

Separation and Quantitation of Aflatoxins M_1 and M_2 Using TFA Derivatization and HPLC

Separation of aflatoxins M_1 and M_2 normally is performed using reversed phase liquid chromatography. These substances can be toxic to low parts-per-billion concentrations. Fluorescence detection is more sensitive, and thus more suited, than ultraviolet detection of these compounds.

Key Words:

- aflatoxins • fluorescence detection
- trifluoroacetic acid derivatization • HPLC

Aflatoxins, carcinogenic compounds produced by the fungi *Aspergillus flavus*, are found in a variety of foods. Aflatoxin B_1 is the most often encountered aflatoxin found in contaminated foods. When dairy cows are fed contaminated feed, a portion of Aflatoxin B_1 is metabolized to aflatoxin M_1 , which ultimately can be found in milk products. Detection of Aflatoxin M_1 at levels of 0.5 parts per billion (ppb) or lower have been required for monitoring contamination in dairy products. Very little data is available on contamination of Aflatoxin M_2 in commercial products.

Separation of aflatoxins M_1 and M_2 typically is performed using reversed phase liquid chromatography (RPLC). The Association of Official Analytical Chemists (AOAC) Method 986.16, *Aflatoxins M_1 and M_2 in Fluid Milk*, specifies an LC-18 column, with a mobile phase consisting of deionized water, isopropanol, and acetonitrile. A SUPELCOSIL™ LC-18 column (25cm x 4.6mm, 5µm particles) can separate aflatoxins M_1 and M_2 in under 8 minutes, with excellent resolution (Figure A). Under the conditions indicated in the figure, aflatoxin M_2 eluted before aflatoxin M_1 .

In samples containing ppm levels of aflatoxins M_1 and M_2 , such as in Figure A, UV detection can be used. At the level shown, the signal-to-noise ratio is still very high. However, a UV detector is not sensitive enough to detect aflatoxins in the parts-per-billion (ppb) range needed for testing of food product contaminants.

A fluorescence detector must be used to detect aflatoxins at the ppb levels regulated in food. When fluorescence detection is used for the same standard mixture of aflatoxins, M_2 responds much more readily than the same concentration of M_1 (Figure B). Since aflatoxin M_1 is the more commonly-found contaminant in milk-based products, it must be converted to a derivative in order to be detected at the desired levels.

Converting aflatoxin M_1 to its fluorescent trifluoroacetic acid (TFA) derivative can be difficult. The reaction of aflatoxin M_1 to its TFA derivative (designated M_{2a}) rarely goes to completion. Figure C shows the separation of a qualitative mixture of the aflatoxins M_{2a} ,

Figure A. Aflatoxins M_1 and M_2 by UV Detection

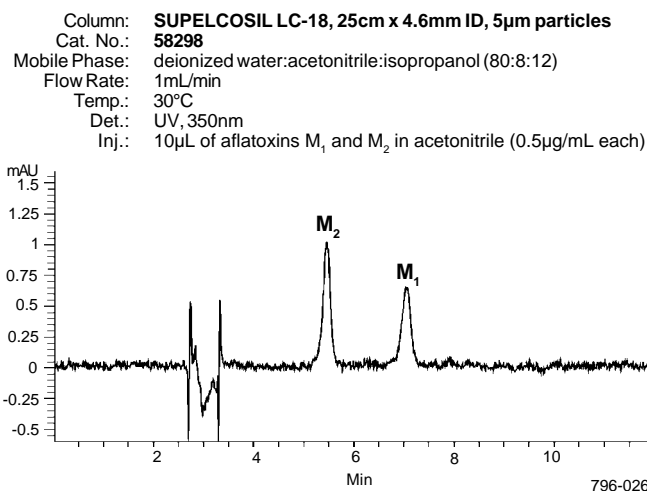
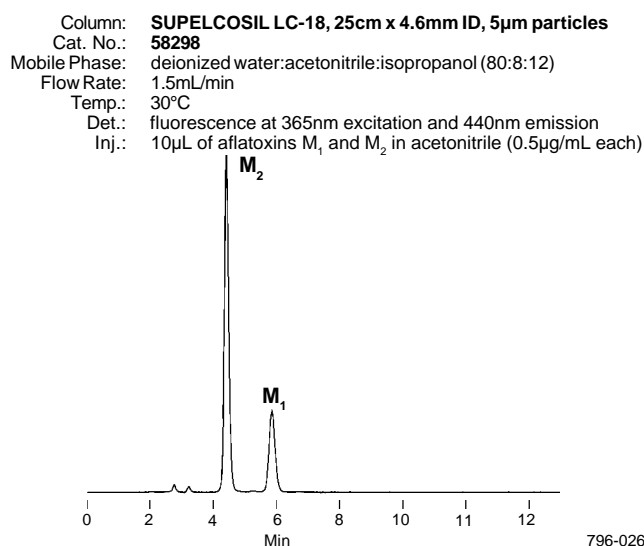


Figure B. Aflatoxins M_1 and M_2 by Fluorescence Detection



M_2 , and M_1 . The extent of the derivatization of M_1 to M_{2a} can be monitored under the RPLC conditions used. As derivatization proceeds, the height of the M_1 peak decreases and the M_{2a} peak increases. The three peaks are well resolved from each other.

