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Note

Rapid determination of aflatoxin M_1 in dairy products by reversed-phase high-performance liquid chromatography

JEAN-MARC FREMY* and BERNADETTE BOURSIER

Laboratoire Central d'Hygiène Alimentaire, Ministère de l'Agriculture, Direction de la Qualité, Services Vétérinaires, 43 Rue de Dantzig, 75015 Paris (France)

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It has been shown that aflatoxin M_1 (AfM_1) is the aflatoxin residue excreted in milk. The amount of excreted AfM_1 in milk corresponds to 0.25–1.5% of the amount of ingested AfB_1[1–4].

The thin-layer chromatographic (TLC) methods available before 1976 could detect AfM_1 levels in dairy products down to 1.0 ppb*. Since then, several methods have been reported which permit lower detection limits. Stubblefield[5] used a silica gel mini-column clean-up procedure, but this procedure has the disadvantage that an important emulsion frequently occurs at the extraction step and that automation for routine control is impossible. Winterlin *et al.*[6] and Beebe and Takahashi[7] reported high-performance liquid chromatographic (HPLC) methods that are interesting for routine control; however, they cannot be used for dried milk and dairy baby food. These HPLC methods[6,7] have detection limits (0.6 and 0.2 ppb) that are higher than that of the TLC method[5] (0.1 ppb).

We now describe a method that involves a rapid clean-up of several dairy products: liquid and dried milk, liquid and powdered whey, caseinate, fresh and ripened cheeses. The detection limit is comparable to that of TLC: 0.1 ppb. The identification of AfM_1 is rapidly confirmed by a chemical reaction with trifluoroacetic acid (TFA), according to Beebe[8].

EXPERIMENTAL

Equipment

The following equipment was used: an oscillatory shaker (Laboral; Prolabo, Paris, France); a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph, equipped with a M6000A pump and a U6K septumless injector; a Schoeffel (Westwood, NJ, U.S.A.) Model 970 fluorescence detector with variable-wavelength excitation, excitation at 360 nm and cut-off filter at 389 nm; and a stainless-steel column (25 cm × 4 mm I.D.), packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.; article No. 9334).

* Throughout this article, the American billion (10^9) is meant.

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Reagents

All solvents were distilled-in-glass solvents for HPLC; the mobile phases were filtered on Millipore (Bedford, MA, U.S.A.) FH (0.5 μm) and HA (0.45 μm) filters.

Sep-Pak silica gel cartridges (Waters Assoc.) and LiChrosorb RP-18 (E. Merck) were used as stationary phases. For Sep-Pak, chloroform–methanol (9:1) was used as the mobile phase, for LiChrosorb water–acetonitrile (72:28).

An AfM_1 standard solution was prepared by dissolving 10 μg of commercially available AfM_1 (Makor, Jerusalem, Israel) in 2 ml methanol; the solution was diluted to 0.5 μg AfM_1/ml.

Extraction

A 50-ml volume of liquid milk was transferred into a 250-ml glass-stoppered erlenmeyer flask to which 125 ml chloroform and 5 g of diatomaceous earth were added.

For powdered milk, a 10-g sample was weighed, and 15 ml water, 125 ml chloroform and 5 g of diatomaceous earth were added.

For cheese, a 10-g sample was weighed, cut in small pieces, and 15 ml water, 125 ml chloroform and 5 g of diatomaceous earth were added.

All samples were shaken slowly for 30 min, using an automatic shaker. The samples were then filtered through filter-paper the filtrate (the volume of which should be recorded) was transferred into a 250-ml round-bottomed flask and evaporated to near dryness, using a 50°C water-bath and a rotary evaporator.

Clean-up

A Sep-Pak cartridge was connected to a 10-ml glass syringe. The residue was dissolved in 2 ml chloroform and transferred to the syringe. The round-bottomed flask was washed twice with 1-ml portions of chloroform and the washings were quantitatively transferred to the syringe. The resulting solution was injected into the Sep-Pak. Then the Sep-Pak cartridge was washed with 2 ml hexane and with 2 ml diethyl ether. The eluates from the Sep-Pak were discarded. A 3-ml volume of chloroform–methanol (9:1) was added to the syringe. The first 1-ml fraction eluted from the Sep-Pak was discarded, and the following 2 ml (containing the AfM_1 fraction) were transferred to a 5-ml vial. The solvent was evaporated to dryness over a 50°C water-bath with a gentle stream of nitrogen. The residue was dissolved in 100 μl of methanol for HPLC analysis.

Standard curve and determination

The following conditions were used to obtain a standard curve: AfM_1 standard solution, 0.5 μg/ml in methanol; flow-rate, 2 ml/min; pressure, 1200 p.s.i.; fluorescence detector, excitation wavelength 350 nm, emission cut-off filter 389 nm, sensitivity 6.30, range 0.01 μA, time constant 5 sec. The calibration curve was obtained by plotting several amounts of AfM_1 (0.5–10 ng) against the observed peak areas.

