

Application News

No.L506

High Performance Liquid Chromatography

Assay of Aflatoxin M₁ in Milk Based on Notification Test Methodology, Using Prominence-i and the RF-20A_{XS} Fluorescence Detector

Aflatoxin M₁ (AFM₁) is a mycotoxin suspected of carcinogenicity in humans that is detected in the milk of mammals that eat food contaminated with aflatoxin B₁. The notification "Handling of Aflatoxin M₁ 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 AFM₁ in milk of 0.5 µg/kg, and came into force on January 23, 2016.

The assay methodology for AFM₁ in milk was included in "Test Methodology for Aflatoxin M₁ 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 AFM₁ in bovine milk using the Prominence-i integrated HPLC and the RF-20A_{XS} fluorescence detector. Under these conditions we were able to measure AFM₁ at a concentration of 1/10th Japan's regulatory level for AFM₁ in milk.

■ Analysis of Standard Aflatoxin M₁ Solutions

Chromatograms obtained after analysis of standard AFM₁ 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 R² of ≥ 0.9999 within the concentration range. These results show the RF-20A_{XS} fluorescence detector can be used to analyze trace quantities of AFM₁ with high sensitivity and high precision.

When the standard AFM₁ 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 AFM₁ in milk (0.005 µg/kg).

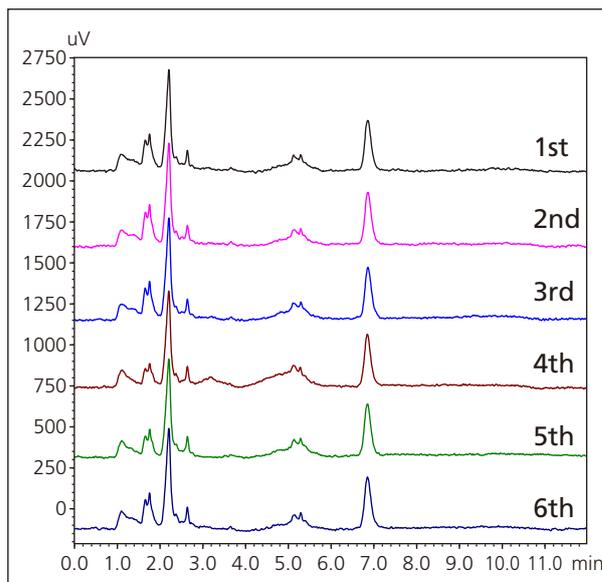


Fig. 1 Chromatograms for Standard AFM₁ Solution Equivalent to 1/100th the Regulatory Concentration (0.1 µg/L, Test Repeated 6 Times)

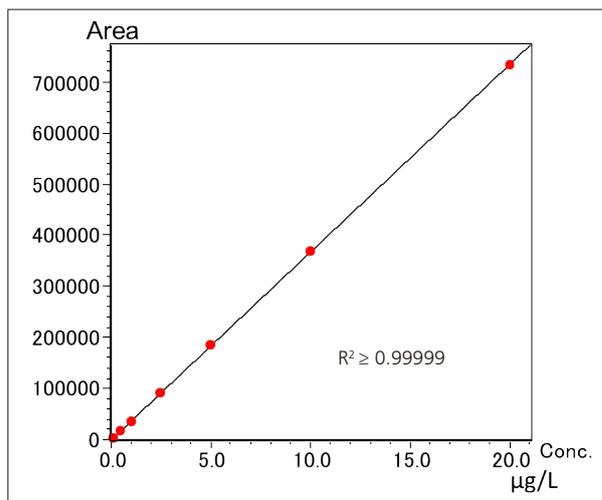


Fig. 2 AFM₁ Calibration Curve (0.1-20 µg/L)

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-20A _{XS} , Ex. at 365 nm, Em. at 435 nm
RF Cell.	:Conventional Cell
Cell Temp.	:25 °C
Injection Volume	:100 µL

■ Analysis of Aflatoxin M₁ in Milk

We analyzed commercially available milk and milk with added AFM₁. AFM₁ 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 methodology²⁾ 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 AFM₁, and (B) is the chromatogram for milk with no added AFM₁.

