISOLATION, IDENTIFICATION AND EXTRACTION OF AFLATOXIN B2 BY CHROMATOGRAPHIC TECHNIQUES

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ABSTRACT

Aflatoxin is the most common and well-studied group of all mycotoxins, which are typically found in the surrounding environment, such as soil and food. Foods most susceptible to aflatoxins include peanuts, corn, and nuts like Brazil nuts and pistachios, as well as certain small grains such as rice. This study aimed to isolate, identify, and extract aflatoxin. An aflatoxin-producing mold from the *Aspergillus* spp. was isolated from the dump yard soil. Isolated colonies were observed and identified morphologically as *Aspergillus flavus*. These exhibit greenish, powdery colonies with white edges, and under microscopic observation, reveal exospores surrounding the conidia. The mold was cultivated on various selective media, with Potato Dextrose Agar supporting optimal growth. Extraction of aflatoxin was achieved through centrifugation and filtration techniques, followed by identification using thin layer chromatography (TLC). This research enhances the understanding of aflatoxin-producing fungi, the types of aflatoxin, and their sources.

KEYWORDS: Aflatoxin, *Aspergillus Flavus*, PDA, TLC

1. INTRODUCTION

Aflatoxins are a type of mycotoxin produced by toxigenic strains of *Aspergillus* (*A. flavus* and *A. parasiticus*), which are usually found in soil, vegetables, and grains, posing health risks through both acute and chronic exposure¹. Aflatoxins are bisfuranocoumarin compounds, characterized by a unique chemical structure featuring two furan rings (furanocoumarin units) connected by a central coumarin ring². Twenty different types of aflatoxins have been identified. The term aflatoxin was first introduced by veterinary scientist William Percy Blount in the early 1960s in a Brazilian groundnut meal^{3, 4}. He observed significant damage during post-mortem examinations of some animals, particularly rats, which showed toxic effects on the liver and the development of liver cancer after being fed groundnut meal⁵. Subsequent studies demonstrated the metabolites synthesized by some strains of *A. flavus*⁵. Aflatoxins, representing a major class of mycotoxins, are ubiquitous contaminants arising from fungal activity during food and feed production, harvesting, storage, and processing⁶. The US Food and Drug Administration (FDA) recognizes aflatoxin as an unavoidable contaminant in food products. Acute exposure can present as nausea, vomiting, abdominal pain, and convulsions, while chronic exposure may result in hepatotoxicity, immunotoxicity, and teratogenicity. Aflatoxin B1 is recognized as a human carcinogen and is a significant cause of hepatocellular carcinoma in developing countries. Various aflatoxin variants exist, with four major types commonly encountered: B1, B2, G1, and G2⁷. As shown in **Figure 1**, Aflatoxin B1 (AFB1) is among the strongest of these compounds and has been experimentally shown to induce hepatocellular carcinoma (HCC) in both humans and animals⁸. The structure of aflatoxin B2 consists of a Hexahydrocyclopenta[c]furo[3,2':4,5]furo[2, 3-h]chromene

skeleton with oxygen functionalities at positions 1, 4, and 11⁹. Aflatoxin B2 is a naturally occurring substance found in *A. flavus* and *A. nomiae*^{9,10}. Aflatoxins G1 and G2, naturally occurring mycotoxins produced by *A. flavus* and *A. parasiticus*, are part of the aflatoxin group of fungal metabolites and exhibit a characteristic light and fluffy crystalline structure, showing green-blue fluorescence under specific lighting conditions. They belong to the chemical class of coumarins. (Source: NTP, 1992)^{11, 12, 13}. In the current investigation, DNA from AFB1-induced trout liver tumors was examined for a single-point mutation in Exon of the two c-ras genes, which had been previously identified from trout liver cDNA 1 of both genes. Using polymerase chain reaction and oligonucleotide hybridization methods, a high proportion (10/14) of the AFB1-initiated tumor DNAs showed evidence of activating point mutations in the trout c-Ki-ras gene. Ten different genotypes of mutant RAs were identified: seven exhibited GGA-GTA transversions in codon 12, two had GGT-GTT transversions in codon 13, and one had a GGA-AGA transition in codon 1220. Nucleotide sequence analysis of cloned polymerase chain reaction products from four of these tumor DNAs provided definitive evidence for two codons 12 GGA - GTA mutations, one codon 12 GGA - AGA mutation, and a single codon 13 GGT - GTT mutation, which aligns with the findings from the oligonucleotide hybridization. No mutations were detected in Exon 1 of a second trout c-ras gene also expressed in the liver, nor in DNA from control livers. The results indicated that the hepatocarcinogen AFB1 induces c-ras gene mutations in trout similar to those observed in rat liver tumors^{14,15,8}.

