FOOD CHEMICAL CONTAMINANTS

Chromatographic Method for the Determination of Aflatoxin M_1 in Cheese, Yogurt, and Dairy Beverages

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The aim of this work was to develop and validate a method to determine aflatoxin M_1 (AFM_1) in cheese, yogurt, and dairy beverages. The method consisted of aqueous methanol extraction, immunoaffinity column purification and isolation, RPLC separation, and fluorescence detection. The four types of cheese samples were classified according to moisture and fat content. The mean recoveries were 71% for cheese at spiked levels from 100 to 517 ng/kg, and 76% for yogurt and dairy beverages spiked at levels from 66 to 260 ng/kg. The mean RSDs were 5.9% for cheese, and 10% for yogurt and dairy beverages. The LOD was 3 ng/kg and the LOQ was 10 ng/kg for all test commodities. To test the applicability of the developed method, a small survey of the presence of AFM_1 in cheese, yogurt, and dairy beverages purchased in Ribeirão Preto-SP, Brazil, was conducted. AFM_1 was detected (>3 ng/kg) in all samples. Twenty cheese samples (83%) were contaminated with AFM_1 in the range of 13–304 ng/kg. In yogurt and dairy beverages, the contamination was lower (13–22 ng/kg) in five samples (42%). The results indicated that the method is adequate for the determination of AFM_1 in these four types of cheese, as well as in yogurt and dairy beverages.

Aflatoxins are toxic metabolites produced by fungi, and are potent liver toxins. Most animal species exposed to these mycotoxins show signs of acute and chronic liver disease. Animals—such as cattle, goats, and sheep—fed rations containing aflatoxin B_1 (AFB_1) excrete aflatoxin M_1 (AFM_1) in their milk (1). The International Agency for Research on Cancer (1993) has classified AFM_1 in Group 2B as possibly carcinogenic to humans.

Milk is an important food in human nutrition because it contains essential constituents, such as proteins with high biological quality, carbohydrates useful for developing nervous systems, and essential fatty acids, vitamins, and minerals. Dairy products, such as cheese, yogurt, and dairy beverages, also have benefits for human health (2, 3).

Milk has great potential for introducing AFM_1 into the human diet. Dairy products derived from milk also can be contaminated with AFM_1 (4). The results of a study in which male Fischer rats were separately given AFM_1 and AFB_1 by stomach tube indicated that AFM_1 hydroxylated derivative has a much lower carcinogenic potency than does the parent substance (5). Another study also reported that AFM_1 was found to be a weak hepatic carcinogen compared to AFB_1 (6). Although AFM_1 is not as hazardous as the parent compound, the U.S. maximum limit is 0.5 μg/kg, largely because milk tends to constitute a large part of the diet of infants and children. AFM_1 is relatively stable during pasteurization, sterilization, preparation, and storage of various dairy products (2, 7). However, there was a report of increasing AFM_1 concentration in cheese as a function of cheese type, technologies, and the amount of water eliminated during processing (7, 8). Minas Frescal and Minas Padrão are Brazilian cheeses—widely consumed—that have about 62 and 41% moisture, and 19 and 16% fat, respectively.

AFM_1 is indirectly controlled by the monitoring of the contamination of AFB_1 in feed. But in Brazil, AFB_1 is regulated only for peanuts and corn. Accurate measurements of AFM_1 in milk and dairy products are important for ensuring food safety and consumer heath protection. At present, there is a Brazilian regulatory limit for AFM_1 in milk, but not in dairy products. The reason could be due to the lack of simple analytical methods for dairy products, or to the lack of surveillance data. LC techniques, TLC, and ELISAs have been developed for the detection and quantitation of AFM_1 in milk and cheese. LC coupled to a fluorescent detector is a common technique for AFM_1 (9). Silica gel, C18, and immunoaffinity columns (IACs) have been used for purification or isolation of the toxin from milk, while only

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Table 1. Classification of cheese samples according to moisture and fat content

Cheese samples	Moisture, g/100 g	Fat, g/100 g
Minas Frescal light	>55	10–24.9
Minas Frescal	>55	25–44.9
Minas Padrão	37–47	26–53
Prato	36–45.9	45–59.9

silica gel and C18 have been used for isolation of AFM_1 from cheese prior to LC separation and quantitation.

