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Application Report
HPLC

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Analysis of Aflatoxins by High Performance Liquid Chromatography with Post-column Bromination

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Introduction

Aflatoxins are a group of naturally occurring mycotoxins produced by two types of mold: Aspergillus flavus and Aspergillus parasiticus. Under favorable temperature and humidity conditions, these fungi grow on certain crops such as peanuts, tree nuts, corn, wheat, and oil seeds (like cottonseed), resulting in the production of aflatoxins.

Aflatoxins have received great attention due to their extremely potent carcinogenic nature in human beings, poultry and livestock. Among the 18 different types of aflatoxins identified, the major members are aflatoxin B_1, B_2, G_1 and G_2. These are usually found together in foods and livestock feeds in various proportions, however, aflatoxin B_1 is the most predominant and is also the most toxic. Their chemical structures are shown in *Figure 1*. It is recognized that aflatoxins are considered "unavoidable contaminants", therefore they are allowed to exist at low levels in many countries.[1]

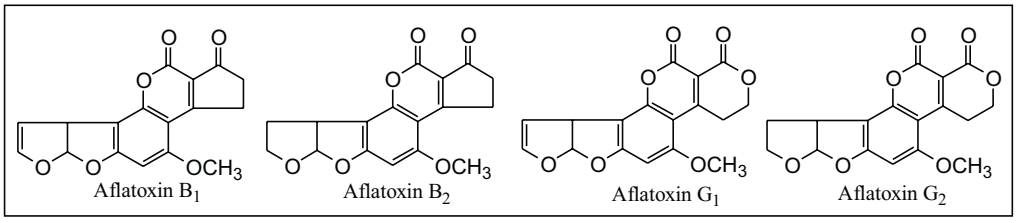


Figure 1. Structures of Aflatoxin B_1, B_2, G_1 and G_2.

Over the years several methods, including enzyme-linked immunosorbent assay (ELISA), TLC, HPLC and LC-MS, etc. have been developed to analyze aflatoxins.[2,3]

High performance liquid chromatography determination of aflatoxins using fluorescence detection has been increasingly used because of its accuracy and ease in automation, but it suffers from the fluorescence quenching of aflatoxin B_1 and G_1 in aqueous solvents. However, it has been observed that reaction of aflatoxins B_1 and G_1 with a variety of reagents such as strong acids or the oxidants, chloramines T, bromine or iodine leads to a significant increase in fluorescence intensity. Various methods including precolumn derivatization with trifluoroacetic acid (TFA), postcolumn derivatization with iodine, and pyridinium bromide perbrominde (PBPB) have been reported. Each of the methods presents several significant drawbacks.[4] Postcolumn derivatization with electrochemically generated bromine method has been successfully used to overcome the requirement of the second pump.[5]

In postcolumn bromination method, reversed phase HPLC column provides good separation of the toxins, and excellent sensitivity can be achieved because aflatoxin B_1 and G_1 are derivatized prior to

the fluorescence detector. The flow from the column passes into an electrochemical cell (Kobra cell), and the derivatizing reagent, free bromine is generated from bromide potassium present in the mobile phase. The amount of bromine produced can be controlled by the generating current. The reaction, as shown in *Figure 2*, takes place at room temperature in a short reaction coil, and following the detector, the flow passes back into the counter-electrode compartment of the Kobra cell to sweep away the reaction products of the electrode. This approach is applicable to routine analyses of samples for aflatoxins in the low ng/g range.[5,6]

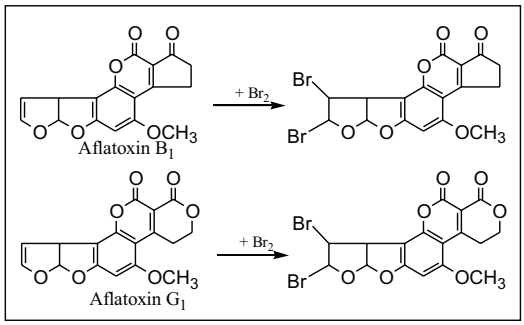


Figure 2. Derivatization reaction of Aflatoxin B_1 and G_1.