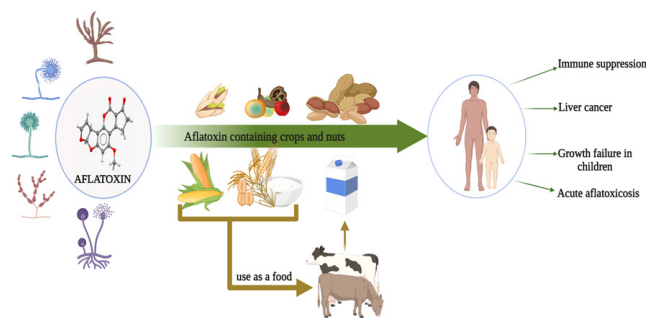


Figure 1: Sources and modes of aflatoxin consumption by humans

2. MATERIAL AND METHODS

2.1. Collection of Soil Sample

Soil samples were procured from the landfill site at Bakrol, Vadodara, with the primary objective of assessing the prevalence of aflatoxigenic fungi. Contaminated dump sites are recognized as potential hotspots for elevated populations of these fungal species. The soil sample was collected in a zip lock bag and moved to the laboratory for further analysis.

2.2. Isolation of Aflatoxin-Producing Fungi

Isolation was carried out by using two different culture media: Potato Dextrose Agar (PDA), and Rose Bengal agar (RBA). PDA (G/L): infusion from potatoes 200, agar 15, dextrose 20, distilled water 1000ml, pH 5.6 ± 0.2 . Rose Bengal Agar (G/L) soya peptone 5.00, dextrose 10.00, monopotassium phosphate 1.00, magnesium sulphate 0.500, rose Bengal 0.050, agar 15.00, distilled water 1000ml, pH 7.2 ± 0.2 . The prepared suspension of the soil sample was inoculated onto all media using the spread plate technique for isolation of aflatoxin-producing fungi. All plates were incubated at 25°C for 24 hours to allow for the growth and isolation of aflatoxin fungal colonies. Fungal colonies were purified by repeated spot streaking to obtain a particular colony of fungi.

2.3. Characterization and Identification of Fungi

The isolated Fungal colonies were subjected to comprehensive morphological and microscopic characterization¹⁶. Morphological characterization was done by studying colony characteristics, and Microscopic identification was done by using the Fungal mounting technique for observing fungal structure under a microscope^{17,18}.

2.4. Production of Aflatoxin

2.4.1. Inoculum preparation

For preparing the *A. flavus* inoculum, the isolated *Aspergillus flavus* culture was inoculated on sterile nutrient slopes and incubated at 30°C for 7 days (Reference)¹⁶. After observing a strong harvest of green conidia, 3 ml of Tween 80 (0.005 v/v%) is added to the inoculation. The spores are scratched with the loop. The inclination was shaken well to prepare a uniform suspension. Thus, spore suspension was used as an inoculated medium for the final production of aflatoxin¹⁹.

2.4.2. Solid state fermentation

For the solid-state fermentation for the production of aflatoxin, Inoculum was added to the production media (Rice meal). The

composition of the media was 50 grams of polished rice and 25 mL of distilled water was placed in a 250 mL flask and allowed to be shaken at 160 rpm at 30°C for two hours. Then, the flasks were sterilized by autoclaving at 121°C and 15 psi for 20 minutes. After autoclaving the rice meal production media, 1 mL of freshly prepared spore suspension of *A. flavus* was added and incubated in a shaker incubator of 160 rpm at 30°C for one week. After 7 days, the flasks showed signs of greenish fungus growth¹⁹ which may indicate the successful growth of aflatoxin fungus and their aflatoxin product¹⁹.

