Automated Extraction of Aflatoxin M_1 from Milk According to AOAC Method 2000.08 Using the Gilson GX-271 ASPEC® System

APPLICATION NOTE FB0916

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Aflatoxin M_1, the main hepatic metabolic product of Aflatoxin B_1, was isolated from milk samples using the GX-271 ASPEC® system with excellent recovery, repeatability, and reproducibility. Automation of AOAC Method 2000.08 with the GX-271 ASPEC® system provided a reliable, hands-off solution for the detection of this potentially carcinogenic food supply contaminant.

Introduction

Aspergillus is both one of the most useful and most harmful fungal genera known. Some species, including *A. niger* and *A. oryzae*, are critical to industrial fermentation processes,[1] while others produce toxic and carcinogenic secondary metabolites known as aflatoxins.

Aflatoxins are mycotoxins produced by fungi of the genus *Aspergillus*, principally the species *A. flavus* and *A. parasiticus*. Aflatoxins are found as contaminants in a variety of staple commodities, including grains, maize, and peanuts. These compounds are quite stable and can survive relatively high temperatures, including pasteurization,[2] and the milk fermentation process,[3] and are known to cause liver damage, reproductive effects and immune suppression. The major aflatoxin species are B_1, B_2, G_1 and G_2, with Aflatoxin B_1 being the most toxic. The two main metabolic products, M_1 (see Figure 1) and M_2, are produced in the liver from B_1 and B_2, respectively. Aflatoxin M_1 (AFM1) is a Group 2B carcinogen (possibly carcinogenic to humans) present in the milk of lactating mammals that ingest food contaminated with aflatoxin B_1.[4]

Figure 1: GX-271 ASPEC®

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The World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), U.S. Department of Agriculture (USDA), and U.S. Food and Drug Administration (FDA), among other organizations, categorize aflatoxin as a serious health risk and have established maximum levels for the occurrence of this toxin in food products. Food testing laboratories face the challenge of meeting regulatory requirements and implementing reliable and reproducible methods for identification of toxins and other hazards in order to ensure a safe food supply.

The AOAC (Association of Official Analytical Chemists) has established a method for detection of Aflatoxin M_1 in milk.[5,6] This method incorporates sample cleanup using an immunoaffinity column and analytical chromatography with fluorometric detection. Sample preparation by this method requires many steps carried out in a precise fashion. The Gilson GX-series of automated solid phase extraction cartridge (ASPEC) liquid handlers was used to automate the sample preparation and cleanup method.

In this application note we examine limits of quantification and detection, repeatability, reproducibility, and recovery. Automation with the GX-271 ASPEC® system provides a reproducible and reliable method for the isolation of AFM1 from milk samples using AOAC Official Method 2000.08.

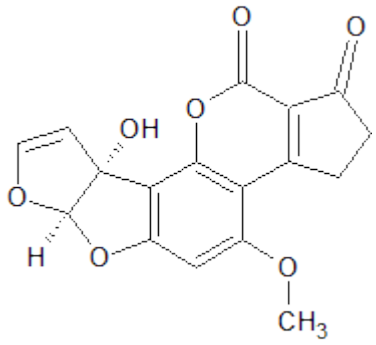

Figure 2: Chemical structure of Aflatoxin M1, CAS No. 6795-23-9

Materials and Methods

Samples and Reagents

Reagents and chemicals were ACS grade quality or better. Aflatoxin M1 standard was obtained from Sigma-Aldrich® (P/N A6428). HPLC grade acetonitrile was obtained from Panreac AppliChem (P/N 361881). All water was purified using a Milli-Q® system or equivalent.

Preparation of sample prior to SPE

Milk samples were heated at 37 ±2°C and centrifuged for 15 minutes at 4000 rpm (2800 x g). After centrifugation, the upper fat layer was discarded and the sample was filtered with filter paper before being transferred to a 50 mL Falcon tube on the bed of the GX-271 ASPEC®.

