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Note

Assay method for aflatoxin in milk

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During the last three years we have been interested in developing a sensitive, reliable method to detect and quantitate aflatoxins in milk. The method has now been successfully tested and used to study the effects of some parameters on the appearance of aflatoxins in milk and the analysis of commercial dairy samples in France obtained during a period of importation of groundnut cakes heavily contaminated by aflatoxins. For these purposes the previously reported techniques were not very satisfactory. For example, the method of Pons *et al.*[1], modified by Stubblefield and Shannon[2], did not permit a sufficiently clean extract for the sensitivity desired; this observation was equally true for the technique of Jacobson *et al.*[3] applied to powdered milk. The major inconvenience of Tuinstra and Bronsgeest's method[4], which offers low detection limits, is the utilization of two-dimensional thin-layer chromatography (TLC). Finally, in our hands, the method elaborated by Gauch *et al.*[5] using an Extrelut column did not produce the high quality clean-up claimed by the authors.

Our method was designed to: (1) use a powerful extraction agent in order to break the anticipated bonds of aflatoxin M1 which is more polar than aflatoxin B1; (2) avoid the employment of a completely water immiscible organic phase for the extraction of aflatoxin from milk, which is probably incomplete and depends on several factors; (3) prevent the formation of a colloidal protein precipitate by contact with certain organic solvents; (4) avoid the adsorption of the mycotoxin on the precipitate of proteins caused by heavy metals; (5) obtain a detection limit of 5 ng/kg, at least for the liquid milk, and 50 ng/kg for powdered milk, with good repeatability.

MATERIALS AND METHODS

Reagents

The solvents were isopropanol, methanol, chloroform, toluene, diethyl ether (0.01 % ethanol) and acetone (Merck "pro analysis", or Carlo Erba RPE or equivalent). Reagent grade epichlorohydrin and glass-distilled water were also used. Celite 545 (Johns-Manville, Denver, CO, U.S.A.) was employed as filter aid. The chromatographic adsorbents were Aluminoxid 90 (aktiv-basisch, Merck, 70–230 mesh) and Kieselgel H 60 (Merck). Crystallized aflatoxins M1 and M2 were obtained from Serva-Feinbiochem (Heidelberg, G.F.R.).

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Apparatus

The following were employed: 500-ml separatory funnels; rotary evaporator with cold-trap; micro-syringes or Microcaps (Drummond, Broomall, PA, U.S.A.); chromatographic columns, fitted with glass valves; thin-layer plates, Merck Si 60-5721; filter-paper, Whatman No. 1; UV lamp, Philips HP WI25; photodensitometer (Model PHI 5; Vernon, Paris, France).

Procedure

Extraction of milk powder. A 10-g amount of milk powder were mixed with 30 ml water, then added to a separatory funnel containing 60 ml isopropanol. The mixture was shaken vigorously for 1 min. Then 50 ml methanol, 10 g Celite and 250 ml chloroform were added successively. After each addition the funnel was shaken and the contents allowed to settle. The clear lower layer was filtered through paper containing 10 g of anhydrous Na_2SO_4, 300 ml of the filtrate being collected. This solution was evaporated *in vacuo* to about 5 ml on a rotary evaporator at 50°C. The remaining solvent consisting mainly of isopropanol was evaporated as an azeotrope by the addition of 30 ml chloroform. The mixture was evaporated to near dryness and the residue dissolved in 30 ml toluene. After concentration to 0.5 ml, another 30 ml toluene were added and then concentrated to 5 ml.

Extraction of liquid milk. A 50-ml sample of milk was heated in a water-bath at 100°C for 5 min. After cooling to 20°C, the mixture was extracted as above.

Alternatively 100 ml of heated milk were concentrated *in vacuo* to about 25 ml in a rotary evaporator. The volume was adjusted to 30 ml by adding water. The concentrate was then treated as described previously.

Column chromatography. The column was prepared by dry packing 10 g of anhydrous sodium sulphate and 1 g alumina in the ascending order. The column was partially filled with toluene and a slurry of 2 g silica gel in the same solvent was poured into it and firmly packed under pressure. Anhydrous sodium sulphate (2 g) was added to the toluene above the settled silica gel. All the interfaces of the packed materials were separated by pieces fo filter-paper.

The milk extract in toluene was passed through the column. The fats and other substances which interfere with TLC were eluted first with 50 ml toluene and then with 50 ml chloroform. The milk aflatoxins were eluted with 100 ml chloroform–acetone (4:1 v/v). The eluate was collected in a 250-ml round-bottomed flask and concentrated *in vacuo*. Before evaporation was complete, the residue was quantitatively transferred with chloroform to a small vial (*ca.* 1 dram) fitted with a tight cap, dried under a stream of nitrogen and redissolved in 100 μl of chloroform ready for TLC.

