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Analysis of aflatoxin M_1 and M_2 in commercial dairy products using high-performance liquid chromatography with a fluorescence detector

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ABSTRACT

Aflatoxin M_1 (AFM_1) and M_2 (AFM_2) in commercial dairy products were analyzed by high-performance liquid chromatography (HPLC) with a fluorescence detector (FLD). To ensure an accurate analysis, two derivatization methods, bromination and aflatoxin–trifluoroacetic acid derivatization (ATD), were compared. The limits of detection (LODs) of the bromination method were 124.42–151.73 ng/kg, and the recovery rates were between 64 and 102%. The detection rates and concentration levels of AFM_1 were 6–74% and 14.48–270.94 ng/kg, respectively. AFM_1 was detected in 74% of milk powder samples and 36% of ice cream samples. The mean values of AFM_1 in milk powder and ice cream samples were 270.94 and 33.16 ng/kg, respectively. In the case of AFM_2, the detection rates were 2–10%, and the concentration levels were 20.62–55.67 ng/kg in milk and milk powder. Among milk and milk powder samples, ultra heat-treated (UHT) milk had lower AFM_1 contamination levels than pasteurized milk.

1. Introduction

Aflatoxins are secondary metabolites of *Aspergillus parasiticus* and *Aspergillus flavus* (Hwang & Lee, 2006). They have carcinogenic, teratogenic, and mutagenic properties (Ali et al., 2005; Cho et al., 2007). Aflatoxin M_1 (AFM_1) is produced by the metabolizing systems of humans and dairy cows with aflatoxin B_1 ingestion (Diaz & Espitia, 2006; EFSA, 2007). The International Agency for Research on Cancer (IARC, 1993) classified AFM_1 as a possible human carcinogen (Group 2B). For this reason, many countries have set limits for AFM_1 in milk, cheese, and baby food (e.g., 500 ng/kg) (Anfossi, Baggiani, Giovannoli, & Giraudi, 2011).

According to the previous studies, TLC (thin layer chromatography), high-performance liquid chromatography (HPLC), and ELISA are widely used for the analysis of aflatoxins (Awad, Ghareeb, & Bohm, 2012; Manetta et al., 2005). Although the TLC method is the AOAC's official method, it is limited in its ability to quantitate aflatoxins accurately (Awad et al., 2012). The ELISA method is a quick AOAC method; however, it has a 20% false-positive rate (Awad et al., 2012). HPLC with a fluorescence detector (FLD) and mass spectrometry (MS) detector is suitable for aflatoxin quantification. However, few studies have used an MS for aflatoxin analysis by estimating particular samples, such as milk and herbal medicines. Recently, LC/MS was used for the qualitative analysis of aflatoxins, and HPLC-FLD was used for the quantitative analysis of aflatoxins (Bognanno et al., 2006; Jang et al., 2007). Many previous studies have analyzed aflatoxins by HPLC-FLD with immunoaffinity extraction. Analytical methods of aflatoxins are usually carried out by HPLC-FLD either with trifluoroacetic acid (TFA) or bromination derivatization (Reiter, Zentek, & Razzazi, 2009).

Though AFM_1 is known as a stable material (Iha, Barbosa, Okada, & Trucksess, 2012), many previous studies have reported that AFM_1 levels are altered during processing and storage. Ultra heat-treated (UHT) milk has been shown to have lower AFM_1 contamination levels than pasteurized milk (Rahimi & Behzadnia, 2012; Zheng et al., 2012). In yogurt, cheese, ice cream, and sherbet, processing and storage have been shown to affect AFM_1 levels in products (Iha et al., 2012; Wiseman & Marth, 1983). On the other hand, the processing and storage of milk probably did not affect aflatoxins, because milk has a very short distribution period from production to consumption.

In this study, various sample preparation methods such as derivatization method and immunoaffinity columns were compared and validated to determine the optimum analytical method for AFM_1 and AFM_2 in various dairy products. In addition, the levels of AFM_1 and AFM_2 in the dairy products (e.g., milk,

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yogurt, milk powder, ice cream, and sherbet) were analyzed by the optimized method.

2. Materials and methods

2.1. Reagents and materials

AFM_1 and AFM_2 standard powder (Enzo Life Sciences, Lausen, Switzerland) with 70% methanol (J.T. Baker, Phillipsburg, NJ, USA) was prepared for the stock solution. Working solutions were prepared as 500, 1,000, 2,000, 5,000, and 10,000 ng/kg for calibration curves. Water and acetonitrile, and methanol were HPLC grade (J.T. Baker). TFA (Sigma–Aldrich, MO, USA) and Kobra cell (K01, Biopharm Rhone, Glasgow, Scotland) were prepared for derivatization.

2.2. Sampling

Milk sampling (including flavored milk) in Seoul, Korea was conducted from June to August 2012. Samples were collected from commercial markets. Yogurt samples were of the drinking yogurt type. Ice cream samples having more than 6% milk lipids were selected, and sherbet samples having less than 3% milk lipids were selected.