For determination, the sample extracts was injected under the same conditions; the injection volume was either 20 or 40 μl, depending on the concentration of AfM_1 in the sample.

Identification

The methanolic solution of the residue employed in the HPLC analysis was evaporated to dryness over a 50°C water-bath under a gentle stream of nitrogen. The residue was dissolved in 100 μl hexane. A 25-μl volume of TFA was added, and the compounds are mixed. After 10 min in the dark at room temperature, the reaction mixture was evaporated to dryness over a 50°C water-bath under a stream of nitrogen. The TFA-treated sample residue was dissolved in methanol; samples of this solution should be injected on the same day as they are derivatized.

Simultaneously, 20- and 40-μl volumes of the AfM_1 standard solution were transferred to a 4-ml vial and evaporated to dryness with minimum heating under a stream of nitrogen. The derivatization solvents were added and the standard treated as the dairy sample.

RESULTS AND DISCUSSION

The Sep-Pak silica cartridge removes much of the lipids and pigments from the sample.

AfM_1 is completely eluted by the elution solvent used. This is a quick and easy clean-up step. The AfM_1 levels in the sample can be estimated easily when they are in the range of the calibration curve (linear response from 0.5 to 15 ng). Table I shows the recoveries of AfM_1 added to dairy product samples in the range 0.1–5.0 ppb. The recoveries were between 80 and 120%, the variation being due mainly to the extraction step (see Table I).

TABLE I

PERCENTAGE RECOVERIES AND DETECTION LIMITS

NM = Detected but recovery not measured precisely ($\approx 110 \pm 25\%$); NR = not recovered, not detected.

Dairy product	*Sample*	*AfM_1 added (ng)*	*Level (ppb)*	*Recovery (%)*	*No. of determinations*
Liquid raw milk	50 ml	25	0.5	71 (±10)	4
		5	0.1	85 (±15)	4
		1	0.05	NM	4
		0.5	0.01	NR	2
Dried milk	10 g	10	1	110 (±10)	3
		5	0.5	90 (±10)	4
		1	0.1	NM	4
		0.5	0.05	NR	3
Cheese	10 g	10	1	60 (± 8)	4
		5	0.5	70 (±15)	3
		1	0.1	NM	4
		0.5	0.05	NR	2

Fig. 1 illustrates a typical chromatogram of 4 ng AfM_1 standard and of 4 ng AfM_1 standard derivatized by the TFA reaction. The retention times of AfM_1 and derivatized AfM_1 are 8.0 and 4 min, respectively.

Fig. 2 shows a chromatogram of a sample of powdered milk in which no AfM_1

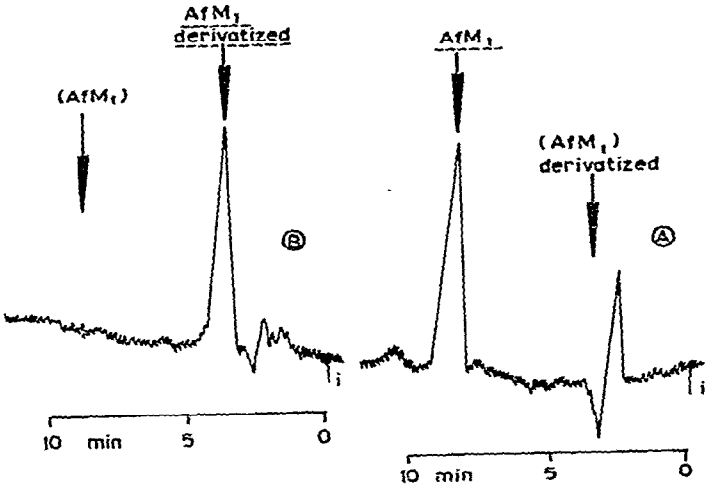

Fig. 1. Chromatograms of AfM_1 standard (4 ng) (A) and of TFA-derivatized AfM_1 standard (4 ng) (B).

was detected, and a chromatogram of the same sample spiked with 0.1 ppb of AfM_1.

Fig. 3 shows chromatograms of a liquid milk sample and a cheese sample.

Fig. 4 illustrates the TFA reaction to confirm the AfM_1 identity in a powdered milk sample. From the fact that the AfM_1 peak disappears, it can be concluded that the TFA reaction is complete.

