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# DETERMINATION OF AFLATOXINS UTILIZING XBRIDGE HPLC COLUMNS

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## BACKGROUND

Aflatoxins are toxic metabolites produced by certain fungi in food and feeds and have been associated with various health risks in livestock, domestic animals and humans worldwide. Potentially harmful effects include aflatoxicosis and carcinogenic effects which have been seen in laboratory animals. Because of their acute toxicological effects in humans, aflatoxins have been studied to a greater degree than most other mycotoxins.

Aflatoxins are produced primarily by strains of Aspergillus flavus and of Aspergilus parasiticus, plus related species of Aspergillus nomius and Aspergillus niger. There are four major aflatoxins, B1, B2, G1 and G2 that are significant as direct contaminants of foods and feeds. There are also two additional aflatoxin metabolites, M1 and M2, which have been isolated and determined to be harmful (Table 1 and Table 2).

| | |
|---|---|
| B1 & B2 | produced by Aspergillus flavis and Aspergillus parasiticus |
| G1 & G2 | produced by Aspergillus parasiticus |
| M1 | metabolite of aflatoxin B1 in humans and animals (exposure in ng can come from mother's milk) |
| M2 | metabolite of aflatoxin B2 in milk of cattle fed on contaminated foods |

*Table 1. Aflatoxin sources*

| | |
|---|---|
| B1 | C17H1206 |
| B2 | C17H1406 |
| G1 | C17H1207 |
| G2 | C17H1407 |

*Table 2. Aflatoxin molecular structures*

These toxins have similar structures and form a unique group of highly oxygenated, naturally occurring herocyclic compounds (Figure 1). Aflatoxins B2 and G2 have been identified as dihydroxy derivatives of B1 and G1 respectively.

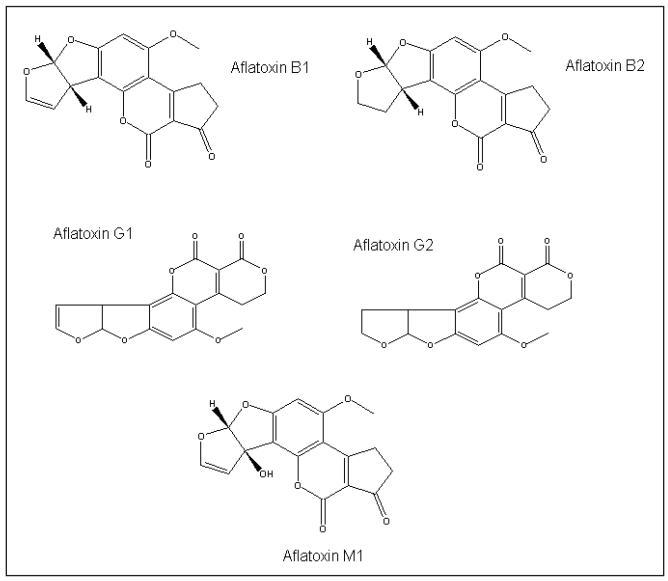


*Figure 1. Aflatoxin molecular structures.*

Crops which are frequently affected by aflatoxins include cereals (wheat, maize, sorghum, rice), oil seeds (peanuts, sunflower, cotton, soybean), spices (chili peppers, black pepper, red pepper, turmeric, ginger) and tree nuts (almond, pistachio, brazil nut, coconut, walnut). Virtually all sources of commercial peanut butter contain minute quantities of aflatoxin but at levels far below the US Food and Drug Administration's recommended limit.

Humans are exposed to aflatoxins when consuming foods contaminated with products of fungal growth. Even though heavily contaminated food is not permitted to enter the food supply in developed countries, concern still exists regarding the possible adverse effects resulting from long-term exposure to low levels of aflatoxins in the foods. Aflatoxins are considered unavoidable contaminants in food and feed, even where good manufacturing practices are in place. The FDA, as well as other regulatory bodies around the word, has established specific guidelines on acceptable levels of aflatoxins in human food and animal feed as seen in Table 3.

