Determination of Aflatoxin M_1 by High-Pressure Liquid Chromatography Using Fluorescence Detection

A high-pressure liquid chromatography method developed for aflatoxins B_1, B_2, G_1, and G_2 in various foods was extended to the determination of a residue metabolite, M_1, in dairy products. Products tested were fluid milk, sour cream, cottage cheese, and buttermilk. The method was found to be rapid, accurate, sensitive, and selective. M_1 was determined at levels as low as 0.3 ppb and confirmed by formation of the trifluoroacetic acid reaction product. Recoveries were 93–125% at 1 ppb in three products.

Aflatoxins, produced by certain fungi, occur naturally in a wide variety of foods. The most often encountered of the group, aflatoxin B_1, is a recognized carcinogen and shows other toxic characteristics. When B_1 is ingested by dairy cows, a portion of it is metabolized to M_1 which may be found in the milk. The metabolite is about 75% as toxic to ducklings as the parent compound (Wogan, 1969). A recent incident involving the wide distribution of cottonseed containing high levels of aflatoxin B_1 caused contamination of milk and milk products with M_1. Determination of M_1 at levels of 0.5 ppb or below in fluid milk and higher levels in other dairy products was necessary. The official (AOAC, 1979) procedure employing two-dimensional thin-layer chromatography (TLC) with visual or densitometric measurement is often complicated by fluorescent extractives not well separated from the M_1 (Van Egmond et al., 1978). A high-pressure liquid chromatography (LC) method (Takahashi, 1977; Beebe, 1978) which has been found by this laboratory to be sensitive and precise for the measurement of aflatoxins B_1, B_2, G_1, and G_2 was extended to include M_1.

The method employs reverse-phase LC separation, followed by fluorescence detection of the aflatoxins and/or their trifluoroacetic acid (TFA) reaction products. Treatment with TFA causes the addition of water across the double bond of the terminal furan ring in B_1 and G_1 to form B_{2a} and G_{2a}, respectively. Figure 1 illustrates these structural changes. This increases the fluorescence of the molecules in the reverse-phase solvent system and decreases their retention times (t_R). Although the exact configuration of the M_1/TFA reaction product has not yet been established, we have noted changes in fluorescence and t_R similar to those of B_1 and G_1 and therefore have assumed similar structural changes. The reaction product, tentatively designated M_{2a}, is three–four times more fluorescent than M_1. Under the conditions described below, we have found the reaction reproducible and quantitative.

EXPERIMENTAL SECTION

Sample Preparation. Dairy products (fluid milk, sour cream, cottage cheese, and buttermilk) were blended with acetone–water (3:1, moisture content of product included in water figure), filtered, treated with lead acetate, followed by sodium sulfate, and refiltered. The filtrate was washed with hexane and extracted with chloroform. Duplicate aliquots of chloroform extract, each equivalent to 20 mL of milk, were placed in vials and evaporated (AOAC, 1975).

Trifluoroacetic Acid Reaction. Hexane (200 μL) was added to the residue in each vial. To one portion, 50 μL of TFA was added, and the vial was capped, mixed, and allowed to react for 15 min. Both portions were then diluted with 5 mL of water–acetonitrile (9:1). The aqueous layers were withdrawn and filtered through a syringe-mounted Millipore filter (type LS). Inject in LC on the same day as derivatized.

Table I. Correlation of Naturally Occuring M_1 in Milk and B_1 in Cottonseed[a]

source	milk M_1, ppb	cottonseed B_1, ppb	$(M_1/B_1) \times 10^{-4}$
A	4.3	5690	7.6
B	0.68	1090	6.2
C	8.8	7400	11.9
D	6.7	7510	8.9
E	6.4	7050	9.1
F	5.0	8840	5.7

[a] Cottonseed homogeneity and feeding rations unknown.

Standard Solutions. M_1 (10 ng, Eureka Labs, Sacramento, CA) and 5 ng of M_2 (Sigma Chemical Co., St. Louis, MO) were placed in each of two vials, evaporated to dryness, and treated as above (hexane omitted).

LC System. The liquid chromatograph used was Spectra-Physics, (Santa Clara, CA) Model 3500B with dual pumps, solvent programer, and 500-μL loop injector. The LC column (3.2 mm i.d. × 25 cm) was packed with 10 μm Spherisorb-ODS. Mobil phases were (A) water and (B) acetonitrile–methanol (3:2). Solvents were filtered through 5 μm Millipore filter and degassed under vacuum. Operating conditions were: temperature, ambient; flow rate, 3.2 mL/min (constant); initial pressure, 2300 psi (automatically varied); gradient, linear from 10% B to 20% B in 20 min (program initiated upon injection). Detection was by Aminco Fluoro-Monitor (American Instrument Co., Silver Spring, MD), excitation at 365 nm and detection above 400 nm, using 10-mV recorder and chart speed of 10 cm/h. Sensitivity was adjusted to give half-scale deflection with 1 ng of M_{2a} equivalent.

RESULTS AND DISCUSSION

Chromatograms in Figure 2 show the separation of the six aflatoxins we have studied. Note that when the TFA reaction is allowed to proceed for 15 min, the M_1, B_1, and G_1 are totally converted to M_{2a}, B_{2a}, and G_{2a}. However, if the reaction is stopped by dilution immediately after the TFA is added, the B_1 and G_1 are converted but the M_1 conversion is not yet detectable. These standard chromatograms include M_2, which became available to us quite recently. With present cleanup methods, M_2 is obscured in the sample chromatogram by a fluorescent artifact.

