The Rapid Determination of Aflatoxin M_1 in Dairy Products

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ABSTRACT

A method for aflatoxin M_1 in dairy products is presented. It decreases the analysis time compared to currently accepted methods. Samples are extracted in a blender or separatory funnel for 1 min with chloroform and saturated sodium chloride solution. The chloroform extracts are partially purified on a small silica gel column (2 g), and M_1 is determined by thin layer chromatography (TLC) and densitometry. Recoveries of M_1 added to milk are about 80%. Recovery of M_1 from cheeses is variable depending on the type of cheese. The method gave results for a naturally contaminated powdered milk comparable to analyses by accepted methods. The determination limit of the method is about 0.1 μg/kg.

INTRODUCTION

Aflatoxin M_1 is a toxic metabolite found in the milk of lactating mammals consuming aflatoxin B_1-contaminated feed. Analytical methods for this aflatoxin were slow in being developed and were poor in quality until 1973, when Pons et al. (1) reported a method for the quantitative determination of M_1 in milk and milk products. Stubblefield and Shannon (2) adapted this method for all dairy products and tested it in an international collaborative study (3) for the Association of Official Analytical Chemists (AOAC). This method was adopted as official first action by the AOAC (4) and has been used extensively by analysts in the United States. The main drawback of this method is the long time required for assay. A full 8 hr day is required for one analyst to assay six samples if the cellulose column chromatography step is used.

In the fall of 1977, a high incidence of B_1-contaminated corn occurred in the southeastern United States (5). Frequent occurrences and high levels of M_1 in milk have been detected from the use of this corn in diets of dairy cattle; consequently, the FDA has established an administrative guideline of 0.5 ppb M_1 in milk (6). Need for a more rapid method for the assay of milk products arose because of the heavy work load imposed by this problem.

Two methods have been published (7-9) that offer low limits of determination and rapid analysis time. Tuinstra and Bronsgeest's method (7) is applicable to fluid milks, and probably to powdered milks, but not to cheese or other solid products. The method of Schuller et al. (8) was developed for milks, but it has been recently modified to include cheeses (9). Both have low limits of determination (0.004-0.05 μg/liter) and have been shown to produce results equivalent to the AOAC method (10,11). However, both utilize minimal cleanup of sample extracts and, instead, incorporate two-dimensional thin layer chromatography (TLC) to separate M_1 from interferences. Although two-dimensional TLC is an effective technique for impure extracts, time for analysis is increased because two developments are required and only one sample can be run on a plate. However, large numbers of dairy samples can be screened for aflatoxin M_1 by two-dimensional TLC if 10 x 10 cm plates are used. In this manuscript, a method is described for determining M_1 in dairy products that has a limit of determination of ca. 0.1 μg/liter or μg/kg and by which analysis of six samples can be completed in less than 2.5 hr.

EXPERIMENTAL PROCEDURES

Materials

All dairy products except raw milk were purchased at local supermarkets. Raw milk was donated by Producers Dairy, Inc., Peoria, Illinois.

Extraction of Dairy Products

Liquid milk (whole and raw) (50 ml) was shaken vigorously with sodium chloride solution (saturated, 10 ml) and chloroform (120 ml in a 250 ml separatory funnel for 60 sec. After the phases separated (ca. 1-2 min), the chloroform layer (bottom) was filtered through paper (S. and S. No. 588 or Whatman 2V, 24 cm) into a 100 ml graduate, and the volume was recorded.

Powdered milk (5 g) was added to a separatory funnel (250 ml), shaken with water (50 ml), and the sodium chloride solution (10 ml). It was then shaken with chloroform which was separated and filtered as described for liquid milk.