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.

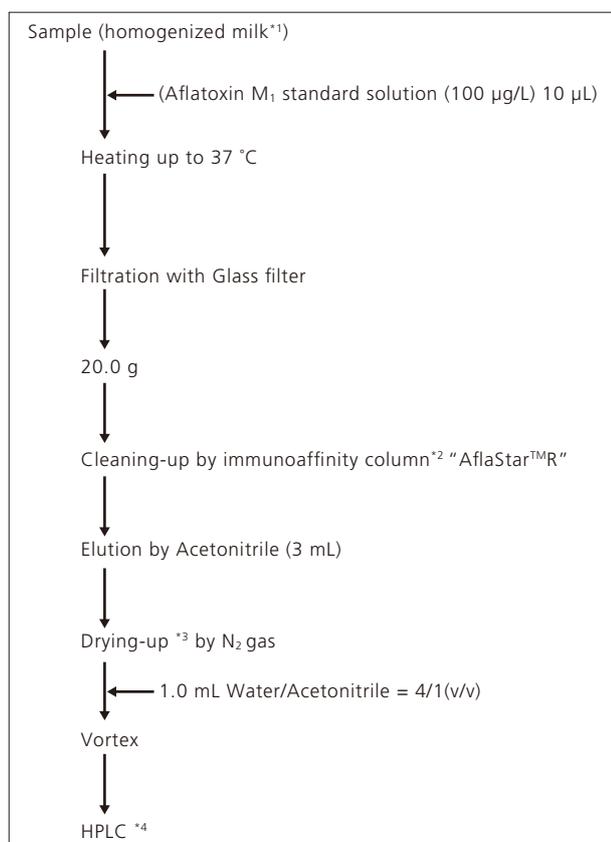


Fig. 3 Milk Pretreatment Procedure

- *1 A pretreatment centrifugation step is needed for raw milk and other milks that are not homogenized. Refer to reference²⁾ for details.
- *2 Refer to the annotations in reference²⁾ for detailed information on use of the immunoaffinity column.
- *3 AFM₁ 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 AFM₁ 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 AFM₁ elution position when milk with no added AFM₁ was analyzed. Using LC/MS/MS to analyze the milk with no added AFM₁ suggested this peak was derived from AFM₁, and the concentration of the substance present was below 1/100th Japan's regulatory level.

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

Eqn. 1 Percentage Recovery Equation

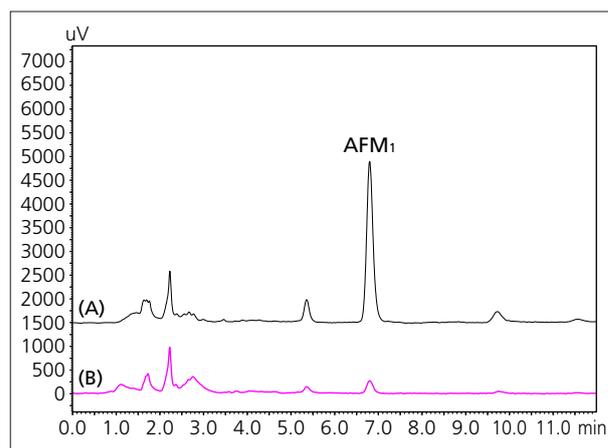


Fig. 4 HPLC Chromatograms for Commercially Available Milk (A) With added standard AFM₁, (B) with no added standard AFM₁

References

- 1) "Handling of Aflatoxin M₁ in Milk" (July 23, 2015, Notification No. 0723-[1] of the Department of Food Safety, PFSB, MHLW)
- 2) "Test Methodology for Aflatoxin M₁ in Milk" (Notification No. 0723-[5] of the Department of Food Safety, PFSB, MHLW)

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