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Note

Sensitive reversed-phase high-performance liquid chromatographic determination of aflatoxin M_1 in dry milk

ALESSANDRO CARISANO* and GIANCARLO DELLA TORRE
Laboratorio Controllo Qualità, STAR S.p.A., 20041 Agrate Brianza MI (Italy)
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When cows eat feeds that have been attacked by fungi such as *Asperfillus flavus* and *A. parasiticus*, their milk contains aflatoxin M_1, produced by the hepatic metabolization of aflatoxin B_1. Since aflatoxin M_1 is as toxic and carcinogenic as aflatoxin B_1, a fast and accurate method for detecting and quantifying this contaminant in milk —particularly in the case of milk used as an infant food— is very important.

Among the various national legislations that have established a provisional or definitive limit for aflatoxin M_1 in milk, Swiss law —which is the most restrictive— has fixed a limit of 10 ng/l for reconstituted powdered milk for babies. Given this, any method selected must be capable of detecting, with a reasonable degree of reliability, quantities of less than 10 ng/l. None of the high-performance liquid chromatographic (HPLC) methods[1–9] for detecting aflatoxin M_1 permits such a degree of sensitivity since, at best, they are capable of detecting 10 ng/l[6].

This paper describes a method that permits reliable quantification of aflatoxin M_1 at levels well below 2 ng/l without having to handle large quantities of samples and solvents.

EXPERIMENTAL

Equipment and column

All the analyses were performed using a liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with two Model 6000A pumps, a Wisp 710B automatic injector, a M721 system controller, a M730 data module and a LS-5 luminescence spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.) with a 20-μl quartz flow cell, excitation wavelength of 358 nm (slit width 15 nm), emission wavelength of 428 nm (slit width 20 nm) and fixed scale = 20 (= sensibility × 20).

The column was a Nova-Pak C_{18}, 5-μm cartridge, 100 × 5 mm I.D., fitted in a RCM 100 compression module (Waters).

Reagents

Aflatoxins M_1 and B_2 were purchased from Makor Chemicals (Jerusalem, Israel).

A standard solution of aflatoxin M_1 was prepared by dissolving 10 μg of it in 10 ml of acetonitrile. A working solution was prepared by diluting 100 μl of this

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standard solution in 10 ml of acetonitrile. A standard solution of aflatoxin B_2 was prepared by dissolving 5 mg of it in 10 ml of acetonitrile–chloroform (8:2). A 10-μl volume of this standard solution was then diluted in 10 ml of acetonitrile. A working solution was prepared by diluting 75 μl of the latter solution in 10 ml of acetonitrile.

All aflatoxin solutions should be stored in a refrigerator at −10°C in well sealed aluminium foil-wrapped containers.

All the reagents were HPLC grade. All the water used was obtained from a Milli-Q water-purification system (Millipore, Bedford, MA, U.S.A.).

A Sep-Pak C_{18} cartridge (Waters) was used for extracting M_1 from milk.

Preparation of analytical solutions

A 50-g amount of powdered milk was weighed into a volumetric flask and adjusted to volume (500 ml) with water. After shaking well to obtain a homogeneous sample, 20 ml of the solution were placed in a flask already containing 100 μl of the working solution of aflatoxin B_2 as internal standard (I.S.). The Sep-Pak C_{18} cartridge was washed with 10 ml of acetonitrile followed by 10 ml of water, using a 10-ml glass syringe and applying sufficient vacuum to the base of the cartridge to create a flow-rate of approximately 5 ml/min.