Hard cheeses (cheddar and Swiss) (50 g) were cubed and blended (low speed ca. 10 sec) in an explosion-proof blender to grate the cheese. Soft cheeses (Edam, Brie, and Camembert) were only cubed and added to the blender bowl. For all cheeses, sodium chloride solution (2.5 ml) and chloroform (100 ml) were added, and the cheese was extracted at low speed for 60 sec. The pureed mixture was filtered through paper into a 100 ml graduate, the filter paper top was closed, and the entire mass compressed

TABLE I

Recovery of Aflatoxin M_1 from Artificially Contaminated Dairy Products[a]

Dairy product	No. samples	Aflatoxin M_1 concentration: Added (μg/liter or μg/kg)	Recovered (μg/liter or μg/kg)	%	Range (μg/liter or μg/kg)	Coeff. var. (%)
Whole milk	10	0.5	0.41	81.4	0.31-0.46	12.5
Raw milk	14	0.5	0.38	76.4	0.28-0.43	19.5
Powdered milk	5	5.0[b]	3.88	80.8	3.00-4.40	14.5
Cheddar cheese	9	0.5	0.31	62.4	0.25-0.37	14.7
Edam cheese	5	0.5	0.27	54.8	0.23-0.30	9.5
Swiss cheese	4	0.5	0.59	117.7	0.49-0.68	13.8
Brie cheese	2	0.5	1.17	234.0	---	---
Camembert cheese	2	0.5	0.99	199	---	---

[a]As determined by TLC and densitometry – one dimension.

[b]This is equivalent to 0.5 μg/liter liquid milk.

against the sides of the funnel to expel maximum filtrate. The filtrate volume was recorded for concentration calculations.

Column Chromatography

This cleanup step was patterned after the one described by Pons and Franz (12). Silica gel 60 (E.M. Merck No. 7734) containing 1% water (13) was washed with methanol and then with chloroform before activation. Silica gel (2 g) was slurried in chloroform in the glass column (1.0 cm I.D. x 30 cm, Econo-columns No. 737-2250, Bio-Rad Laboratories, Richmond, California 94804) fitted with a Luer nylon stopcock (Bio-Rad Laboratories No. 732-9009). Anhydrous sodium sulfate (granular) (1.5-2 g) was added to the chloroform above the settled silica gel.

The entire milk or cheese filtrate was drained through the column into a 150 ml beaker. The graduate and finally the inside of the column was rinsed with chloroform and drained through the column. The column was then washed with acetic acid/toluene (1:9, v/v) (25 ml) and with acetonitrile ether/hexane (2:3:5, v/v/v), and the washes were discarded. Aflatoxin M_1 was eluted with acetone/ chloroform (1:4, v/v) (30 ml) into a 125 ml Soxhlet flask and the eluent was evaporated in vacuo. The dry residue was transferred quantitatively with chloroform to a 1 dram vial fitted with a Teflon-lined screw cap, and the extract evaporated to dryness under nitrogen for TLC.

Thin Layer Chromatography

TLC of extracts, densitometry of developed plates, and calculations of M_1 concentrations were as described previously (2). Several sizes of TLC plates were used (10 x 10 cm, 10 x 20 cm, and 20 x 20 cm). All were prepared with adsorbosil-1 (Applied Science Laboratories).

Method Recovery and Comparison Studies

Uncontaminated dairy products were spiked with 0.5 ml standard aflatoxin M_1 solution (0.05 μg/ml in acetonitrile) to give a 0.5 μg/liter or μg/kg level. The spiked products were assayed as described above, and M_1 recoveries were calculated. Artificially contaminated powdered milk was prepared by adding M_1 to whole milk at 0.5 μg/liter level after which the milk was freeze-dried. The dried powder was weighed and the contamination level calculated at 4.8 μg/kg. Naturally contaminated powdered milk was assayed in quintuplet by the method above, the AOAC method (14), and the method of Schuller et al. (8).

RESULTS AND DISCUSSION

Recovery of aflatoxin M_1 from artificially contaminated dairy products is presented in Table I. Values for milk (whole, raw, and powdered) averaged ca. 80% with a satisfactory coefficient of variation (CV) (ca. 15%) considering the low level tested – 0.5 μg/liter. Interestingly, when powdered milk was spiked directly, little M_1 was recovered by the method. Some type of binding occurred that was independent of the spiking solvent (chloroform, methanol, or acetonitrile). However, when the liquid milk was spiked and freeze-dried, recoveries were as shown in the Table. Recoveries of M_1 from cheddar and Edam cheeses were low, ca. 58%; and high recovery values, exceeding 100%, were found for Swiss, Brie, and Camembert cheeses. The low values obtained with cheddar and Edam cheese may indicate a problem similar to that observed with the powdered milk. Also, there may be interferences present in cheese extracts which quench or enhance the fluorescence of the M_1 zone. The data obtained in the analyses of cheeses strongly suggest that two-dimensional TLC as described by Schuller et al. (8) should be used for these dairy products.