Our goal was to develop and validate a method using IAC as a cleanup column for the determination of AFM_1 in cheese, yogurt, and dairy beverages.

Materials

Dairy Products

Twenty-four cheese samples, six yogurt samples, and six dairy beverages were purchased from supermarkets in Ribeirão Preto-SP, Brazil. The cheese samples were classified into four categories depending on their moisture and fat contents (Table 1); six in each category were ground and homogenized before analysis.

AFM_1 Added Materials (for Recovery Study)

An appropriate amount of AFM_1 was added to 8 g test samples (control material containing $AFM_1 < 3$ ng/kg) to obtain AFM_1 levels ranging from 66 to 517 ng/kg. Spiked test samples were mixed and kept at room temperature (22°C) for 1 h, then analyzed for AFM_1.

Equipment and Supplies

(**a**) *IAC column*.—AflaStar Fit 3 (Romer Labs, Tulin, Austria).

(**b**) *LC system*.—Shimadzu Instruments (Kyoto, Japan) with a fluorescence detector and a Rheodyne L.P. (Cotati, CA) injector with a 50 µL loop. *LC operating conditions*: Mobile phase: water–acetonitrile (6 + 4, v/v). Flow rate: 0.7 mL/min. Fluorescence detector set at excitation and emission wavelengths of 330 and 460 nm, respectively. Column: Shim-pack CLC-ODS (M), 4.6 × 250 µm, 5 µm (Shimadzu).

(**c**) *Spectrophotometer*.—Hitachi (Tokyo, Japan).

(**d**) *Vortex mixer*.—Fanem (São Paulo, Brazil).

(**e**) *Centrifuge*.—Fanem.

(**f**) *Column manifold*.—Supelco (Bellefonte, PA).

(**g**) *Orbital shaker*.—DS-500E, VWR (West Chester, PA).

(**h**) *Column reservoir 60 mL plastic syringe*.—Supelco.

(**i**) *Glass microfiber filter paper*.—Whatman 934AH (Clifton, NJ).

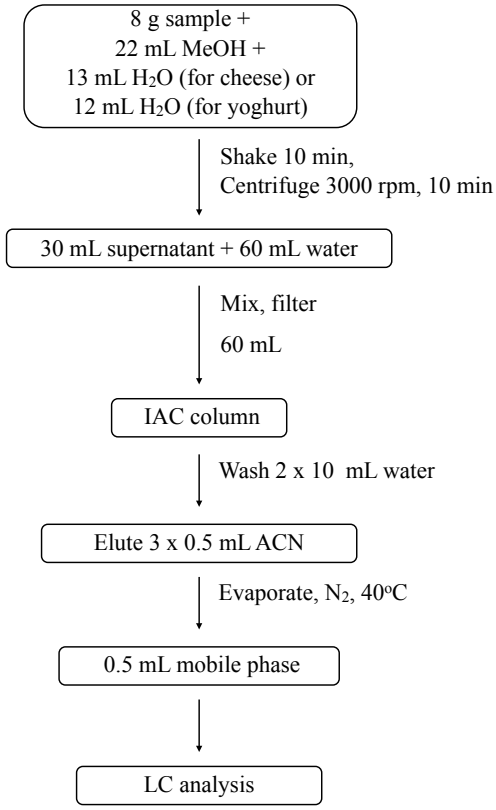

Figure 1. Procedure for determination of AFM_1 in dairy products.

Reagents

(**a**) *Solvent and reagents*.—LC grade methanol, acetonitrile, and MilliQ water.

