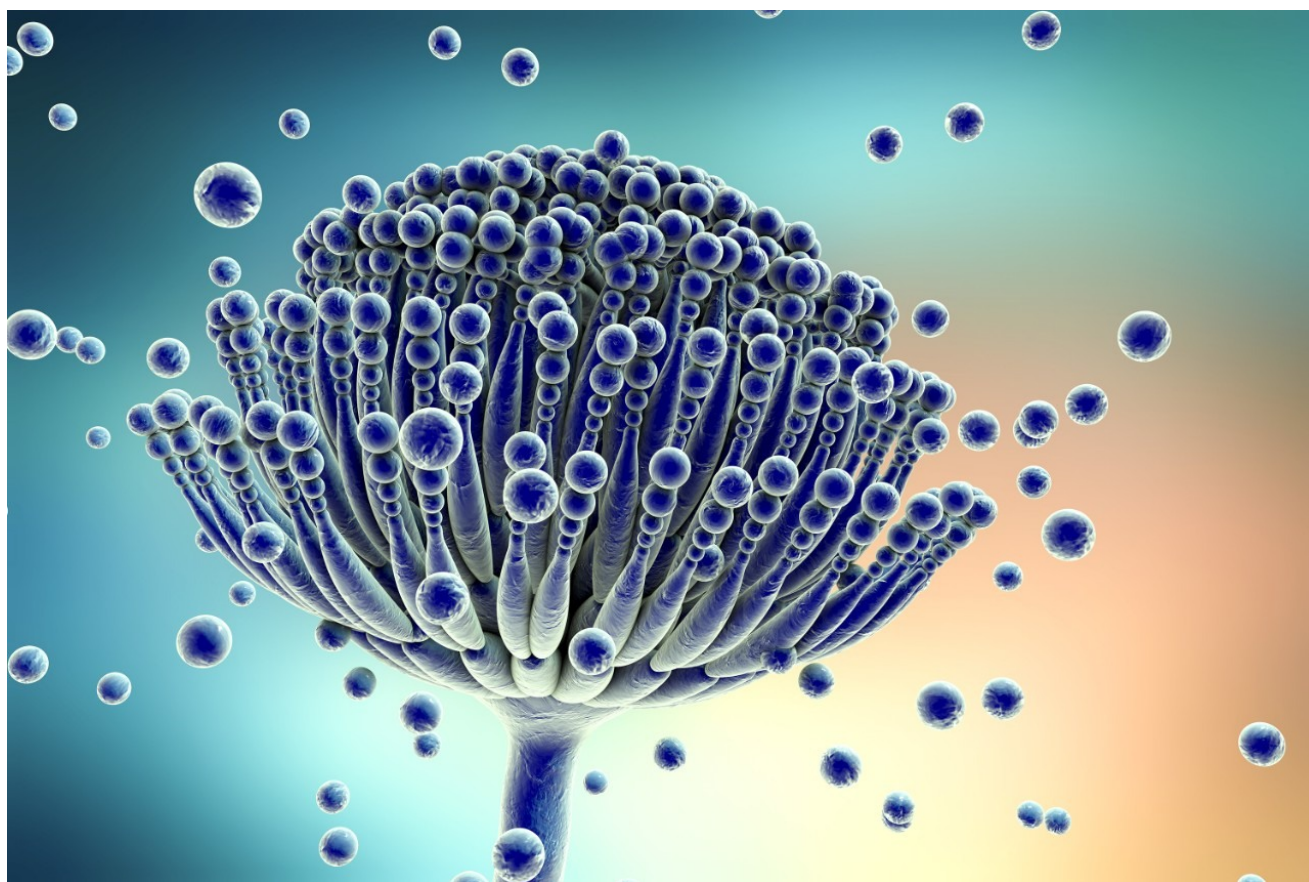


## Determination of Aflatoxins Utilizing XBridge HPLC Columns

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### Abstract

This application note evaluates XBridge C<sub>18</sub> columns for their ability to accurately separate aflatoxins which

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provides excellent resolution and quantification at a picogram level.

