Application of a Diphasic Dialysis Technique to the Extraction of Aflatoxins in Dairy Products

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ABSTRACT

A new method is described for the extraction of aflatoxins from milk and milk products based on a diphasic dialysis technique, followed by detection and quantification of aflatoxins by TLC. Recovery was 65 to 99% for aflatoxins B_1, G_1, and M_1, and the detection limit in milk and yogurt was .01 to .02 ppb for all aflatoxins studied. In cheese, the detection limit was .03 to .04 ppb for aflatoxins B_1 and G_1 and .1 ppb for aflatoxin M_1.

The main advantages of this technique are its simplicity; sensitivity; very economical use of reagents, thereby minimizing environmental pollution; efficiency, allowing the quick processing of a high number of samples; use by laboratories with little equipment; and a sensitivity comparable with that of much more sophisticated techniques.

(**Key words**: technique, dialysis, aflatoxins, dairy products)

INTRODUCTION

The chemical properties of aflatoxins, such as their molecular weight, solubility, polarity, and capacity for emitting fluorescence under different wavelengths of ultraviolet light, have led to the use of various physicochemical techniques for their extraction, purification, detection, and quantification. In recent years, much work has been carried out on the final phase of the process, i.e., separation, detection, and quantification. Two techniques are currently in use, TLC and HPLC. Although these techniques are extremely sensitive, allowing the detection of aflatoxins in quantities as low as picograms (2, 3, 8), they can only be applied after the samples have passed through complex extraction and purification systems to eliminate interfering substances.

Traditionally, extracts have been purified by different methods, according to the nature of the substrate in which the aflatoxins were found. These methods included partitioning using immiscible solvents, separation in chromatography columns, formation of complexes with metals, and precipitation systems (3). Of these, the most commonly used have been combinations of the first two techniques, using various solvents and columns.

However, these extraction and purification techniques need further improvement to reduce the time taken and the amount of toxic solvents used and to create methods that may be applied simultaneously to different mycotoxins in different substrates (8). This need has led to the development of fast purification systems in the form of commercial kits (12) and, more recently, to the use of various membrane techniques for the purification of extracts (10, 11).

To meet these requirements, our laboratory has developed a diphasic dialysis technique (5) that can be applied to the extraction of aflatoxins from milk, yogurt, and cheese. This technique is based on the use of semipermeable membranes that separate two immiscible solvents; for dairy products, there is an aqueous phase containing the aflatoxins and a chloroform phase. Aflatoxins can be separated from other substances because of their lower molecular weight and greater solubility in the organic solvent than in the aqueous phase. Because the two phases are separated by the membrane, the problems of protein precipitation and formation of lipid emulsions, which occur in traditional partition in funnels, are avoided.

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MATERIALS AND METHODS

Materials

The dialysis tube of regenerated cellulose had an average pore diameter of 24 Å and an exclusion limit between 8000 and 15,000 Da. The tube had a diameter of 3 cm, and its length was 60 cm. (Visking dialysis tubing 20/32; Serva, Feinbiochemical, Heidelberg, Germany).

Chloroform, methanol, acetone, isopropanol, and anhydrous sodium sulfate were all analytical grade.

Standard aflatoxins M_1, G_1, and B_1 (Sigma Chemical Co., St. Louis, MO) were dissolved in chloroform at 10 and 1 μg/ml (aflatoxin M_1) and 50 and .5 μg/ml (aflatoxins G_1 and B_1), deep-frozen (–80°C), and calibrated photometrically (official method 979.44 of AOAC) (2).

For the Adye and Mateles' medium (1), we dissolved in 1 L of distilled water 50 g of glucose, 3 g of $(NH_4)_2SO_4$, 10 g of KH_2PO_4, 2 g of $MgSO_4 \cdot 7H_2O$, 1 ml of salt solution A (70 mg of $Na_2B_4O_7 \cdot 10H_2O$, 50 mg of $(NH_4)_6Mo_7O_2 \cdot 4H_2O$, 11 mg of $MnSO_4 \cdot H_2O$, and 30 mg of $CuSO_4 \cdot 3H_2O$ in 100 ml of distilled water), and 1 ml of salt solution B (1 g of $Fe_2(SO_4)_3 \cdot 6H_2O$ and 1.76 g of $ZnSO_4 \cdot 7H_2O$ in 100 ml of distilled water). The mixture was sterilized at 110°C for 10 min. The final pH was 4.5.

Preparation of Samples

The extraction technique was tested on three different samples (milk, yogurt, and cheese) contaminated with aflatoxins B_1, G_1, and M_1 at concentrations of .050 ppb of sample in milk, .047 ppb in yogurt, and .10 ppb in cheese.