One investigator (Stubblefield, 1987 — see References) has studied the formation of the M_{2a} derivative, and identified the need for silylated vials and an increase in reaction temperature to 40°C to achieve a complete derivatization. Also, the AOAC has adopted a TFA derivatizing procedure (Table 1) that specifies the use of silylated vials and a reaction temperature of 40°C.

Figure D shows a separation of aflatoxins M_1 and M_2 after derivatization with TFA. Note the increased response to fluorescence detection for a 0.25µg/mL concentration of aflatoxin M_{2a} . Derivatization procedures and efficiencies may vary depending on sample type and other factors. Analysts must evaluate procedures for their particular sample matrix to ensure complete extraction, quantitation, and reproducibility.

Figure C. Qualitative Mixture of Aflatoxins M_1 , M_2 , and M_{2a}

Column: **SUPELCO SIL LC-18, 25cm x 4.6mm ID, 5µm particles**
 Cat. No.: **58298**
 Mobile Phase: deionized water:acetonitrile:isopropanol (80:8:12)
 Flow Rate: 1.5mL/min
 Temp.: 30°C
 Det.: fluorescence at 365nm excitation and 440nm emission
 Inj.: 10µL of Qualitative Mix — aflatoxins M_1 , M_2 , and M_{2a}

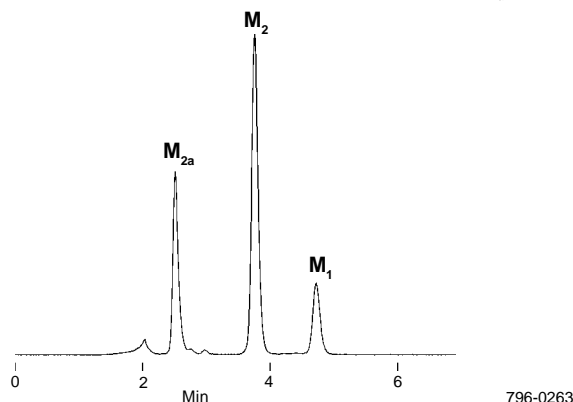


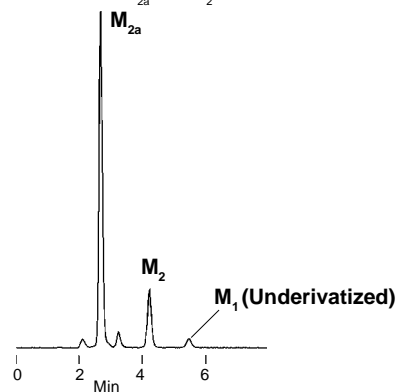
Table 1. TFA Derivatization Procedure for Aflatoxin M_1 *

- Evaporate samples to dryness under nitrogen in silylated vial.
- Add 200µL of hexane and 200µL of trifluoroacetic acid to the dried residue. Standards should be diluted in a solution of acetonitrile: benzene (10:90). Add 50µL of the working standard to the hexane:TFA solution.
- Shake on a vortex mixer for 5-10 sec.
- Let the mixture stand 10 min at 40°C in a heating block or bath.
- Evaporate to dryness under nitrogen on a steam bath or heating block at <50°C.
- Add 2mL of deionized water:acetonitrile (75:25) to the vial and dissolve the residue.
- Shake well on a vortex mixer.
- Filter through a 0.25µm syringe filter tip.
- Inject the sample onto an LC column.

*AOAC Method 986.16, Official Methods of Analysis, 16th edition.

Figure D. TFA-Derivatized Aflatoxin M_{2a}

Column: **SUPELCO SIL LC-18, 25cm x 4.6mm ID, 5µm particles**
 Cat. No.: **58298**
 Mobile Phase: deionized water:acetonitrile:isopropanol (80:8:12)
 Flow Rate: 1.5mL/min
 Temp.: 30°C
 Det.: fluorescence at 365nm excitation and 440nm emission
 Inj.: 10µL of aflatoxins M_{2a} and M_2



We offer a complete line of HPLC columns, analytical standards, reagents, and accessories for safe and efficient analyses of aflatoxins. Our standards for aflatoxins M_1 and M_2 in acetonitrile include a Certificate of Analysis that gives the analytical concentration and percent purity of the starting material.

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| Supelguard™ LC-18 Guard Column Kit 2cm x 4.6mm guard column, holder, connecting hardware | 59554 |
| Supelguard LC-18 Replacement Columns, pk. of 2 | 59564 |
| Aflatoxin M_1 Standard 10µg/mL in 1mL acetonitrile | 46319 |
| Aflatoxin M_2 Standard 1µg/mL in 1mL acetonitrile | 46910-U |
| Trifluoroacetic Acid Reagent (TFA) 10mL | 33077 |
| 25mL | 33075 |

References

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 Ferguson-Foos, J. and J. Warren, *J. Assoc. Off. Anal. Chem.*, **67**: 6 (1984).
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