The reconstituted milk was then passed through the cartridge, by applying a vacuum to give a flow-rate of about 5 ml/min. The loaded cartridge was then washed with 10 ml of water, 10 ml of 10% basic acetonitrile (ammonia–acetonitrile–water, 1:10:90), 10 ml of 10% acidic acetonitrile (acetic acid–acetonitrile–water, 1:10:90) and finally 15 ml of water. The washings were discarded and air was sucked through the cartridge for 1 min. It was then eluted with 10 ml of acetonitrile and the eluate collected in a 15 ml conical tube, equipped with a screw-cap. The eluate was evaporated to dryness at 40°C using a gentle flow of nitrogen. To the residue were added 200 μl of hexane and 50 μl of TFA (trifluoroacetic acid) in order to derivatize the aflatoxin M_1. The tube was closed with the cap, the contents mixed well using a vortex mixer and left in an oil-bath for 10 min at 40°C. The mixture was evaporated to dryness using a gentle flow of nitrogen for 7 min at room temperature. To the residue were added 200 μl of acetonitrile–water (30:70) and mixed well using a vortex mixer. A 50-μl volume of working aflatoxin M_1 solution and 100 μl of working aflatoxin B_2 solution were placed in another conical tube. This solution was evaporated to dryness and the residue derivatized using hexane and TFA as for the sample. The final residue was taken up with 200 μl of acetonitrile–water (30:70) and mixed well with the vortex mixer.

Chromatographic procedure

The Wisp was set to inject 50 μl of each sample and to continue the analysis for 28 min. The solvents used to create the gradient elution were (A) methanol–water (10:90) and (B) methanol–water (65:35). The following program was used: linear gradient from 0 to 1 ml/min in 1 min with solvent A; linear gradient from 0 to 45% B in 9 min; linear gradient from 45 to 50% B in 13 min; isocratic at 50% B for 5 min. To recondition the column, the solvent A is passed for 10 min at a rate of 1 ml/min.

RESULTS AND DISCUSSION

The introduction of aflatoxin B_2 as an I.S. is extremely important since, given the need for a certain amount of manipulation of the sample for clean-up, the presence of the I.S. makes it unnecessary to go through a tedious procedure of rinses, for complete recovery, and to measure exactly the volume of the solutions to be analyzed. Aflatoxin B_2 is particularly suitable as an I.S. since it has intense fluorescence at the wavelengths used to detect aflatoxin M_1. It is not present in milk since any aflatoxin B_2 contained together with aflatoxin B_1 in feeds is metabolized hepatically to aflatoxin M_2 which is more polar than B_2 and thus leaves the C_{18} reversed-phase column much earlier than aflatoxin B_2.

With the gradient used, aflatoxin M_{2a} (resulting from the derivatization of M_1) and aflatoxin B_2 appear in a zone with no appreciable peaks due to extraneous fluorescent substances which are inevitably present in milk, even after exhaustive clean-up (Figs. 1 and 2). Nevertheless the presence of foreign substances is limited since the Sep-Pak C_{18} extraction and clean-up method proposed by Takeda[4], was found to be very effective in eliminating fluorescent inferfering substances, such as B-complex vitamins.

We also derivatized aflatoxin M_1 to aflatoxin M_{2a}, using hexane and TFA, according to the method proposed by Hisada *et al.*[6], not only because the fluorescence of M_{2a} is approximately six times greater than that of M_1, from which it is derived —thus permitting the achievement of greater sensitivity— but also because this makes it possible to differentiate M_1 from fluorescent interfering substances which might be present in milk from cows fed with citrus-fruit residues.

When the preparation of a sample or standard has been started, the process must be continued until dissolution of the derivative in acetonitrile–water (30:70) since, if operations are interrupted prior to this point, aflatoxin M_1 may be lost.

The derivative, dissolved in 200 ml of acetonitrile–water (30:70), can be kept for at least a week prior to analysis, in a refrigerator away from the light.