2.5. Extraction of Aflatoxin

The extraction of aflatoxins was performed following methodologies. Initially, 50 mL of deionized water (DW) was introduced into the culture flask containing the fungal growth medium. The diluted culture broth was then subjected to filtration through standard filter paper to remove fungal biomass and particulate matter. Subsequently, the aflatoxin-containing filtrate was centrifuged at 160 rpm for 15 minutes to further clarify the supernatant. The supernatant was carefully decanted, and 100 mL of chloroform was added to facilitate liquid-liquid extraction of the aflatoxins. The mixture was thoroughly agitated to ensure comprehensive partitioning of the analytes into the organic phase. Upon allowing the mixture to settle, distinct aqueous and chloroform layers formed. The chloroform layer, containing the extracted aflatoxins, was then separated and passed through a funnel packed with anhydrous sodium sulfate to remove any residual water. The anhydrous chloroform extract was collected in a clean petri dish and subjected to evaporation at 30°C using a hot plate evaporator until complete dryness. The resulting crude aflatoxin residue was then reconstituted in a precise volume of chloroform for subsequent quantitative and qualitative analyses.

2.6. Determination of Aflatoxin

The detection of aflatoxin was confirmed by employing the Thin-layer chromatography (TLC) technique. TLC is generally based on the classic chromatography principle where mixture components are separated between a fixed stationary phase and a liquid mobile phase by differential affinities between the two phases, in this study Silica gel works as a Stationary phase and Benzene: Acetone: Acetic acid 18: 2: 2 works as a mobile phase. After running the mobile phase, allow it to dry and observe the TLC plate under UV light (long-wave) to determine the presence of either blue or green fluorescent color on the TLC plate. The R_f value of aflatoxin was determined and compared to the standards of aflatoxins. (Aflatoxin B1 = 0.80, B2 = 0.67, G1 = 0.59, G2 = 0.48 ± 0.02).

3. RESULT AND DISCUSSION

3.1. Isolation of Aflatoxin-Producing Fungi

Mycological observations were conducted following a 48-hour incubation period to assess the proliferation of aflatoxin-producing fungi. A total of 47 discrete fungal colonies were enumerated on the Rose Bengal Agar (RBA) master plate. Concurrently, the Potato Dextrose Agar (PDA) master plate yielded 19 distinct colonies. On both culture media, the predominant morphological characteristic observed was the presence of greenish, powdery fungal colonies with white edges. These colonial characteristics are highly suggestive of species

within the *Aspergillus* genus, specifically *Aspergillus flavus* or *Aspergillus parasiticus*, both recognized for their aflatoxigenic capabilities. (Refer to Figure 2 for visual representation).

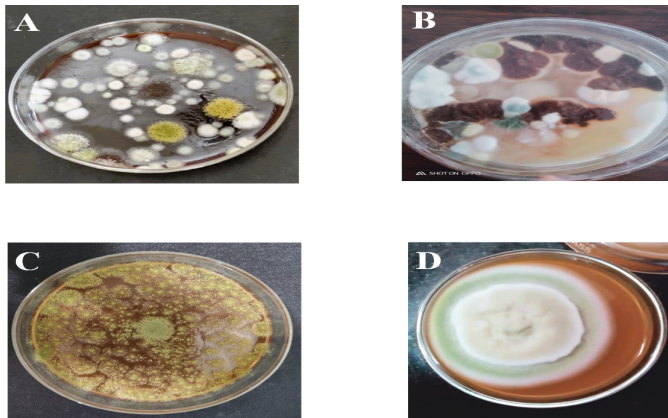


Figure 2: Master plate and isolated fungi, **A.** RBA master plate which contains a total of 47 fungal colonies, **B.** PDA mother plate, which contains 19 colonies, **C.** Isolated pure powdery greenish colony of fungi, **D.** isolated lemony greenish fungal colony containing rough white margin.

3.2. Morphological and microscopic identification of *Aspergillus flavus*

Microscopic and morphological characteristics are consistent with *Aspergillus flavus*. On Potato Dextrose Agar (PDA), the fungal colony exhibited a light green pigmentation at the center, radially bordered by a regular white margin, achieving an approximate diameter of 35 mm. Under microscopic examination (as depicted in Figure 3), the conidiophores of the *A. flavus* isolates were observed to be colorless, thick-walled, and conspicuously roughened, bearing globose to sub-globose vesicles. The conidia were uniformly globose, thin-walled, and exhibited a slightly rough texture. These detailed macroscopic and microscopic features, including colony morphology and the distinctive characteristics of conidiophores, vesicles, and conidia, collectively facilitated the definitive identification of the isolated fungi as *Aspergillus flavus*, aligning with established morphological criteria for this aflatoxin-producing species.