(**b**) *AFM_1*.—From *Aspergillus flavus*, A6428 (Sigma Chemical Co., St. Louis, MO). (*1*) *AFM_1 stock standard solution (510 ng/mL)*.—Prepare stock solution in acetonitrile and determine concentrations according to AOAC *Official Methods*^{SM} **986.16, 971.22**, and **970.44** (10). (*2*) *AFM_1 working standard solutions*.—Prepare appropriate portions of the stock standard solution of AFM_1 by evaporating and diluting with mobile phase to give concentrations of 0.2, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 ng/mL. (*3*) *AFM_1 spiking solutions*.—Prepare appropriate portions of the stock solution of AFM_1 by evaporating and diluting with methanol to give the concentration of 50 µg/mL.

Summary

Figure 1 shows an outline of the procedure, which consisted of aqueous methanol extraction, IAC purification and isolation, RPLC separation, and fluorescence detection.

Extraction

Weigh 8.0 g test portion in a 50 mL centrifuge tube. For cheese, add 22 mL methanol and 13 mL water. For yogurt and dairy beverages, add 22 mL methanol and 12 mL water. Shake at 400 rpm for 10 min, then centrifuge at 3000 rpm for 10 min. Aspirate and discard the upper oil layer. Place 30 mL supernatant into a 125 mL Erlenmeyer flask, add 60 mL water, and mix. Pass the mixture through glass microfiber paper to collect 60 mL filtrate (approximately 4.6 g test portion) into a 100 mL graduate cylinder, and proceed immediately with IAC chromatography.

IAC Isolation

After being removed from storage at 4°C, the IAC should be equilibrated to room temperature for at least 15 min before use. Remove the top cap from the column and connect it to the reservoir of the column manifold. Remove the bottom cap from the column and let liquid in the column pass through until it is about 2–3 mm above the column bed. Pass 60 mL filtrate into the column reservoir, letting the filtrate flow through the IAC by gravity. Add 10 mL water to the column reservoir when the liquid level is 2 mm above the column packing. Wash the column with an additional 10 mL water. Let the column run dry, then force 10 mL air through the column with a syringe. Elute with 0.5 mL methanol, collect AFM_1 in a 4 mL vial. Let it drip freely, allowing the column to run dry; elute twice again with an additional 0.5 mL methanol and collect it into the same vial. Let the column run dry and force 10 mL air through the column. Evaporate the eluate to dryness under a stream of nitrogen at 40°C. Add 0.5 mL LC mobile phase to the residue. Vortex for 1 min, and inject 0.05 mL to perform the LC analysis.

LC Analysis

Inject 0.05 mL reagent blank, AFM_1 working standards, or test solution into the LC column. Identify AFM_1 peaks in the test solution by comparing the retention time with those of the standards. In our work, the retention time of AFM_1 was approximately 7 min (Figure 2), and the peaks were baseline-resolved. Construct standard curves of AFM_1, and determine the concentration of AFM_1 in the test solution from the standard curve.

Standard Curves

Standard curves should be prepared for AFM_1 with the working standard solutions containing the AFM_1. These solutions cover the range of 0.2–10.0 ng/mL AFM_1. Construct the standard curve prior to analysis and check the plot for linearity by examining the correlation coefficient (R^2 >0.99) of concentrations and responses. If test solution area response is outside (higher than) the standard range, the purified test extract should be diluted with LC mobile phase and reinjected into the LC column.

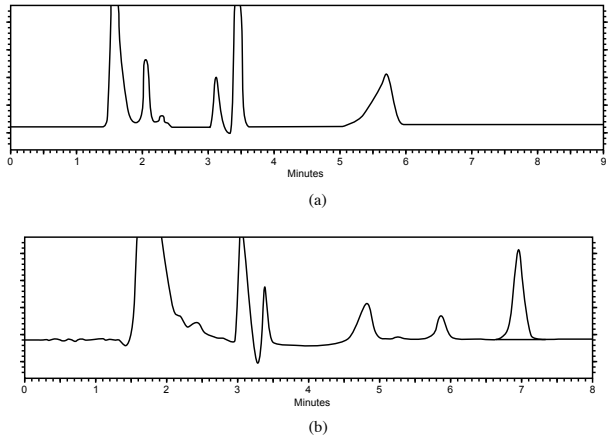

Figure 2. LC chromatogram of (a) blank cheese sample and (b) cheese sample naturally contaminated with 94 ng AFM_1/kg.

