

Separation and Quantitation of Aflatoxins B and G Using HPLC

Monitoring grain and dairy foods for the presence of aflatoxins is important to ensuring consumer safety. HPLC is the method of choice for separating these potentially carcinogenic substances. Detection normally is performed by fluorescence spectroscopic methods on TFA derivatives of aflatoxins.

Key Words:

● aflatoxins ● fluorescence detection ● HPLC

Aflatoxins are a group of highly oxygenated heterocyclic compounds with closely related structures (Figure A). These substances are produced by the growth of *Aspergillus flavus* on food products when sufficient moisture is present. Foods naturally contaminated with aflatoxins include corn, barley, cottonseed meal, peanuts, soybeans, wheat, and rice. Monitoring of a variety of foods is necessary to ensure consumer safety.

Aflatoxins were discovered in the 1960s in Great Britain, when thousands of turkey poults died with severe lesions of the liver. The disease was traced to substances contaminating Brazilian peanut meal used in poultry feed. A single feed company was known to be the common supplier of the peanut meal involved in the outbreak. Using thin layer chromatography, scientists identified at least four related compounds that caused acute

toxicity and liver carcinogenicity in duckling feeding trials. Aflatoxins were characterized as B (blue fluorescence) and G (green fluorescence).

Four aflatoxins, B₁, B₂, G₁, and G₂, are synthesized by *A. flavus*. In cases of contamination, aflatoxin B₁, the most toxic and most carcinogenic, is almost always present.

Preparation of Standards

Because aflatoxins are potential carcinogens, extreme handling precautions are warranted. Safety measures include protective masks and gloves, disposable protective covers for work surfaces, and thorough cleaning of glassware with bleach or other powerful oxidants.

Solutions of aflatoxins are not particularly stable unless prepared in specific solvents and packaged under conditions that exclude light. Properly prepared stock solutions can be diluted to working standard concentration levels with the solvents of choice and held for brief periods. Supelco offers a variety of mycotoxin standards in solution to meet your analytical needs (see our general catalog). These solutions can be used as is, or maintained as stock solutions for preparing working standards.

The concentration of aflatoxin standard solutions is determined by using UV spectroscopy. Standard solutions of mycotoxins are measured throughout the production process to determine the true analytical concentration of the particular mycotoxin as outlined in The Association of Official Analytical Chemists (AOAC) 16th Edition, Methods of Analysis. The absorbance at the maximum wavelength is measured and compared to the molar absorptivity:

$$\mu\text{g/mL of Aflatoxin} = \frac{\text{Absorbance} \times \text{Molecular Weight} \times 1000}{\text{Molar Absorptivity}}$$

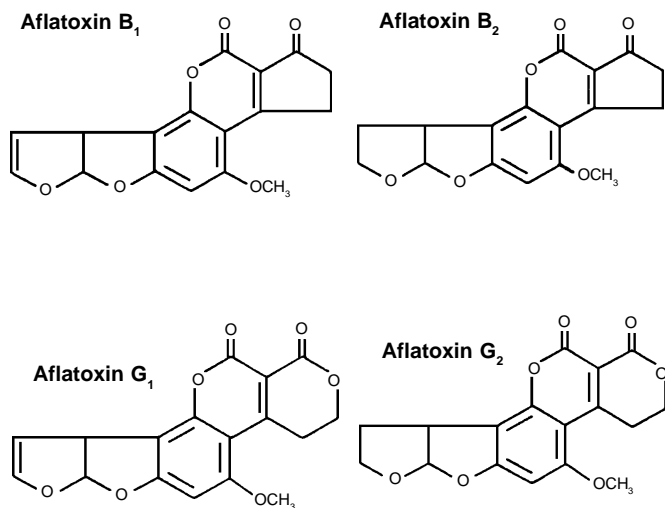
Using this formula and the information in Table 1, the concentra-

Table 1. Aflatoxin Data

Each aflatoxin in benzene:acetonitrile (98:2)

Aflatoxin	CAS No.	Formula	Mol. Wt.	Wavelength (nm)	Molar Absorptivity
B ₁	1162-65-8	C ₁₇ H ₁₂ O ₆	312	350	19,800
B ₂	7220-81-7	C ₁₇ H ₁₄ O ₆	314	350	20,900
G ₁	1165-39-5	C ₁₇ H ₁₂ O ₇	328	350	17,100
G ₂	7241-98-7	C ₁₇ H ₁₄ O ₇	330	350	18,200