Figure 3 shows successive injections of TFA-treated standard, nontreated standard, TFA-treated sample, and nontreated sample. Comparison of peak heights allows quantitation and the peak shift provides identification. The change of peak height and retention time between the M_1 and M_{2a} in the treated and nontreated portions is visible even if the product contains some background fluorescent materials. Most of the dairy products analyzed

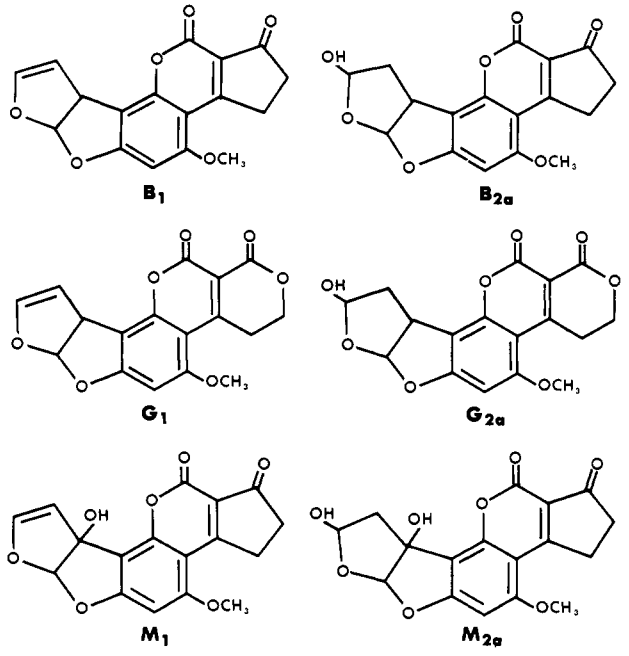

Figure 1. Chemical structures of some aflatoxins.

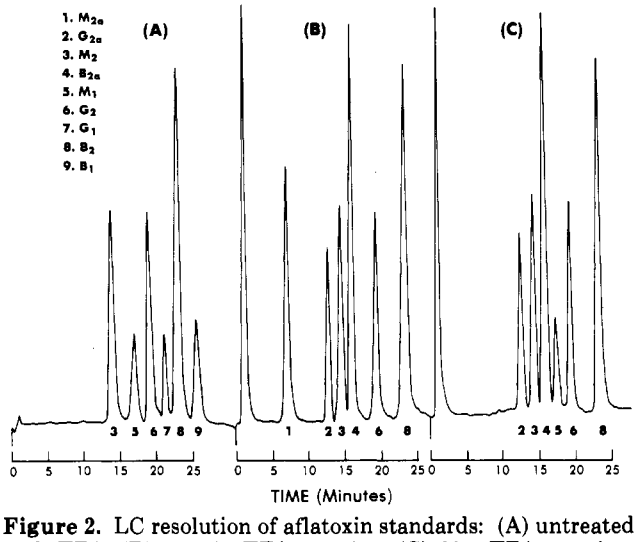

Figure 2. LC resolution of aflatoxin standards: (A) untreated with TFA, (B) 15-min TFA reaction, (C) 30-s TFA reaction. Amounts injected: (1, 3, 5) 1 ng, (2, 4, 6, 8) 0.5 ng, (7, 9) 15 ng each. LC parameters are as described in the text.

contained a fluorescent artifact which eluted just prior to the M_1. The height and t_R of this peak did not change with the addition of TFA. With isocratic elution, this artifact may interfere with M_1, requiring further cleanup of the sample extracts.

During the investigation of the aforementioned incident, a number of milk samples were analyzed for M_1 along with the associated cottonseed which had been fed at the rate of about 4 lb daily per cow along with other unknown feed for approximately one month. Table I lists the B_1 and M_1 contents of related samples. Although this was not a

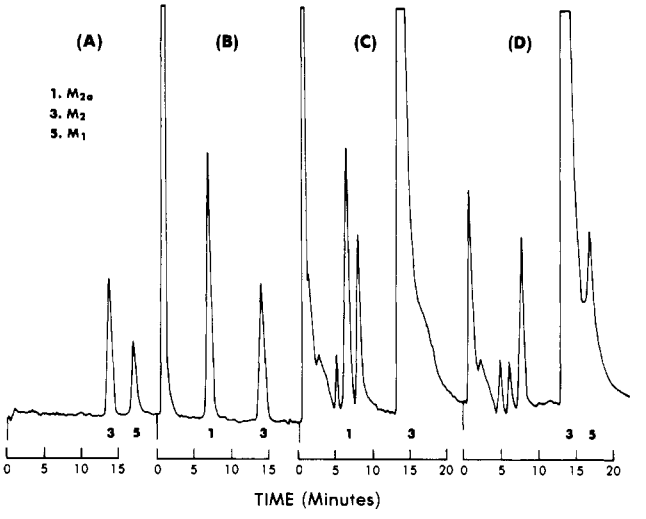

Figure 3. LC determination of 1 ppb aflatoxin M_1 in milk: (A) standards untreated with TFA, (B) standards with 15-min TFA reaction, (C) milk sample with TFA, (D) milk sample, no TFA.

controlled feeding study, there appears to be definite correlation between B_1 feeding levels and M_1 residue levels.

A limited recovery study was done using one sample each of sour cream, buttermilk, and milk. At the level of 1 ppb added M_1, the recoveries were 110, 125, and 93%.

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