Quantitation and Calculation

Quantitation of AFM_1 should be performed by measuring the peak area at the AFM_1 retention time and comparing it with the standard curve. Plot the peak area (response, *y*-axis) of AFM_1 standard against the concentration (ng/mL, *x*-axis) and determine the slope (S) and *y*-intercept (a). Calculate the level of toxin in the test sample with the following formula:

$$\text{Toxin, ng/kg} = ([(R - a)/S] \times V/W) \times F \times 1000$$

where R is the test solution peak area, V is the final volume (mL) of the injected test solution, and F is the dilution factor. F is 1 when V is 0.5 mL; W is 4.6 g test sample passed through the IAC.

Recovery Experiments

For recovery studies, replicates of four AFM_1-free cheese test samples were spiked at levels ranging from 100 to 517 ng/kg; yogurt and dairy beverages were spiked at levels ranging from 66 to 260 ng/kg. All spiked test portions were kept at room temperature for at least 1 h before analysis. The recovery calculations were made in two forms:

(*1*) *Without correction of water content*.—Made using volume extractions of 35 mL for cheese (22 mL methanol + 13 mL water) and 34 mL for yogurt (22 mL methanol + 12 mL water); and

(*2*) *With correction of water content*.—Made using volume extractions of 35 mL for cheese plus moisture in the sample (% moisture × 8 g) and 34 mL for yogurt plus moisture in the sample (% moisture × 8 g).

Table 2. Recoveries of AFM_1 added to cheeses

Sample[a]	AFM_1 spiked, ng/kg	Recovery, %[b]			Protein %	Moisture %	Recovery, %[c]
		Mean	SD	RSD			Mean
Minas Frescal light cheese	243	65	3	5	13	72	76
Minas Frescal cheese	100	80	6	8	10	72	96
Minas Frescal cheese	217	79	5	6	10	72	93
Minas Frescal cheese	400	86	2	2	10	72	101
Minas Padrão cheese	250	61	6	10	21	45	67
Prato cheese	131	68	6	9	25	42	73
Prato cheese	251	66	3	4	25	42	70
Prato cheese	517	65	2	3	25	42	69
Mean		71	4.1	5.9			80
Yogurt	66	74	6	8	2.7	91	98
Dairy beverage	136	76	9	11	2.5	84	95
Dairy beverage	260	78	9	12	2.5	84	85
Mean		76	8.0	10.0			93

[a] $n = 4$.
[b] Recovery calculated without correction for water content.
[c] Recovery calculated with correction for water content.

Results and Discussion

In Figure 2a, the LC chromatogram of the blank cheese sample shows no peaks near the AFM_1 peak area. This indicates that the method provides test extracts that are free of interferences for LC analysis.

The equation of the calibration curve used for the determination of the linearity of the method was calculated by least-squares linear regression, and was linear from 0.2 to 10 ng/mL, with R^2 of 0.9992, and the linear regression equation was $y = 40\ 100x$ (the intercept was set at zero because the intercepts in most cases were less than 1% of y).

The moisture in cheese samples was 42–72%; in yogurt and dairy beverages, it was 84–91%. In order to obtain a low-fat test extract of AFM_1 from high-fat dairy products, a mixture of methanol–water at a ratio of 55:45 (v/v) was used as the extraction solvent. Water and methanol were added separately. Different volumes of water and methanol were added to cheese and to yogurt and dairy beverages because of the different water content in the test portions.