Figure A. Aflatoxin Chemical Structures



796-0024, 0025, 0026, 0027

Table 2. AOAC Aflatoxin Extraction Methods*

Food Products	AOAC Method	Extraction Solvent	Aflatoxins Detected
White & yellow corn; peanut & cottonseed meals; peanut butter; almonds; mixed feeds; pistachio nuts	975.36	Acetone:water (85:15)	B ₁ , B ₂ , G ₁ , G ₂
White & yellow corn; raw shelled peanuts	979.18	Methanol:water (80:20)	Total aflatoxins
Whole cottonseed; peanut butter; corn; raw peanuts	990.34	Methanol:water (80:20)	B ₁ , B ₂ , G ₁
Peanuts & peanut products	968.22	Chloroform	B ₁ , B ₂ , G ₁ , G ₂
Peanut butter	991.45	Acetonitrile:water (50:50)	B ₁ , B ₂ , G ₁ , G ₂
Cocoa beans	971.23	Hexane (AgNO ₃)	B ₁ , B ₂ , G ₁ , G ₂
Coconut; copra; copra meal	971.24	Chloroform / sodium chloride / water	B ₁ , B ₂ , G ₁ , G ₂
Corn	993.16	Methanol:water (80:20)	B ₁ , B ₂ , G ₁
Corn	972.26	Chloroform	B ₁ , B ₂ , G ₁ , G ₂
Corn; peanuts	993.17	Methanol:water (85:15)	B ₁ , B ₂ , G ₁ , G ₂
Corn	990.32	Methanol	B ₁
Corn; peanut butter	990.33	Methanol:0.1N HCl	B ₁ , B ₂ , G ₁ , G ₂
Corn; raw peanuts; peanut butter	991.31	Methanol:water (70:30)	B ₁ , B ₂ , G ₁ , G ₂
Cottonseed products	980.2	Acetone:water	B ₁ , B ₂ , G ₁ , G ₂
Corn; almonds; brazil nuts; peanuts; pistachio nuts	994.08	Acetonitrile:water (90:10)	B ₁ , B ₂ , G ₁ , G ₂
Cottonseed products; mixed feed	989.06	Methanol:water (55:45)	B ₁
Eggs	978.15	water / sodium chloride / acetone	B ₁
Pistachio nuts	974.16	Methanol:water (55:45)	B ₁ , B ₂ , G ₁ , G ₂
Soybeans	972.27	Chloroform	B ₁ , B ₂ , G ₁ , G ₂
Dairy products	974.17	Chloroform	M ₁
Milk; cheese	980.21	Chloroform	M ₁
Liver	982.24	Methylene chloride	B ₁ , M ₁
Fluid milk	986.16	water / SPE	M ₁ , M ₂

*AOAC Official Methods of Analysis, 16th Ed., 1995.

tion of an aflatoxin can be determined.

Sample Extraction

Extraction methods are based on the solubility of aflatoxins in different organic solvents such as chloroform, methanol, acetone, acetonitrile, and benzene. The AOAC Methods of Analysis Manual lists a host of collaboratively-studied methods for a variety of food products. Food-based samples, AOAC method numbers, extraction solvents used, and the aflatoxins detected using the method are listed in Table 2.

Chromatography

HPLC is the preferred method for analyzing aflatoxins. A variety of separation and quantitation modes using reverse-phase chromatography (RPLC) have been developed. RPLC employs a nonpolar bonded silica surface and a polar mobile phase. For the analysis of aflatoxins, silica-based HPLC columns bonded with C8 or C18 groups are used with mobile phases consisting of binary or ternary mixtures of polar solvents. Commonly used solvent mixtures include deionized water, methanol, and acetonitrile. In the reversed phase mode, the elution order of the common aflatoxins is G₂, G₁, B₂, and B₁.

Aflatoxins may be separated and detected by UV detection (Figure B, first chromatogram). However, the sensitivity is not sufficient to detect these compounds at the parts per billion (ppb) concentrations required for food analyses. With a fluorescence detector (Figure B, second chromatogram), aflatoxins G₂ and B₂ both fluoresce more intensely than aflatoxins G₁ and B₁.