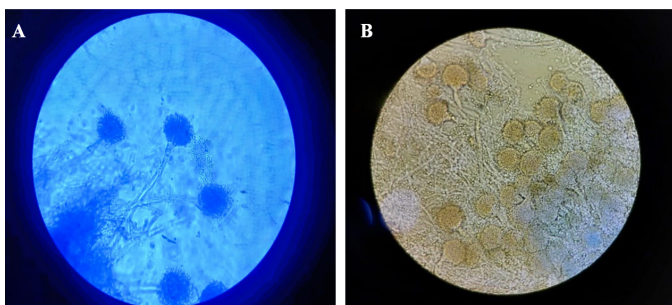


Figure 3: Mounting of fungi(*A. flavus*), **A.** Mounted with LPCB (phenol, lactic acid, glycerol, cotton blue or trypan blue, and water) Observed the globe exospore and hyphae, **B.** natural color of conidiophore using tape touch method.

3.3. Extraction of aflatoxin

Optimal aflatoxin biosynthesis was achieved on the seventh day of incubation under controlled environmental conditions.

These conditions included a pH range of 5 to 6, a constant temperature of 30°C, and continuous agitation at 100 to 120 rpm within a shaker incubator. Following this optimal production phase, the aflatoxin-containing culture medium was subjected to initial clarification by filtration through filter paper. After filtration, specific chemical agents were introduced to the filtrate to prepare it for solvent extraction. The prepared solution was then centrifuged at 10,000 rpm for 5 minutes. This high-speed centrifugation resulted in the formation of two distinct layers: an upper aqueous phase and a lower chloroform phase, as visually represented in **Figure 4A**. The chloroform layer, identified as the organic phase, contained the partitioned aflatoxins. The aflatoxin-rich chloroform layer was carefully separated from the aqueous phase and transferred to a sterile Petri dish. To isolate the crude aflatoxin, the chloroform was evaporated at a controlled temperature of 30°C for 25 minutes. After this period, complete evaporation of the chloroform was observed, leaving a discernible aflatoxin residue, as shown in **Figure 4 B**.

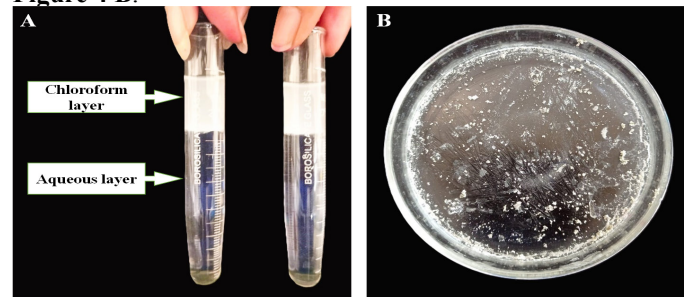


Figure 4: Extraction of aflatoxin . **A.** Extraction of aflatoxin by applying liquid-liquid extraction method and separating two-layer chloroform layer and Aqueous layer. **B.** Observed aflatoxin residue by using the evaporation method

3.4. Detection of Aflatoxin by TLC Chromatography Technique

The plate was visualized under the UV light imager which showed a blue fluorescent image. The TLC result also showed the mobility of the blue fluorescent image on the TLC plate. The calculated Rf value was observed to be **0.66** while the standard aflatoxin B2 showed the Rf value of **0.67**. Therefore, the Rf value again confirms the successful production of aflatoxin from the steamed rice in **Figure 5**,

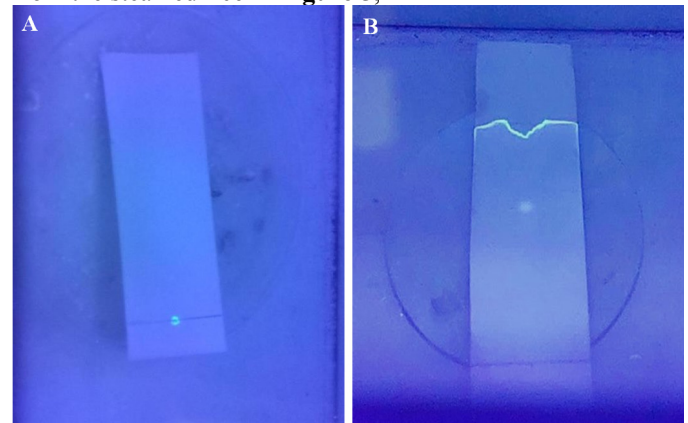


Figure 5: Aflatoxins fluoresce under UV light, **A.** Before running the sample **B.** After running the sample of Aflatoxin, it fluoresced under UV light on Thin layer chromatography

