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RESEARCH ARTICLE

Development of Analytical Method Protocol for the Separation and Quantitation of Aflatoxins in Almond

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Abstract: Aflatoxins are mycotoxins, structurally related compounds produced as secondary metabolites by *Aspergillus* molds, primarily *flavus* and *parasiticus*. Aflatoxins occur naturally in peanuts, cottonseed, corn and dried chili pepper, as well as many mixed or processed foods and feeds. A simple, sensitive and robust HPLC method with post-column derivatization and Ultraviolet detection was used to analyze Aflatoxins B1, B2, G1 and G2 in ground peanuts. Although more than a dozen aflatoxins exist, the four major toxins of interest are B_1, B_2, G_1 and G_2. They are designated according to their absorption properties in the UV region of the spectrum. Aflatoxin B_1 and B_2 emit radiation corresponding to blue wavelength, while G_1 and G_2 emit yellow-green wavelength. The extracts were cleaned using solid phase extraction method (SPE) preferably over immuno affinity columns (IAC) as the former give better recovery of aflatoxins than the latter one even with most challenging matrices and other methods like soxhlet extraction and accelerated solvent extraction. Further no interferences were found to be present after the cleanup of the sample matrix.

Keywords: Mycotoxins, Aflatoxins, molds, RP-HPLC, Vomitoxin, A. Flavus, Fusarium

Introduction

Aflatoxins are a group of related bisfuranocourmin compounds produced by fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. The term aflatoxin is derived from *Aspergillus* (A-) *flavus* (-fla-) and toxin. It has been reported that, out of the known strains of *pergillus parasiticus*, only about one-half produce toxins. There are fourteen known aflatoxins but most of these are metabolites formed endogenously in animals. The well-known ones among these are aflatoxin (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 FLG1) and aflatoxin G2 (AFLG2)[1,2].

Experimental

Sample matrices for the analysis are prepared and cleaned up in the stepwise manner. Different varieties of almonds were ground on exposure to humid environmental conditions

followed by grinding. The ground samples were then dispersed in (A) water; (B) acetonitrile; (C) methanol; (72:14:14, A:B:C) followed by sonication using sonicator bath for 10 minutes so as to have maximum solute transfer and homogenization. Further, for sample clean up and for maximum recovery Supel Tox AflaZea SPE Cartridges were used and thereafter sample was injected in the column. The samples and standards were prepared meticulously using requisite quantities of HPLC grade solvents. The calibration of the system was done for ensuring accuracy and precision in the results.

Aflatoxins B1 and B2 are so designated because of their strong blue fluorescence under ultraviolet light and aflatoxins G1 and G2 show greenish yellow fluorescence[1]. Aflatoxicosis (ill effect of aflatoxin) causes acute liver damage, liver cirrhosis, induction of tumors, impaired central nervous system, skin disorders and hormonal defects[3-5].

The fungus *Aspergillus* grows in soil and decaying vegetation and can colonize and contaminate crops with aflatoxins before harvest or during storage. Aflatoxins are toxic and highly carcinogenic substances, and the presence of aflatoxins B1, B2, G1 and G2 (structures shown in Figure 1) in a variety of processed and unprocessed foods is regulated in countries around the world[2]. The European commission has set maximum levels for aflatoxin B1 between 2.0 and 8.0 µg/kg and for the sum total of all four of these toxins between 4.0 and 15.0 µg/kg in crops such as nuts, groundnuts, grains and dried fruits[6-9]. The mycotoxin aflatoxin is known to be a potent carcinogen[2]. The U.S. Food and Drug Administration has set action levels (levels where the FDA will take legal action to remove products from the market) of 20 ppb (µg/kg) for the sum total of the four aflotoxins in foods such as corn, peanuts, brazil nuts and pistachios as well as other foods[10-15].

Figure 1. (a) Almond sample exposed to humid atmospheric conditions for one week (b) Almond sample solution (Before filtration)

The traditional method for aflatoxins analysis in grains includes soxhlet extraction, sample clean-up using solid-phase extraction (SPE) and separation, identification and quantification using high-performance liquid chromatography (HPLC). Because of the time-consuming extraction and clean-up steps, sample throughput is limited using this technique[5-12]. The structural formula for the aflatoxins is as shown in Figure 2.

A given amount of Almond (dry fruit) as purchased from local market was when exposed to humid atmospheric conditions for a given period facilitates the fungal growth. The nuts with the fungal growth were then suspended in the solvent as methanol/water mixture followed by light scrapping for ridding off the metabolites generated by fungi. The aliquot then was sonicated in a sonicator bath for about 10 minutes for the purpose of homogenization. The mixture was then filtered using a vacuum filter with the help of 0.2 µ filter such that extract containing potential aflatoxins can be separated from the homogenized and filtered mixture.