Aflatoxins separated using normal phase chromatography elute in reverse order (Figure C). This difference in elution order can be used as a confirmation that the aflatoxins of interest are indeed present in the sample. In the normal phase mode, the surface of the silica is polar, and separation is performed using a nonpolar solvent in the mobile phase. A fluorescence detector was used.

Using the normal phase mode, we found that placing the sample in a benzene:acetonitrile solvent eliminated miscibility problems

Figure B. Reversed Phase HPLC Separation of Aflatoxins

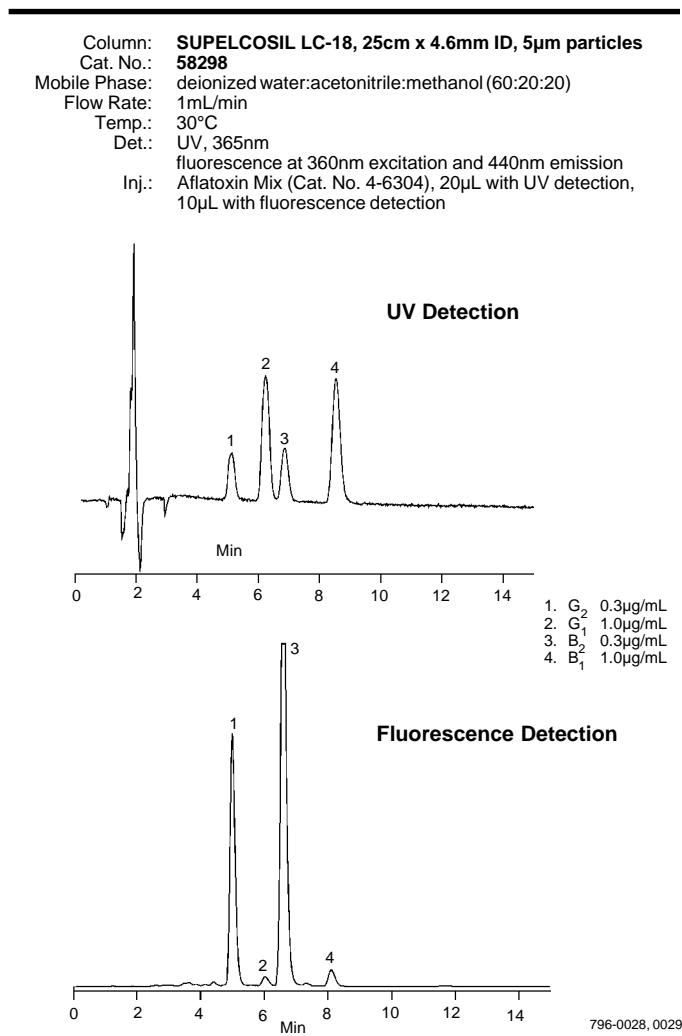
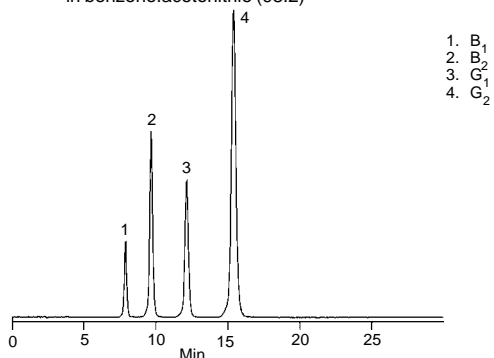


Figure C. Normal Phase HPLC Separation of Aflatoxins Using Fluorescence Detection

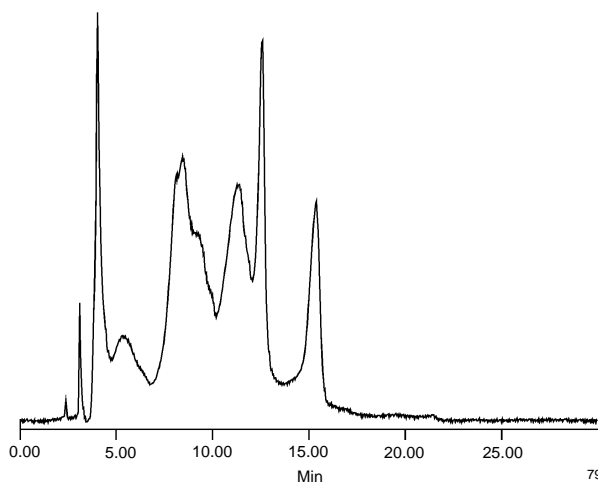
Column: **SUPELCO SIL LC-Si, 25cm x 4.6mm ID, 5µm particles**
 Cat. No.: **58295**
 Mobile Phase: toluene:ethyl acetate:formic acid:methanol (90:6:2:2)
 Flow Rate: 1.5mL/min
 Temp.: 30°C
 Det.: fluorescence at 365nm excitation and 425nm emission
 Inj.: 10µL of 0.75µg/mL each of B₁, B₂, G₁, & G₂ in benzene:acetonitrile (98:2)