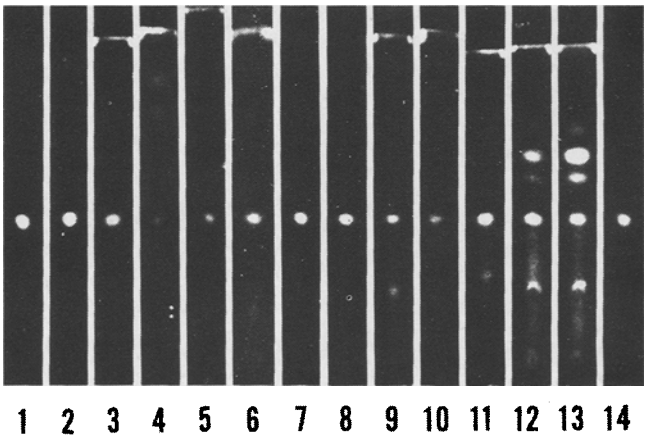

FIG. 1. Composite photograph of several TLC plates of various artificially contaminated dairy products (0.5 μg/kg) shown left to right; 1,2 – std M_1, 3 – whole milk, 4 – whole milk (0.1 μg/kg), 5 – raw milk, 6 – powdered milk, 7,8 – std M_1, 9 – cheddar cheese, 10 – Edam cheese, 11 – Swiss cheese, 12 – Camembert cheese, 13 – Brie cheese, and 14 – std M_1. All extracts were prepared and chromatographed by the method described.

A composite TLC photograph of various dairy products is shown in Figure 1. This picture reveals the absence of interfering substances in milk extracts and the numerous zones in the extracts from cheeses. The small silica gel column of Pons and Franz (12) modified for M_1 does an excellent job in cleaning up the extracts of milk. In addition, it accomplishes the purification quickly, <45 min, and it is economical because small volumes of solvents are used.

A naturally contaminated powdered milk was assayed by three different methods – AOAC (14), Schuller (8), and the NRRC method described in this paper. The results are compared in Table II. The values are very similar and indicate that the methods give comparable results. The precision of the Schuller method is superior, but this method utilizes a 30 min extraction with a wrist-action shaker; therefore, a more efficient extraction occurs. The AOAC method blends the samples 3 min; whereas, the method described in this paper only requires a 1 min

TABLE II

Analyses Data for Aflatoxin M_1 in Naturally Contaminated Powdered Milk by Three Methods[a]

Method	No. samples	Aflatoxin M_1 concentration			
		Average (μg/kg)	Range (μg/kg)	Std. dev. (μg/kg)	Coef. variation (%)
Schuller	5	4.80	4.36-5.00	± 0.26	5.44
AOAC	5	4.85	4.45-5.30	± 0.60	12.40
NRRC	5	5.08	3.82-5.74	± 0.78	15.35

[a]The methods of Schuller et al. (8), the AOAC (14), and Stubblefield (NRRC) as described in text. Measurement of M_1 by TLC and densitometry.

partition, and yet the CV is less than 15%. This method has a limit of determination no greater than 0.1 μg/kg or μg/kg (Fig. 1), which is one-fifth of the current administrative guideline set by the FDA for fluid milk (6). Other dairy commodities may have different limits of determination depending upon the interferences. The method is fast, since six milk samples can be completely assayed in less than 2.5 hr. Finally, it is simple, economical, and reliable. Everything considered, this method would be ideal for the analyst who routinely assays large numbers of dairy samples for aflatoxin M_1.

Note

Prior to publication of this manuscript, a collaborative study on the NRRC method was conducted by the AOAC and IUPAC. It was found that the chloroform needed to be heated to 35 C prior to extraction of both fluid and powdered milks or emulsions were encountered. Also, a 10-ml hexane rinse of the silica gel column after the acetic acid/toluene (1 + 9) wash was necessary to remove excess acetic acid. This step kept aflatoxin M_1 from being eluted during the elution with acetonitrile/ether/hexane (2 + 3 + 5).

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