2.3. Determination of validation values for the analytical method

Tests to determine recoveries, coefficient of variation (CV, %) values, Z-scores, limits of detection (LODs), limits of quantitation (LOQs), linearity, and *R*-squared (R^2) values were conducted. Intra- and inter-day recovery and CV tests were performed with each representative sample. Tests to determine LODs of aflatoxins were carried out by 3.3 × sigma (σ)/slope factor of calibration curve. Sigma was obtained by determining the standard deviation of the *y*-intercept of seven specific calibration curves. Each specific calibration curve was constituted by three-point concentrations. Linearity and R^2 values were calculated using each aflatoxin standard curve for quantification. Method detection limits (MDLs) and method detection quantitation (MDQ) values were calculated by LOD/(dilution rate × sample weight) and 3 × MDL, respectively.

2.4. Aflatoxin extraction for milk, yogurt, and sherbet samples

First, 20 g of sample with 20 ml of methanol was put into a 50-ml conical tube. In the case of sherbet, it was melted and filtered by a sieve. It was then added to 0.5 g of NaCl and shaken at 250 rpm for 1 h. It was centrifuged at 6500 rpm (10 min) and filtrated using a 45-μm syringe filter. Next, 10 ml of filtered solution was diluted by 10 ml of water, and 20 ml of the diluted aflatoxin extracted solution was loaded into an immunoaffinity column. After that, 10 ml of water was loaded for cleaning. The immunoaffinity column was then air-dried for 10 min. Finally, 1 ml of methanol was loaded into the column for aflatoxin elution.

2.5. Aflatoxin extraction for milk powder samples

First, 10 g of sample with 40 ml of 70% (v/v) methanol was put into a 50-ml conical tube. It was added to 0.5 g of NaCl and shaken at 250 rpm for 1 h. It was centrifuged at 6500 rpm (10 min) and filtrated using a 45-μm syringe filter. Next, 10 ml of filtered solution was diluted by 10 ml of water, and 20 ml of the diluted aflatoxin extracted solution was loaded into an immunoaffinity column. After that, 10 ml of water was loaded for cleaning. The immunoaffinity column was air-dried for 10 min. Finally, 1 ml of methanol was loaded into the column for aflatoxin elution.

2.6. Aflatoxin extraction for ice cream samples

Ice cream samples were first melted and filtered by a sieve. Then, 20 g of ice cream filtered of toppings and fruit was put in a 50-ml conical tube with 20 ml of methanol. It was added to 0.5 g of NaCl and shaken at 250 rpm for 1 h. Before shaking, 5 ml of hexane was added, and the results were compared with those when 1 drop of Tween 80 was added. It was centrifuged at 6500 rpm (10 min) and filtrated using a 45-μm syringe filter. Next, 10 ml of filtered solution was diluted by 10 ml of water, and 20 ml of the diluted aflatoxin extracted solution was loaded into an immunoaffinity column. After that, 10 ml of water was loaded for cleaning. The immunoaffinity column was air-dried for 10 min. Finally, 1 ml of methanol was loaded into the column for aflatoxin elution.

2.7. Aflatoxin–TFA derivatization (ATD) method

Eluted aflatoxin extracts were dried by nitrogen gas at 40 °C. For the derivatization, 0.2 ml of TFA with 1 ml of hexane was added, and it was then stored for 20 min in a darkroom. After that, 50% (v/v) methanol was mixed to 1 ml using a vortex, and the bottom layer was then injected for analysis.

2.8. Analytical conditions of HPLC-FLD analysis

A Waters 1525 system (Milford, MA, USA) and 474 FLD (Milford, MA, USA) were used. For the ATD method, the mobile phase was acetonitrile:water (7:3, v/v), and the column was an Agilent XDB-C18 (250 mm × 4.6 mm and 5 μm: Palo Alto, CA, USA). Wavelengths for excitation and emission were 360 nm and 450 nm, respectively. For bromination, the mobile phase was a 0.001 M KBr mixture of water, methanol, and acetonitrile (6:2:2, v/v) with 350 μl of 4 M nitric acid for the 1 L mobile phase, and the column was an Agilent Sb-Aq (250 mm × 4.6 mm and 5 μm: Palo Alto, CA, USA). Wavelengths for excitation and emission were 365 nm and 435 nm, respectively. In both conditions, the column temperature was 40 °C, and the flow rate was 1 ml/min. The injection volume was 20 μl.

2.9. Internal quality control

To ensure an accurate analysis, internal quality control was performed. When the analysis of 10 samples was done, 10 ng/g

Table 1
Limit of detection (LOD), Limit of quantification (LOQ), Linearity, and *R*-squared (R^2) values of aflatoxin–trifluoroacetic acid derivatization (ATD) and bromination methods.

	Type of aflatoxin	LOD (ng/kg)	LOQ (ng/kg)	Linearity	R^2
Bromination	AFM_1	125.42	418.05	$y = 5820.5x + 635.34$	0.9988
	AFM_2	151.73	505.77	$y = 4224.1x + 137.16$	0.9951
Aflatoxin–trifluoroacetic acid derivatization (ATD)	AFM_1	57.78	263.91	$y = 5048.7x + 4297.4$	0.9906
	AFM_2	189.27	879.71	$y = 4770x + 5362$	0.9819