Because aqueous methanol was used as an extraction solvent, the water content of samples may increase the volume of the extract. Therefore, calculations of recoveries for the added AFM_1 from spiked test samples were performed by two different procedures, i.e., with and without corrections for the water content of the test samples. Table 2 gives recovery results for AFM_1 added to a test portion, without corrections for water content. The recoveries of AFM_1 ranged from 61 to 86%. The mean recovery from spiked cheese was 71% (without correction for water content); in yogurt and dairy beverages, it was 76%. The mean recovery corrected for water content was 80% in cheese and 93% in yogurt and dairy beverages. The mean RSD_r of recoveries was about 5.9 and 10%, for cheese and yogurt, respectively. Results indicate that the performance of this method is similar to that of the AOAC *Official Methods*^{SM} for AFM_1 in milk (10).

The LOD of 3 ng/kg was determined using the mean value of replicates of blank (cheese, yogurt, and dairy beverages) test portion analysis (four analyses) plus two SDs (background signal of blank, 95% one side confidence interval). The LOQ was 10 ng/kg (approximately 3.3 times the LOD; 11). The results indicated that the method is adequate for the determination of AFM_1 in cheese, yogurt, and dairy beverages.

The methods for the determination of AFM_1 in cheese often require the use of a toxic solvent—such as hexane, chloroform, or dichloromethane—as extraction solvents (12–15). A method for AFM_1 in yogurt was published using dichloromethane as the extraction solvent; the extract was evaporated and the residues were dissolved in methanol and water (15). Fat was eliminated with hexane before IAC cleanup.

The method presented uses solvents that are environmentally friendly. Parker and Tothill (16) developed a microelectrode array immunosensor for AFM_1 that measures AFM_1 in milk directly without extraction. However, that method requires the use of expensive equipment, and the performance of the method has not been validated by an international collaborative

Table 3. AFM_1 in dairy product samples purchased in the region of Ribeirão Preto-SP, Brazil

	Range of AFM_1 concentration, ng/kg (%)				
Sample[a]	<10	11–50	51–100	101–250	251–500
Minas Frescal light cheese	3 (50)	1 (17)	2 (33)	0	0
Minas Frescal cheese	1 (17)	3 (50)	1 (17)	1 (17)	1 (17)
Minas Padrão cheese	4 (67)	2 (33)	0	0	0
Prato cheese	3 (50)	2 (33)	0	1 (17)	0
Dairy beverages	0	6 (100)	0	0	0
Yogurt	2 (33)	4 (67)	0	0	0

[a] $n = 6$.

study. Consequently, the immunosensor method is not widely used for routine analysis.

A small survey for the occurrence of AFM_1 in cheese, yogurt, and dairy beverages purchased in Ribeirão Preto was conducted. Results of the survey are given in Table 3. AFM_1 was detected (>3 ng/kg) in all samples; 20 cheese samples (83%) were contaminated with AFM_1 in the range from 13 to 304 ng/kg. In yogurt, the contamination was lower in five (42%) samples (13–22 ng/kg).

As the sources of aflatoxin contamination in animal feedstuffs may vary geographically (17), the incidence and occurrence of AFM_1 vary in different countries. There are previous studies on the occurrence of AFM_1 in cheese in Slovenia (18), Libya (19), and Turkey (7, 20), and in yogurt, in Portugal (21) and Turkey (7, 22).

In Brazil, the incidence of AFM_1 in Minas Frescal and Padrão cheeses were not similar. De Sylos et al. (23) detected no mycotoxins in 12 Minas cheese samples. Prado et al. (24) analyzed 57 Minas cheese samples and found AFM_1 at 20–6920 ng/kg in 45 samples. Franco et al. (25) detected AFM_1 in 15 out of 24 samples at 30–1005 ng/kg; three cheeses contained >5000 ng/kg. DeSylos et al. (23) reported no AFM_1 contamination in yogurt; this could be because the LOD of the method of analysis was higher than that of the method presented here.

Conclusions

There is no Brazilian regulatory limit for AFM_1 in cheese, yogurt, and dairy beverages. The results of our study show that AFM_1 is in dairy products at levels below 304 ng/kg. Because the data are limited, it is important to continue surveillance in order to achieve a more comprehensive picture of the situation.

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