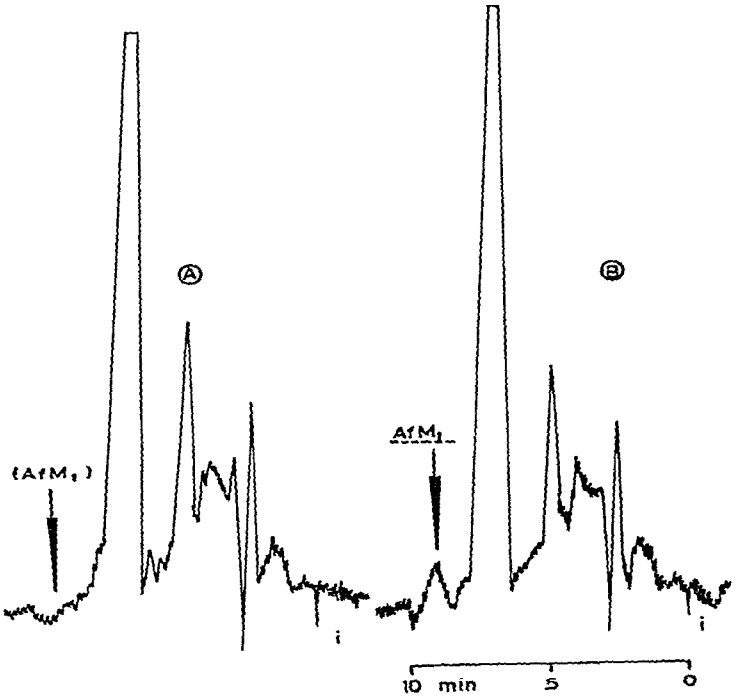

Fig. 2. Chromatograms of a sample of powdered milk (A) and of the same sample spiked with 0.1 ppb AfM_1 (B).

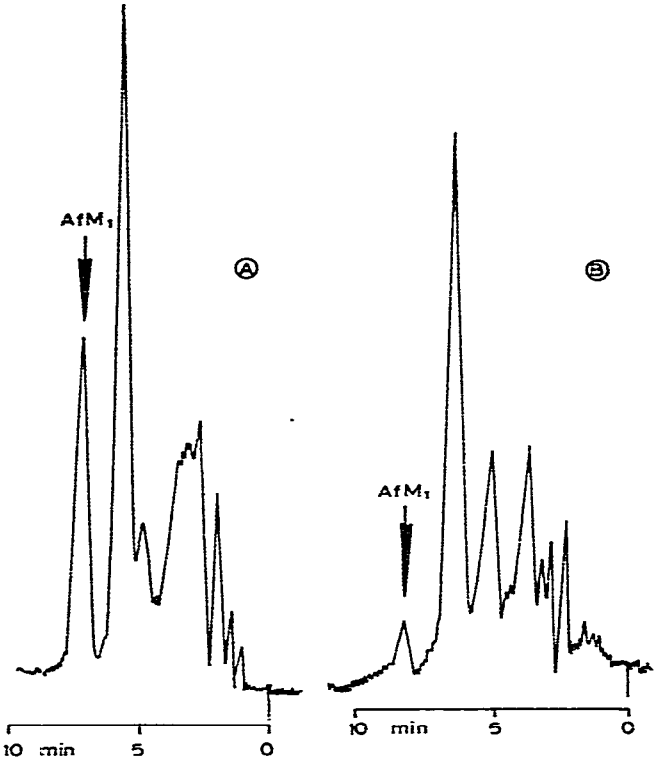

Fig. 3. Chromatograms of liquid raw milk containing 0.5 μg/l AfM_1 (A), and of Camembert cheese containing 0.2 μg/kg AfM_1 (B).

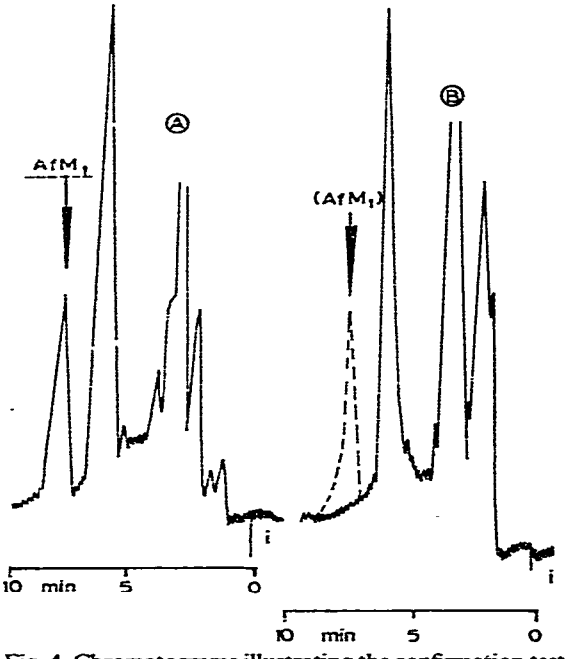

Fig. 4. Chromatograms illustrating the confirmation test of the AfM_1 identity. A, Naturally contaminated powdered milk (1.2 ppb); B, as A, but after TFA derivatization.

In conclusion, the method described is suitable for the rapid determination of AfM_1 in dairy products. The complete analysis, including the confirmation of the AfM_1 identity, can be performed in 3 h. The method is both simple and cheap. AfM_1 can be detected at 0.1 ppb levels in cheese, dried whey and dried milk (results can be given in μg/l: 0.01 μg/l reconstituted 10% liquid milk) and at 0.05 ppb levels in liquid raw milk.

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