SHIMADZU
Excellence in Science

Application News

No.L506

High Performance Liquid Chromatography

Assay of Aflatoxin M_1 in Milk Based on Notification Test Methodology, Using Prominence-i and the RF-20Axs Fluorescence Detector

Aflatoxin M_1 (AFM_1) is a mycotoxin suspected of carcinogenicity in humans that is detected in the milk of mammals that eat food contaminated with aflatoxin B_1. The notification "Handling of Aflatoxin M_1 in Milk" issued on July 23, 2015 (Notification No. 0723-[1] of the Department of Food Safety, PFSB, MHLW) [1] sets a regulatory level for AFM_1 in milk of 0.5 µg/kg, and came into force on January 23, 2016.
The assay methodology for AFM_1 in milk was included in "Test Methodology for Aflatoxin M_1 in Milk" (Notification No. 0723-[5] of the Department of Food Safety, PFSB, MHLW) [2], which was announced on the same day and describes two test methodologies.

(1) Test method consisting of quantitation by HPLC with attached fluorescence detector and confirmation by LC/MS or LC/MS/MS.
(2) Screening method using an assay kit.
We describe an analysis of commercially available milk that is compliant with test method (1). We analyzed for AFM_1 in bovine milk using the Prominence-i integrated HPLC and the RF-20Axs fluorescence detector. Under these conditions we were able to measure AFM_1 at a concentration of 1/10th Japan's regulatory level for AFM_1 in milk.

Analysis of Standard Aflatoxin M_1 Solutions

Chromatograms obtained after analysis of standard AFM_1 solutions (0.1 µg/L, equivalent to 1/100th the regulatory concentration) are shown in Fig. 1, and the analytical conditions used are shown in Table 1. The relative standard deviation (%RSD) of peak areas after repeating analysis six times was 3.4 %. Fig. 2 shows the calibration curve for 0.1 to 20 µg/L. Good linearity was achieved with a contribution ratio R^2 of ≥ 0.9999 within the concentration range. These results show the RF-20Axs fluorescence detector can be used to analyze trace quantities of AFM_1 with high sensitivity and high precision.
When the standard AFM_1 solution of 0.1 µg/L is processed according to the pretreatment procedure shown in Fig. 3, which follows the notification methodology, it produces a sample equivalent to 1/100th the regulatory level for AFM_1 in milk (0.005 µg/kg).

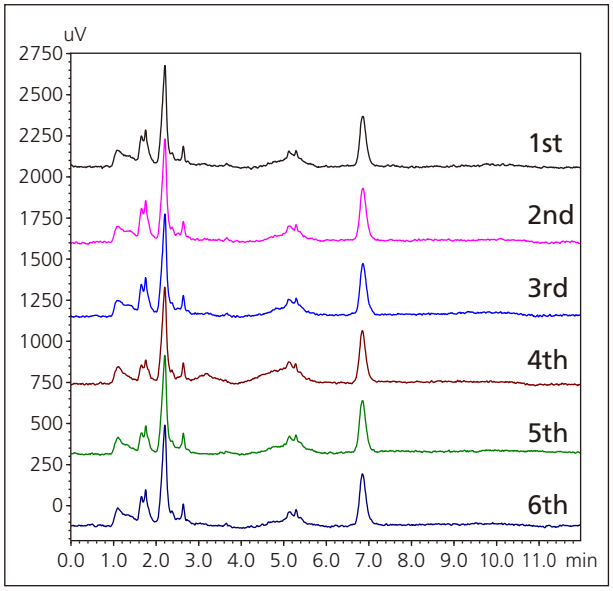

Fig. 1 Chromatograms for Standard AFM_1 Solution Equivalent to 1/100th the Regulatory Concentration (0.1 µg/L, Test Repeated 6 Times)

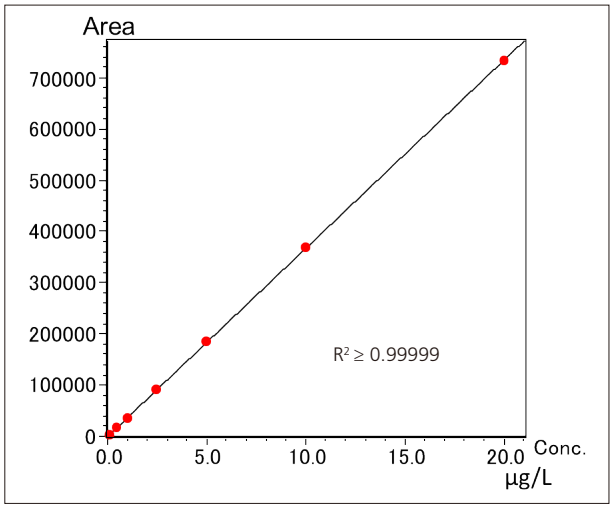

Fig. 2 AFM_1 Calibration Curve (0.1-20 µg/L)

Table 1 HPLC Analytical Conditions

System	:Prominence-i
Column	:Shim-pack VP-ODS (150 mm L. × 4.6 mm I.D., 5 µm)
Mobile Phase	:Water/Acetonitrile = 3/1(v/v)
Flowrate	:1.0 mL/min
Column Temp.	:40 ˚C
Detection	:RF-20Axs, Ex. at 365 nm, Em. at 435 nm
RF Cell.	:Conventional Cell
Cell Temp.	:25 ˚C
Injection Volume	:100 µL

Analysis of Aflatoxin M_1 in Milk

We analyzed commercially available milk and milk with added AFM_1. AFM_1 was added to make up a concentration of 0.05 µg/kg in milk (1/10th the regulatory level), and pretreatment was performed according to the notification methodology.[2] The pretreatment procedure is shown in Fig. 3. Refer to the notification methodology [2] for further details.