## Action Levels for Aflatoxins

### United States (FDA) Action Levels (B1, B2, G2, G2, M1)

| Food Stuff | Level | Regulation |
|---|---|---|
| All products - except milk - designated for humans | 20 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |
| Milk | 0.5 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |
| Corn for immature animals and dairy cattle | 20 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |
| Corn for breeding beef cattle, swine and mature poultry | 100 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |
| Corn for finishing swine | 200 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |
| Corn for finishing beef cattle | 300 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |
| Cottonseed meal (as feed ingredient) | 300 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |
| All feedstuff other than corn | 200 ng/g | Policy Guides 7120.26, 7106.10, 7126.33) |

### EU Action Levels

| Food Stuff | Level | Regulation |
|---|---|---|
| M1 | | |
| Milk | 50 ng/l (ppt) | VO [(EG) Nr. 466/2001] |
| Newborn baby formula and infants formula, inluding newborn baby milk formula and follow-on milk | 25 ng/kg (ppt) | VO [(EG) Nr. 683/2004] |
| Dietary foods for special medical purposes intended specifically for infants | 25 ng/kg (ppt) | VO [(EG) Nr. 683/2004] |
| B1 | | |
| Food, peanuts, shell fruits, dried fruits, cereals | 2 µg/kg (ppb) | VO [(EG) Nr. 2174/2003] |
| Maize (unprocessed) and spices | 5 µg/kg (ppb) | VO [(EG) Nr. 2174/2003] |
| Cereal and other complementary foods for infants and small children | 100 ng/kg (ppt) | VO [(EG) Nr. 683/2004] |
| Dietary foods for special medical purposes intended specifically for infants | 100 ng/kg (ppt) | VO [(EG) Nr. 683/2004] |
| B1, B2, G1, G2 | | |
| Spices (e.g. chilli, paprika, pepper, nutmeg, ginger and turmeric) | 10 µg/kg (ppb) | VO [(EG) Nr. 2174/2003] |
| Cereals | 4 µg/kg (ppb) | VO [(EG) Nr. 2174/2003] |
| Peanuts, shell fruits, dried fruits for direct consumption or as food ingredients | 4 µg/kg (ppb | VO [(EG) Nr. 2174/2003] |
| Dietary foods for infants and young children | 50 ng/kg (ppt) | VO [(EG) Nr. 683/2004] |

### Japan Action Levels

| Food Stuff | Level | Regulation |
|---|---|---|
| B1 | | |
| All foods | 10 µg/kg | Food Sanitation Law |
| Compound feeds for cattle (except calves, dairy cows), pigs (except piglets), chicken (except young chicken, broilers) and quails | 20 µg/kg | Food Sanitation Law |
| Compoung feeds for calves, dairy cows, piglets, young chicken and broilers | 10 µg/kg | Food Sanitation Lawt |

*Table 3. Aflatoxin regulatory action limits.*

In addition to the health ramifications, there is also an economic impact attributable to aflatoxin growth linked directly to crop and livestock losses as well as indirectly to the cost of governmental programs designed to reduce risks to animal and human health. The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxins, of which the most widespread are the aflatoxins. Losses attributable to aflatoxin contaminated feeds include death and the lesser effects of immune system suppression, reduced growth rates and losses in feed efficiency. Other adverse economic effects of aflatoxin include lower yields for food and fiber crops. Because of these dramatic effects, most countries have taken action to mitigate mycotoxins in general and aflatoxins specifically (Table 4).

## Standarized Testing Methods for Aflatoxins

**AOAC Official Methods for Mycotoxins**

| Official Method | Authority | Mycotoxin | First Action | Method and Publishing Citations |
|---|---|---|---|---|
| 991.31 | AOAC | Aflatoxin | 1991 (Final Action 1994) | Aflatoxins in Corn, Raw Peanuts and Peanut Butter: Immunoaffinity Column Method [J. of AOAC Int. 74(1) 81-88, 1991] |
| 999.07 | AOAC | Aflatoxin B1 and Total Aflatoxins | 1999 | Aflatoxin B1 and Total Aflatoxins in Peanut Butter, Pistachio Paste, Fig Paste, and Paprika Powder: Immunoaffinity column Liquid Chromatography with Post-Column Derivitization [J. of AOAC Int. 83(2) 320-40, 2000] |
| 2000.08 | AOAC | Aflatoxin M1 | 2000 | Aflatoxin M1 in Liquid Milk: Immunoaffinity Column by Liquid Chromatography [J. of AOAC Int. 84(2) 437-43, 2001] |
| 2000.16 | AOAC | Aflatoxin B1 | 2000 | Aflatoxin B1 in Baby Food by Immunoaffinity Column and HPLC [J. of AOAC Int. 84(4) 1116-1123, 2001] |
| 20003.02 | AOAC | Aflatoxin B1 | 2003 | Immunoaffinity Column Cleanup with Liquid Chromatography Using Post-Column Bromination for Determination of Aflatoxin B1 in Cattle Feed: Collaborative Study [Journal of AOAC Int. 86(6) 1179-1186, 2003] |