796-0030

Figure D. Aflatoxins in Methanol: Normal Phase HPLC and Fluorescence Detection

Column: **SUPELCO SIL LC-Si, 25cm x 4.6mm ID, 5µm particles**
 Cat. No.: **58295**
 Mobile Phase: toluene:ethyl acetate:formic acid:methanol (90:6:2:2)
 Flow Rate: 1.5mL/min
 Temp.: 30°C
 Det.: fluorescence at 365nm excitation and 425nm emission
 Inj.: 10µL of Aflatoxin Mix (Cat. No. 4-6304) in methanol



796-0064

(Figure C). The aflatoxin standards shown in Figure D were dissolved in methanol. The poor chromatography was caused by the incompatibility of the mobile phase and the methanol solvent used to dissolve the aflatoxin standards. Choosing a solvent that is compatible with the sample and standard is important.

Trifluoroacetic Acid (TFA) Derivatives

While UV detectors are not sensitive enough to detect aflatoxins at the ppb concentrations required for foods, fluorescence detectors can achieve the desired levels. As Figure B shows, aflatoxins B₁ and G₁ do not respond well to fluorescence in the range used. Derivatization with TFA (Table 3) converts aflatoxins B₁ and G₁ to form B_{2a} and G_{2a}, respectively, enhancing their fluorescence. Treatment with TFA causes the addition of water across the

terminal furan ring in B₁ and G₁. This increases fluorescence of the molecules in the reversed phase solvent system and decreases retention times (Figure E).

The reversed phase method used to analyze both the aflatoxins and the TFA derivatives can be used to monitor the extent of the conversion of B₁ to B_{2a} and G₁ to G_{2a}, assuring that the derivatization process has been carried to completion. A reversed phase SUPELCO SIL™ LC-18 column resolved the six components of an aflatoxin mix to the baseline (Figure F).

We offer a complete line of aflatoxin standards, reagents, and columns for aflatoxin analysis. Our standards are evaluated to determine concentration and stability of the analytes of interest. SUPELCO SIL LC-18 columns provide excellent separation of aflatoxins in both derivatized and underivatized samples, allowing you to monitor the extent of the derivatization of aflatoxins B1 and G1. SUPELCO SIL LC-Si columns can be used in the normal phase chromatographic mode as a confirmation of the identity of the aflatoxins of interest.

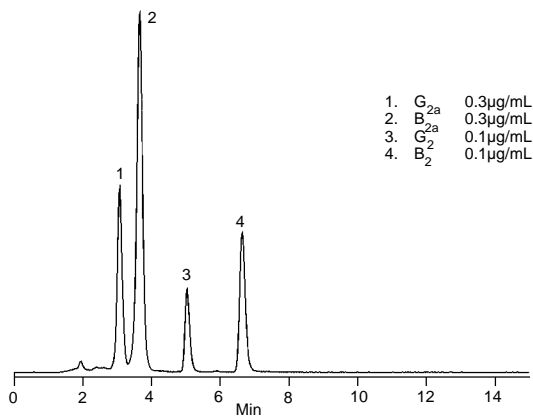
Table 3. Aflatoxin Derivatization Procedure*

- Evaporate solution containing aflatoxin standards to dryness using nitrogen in screw cap vial.
- Add 200µL of hexane to redissolve aflatoxins.
- Add 50µL of trifluoroacetic acid, cap, and vortex for 30 sec.
- Let stand 5 min.
- Add 1.95mL of deionized water:acetonitrile (9:1).
- Vortex for 30 sec.
- Allow layers to separate.
- Remove aqueous layer (lower layer) containing aflatoxins.
- Filter through a 0.45µm syringe filter tip.
- Inject sample onto LC column.