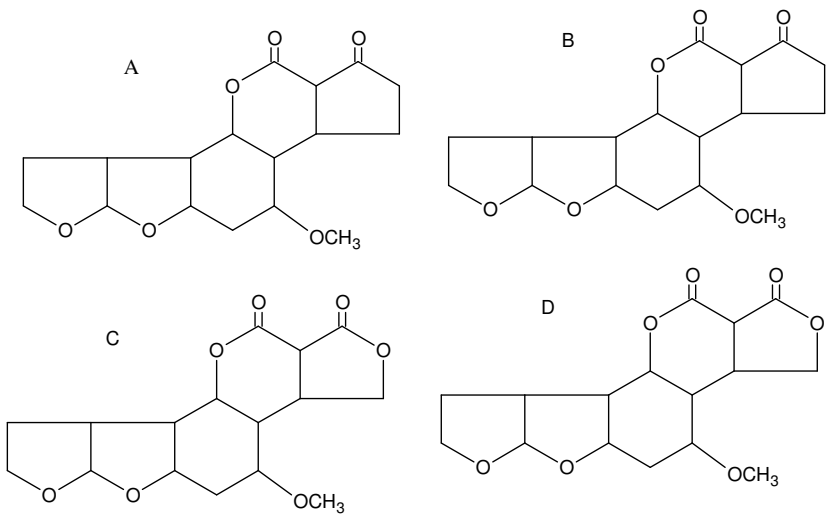

Figure 2. Molecular structures of aflatoxins (A) Aflatoxin B1, (B) Aflatoxin B2, (C) Aflatoxin G1 and (D) Aflatoxin G2

The extract in the form of filtrate is then first tested for identifying wavelength of maximum absorption. (λ_{max}) using UV-Visible spectrophotometer (UV–1650PC) interfaced with the software UV Probe. The extract was further diluted with Milli-Q water and the diluted extract was injected (20 µL) using a Hamilton Micro syringe to LC chromatograph (LC–10AT Vp). A method protocol has been developed to separate the aflatoxins with better resolution efficiency, recovery and quantitation.

The system used LC-10AT VP with a UV detector, is interfaced with a software Spinchrome. Replicate measurements were taken to test for the reproducibility in results by computing standard deviation. Both the instruments were calibrated using official methods prior to making measurements to ensure maximum accuracy. After getting the chromatogram for the separated metabolites with the corresponding retention characteristics the identification of separated metabolites (Aflatoxins) was done by further characterization using external standard method. The standard chromatogram for the aflatoxins is as shown in Figure 3. It shows the calibration curve for external standard aflatoxin, the concentration of which was varied between 0.1 ng/mL to 100 ng/mL. The solution of aflatoxins in methanol/water/acetonitrile mixture as an external standard was prepared, sonicated for 5 minutes and then 20 µL of it was injected and the chromatogram obtained is as shown in Figure 3. Replicate measurements were taken to check for precision and accuracy of the results. Further inter-day and intra-day measurements were also taken and STD and RSD was also calculated which was found to be well within limits. The retention time at which aflatoxins as a standard gets eluted and appears in the form of peak in the chromatograph, is in semblance with that for analytes of interest like aflatoxins G1, G2, B1, B2 *etc*. separated in the sample chromatogram as shown in Figure 4.

The standards were obtained from Sigma Aldrich. The purity of the compounds was greater than 99%. Further HPLC-grade methanol was obtained from Merck. Water was purified in-house with a Milli-Q system (Millipore, Tokyo, Japan). The method protocol developed for the separation analysis of Aflatoxins in Almond samples using RPHPLC – UV is as follows:

HPLC system : LC – 10AT VP (Shimadzu Make)

Detector : SPD – 10 vp (UV detector)
Column : C_{18} ODS), 250x4.6x5 µ
Mobile phase : (A) water; (B) acetonitrile; (C) methanol; (72:14:14, A: B: C)
Flow rate : 1 mL/min
Sample volume : 20 µL
Temperature : Ambient
Wavelength : 270 nm