Methods

Solid Phase Extraction	
Instrumentation	GX-271 ASPEC®
Cartridge	VICAM® Afla M_1™ HPLC
Load	50 mL pre-treated sample at 1.5 mL/min
Wash	20 mL water at 3 mL/min; air push of 24 mL at 40 mL/min
Elute	2 mL acetonitrile at 1 mL/min
Elute	2 mL acetonitrile at 1 mL/min; 20 mL air push at 40 mL/min

Solid phase extraction was automated using a GX-271 ASPEC® controlled with Gilson TRILUTION® LH software. Afla M_1 HPLC cartridges from VICAM® (part number G1007) were used for affinity purification of Aflatoxin M_1 as follows: 50 mL of pre-treated milk was loaded onto a cartridge at a flow rate of 1.5 ml/min. Cartridges were washed with 20 mL of water at 3 mL/min, followed by an air push (24 mL at 40 mL/min flow rate). Two rounds of elution were carried out, each with 2 mL acetonitrile applied at 1 mL/min. This was followed by an air push (20 mL air at 40 mL/min flow rate). The 4 mL of collected extract was evaporated to dryness in a water bath at 50°C under a gentle stream of nitrogen. The dry extract was then dissolved in 500 µL of mobile phase (water/acetonitrile, 67:33, v/v), filtered through a syringe filter of modified PTFE membrane, and frozen until HPLC analysis.

HPLC	
Instrumentation	Shimadzu HPLC Prominence®: System Controller CBM-20A System with fluorescence detection; Degassing Unit DGU-20A5; Solvent Delivery Unit LC-20AT; Autosampler SIL-10AF; Column Oven CTO-20A; Fluorescence Detector RF-20A
Column	Shimadzu® RP C18, 5 µm, 250 x 4.6 mm (Shimadzu® P/N 228-34937-92)
	Shimadzu® C18 guard column, 5 µm, 10.0 x 4.0 mm (Shimadzu® – P/N 228-34938-91)
Gradient	Water/Acetonitrile 67:33; 1.0 mL/min
Injection Volume	50 µL
Detection	Fluorescence detection; excitation/emission: 365/435 nm

Results and Discussion

Sample cleanup and extraction of Aflatoxin M1 from milk samples was automated using the GX-271 ASPEC® system. Immunoaffinity cartridges (Afla M1 HPLC cartridges from VICAM®) were placed in a Gilson DEC rack, a mobile rack that is used for automated solid phase extraction. The GX-271 ASPEC® can automatically load, condition, and wash the column, followed by eluting the compound(s) of interest. The automated procedure is diagrammed in Figure 3.

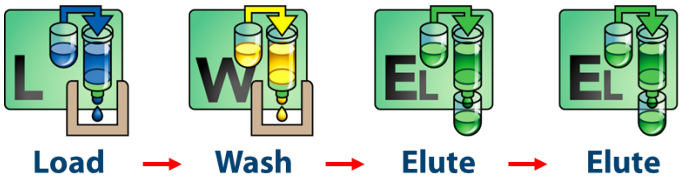

Figure 3: Schematic of the SPE process in TRILUTION® LH software.

Repeatability, Reproducibility, and Recovery

Repeatability, reproducibility, and recovery were assessed from the results obtained by two different analysts on two different days. Analysis by HPLC was performed in triplicate with three replicate samples at three different concentration levels. A representative HPLC trace is shown in Figure 4.