**CEN (European Committee for Standardization) Methods for Mycotoxins**

| Official Method | Authority | Mycotoxin | First Action | Method and Publishing Citations |
|---|---|---|---|---|
| EN-12955 | CEN | Aflatoxin | July 1999 | Foodstuffs - Determination of aflatoxins B1, and the sum of aflatoxin B1, B2, G1 and G2 in cereals, shell-fruits and derived products - High performance liquid chromatographic method with post column derivatization and immunoaffinity column cleanup |
| EN-14123 | CEN | Aflatoxins | June 2003 | Foodstuffs - Determination of aflatoxin B1, and the sum of aflatoxins B1, B2, G1 and G2 in peanuts, pistachios, figs and paprika powder - High performance liquid chromatographic method with post column derivatization and immunoaffinity column cleanup |

*Table 4. Standardized Methods for aflatoxins.*

Numerous analytical methods based on high-performance liquid chromatography (HPLC) have been developed for detecting and quantifying aflatoxins. This report describes two improved analytical methodologies for the identification of aflatoxins using XBridge™ $C_{18}$ HPLC columns.

## EXPERIMENTAL

### Example One

By far, the most commonly used laboratory methods for aflatoxins utilize High Performance Liquid Chromatography (HPLC) with fluorescence detection. In this example, a standard aflatoxin mixture was used to evaluate the ability of the XBridge $C_{18}$ HPLC column to separate these compounds.

**Sample Preparation**

A commercial mixture of aflatoxins B1, B2, G1 and G2. A final solution was appropriately prepared to create the final concentrations:

B1: 250 pg/mL

B2: 25 pg/mL

G1: 250 pg/mL

G2: 25 pg/mL

| HPLC Conditions | |
|---|---|
| Column | XBridge $C_{18}$, 4.6 x 150 mm, 5 µm |
| Part Number | 186003009 |
| Mobile Phase | Water/MeOH, 70/30, v/v |
| Flow Rate | 0.6 ml/min |
| Injection Volume | 10 µl |
| Column Temp. | 40 °C |
| Detection | λex: 365 nm, λem: 455 nm |
| Instrument | Waters Alliance® 2695 with fluorescence detector |

### Results

Utilizing an excitation wavelength of 365 nm and an emission wavelength of 455 nm, all of the standard aflatoxins were separated and identified using the XBridge $C_{18}$ column (Figure 2).

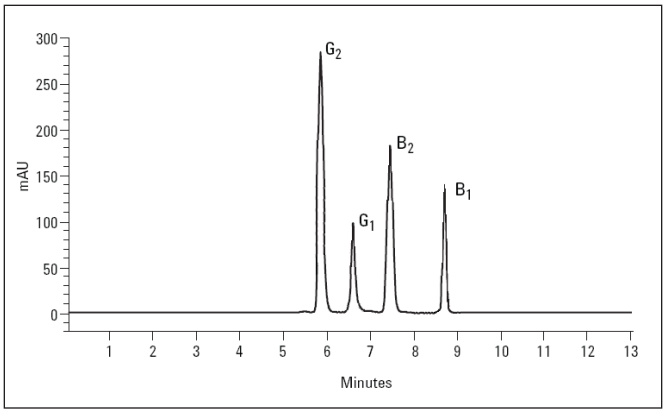


*Figure 2. Chromatogram of aflatoxin standard mixture. Chromatogram provided courtesy of the University of Iowa.*

### Example Two

Humans are exposed to aflatoxins in a wide variety of food stuffs. In this example, a sample of commercially available red pepper was obtained and analyzed for mycotoxins, using the XBridge $C_{18}$ HPLC column.