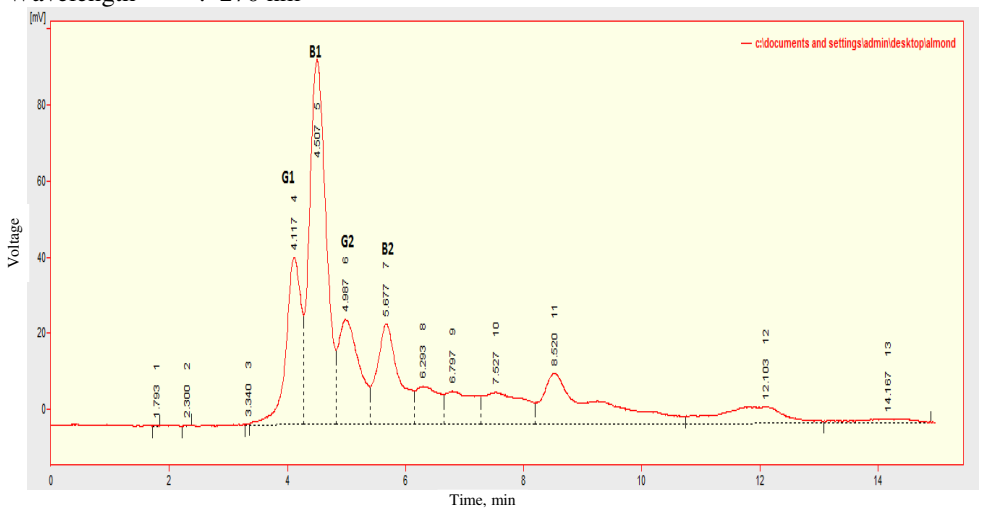

Figure 3. Standard chromatogram of almond

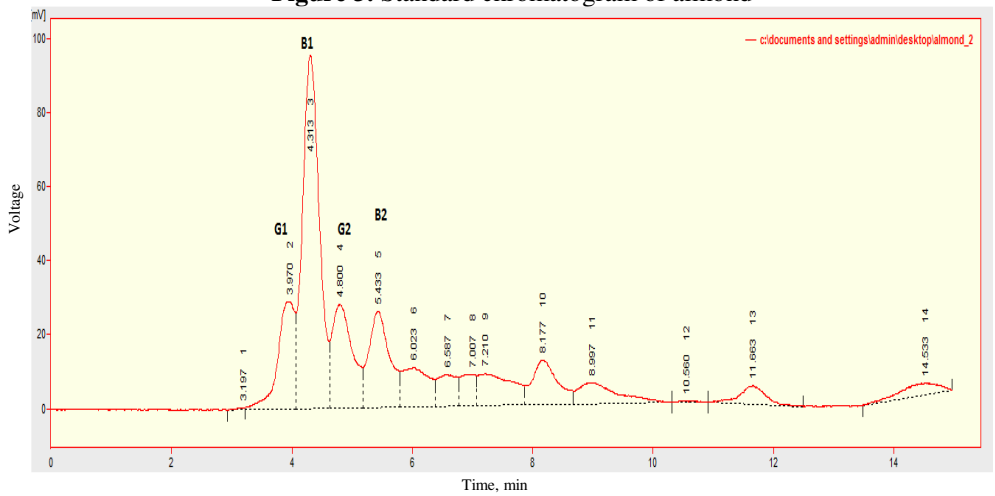

Figure 4. Raw almond chromatogram

Results and Discussion

In this study, aflatoxins in dry fruit (Almond) using RP-HPLC were successfully separated, and purified using multi step process. In the initial screening crude extract obtained from it with fungal growth was purified using vacuum filtration followed by its dilution using Millipore water, Water, Acetonitrile & Methanol & (72:14:14, v/v) is used as a solvent for

extraction by RP-HPLC technique. The extract first was tested for finding out absorption maximum and was found to be 270 nm. The extract (Sample) of raw Almond was then injected onto a C18 RP-HPLC column for getting chromatogram which is as shown in Figure 3. By injecting the volume of sample 20 µL keeping the flow rate 1 mL/min.at wavelength 270 nm. The confirmation of the 4 separated components at the retention time 3.970, 4.313, 4.800 and 5.455 respectively. Presence of these 4 separated aflatoxins further confirmed and characterized by using external standard of aflatoxins (Sigma Aldrich) .The retention times of external standard was found 4.117, 4.507, 4.907 and 5.577, Aflatoxin G1, B1, G2 and B2 respectively (Figure 3), which match with the component /aflatoxin peaks having the retention time 3.970, 4.313, 4.800 and 5.455 (Aflatoxin G1, B1, G2 and B2) in the sample chromatogram as shown in Figure 4. As the retention time of sample chromatograms matches with the retention times of external standard of aflatoxins which confirm the presence of aflatoxin G1, B1, G2 and B2 in the sample of almond.

Figure 5 indicates presence of 4 aflatoxin peaks of raw Almond which is exposed to humid atmospheric conditions for a week. The retention times of these peaks 4.697, 5.087, 5.880 and 6.397, which matches with the retention times of external standard of aflatoxins which confirm the presence of aflatoxin G1, B1, G2 and B2 in the sample of almond.

The test procedure is made up of 3 steps, *i.e*., sampling, sample preparation and analysis. The linearity in the calibration curve was evaluated and was found to be very good with correlation coefficients (r^2) greater than 0.999. The overlay of the chromatograms having 3 runs of the sample which shows the RED chromatogram for FIRST RUN, PINK chromatogram for SECOND RUN and GREEN chromatogram for THIRD RUN which is as shown in Figure 6.