Thin-layer chromatography. TLC was performed on silica gel plates heated at 110°C for 20 min. 20-μl aliquots of the extract, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ng of aflatoxin M1 were spotted. A further 10 μl of the extract were spotted on a 0.3 ng of M1 which serves as an internal standard. (The disposition of this standard helps to discriminate aflatoxin M1 from other blue fluorescent spots given by extracts of some milks.) The plate was developed for 12 cm in an unlined tank containing diethyl ether–epichlorohydrin–methanol–water (90:6:3:1 v/v) which had been equilibrated for 2 h.

If aflatoxin M1 was judged to be present in a sample without internal standard,

the mycotoxin was quantitated by matching its fluorescence with a standard spot. If the fluorescence intensity of the sample spot exceeded that of the highest standard, TLC was repeated after dilution of the extract, taking into consideration the 30% of extract already used. If no aflatoxin was detected in the 20-μl sample spot the experiment was repeated with a 50-μl aliquot.

Quantitative densitometry was performed as described by Gauch *et al.*[5].

Confirmation. To confirm the nature of the fluorescent material the test elaborated by Przybylski[6] or the modification proposed by Van Egmond *et al.*[7] was used.

Calculation of the aflatoxin M1 concentration. The value given by the chromatogram corresponds to the amount of the aflatoxin contained in 300 ml of organic extract. Since the total volume of the recoverable organic phase is 360 ml for dry milk and 345 ml for liquid milk, it is necessary to introduce factors of 1.2 and 1.15 respectively for calculation of the aflatoxin level in the analysed material.

RESULTS AND DISCUSSION

The described method has been used to analyze about 1000 samples of dry or fluid milks. Several other experiments have been conducted with artificially contaminated products.

Recovery of aflatoxin M1

Samples (0.5 g) of milk powder without aflatoxin were contaminated by adding titrated solutions of aflatoxin M1. After evaporation of solvent, each of these samples was incorporated either in solutions of powdered milk (9.5 g in 30 ml distilled water) or in liquid milks without any aflatoxin.

The results in Table I show that for aflatoxin levels of 20–800 ng/l (natural or reconstituted milk) the proposed method gives average recoveries of 98.2%.

TABLE I

RECOVERY (%) OF AFLATOXIN M1 ADDED TO MILK

*Aflatoxin M1 added** (ng/l)	*Dried milk*		*Liquid milk*	
	No. of tests	*Average recovery***	*No. of tests*	*Average recovery*
20	7	96	6	105
50	7	98	6	96
100	6	95	6	95
250	2	103	2	97
800	6	98	5	99

* Natural or reconstituted milk.

** Calculated (see text).

Repeatability

The present results using the described method were obtained by only one group of analysts. On the other hand, these results all relate to naturally contaminated milks. Table II shows the slight scattering of the values even when the contamination level is low.

TABLE II

PRECISION FOR NATURALLY CONTAMINATED MILKS

m = Average value; s = standard deviation.

Sample	*No. of tests*	*m (ng/kg)*	*s*	*s/m (%)*
Dried milk				
a	6	11,033	312	2.8
b	8	4975	134	2.7
c	5	2210	72	3.3
d	6	104	11	10.7
e	8	75	9	12.2
Liquid milk				
1	6	510	15.6	3.0
2	8	372	9.3	2.5
3	6	122	6.4	5.3
4	8	71	4.8	6.8
5	8	44	6.0	13.3

Sensitivity

The detection limits for aflatoxin M1 are 5 ng/l for liquid milk and 25 ng/kg for powdered milk. It should be noted that the values for aflatoxin M are the totals for aflatoxins M1 and M2, which are not separated by the TLC phase employed.

On several occasions we have verified that M2 represents only a small fraction of the total aflatoxin M and therefore this method of expressing the results does not overestimate the (potential) health risk.

TABLE III

DETERMINATION OF AFLATOXIN M1 (ng/kg) IN NATURALLY CONTAMINATED MILKS BY THE METHOD OF STUBBLEFIELD (A) AND BY THE PROPOSED METHOD (B)

The values are means from two assays for each sample. T = Trace; ND = not detected.

Liquid milks			*Dried milks*		
Sample	*A*	*B*	*Sample*	*A*	*B*
I	250	510	A	9100	11,650
II	200	362	B	8500	11,125
III	100	235	C	4650	5875
IV	150	210	D	2950	4950
V	100	175	E	2050	2475
VI	100	160	F	400	450
VII	ND	105	G	200	275
VIII	T	80	H	100	225
IX	ND	42	I	T	160
X	ND	20	J	ND	125
XI	ND	15	K	ND	75
			L	ND	62
			M	ND	50
			N	ND	47

Comparison with the Stubblefield method

During the development of our method, an assay for aflatoxin M1 was described by Stubblefield[8]. It was therefore of interest to compare results obtained by the two methods using the same samples. Experiments were performed with naturally contaminated liquid and dried milks. The results in Table III show, chiefly for samples of liquid milk, some of the difficulties of applying the Stubblefield method.

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