## Introduction

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 *Aspergillus 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 <i>Aspergillus flavus</i> and <i>Aspergillus parasiticus</i>
G1 & G2	produced by <i>Aspergillus parasiticus</i>
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	C <sub>17</sub> H <sub>12</sub> O <sub>6</sub>
B2	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>
G1	C <sub>17</sub> H <sub>12</sub> O <sub>7</sub>
G2	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>

Table 2. Aflatoxin  
molecular structures

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

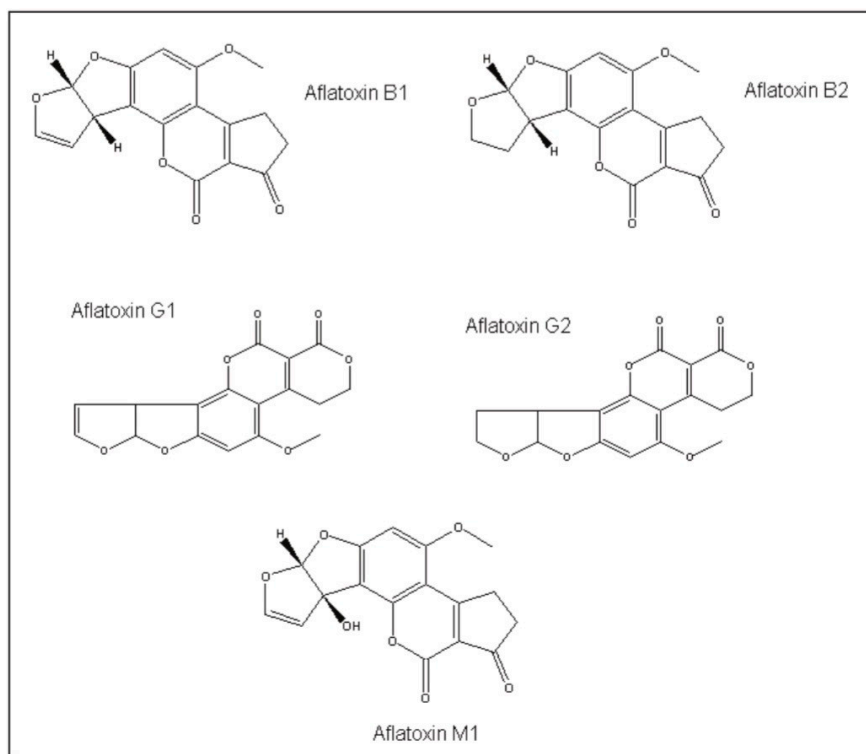


Figure 1. Aflatoxin molecular structures.

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 world, 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
<b>M1</b>		
Milk	50 ng/l (ppt)	VO [(EG) Nr. 466/2001]
Newborn baby formula and infants formula, including 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]
<b>B1</b>		
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]
<b>B1, B2, G1, G2</b>		
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
Compound feeds for calves, dairy cows, piglets, young chicken and broilers	10 µg/kg	Food Sanitation Law

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).

## Standardized Testing Methods for Aflatoxins

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 Derivatization [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]

AOAC Official Methods for Mycotoxins

## 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<sub>18</sub> 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<sub>18</sub> 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 <sub>18</sub> , 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	λ <sub>ex</sub> : 365 nm, λ <sub>em</sub> : 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<sub>18</sub> Column (Figure 2).

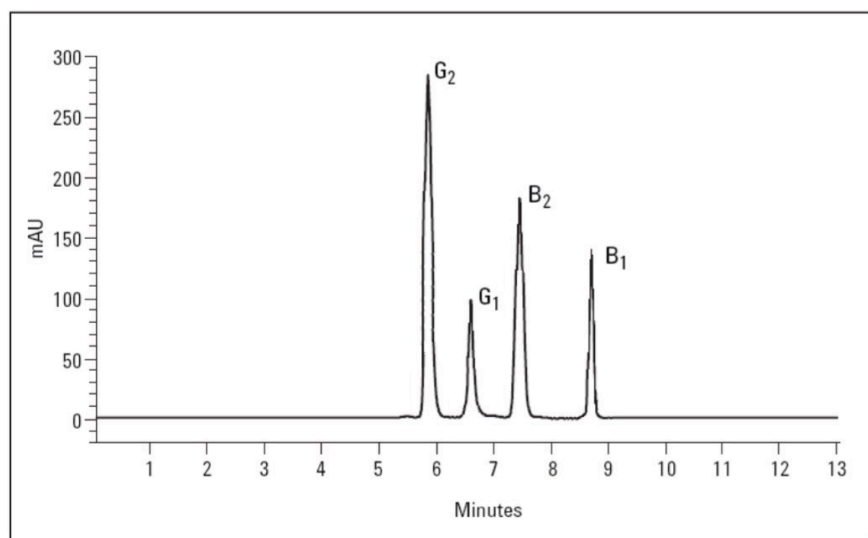


Figure 2. Chromatogram of aflatoxin standard mixture. Chromatogram provided courtesy of the University of Iowa.