3.5. Calculation of Rf value

Rf value: - Distance traveled by sample / Distance traveled by solvent

$$= 3.2 / 4.8$$

$$= 0.66 \text{ (Rf value of aflatoxin)}$$

This Rf value matches the standard Rf value of Aflatoxin B2 (0.67).

4. DISCUSSION

This study highlights the isolation and identification of *Aspergillus flavus* from landfill soil and confirms its ability to produce aflatoxin B2 when cultured on polished rice under controlled fermentation conditions. Morphological and microscopic observations corroborated previous findings by Khan (2021), who emphasized the distinctive colony characteristics and conidial structures typical of *A. flavus*, such as roughened conidiophores and globose vesicles²⁰. The use of Potato Dextrose Agar (PDA) as a cultivation medium resulted in optimal fungal growth, aligning with the findings of Okayo et al. (2020), who reported PDA to be effective for isolating toxigenic strains of *A. flavus* from groundnuts¹⁶. The observed predominance of fungal colonies on Rose Bengal Agar (RBA) further supports its selective inhibitory effect on bacterial contaminants, as noted in earlier mycological studies.

A significant aspect of this study is the utilization of polished rice as a substrate for solid-state fermentation. This aligns with classic research by Shotwell et al. (1966), who demonstrated that rice is a highly suitable substrate for aflatoxin production due to its rich carbohydrate content and favorable moisture-holding capacity¹⁹. Thin Layer Chromatography (TLC) analysis revealed an Rf value of 0.66 for the extracted toxin, closely matching the standard Rf of 0.67, confirming the identity of aflatoxin B2. This correlates well with previous TLC-based studies such as those by Trucksess et al. (1984), who validated TLC as a reliable and cost-effective technique for aflatoxin detection in field and laboratory conditions.²¹ Comparatively, other detection methods, such as High-Performance Liquid Chromatography (HPLC) and ELISA offer greater sensitivity and quantification (Shephard, 2008), but they require more complex instrumentation and are not always accessible in low-resource settings. Therefore, the TLC method employed in this study remains a practical tool for routine screening of aflatoxins in developing regions²².

5. CONCLUSION

This study successfully isolated and identified *Aspergillus flavus* from landfill soil, confirming its capacity to produce aflatoxin B2 through morphological, microscopic, and chromatographic techniques. Potato Dextrose Agar (PDA) proved to be the most effective medium for fungal cultivation, while polished rice supported optimal aflatoxin biosynthesis under solid-state fermentation conditions. Extraction using chloroform followed by Thin Layer Chromatography (TLC) confirmed the presence of aflatoxin B2, as indicated by an Rf value of 0.66, which closely matches the standard Rf value of 0.67. These findings demonstrate the efficacy of simple culture and chromatographic methods for the detection and characterization of aflatoxins, highlighting the potential risk associated with

fungal contamination in food substrates, particularly polished rice. Further studies involving quantitative aflatoxin analysis and molecular tools may enhance monitoring strategies and inform food safety regulations.