An aqueous solution was obtained by contamination of a clean, thoroughly dried flask with a quantity of each aflatoxin in chloroform solution, evaporation to dryness, and addition of 50 ml of Adye and Mateles' medium (1). The flask was then shaken for 30 min. Tubes containing 3 ml of the resulting solution were stored at –80°C until use. The quantity of aflatoxins present in the contaminating solution was calculated before tests: a 1-ml sample was shaken in 50 ml of chloroform for 30 min, and the extract was passed through a separating funnel to separate the phases, after which the chloroform phase was filter-dried with anhydrous $NaSO_4$. Finally, the extract was concentrated to 400 μl in a rotary evaporator. Determination and quantification of aflatoxins were carried out by TLC and fluorodensitometry. In this way, an aflatoxin solution of approximately 2.5 ng/ml was obtained. Contamination of samples with the aqueous solution was then carried out.

Contamination of Milk. Commercial UHT whole (3.3% fat) milk was used. Aqueous aflatoxin solution was added to give an aflatoxin concentration of .050 ppb. Samples of 50, 250, and 1000 ml were analyzed.

Contamination of Yogurt. Yogurt was prepared in the laboratory from the contaminated milk; milk was preheated to 40°C and placed in 100-ml containers, 90 ml in each. To thicken the contaminated milk, 4 g of dried milk were added with 1% of starter (commercial yogurt). The mixture was shaken and then incubated at 45°C for 8 h, and the finished product was stored at 4°C until use. Fifty grams of yogurt were used for each extraction.

Contamination of Cheese. Reference solutions of aflatoxins in chloroform were used as follows. Fifty-gram samples of commercial semimature hard Manchego-type cheese (40% fat in DM cheese) were used. Sufficient solution was dropped onto each sample to achieve contamination of .1 ppb, and samples were warmed at 37°C for 15 min to evaporate the chloroform. Samples were then blended with 70 ml of 1.3% $NaHCO_3$, following the technique of Domínguez et al. (4), to obtain a consistent paste.

Extraction of Aflatoxins

Each contaminated sample was placed in a 1-L Erlenmeyer flask. Extraction consisted of pouring 70 ml of chloroform into a dialysis tube (60 cm long) that had been previously soaked in water. The tube was then closed and placed in the flask containing the sample, which was shaken at 120 rpm for 2, 3, 5, 8, or 18 h. The tube then was removed from the flask, opened, and emptied into a separatory funnel by which the chloroform phase was separated. This phase was then filter-dried through anhydrous $NaSO_4$. The dried extract was transferred to a round-bottomed flask and

concentrated on a rotary evaporator to a final volume of 200 μl for milk and yogurt and 500 μl for cheese.

Determination and Quantification of Aflatoxins

Determination of aflatoxins was made by TLC using high resolution plates (10 × 10 cm) with no fluorescence indicator (Merck, Darmstadt, Germany). These plates were developed in an unsaturated chamber using several developing solvents (vol/vol): chloroform: acetone, 176:24; chloroform:acetone:isopropanol, 170:20:10; chloroform:methanol, 95:5, and chloroform:methanol, 90:10.

Quantification of aflatoxins was carried out with a densitometer Camag TLC Scanner II equipped with Cats 3 software (Camag, Muttenz, Switzerland) and using an excitation wavelength of 350 nm.

Detection Limit

Detection limit was the lowest concentration of contamination that could be measured by the diphasic dialysis technique.

The detection limit for milk was determined by using contaminated milk at .05 ppb of each aflatoxin investigated. From this milk, different dilutions, corresponding to different contamination concentrations, were obtained.

Cheese samples were contaminated with various dilutions of the aflatoxin standard solutions. In this way, cheese samples with different contamination concentrations were obtained.

The aflatoxin extraction and quantification were performed as described before to determine the detection limit of the technique.

RESULTS AND DISCUSSION

Table 1 shows the results of the experiments performed after optimal conditions had been determined for the different substrates. To establish these conditions, various factors had to be taken into account: extraction solvent used, influence of sample size on percentage of recovery, extraction time for different sample sizes, effect of the pH of the product, and developing solvent used for chromatography.

Various combinations of extraction solvent were tried: chloroform:acetone (50:20, 35:35, 54:16; vol/vol) and chloroform (70 ml), but the best results were obtained from pure chloroform.

For sample size and extraction time, 50 g in 5 h was considered to be optimal because, even with increased time, percentage of recovery in milk and yogurt did not rise appreciably. However, changes to the extracts occurred that made them more difficult to process, namely, an increasing aqueous phase and increased fat in the tubing.