*AOAC Method 990.33, Official Methods of Analysis, 16th Ed., 1995.

Figure E. HPLC of TFA-Derivatized Aflatoxins

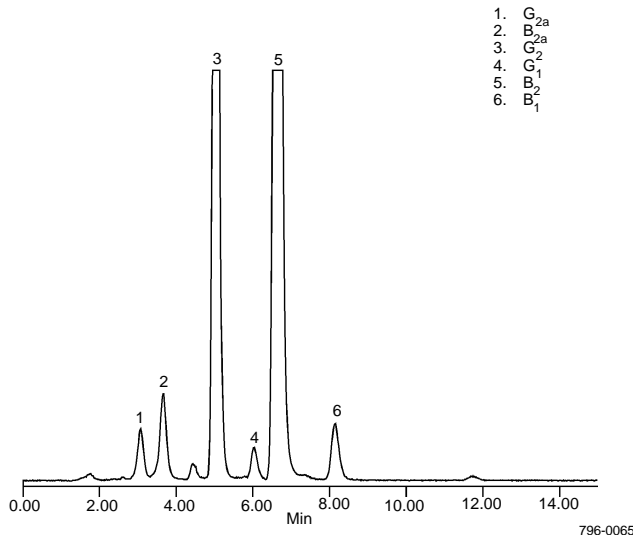
Column: **SUPELCO SIL LC-18, 15cm x 4.6mm ID, 5µm particles**
 Cat. No.: **58230-U**
 Mobile Phase: deionized water:acetonitrile:methanol (60:20:20)
 Flow Rate: 1mL/min
 Temp.: 30°C
 Det.: fluorescence at 360nm excitation and 440nm emission
 Inj.: 10µL of Aflatoxin Mix (Cat. No. 46304-U)



796-0031

Figure F. HPLC of Underivatized and Derivatized Aflatoxins

Column: **SUPELCO SIL LC-18, 25cm x 4.6mm ID, 5µm particles**
 Cat. No.: **58298**
 Mobile Phase: deionized water:acetonitrile:methanol (60:20:20)
 Flow Rate: 1mL/min
 Temp.: 30°C
 Det.: fluorescence at 360nm excitation and 440nm emission
 Inj.: 10µL



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 For information on the analysis of aflatoxins M₁ and M₂, request Application Note 102 (Publication Number 396102).
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Ordering Information:

Description	Cat. No.
SUPELCO SIL HPLC Columns	
25cm x 4.6mm ID, 5µm particle	
LC-18	58298
LC-Si	58295
15cm x 4.6mm ID, 5µm particle	
LC-18	58230
LC-Si	58200
LC-18 Guard Column Kits	
2cm x 4.6mm guard column, holder, connecting hardware	
LC-18	59554
LC-Si	59550
Replacement Columns, pk. of 2	
LC-18	59564
LC-Si	59560
Trifluoroacetic Acid Reagent (TFA)	
25mL	33075
100mL	33076
10 x 1mL	33077
Iso-Disc™ Syringe Tip Nylon Filters	
25mm x 0.45µm pores, pk. of 50	59230

Aflatoxin Standards

These quantitative standards are designed for use in accordance with AOAC Method 970.44.■

Aflatoxin B and Aflatoxin G Standards

Each ampul contains 1µg B₁, 1µg G₁, 0.3µg G₂.

Qty.	Solvent	Cat. No.
5 x 1mL	Benzene:acetonitrile (98:2)	46300-U
5 x 1mL	Methanol	46304-U
5mL	Methanol	46303

Aflatoxin B and Aflatoxin G Standards

Each 3µg/mL in 1mL benzene:acetonitrile (98:2)

Description	CAS No.	Cat. No.
Aflatoxin B ₁	1162-65-8	46323
Aflatoxin B ₂	7220-81-7	46324-U
Aflatoxin G ₁	1165-39-5	46325-U
Aflatoxin G ₂	7241-98-7	46326-U

Aflatoxin M Standards

At indicated concentrations in 1mL acetonitrile.

Description	CAS No.	Conc.	Cat. No.
Aflatoxin M ₁	16795-23-9	10µg/mL	46319
Aflatoxin M ₂	6885-57-0	1µg/mL	46910-U

Caution: Mycotoxins may be carcinogenic and, therefore, should be handled only by qualified personnel.

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