Recovery, accuracy and precision

To determine the average recovery and precision possible using the method described, we analyzed six samples of milk, which did not contain any detectable quantities of aflatoxin M_1, to which 10 ng/l of M_1 were added, and six samples to

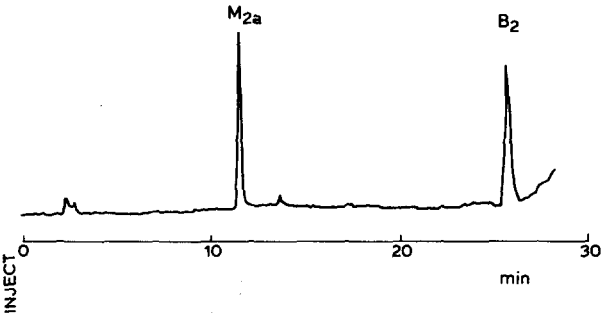

Fig. 1. Chromatogram of a standard solution of 300 pg aflatoxin M_1 corresponding to 15 ng/l. Chart speed = 0.5 cm/min.

TABLE I

RECOVERY OF AFLATOXIN M_1 ADDED TO A RECONSTITUTED MILK

Sample No.	*Amount of aflatoxin (ng/l)*		*Recovery (%)*
	Added	*Found*	
1	10	9.27	92.7
2		8.86	88.6
3		9.75	97.5
4		9.90	99.0
5		9.50	95.0
6		8.11	81.1
Mean ± S.D.		9.23 ± 0.66	92.3
7	25	25.43	101.7
8		25.55	102.2
9		26.55	106.2
10		24.73	98.9
11		25.88	103.5
12		25.12	100.5
Mean ± S.D.		25.49 ± 0.62	102.2

which 25 ng/l had been added. As is seen in Table I, the recoveries were 92.3 and 102.2% respectively, with an average of 97.2%. Table I also shows the precision of the method, the mean coefficient of variation (standard deviation/average · 100) being 4.8%.

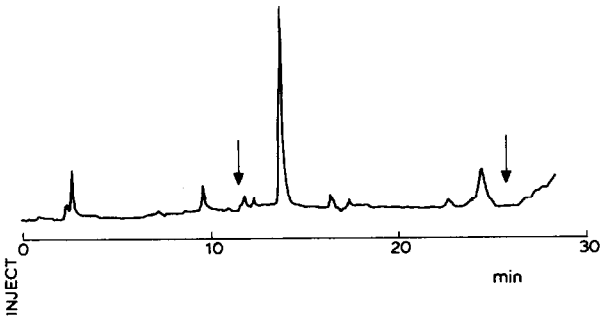

Fig. 2. Chromatogram of a reconstituted milk with no detectable aflatoxin M_1. The arrows indicate where aflatoxin peaks would be found if present. Chart speed = 0.5 cm/min.

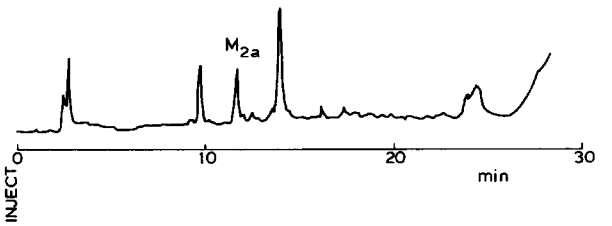

Fig. 3. Chromatogram of a reconstituted milk to which 5 ng/l of aflatoxin M_1 had been added. Chart speed = 0.5 cm/min.

Linearity and detection limit

Fig. 3 shows that the detection limit, expressed as the minimum quantity of aflatoxin M_1 present in the solution analyzed equivalent to three times the signal-to-noise ratio, corresponds to 1 ng/l of reconstituted milk.

The linearity of response of the aflatoxin M_1, derivatized to M_{2a}, was found to be excellent in the tested range: 2.5–50 ng/l of reconstituted milk.

CONCLUSIONS

The method described enables the determination in milk, reconstituted from powdered milk, of aflatoxin M_1 in quantities of less than 2 ng/l. It is sufficiently precise and accurate. The separation of aflatoxin M_1 from milk and its clean-up are performed using only a Sep-Pak C_{18} cartridge and modest quantities of solvents.

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