An AflaStar™ R* immunoaffinity column from Romer Labs was used to remove contaminant constituents. The chromatograms obtained after analysis of these samples are shown in Fig. 4. (A) is the chromatogram for milk with added AFM_1, and (B) is the chromatogram for milk with no added AFM_1.

The analytical conditions were the same as those used in Fig. 1, which are shown in Table 1.

* "AflaStar" is a registered trademark of Romer Labs.
The AflaStar™ R can be purchased from Shimadzu GLC Ltd.

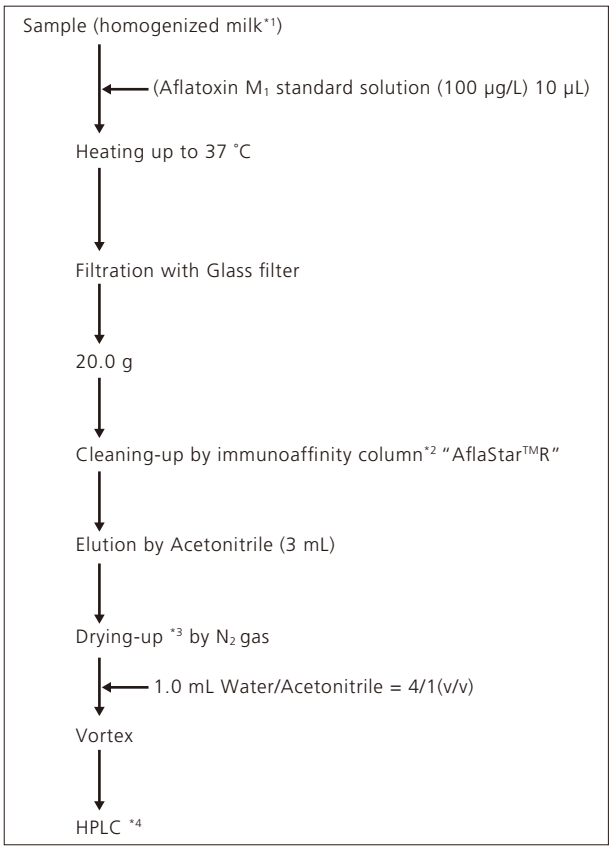

Fig. 3 Milk Pretreatment Procedure

*1 A pretreatment centrifugation step is needed for raw milk and other milks that are not homogenized. Refer to reference [2] for details.
*2 Refer to the annotations in reference [2] for detailed information on use of the immunoaffinity column.
*3 AFM_1 can adhere to the container during drying, so it is recommended that silane-treated containers be washed with 20 % to 30 % aqueous acetonitrile then dried before use.
*4 AFM_1 can adhere to glass containers used to hold samples for HPLC even when these containers have been treated with silane, so it is recommended that plastic containers be used.

The percentage recovery calculated according to Eqn. 1 shown below was 98 %. We found that using the RF-20Axs fluorescence detector allows for analysis at concentrations 1/10th the regulatory level with high sensitivity and good precision.

A small peak was observed at the AFM_1 elution position when milk with no added AFM_1 was analyzed. Using LC/MS/MS to analyze the milk with no added AFM_1 suggested this peak was derived from AFM_1, and the concentration of the substance present was below 1/100th Japan's regulatory level.

$$\text{Recovery rate (\%)} = \frac{\text{(Peak area of milk with added standard AFM}_1\text{)} - \text{(Peak area of milk with no added standard AFM}_1\text{)}}{\text{Peak area of standard AFM}_1\text{ sample}} \times 100$$

Eqn. 1 Percentage Recovery Equation

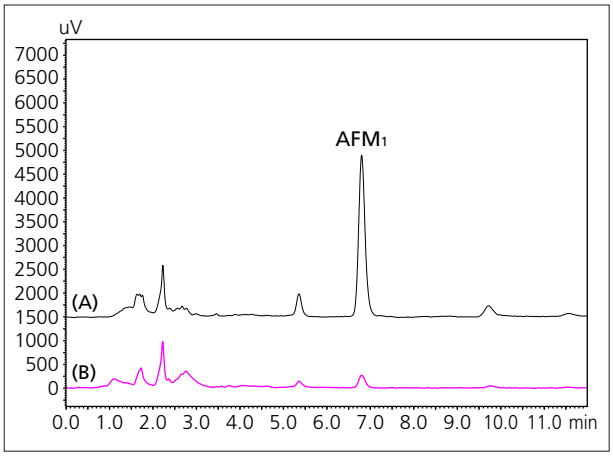

Fig. 4 HPLC Chromatograms for Commercially Available Milk (A) With added standard AFM_1, (B) with no added standard AFM_1

References
1) "Handling of Aflatoxin M_1 in Milk" (July 23, 2015, Notification No. 0723-[1] of the Department of Food Safety, PFSB, MHLW)
2) "Test Methodology for Aflatoxin M_1 in Milk" (Notification No. 0723-[5] of the Department of Food Safety, PFSB, MHLW)

First Edition: Jun. 2016

Shimadzu Corporation
www.shimadzu.com/an/