Nevertheless, extraction time had to be increased from 5 to 18 h for cheese to achieve good recovery, perhaps because of the difficulties involved in creating a perfect mixture with the bicarbonate solution. Not only was total

TABLE 1. Recovery of aflatoxins B_1, G_1, and M_1 from inoculated milk, yogurt, and cheese samples extracted by a diphasic dialysis technique using a chloroform solvent.[1]

Product		Sample size	Contamination	Aflatoxin	Recovery			
					Mean	Range	Mean	CV
	(n)		(ppb)		(ppb)		(%)	
Milk	8	50 ml	.050	B_1	.032	.025–.038	64.8	11.9
				G_1	.033	.029–.042	68.2	12.1
				M_1	.048	.038–.055	96.5	12.7
Yogurt	5	50 g	.047	B_1	.039	.033–.046	86.7	13.6
				G_1	.046	.035–.050	99.0	16.6
				M_1	.046	.036–.055	97.8	18.2
Cheese	5	50 g	.10	B_1	.067	.053–.095	67.5	17.0
				G_1	.084	.066–.090	84.1	14.0
				M_1	.093	.073–.100	93.5	15.0

[1]Extraction conditions: shaken at 120 rpm for 5 h (milk and yogurt) or 18 h (cheese).

volume increased when the bicarbonate solution was added, but the aflatoxins also were captured by the casein granules in the cheese.

Figure 1 shows the influence of extraction time at different milk volumes on the percentage of recovery of aflatoxin M_1. When extraction time was increased, the percentage of recovery was higher. However, when large volumes of milk were used, the percentage of recovery was lower than in smaller volumes. Nevertheless, more aflatoxin was extracted from large milk volumes. This result is important when the diphasic dialysis technique is standardized for each particular laboratory.

In our previous studies (unpublished data), effect of pH on percentage of recovery was tested, and the normal pH conditions of the products were the most suitable because they gave the best recovery without altering the extract; thus, the process was simplified further. Milk at its natural pH (ca. 6.7) gave 96.5% recovery of aflatoxin M_1, but higher or lower pH led to lower recovery (56% at pH 7.7, 82% at pH 5.7, and 70% at pH 4.7) and produced changes in the extract from precipitation.

Various combinations of developing solvent were tested, and aflatoxins were best separated by use of a mixture of 95:5 (vol/vol) chloroform:methanol (7). This solvent system is also suitable for the simultaneous determination of all three aflatoxins (B_1, G_1, and M_1) in the same extract. For cheese, however, the occasional presence on the plate of substances interfering with aflatoxin M_1 made quantification difficult near .10 ppb, so other solvents were tested for the separation of this mycotoxin. Best results were obtained from a 90:10 (vol/vol) mixture of chloroform: methanol, and this solvent is recommended for quantification of the aflatoxin M_1 at contamination approaching .10 ppb, the detection limit.

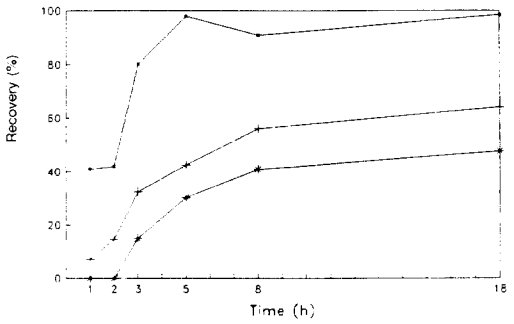

Figure 1. Influence of time on recovery of aflatoxin M_1 from different volumes of milk contaminated at .050 ppb: 50 ml (■), 250 ml (+), and 1000 ml (*).

Diphasic dialysis is less effective in cheese because the preparation of cheese samples is more complex and also because of the need to concentrate the extract to a greater volume (500 μl) to eliminate interferences on the plates.

The present results show that both percentage of recovery and detection limit are comparable with those of other techniques. Stubblefield (9) reported 80% recovery and .1 ppb detection limit for aflatoxin M_1 in milk. Domínguez et al. (4) reported 92.8% recovery and a detection limit of .02 ppb for aflatoxin M_1 in milk and yogurt. Gauch et al. (6) showed 78% recovery and a detection limit of .005 ppb for aflatoxin M_1 in milk, and Tuinstra et al. (10, 11) showed 50% recovery and a detection limit of .02 ppb for aflatoxin M_1 in milk.

CONCLUSIONS

The results of this work showed that the diphasic dialysis technique allowed the extraction of aflatoxin from different dairy products with low contamination.

There are several other advantages to this new technique. It is simple because the number of stages is greatly reduced. It is economical because of the reduced use of reagents and other materials. Aflatoxins can be extracted from different products by making only the minimal changes required by the physical nature of the product. The extraction method, combined with more sensitive detection techniques, such as HPLC, may improve detection limits and may achieve suitable conditions for its application to other mycotoxins. This aspect is currently under study in our laboratories.

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