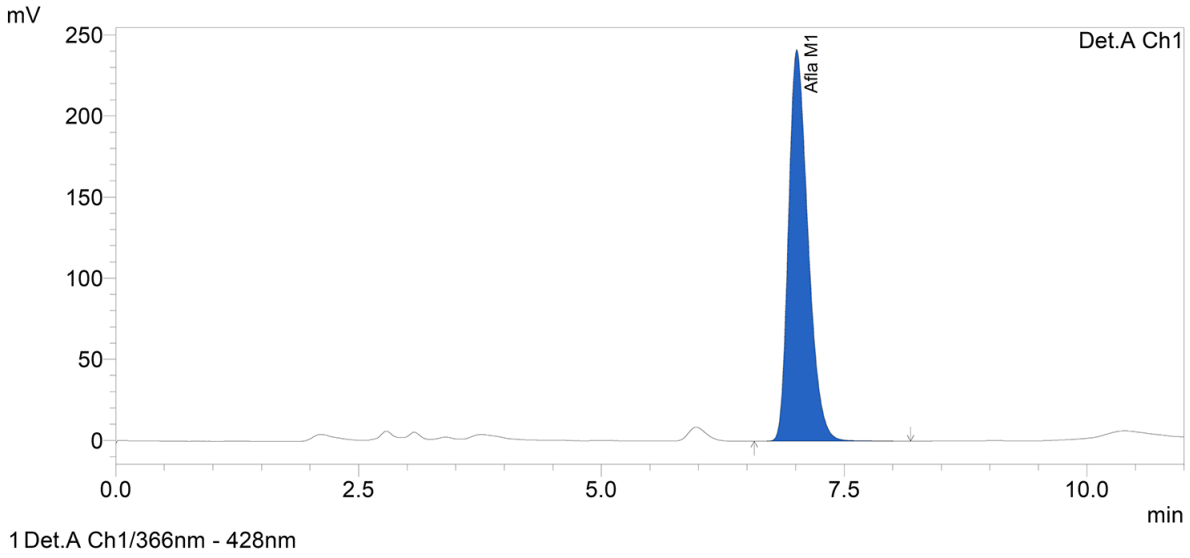

Figure 4: Representative HPLC chromatogram from this study with an Aflatoxin M_1 peak at ~7 minutes.

A summary of the results of the repeatability, reproducibility, and recovery study is presented in Table 1. These values are in agreement with the published relative standard deviation numbers from the AOAC formal collaborative studies.[7]

Table 1: Repeatability, reproducibility, and recovery values for Aflatoxin M_1.

Concentration (µg/L)	*Repeatability RSD_r(%)*	*Reproducibility RSD_R(%)*	*Recovery (%)*
0.12	13.42	13.42	110
0.40	7.62	12.47	104
0.70	7.83	7.83	107

Detection and Quantification Limits

The limit of detection was determined to be three times the standard deviation of the intercept divided by the slope from the calibration curve used in the linearity assessment. The limit of quantification was taken as the lowest point of the linear range of the method.[8]

Table 2: Detection and quantification limits for Aflatoxin M_1.

Aflatoxin M_1	
Limit of Detection (µg/L)	0.02
Limit of Quantification (µg/L)	0.12

Conclusions and Benefits

While ELISA-based techniques can permit easy detection of the presence of mycotoxins, the methods are subject to false positive results. Analysis by HPLC after cleanup with immunoaffinity columns is therefore required for precise quantitation of the toxins. The chromatographic methods require extensive sample preparation steps and well-trained personnel. This application note shows the advantage of automating sample cleanup using the GX-271 ASPEC®:

- Precise and reproducible loading of large volume samples (50ml)
- Compatibility with commonly used labware (Falcon tubes)
- Multiple elution steps to improve recovery
- Unattended sample preparation frees skilled personnel for more valuable tasks
- Recovery, repeatability, and reproducibility in accordance with AOAC Official Method 2000.08

The GX-271 ASPEC® is compatible not only with Gilson's pre-capped Silica, C18, SCX, WCX, HLB and other SPE cartridges, but also with all 1 mL, 3 mL and 6 mL commercial cartridges, and can therefore be used for any solid phase extraction procedures in the laboratory.

References

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Acknowledgements

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Trademarks

Ordering Information

Part Number	*Description*
2614007	GX-271 ASPEC®