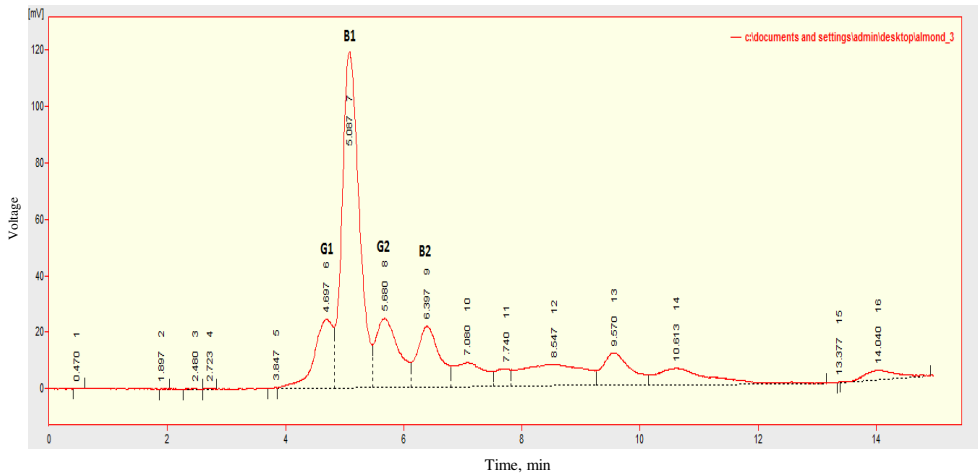

Figure 5. Chromatogram for raw almond exposed to humid atmospheric conditions for a week

Although there is some time lag between a peak or two the prominent ones meant for identified aflatoxins are very well in harmony with those appear in standard chromatogram. The method showed acceptable linearity and precision.

The limit of detection allows the presence of aflatoxins in food with maximum acceptable levels of 2 µg/kg for aflatoxin B1 and 4 µg/kg for the sum total of the aflatoxins that is G1, G2, B2 and other toxins.

Table 1. Retention times of aflatoxin peaks of raw almond and aflatoxin standard solution

Runs	Retention times			
	Peak1	Peak2	Peak3	Peak4
Chromatogram 1(**Standard**)	**4.117**	**4.507**	**4.907**	**5.577**
Chromatogram 2(Sample)	**3.970**	**4.313**	**4.800**	**5.455**
Chromatogram 3(Sample)	**4.007**	**5.097**	**5.600**	**6.307**

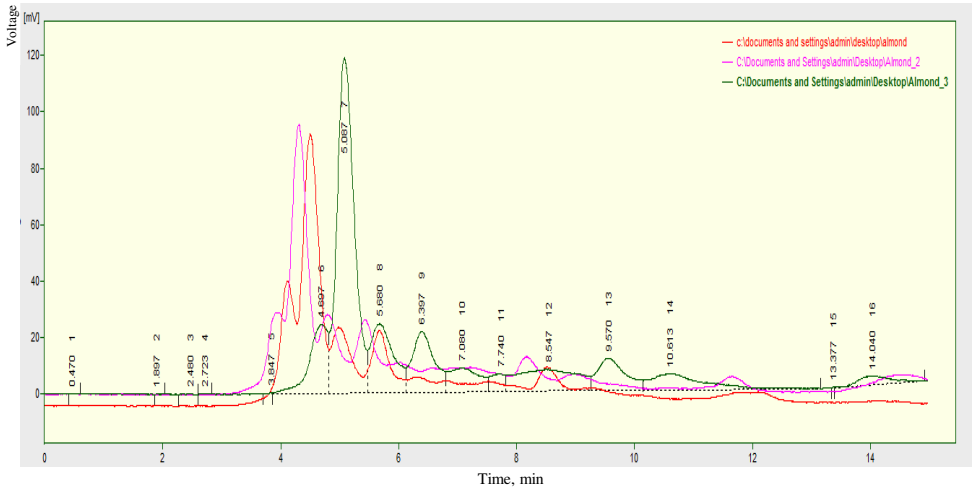

Figure 6. Overlay chromatogram of almond

The retention times of external standard was found 4.117, 4.507, 4.907 and 5.577 which are matches with the component peaks of raw Almond, having retention times 3.970, 4.313, 4.800 and 5.455 as shown in Figure 4. This confirms the presence of aflatoxin G1,B1, G2 and B2 in the sample of raw almond.

Conclusion

The RP-HPLC method described here was suitable for the determination of mycotoxin, aflatoxins in dry fruit (Almond) due to its high sensitivity and high selectivity of liquid Chromatography. The quantitative and qualitative analysis of aflatoxins can be done by developing an analytical method effectively and efficiently even at trace level and its validation can be done using a standard using RP-HPLC as a analytical tool which is most sought after. The developed method protocol can further be used for the separation and analysis of other classes of mycotoxins as well.

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