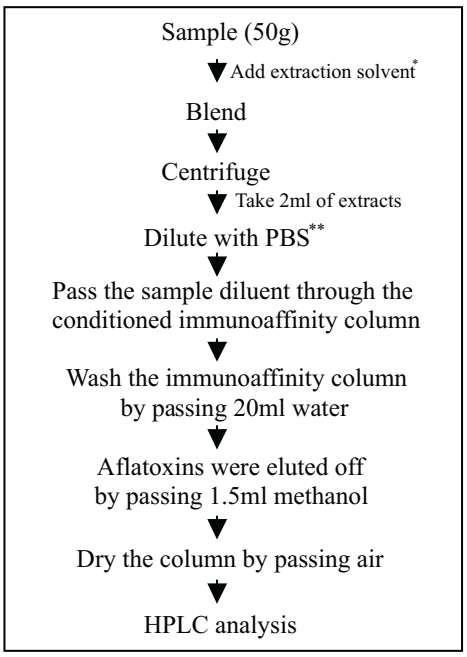


Figure 3. Pretreatment of samples.

**100ml 60% acetonitrile/water (v/v) extraction is recommended for commodities such as animal feed, herbs and spices. 80% methanol/water (v/v) extraction is recommended for nuts, figs, maize, cereals and cumin.*

*** PBS (phosphate buffered saline solution) - pH 7.4. Dissolve 0.20g KCl, 0.20g KH_2PO_4, 1.16g anhydrous Na_2HPO_4, and 8.00g NaCl in 900ml water. Adjust to pH 7.4 with 0.1M HCl or NaOH and dilute to 1L. (Commercial PBS tablets may be used).*

Experimental[7]

The sample was extracted with suitable solvent. After dilution and filtration, the sample extract is passing through immunoaffinity column, which contains a gel suspension of monoclonal antibody covalently attached to a solid support. Any aflatoxins present in the sample are bound to the antibody within the column, the column is then washed with water to remove extraneous non-specific material, and the toxins are released from the column using methanol or acetonitrile. The eluate can then be analyzed by HPLC. The pretreatment procedure is shown in *Figure 3*. The procedures might be varied slightly between immunoaffinity column manufacturers.

Results and Discussion

Aflatoxins elute in the order G_2, G_1, B_2 and B_1 with retention times of ca 5, 6, 8, and 10 min, respectively at the same fluorescence emission wavelength in a single run, and they are baseline resolved as shown in *Figure 4*. A comparison of the chromatograms of aflatoxins obtained *(a)* without Kobra cell and *(b)* with Kobra cell is given in *Figure 5*. It is observed that the fluorescence intensity of aflatoxins B_1 and G_1 are increased by using Kobra cell, while the peak area of B_2 and G_2 are similar in two chromatograms.

A good linearity was achieved with regression of 0.9999 (*Figure 6a-6d*). Detection for the four aflatoxins could easily reach to below ng/ml levels under the conditions employed as shown in *Figure 9*. The limit of detection (signal-to-noise ratio=3) for aflatoxins B_1, B_2, G_1 and G_2 are 0.03, 0.08, 0.01 and 0.03ng/ml, respectively. Peanuts and chilli were analyzed and the results are displayed in *Figure 7* and *Figure 8*.

Attempts were made to analyze aflatoxins by Shim-pack FC-ODS (4.6mmID x 30mmL) column, which is a fast LC column packed with 3 µm porous high purity silica. Shim-pack FC-ODS is a short column,

and its particle size is smaller than that of traditional columns. According to Van Deemter's plot, smaller particles provide increased efficiency of separation because columns packed with smaller particles have less interstitial space than those packed with larger particles, therefore, the analytes can move more easily from particle to particle and there are more interaction points within the packed bed. This process results in a more efficient mass transfer and more theoretical plates. Hence shorter columns can be applied to obtain a faster analysis without sacrificing the resolution. By adjusting the concentration of methanol in mobile phase, it is able to reduce the analysis time to ca 7 min as shown in *Figure 10*.

Table 1. Analytical conditions.

Instrument	:	Shimadzu HPLC
Column	:	Shim-Pack VP-ODS (4.6mmID x 150mmL)
Flow rate	:	1.0 ml/min
Temperature	:	40ºC
Injection volume	:	10 µl
Mobile phase	:	A / B = 55/45 *A. water containing 216.4 mg KBr & 159.1 µl HNO_3* *B. methanol*
Current	:	100 µA
Detection	:	Fluorescence detector Ex:360 nm; Em:425 nm

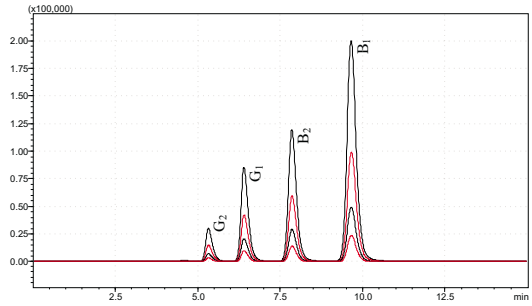


Figure 4. Chromatograms of aflatoxins G_2, G_1, B_2 and B_1.