REFERENCES

1. Okayo RO, Andika DO, Dida MM, K'otuto GO, Gichimu BM. Morphological and Molecular Characterization of Toxigenic *Aspergillus flavus* from Groundnut Kernels in Kenya. *Int J Microbiol.* 2020;2020. doi:10.1155/2020/8854718
2. Natesan P, Veterinary TN, Srinivasan MR, Veterinary TN. AN IN SILICO APPROACH FOR THE EVALUATION OF CELL WALL AN IN SILICO APPROACH FOR THE EVALUATION OF CELL WALL COMPONENTS OF PROBIOTICS AS BIOLOGICAL ADSORBERS OF. 2023;(November):17-28.
3. Pickova D, Ostry V, Toman J, Malir F. Aflatoxins: History, significant milestones, recent data on their toxicity and ways to mitigation. *Toxins (Basel).* 2021;13(6):1-23. doi:10.3390/toxins13060399
4. Benkerroum N. Mycotoxins in dairy products: A review. *Int Dairy J.* 2016;62:63-75. doi:10.1016/j.idairyj.2016.07.002
5. Zhang K, Wong JW, Krynsky AJ, Trucksess MW. Perspective on advancing fda regulatory monitoring for mycotoxins in foods using liquid chromatography and mass spectrometry (review). *J AOAC Int.* 2016;99(4):890-894. doi:10.5740/jaoacint.16-0116
6. Cary JW, Klich MA, Beltz SB. Characterization of aflatoxin-producing fungi outside of *Aspergillus* section *Flavi*. *Mycologia.* 2005;97(2):425-432. doi:10.3852/mycologia.97.2.425
7. Eaton DL, Gallagher EP. Mechanisms of aflatoxin carcinogenesis. *Annu Rev Pharmacol Toxicol.* 1994;34(September):135-172. doi:10.1146/annurev.pa.34.040194.001031
8. Pavão AC, Neto LAS, Neto JF, Leão MBC. Structure and activity of aflatoxins B and G. *J Mol Struct THEOCHEM.* 1995;337(1):57-60. doi:10.1016/0166-1280(94)04104-Z
9. Klich MA. Environmental and developmental factors influencing aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*. *Mycoscience.* 2007;48(2):71-80. doi:10.1007/s10267-006-0336-2
10. Horn BW, Moore GG, Carbone I. Sexual reproduction in aflatoxin-producing *Aspergillus nomius*. *Mycologia.* 2011;103(1):174-183. doi:10.3852/10-115
11. Podgórska-Kryszczuk I, Pankiewicz U, Sas-Paszt L. Biological Control of *Aspergillus parasiticus* and *Aspergillus ochraceus* and Reductions in the Amount of Ochratoxin A and Aflatoxins in Bread by Selected Non-Conventional Yeast. *Foods.* 2023;12(20). doi:10.3390/foods12203871
12. Castellá G, Bragulat MR, Cabañes FJ. Fumonisin production by *Fusarium* species isolated from cereals and feeds in Spain. *J Food Prot.* 1999;62(7):811-813. doi:10.4315/0362-028X-62.7.811
13. Benkerroum N. Retrospective and prospective look at aflatoxin research and development from a practical standpoint. *Int J Environ Res Public Health.* 2019;16(19). doi:10.3390/ijerph16193633
14. Sargeant K, Carnaghan RBA. Groundnut Toxicity in Poultry: Br Vete J. 1963;119(4):178-184. doi:10.1016/s0007-1935(17)42454-7
15. © 1961 Nature Publishing Group. Published online 1961.
16. Okayo RO, Andika DO, Dida MM, Otuto GOK, Gichimu BM. Morphological and Molecular Characterization of Toxigenic *Aspergillus flavus* from Groundnut Kernels in Kenya. 2020;2020. doi:10.1155/2020/8854718
17. Khan R. Morphology of *Aspergillus flavus* Morphology of *Aspergillus flavus*. *ReserachGate*. Published online 2021:1-7.
18. Geiser DM, Dorner JW, Horn BW, Taylor JW. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus*

- and *Aspergillus oryzae*. Fungal Genet Biol. 2000;31(3):169-179. doi:10.1006/fgbi.2000.1215
19. Shotwell OL, Hesseltine CW, Stubblefield RD, Sorenson WG. Production of aflatoxin on rice. Appl Microbiol. 1966;14(3):425-428. doi:10.1128/aem.14.3.425-428.1966
 20. Khan R, Ghazali FM, Mahyudin NA, Samsudin NIP. Biocontrol of aflatoxins using non-aflatoxigenic *aspergillus flavus*: A literature review. J Fungi. 2021;7(5). doi:10.3390/jof7050381
 21. Trucksess MW, Brumley WC, Nesheim S. Rapid quantitation and confirmation of aflatoxins in corn and peanut butter, using a disposable silica gel column, thin layer chromatography, and gas chromatography/mass spectrometry. J Assoc Off Anal Chem. 1984;67(5):973-975. doi:10.1093/jaoac/67.5.973
 22. Seymour Shephard G. Determination of mycotoxins in human foods. Chem Soc Rev. 2008;37(11):2468-2477. doi:10.1039/b713084h