## 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<sub>18</sub> 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 <sub>18</sub> , 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	λ <sub>ex</sub> : 333 nm, λ <sub>em</sub> : 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

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analyzed. The XBridge C<sub>18</sub> 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.

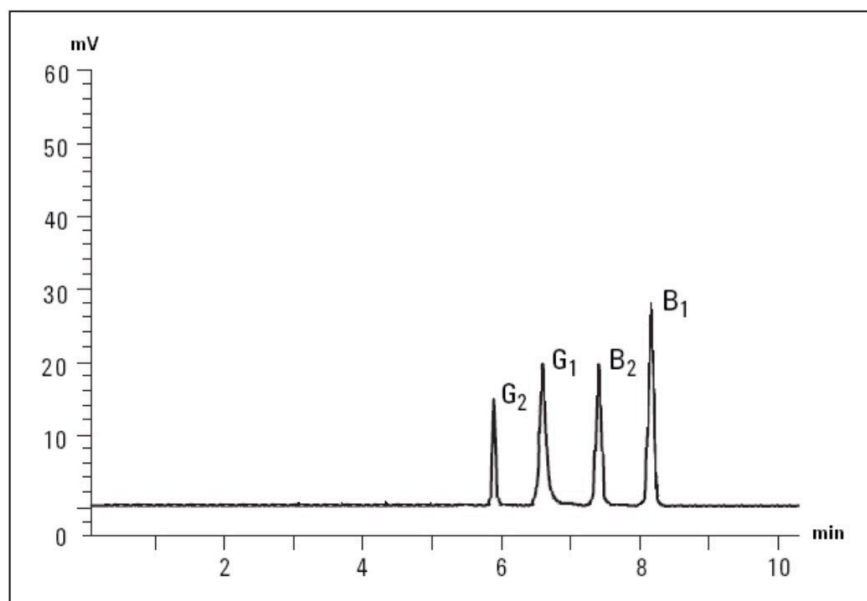


Figure 3. Chromatogram of prepared aflatoxin standard mixture: G<sub>2</sub>, 0.2 ng/mL; G<sub>1</sub>, 0.4 ng/mL; B<sub>2</sub>, 0.2 ng/mL; B<sub>1</sub>, 0.4 ng/mL. Chromatogram provided courtesy of Istanbul University.

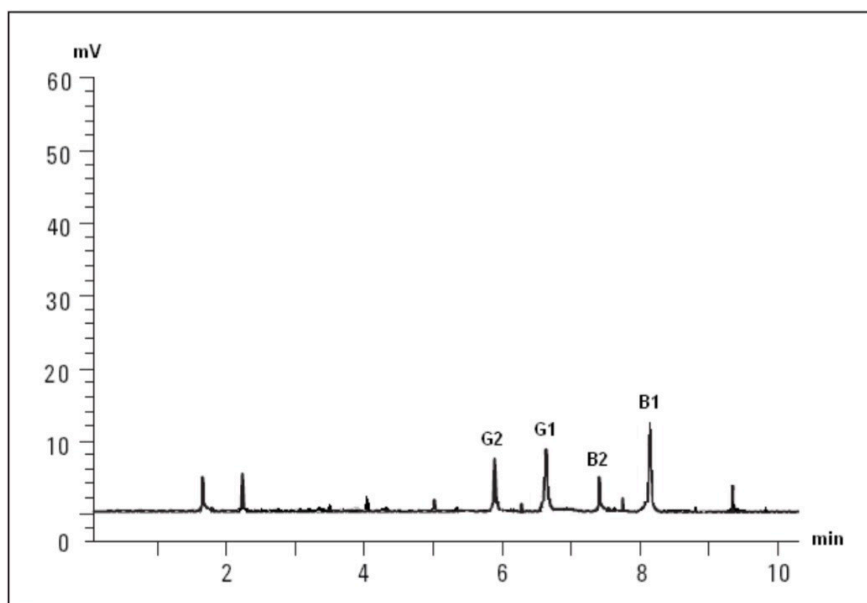


Figure 4. Chromatogram of aflatoxins in red pepper extract. Chromatogram provided courtesy of Istanbul University.

## Conclusion

The ability of aflatoxins to cause cancer and related diseases in humans and impart broad economic impact worldwide has made them a primary mycotoxin of concern and widely analyzed. In these studies XBridge C<sub>18</sub> 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.

## Featured Products

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- [2475 Fluorescence \(FLR\) Detector <https://www.waters.com/514434>](https://www.waters.com/514434)

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