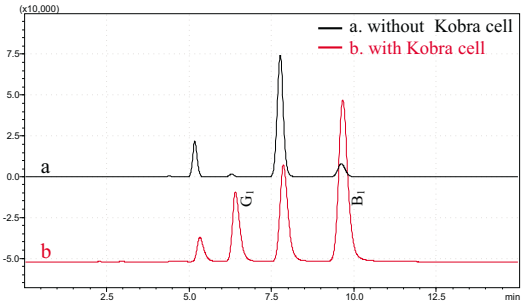
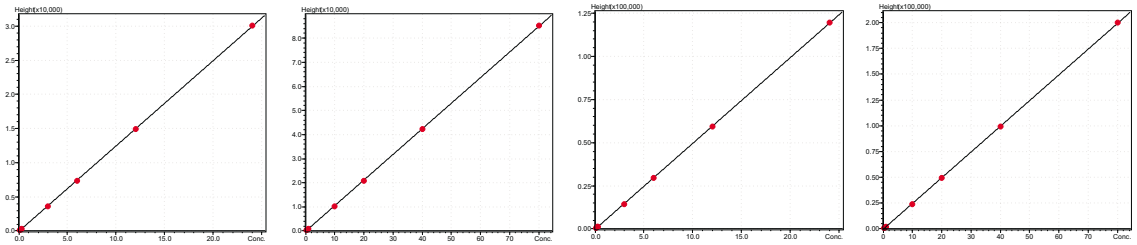


Figure 5. Comparison of chromatograms of using Kobra cell and without Kobra cell.



(a) G_2 *(b) G_1* *(c) B_2* *(d) B_1*

Figure 6. Calibration curves of aflatoxin G_2, G_1, B_2 and B_1.

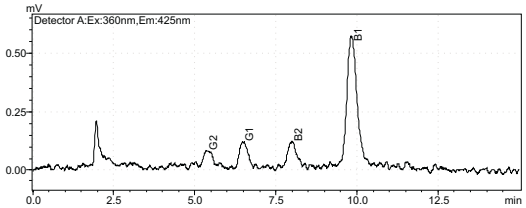


Figure 7. Chromatogram of peanuts. Aflatoxins G_2: 0.09 ng/ml; G_1: 0.13 ng/ml; B_2: 0.03 ng/ml; B_1: 0.24 ng/ml.

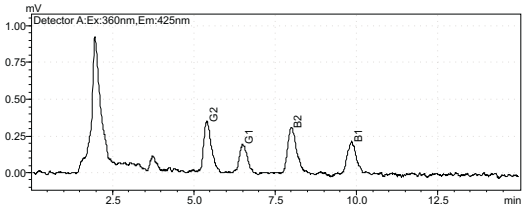


Figure 8. Chromatogram of chilli. Aflatoxins G_2: 0.33 ng/ml; G_1: 0.20 ng/ml; B_2: 0.08 ng/ml; B_1: 0.09 ng/ml.

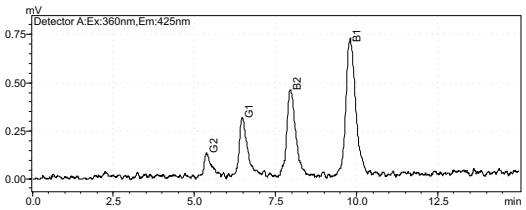


Figure 9. Detection of low concentrations of aflatoxins G_2: 0.10 ng/ml; G_1: 0.33 ng/ml; B_2: 0.10 ng/ml; B_1: 0.33 ng/ml.

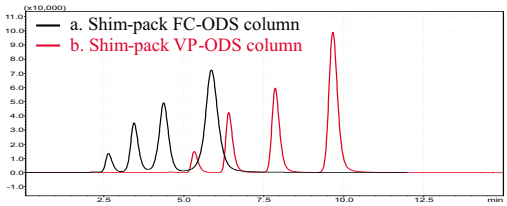


Figure 10. Comparison of chromatograms obtained by Shim-pack VP-ODS and Shim-pack FC-ODS column.

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