**Sample Preparation**

A commercial aflatoxin standard was obtained. Working standard solutions were prepared daily.

An extraction of the sample was performed utilizing the following: 50 g of sample was added to 5 g NaCl, extracted with 300 mL methanol:water (80:20, v/v) in a mixer. Filtered extract was diluted with 60 mL PBS and 65 mL of diluted filtrate was applied to an immunoaffinity column conditioned with PBS. The column was washed with 10 mL water and air applied until dry. Aflatoxins were then eluted by applying 1.20 mL of methanol to the column. The eluate was diluted with 1.50 mL water.

| HPLC Conditions | |
|---|---|
| Column | XBridge $C_{18}$, 4.6 x 250 mm, 5 µm |
| Part Number | 186003117 |
| Mobile Phase | acetonitrile/water/methanol , 17:54:29, v/v/v |
| Flow Rate | 1 mL/min |
| Injection Volume | 100 µl |
| Column Temp. | 40 °C |
| Detection | λex: 333 nm, λem: 460 nm |
| Instrument | Waters Alliance 2695 with a fluorescence detector |

## Results

Utilizing the described chromatographic conditions, a standard test mixture and an extract of red pepper were analyzed. The XBridge $C_{18}$ column provided excellent separation of the analytes of interest (Figure 3 and Figure 4). Toxins in the extract were identified based upon a comparison to the standard chromatogram.

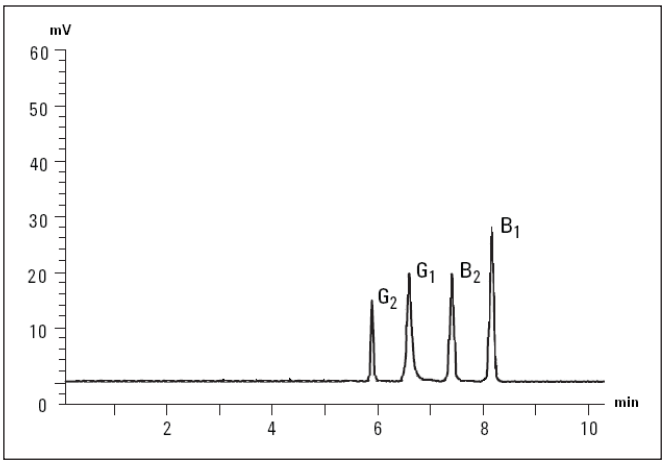


*Figure 3. Chromatogram of prepared aflatoxin standard mixture: G2, 0.2 ng/mL; G1, 0.4 ng/mL; B2, 0.2 ng/ml; B1, 0.4 ng/mL. Chromatogram provided courtesy of Istanbul University.*

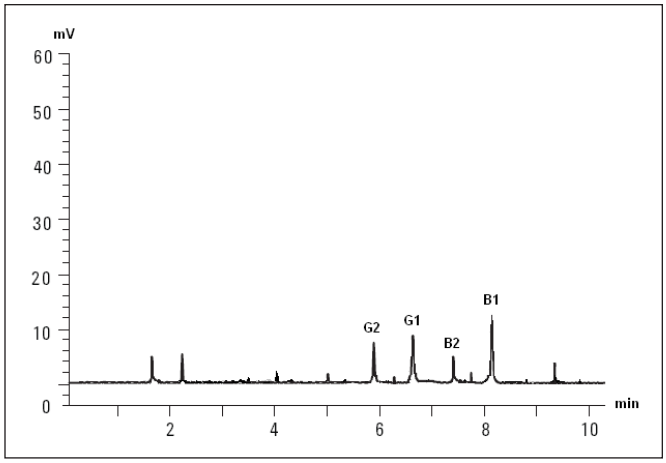


*Figure 4. Chromatogram of aflatoxins in red pepper extract. Chromatogram provided courtesy of Istanbul University.*

## CONCLUSION

The ability of aflatoxins to cause cancer and related diseases in humans and impart broad economic impacts worldwide has made them a primarily mycotoxin of concern and widely analyzed. In these studies, XBridge $C_{18}$ columns were evaluated for their ability to accurately separate aflatoxins. Providing excellent resolution and quantification at a picogram level, these columns are shown to be well suited for trace level analysis of these analytes.

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