ANALYSIS OF AFLATOXIN M_1 IN LIQUID AND POWDERED MILK

P. L. SCHULLER, C. A. H. VERHÜLSDONK and W. E. PAULSCH

National Institute of Public Health, P.B.1, Bilthoven, The Netherlands

ABSTRACT

A simple and rapid isolation procedure based upon the so-called Celite procedure for aflatoxin B_1 determination in peanut products can be used in combination with two-dimensional thin-layer chromatography for the determination of aflatoxin M_1 in liquid milk and milk powder. The improved separation of the aflatoxin M_1 from the interfering substances by this technique on the thin-layer chromatogram permits the measurement of aflatoxin M_1 with a fluorodensitometer. The method is sensitive to 0.05 μg aflatoxin M_1 per litre of liquid milk and to 0.5 μg per kilogramme of milk powder.

It is now well known that cows fed rations containing small amounts of aflatoxin B_1 excrete in their milk a metabolite (aflatoxin M_1), the toxicity of which is of the same order as that of aflatoxin B_1 [1-7]. Laboratory experiments suggest[8, 9] that aflatoxin M_1 may also be carcinogenic and therefore its presence in milk is undesirable. These findings have created the need for accurate and sensitive analytical methods for the estimation of aflatoxin M_1 in milk at low levels. Several authors have noticed that the amount of aflatoxin M_1 excreted in the milk bears a linear relationship to the amount of aflatoxin B_1 ingested[6, 10, 11]. It is not surprising that the Commission of the European Economic Community, in common with other countries, has proposed a directive in which maximum levels of aflatoxin B_1 in different fodders have been fixed.

Now it seems to us that intensive screening of industrially prepared feed for aflatoxin contamination as the only control method is not sufficient to ensure that the aflatoxin contamination of milk is negligible. The reason for this is that in isolated areas farmers prepare and store their own feed under less favourable conditions and it is necessary to control the food itself, in this case milk and milk products, for the absence of aflatoxins.

In the analysis of milk for aflatoxin M_1 a dried powdered sample is commonly used[12-14]. Neumann-Kleinpaul and Terplan[15] have recently reviewed the methods. We are of the opinion that a direct analysis of fluid milk for aflatoxins is desirable in order to avoid uncertainties of losses due to drying and/or prolonged storage[16]. The very few chemical methods described for assaying the aflatoxin content of liquid milk are those described by Van der Linde[3], Roberts and Allcroft[17], Brewington[18] and Jacobson[19].

We agree with Roberts and Allcroft's criticism of the method published by Van der Linde. A disadvantage of the method described by Roberts and Allcroft is that the evaporation of the water–acetone extract is accompanied by the formation of foam making the procedure very time consuming. We have found the method of Jacobson[19] the most sound; however a great deal of time, material and manipulation is required for its completion. For this reason it seems that the method of Jacobson has limited value in practice. It was worth checking the applicability of the so-called Celite procedure, a method for the determination of aflatoxin B_1 in peanut products[20], to the problem of the determination of aflatoxin M_1 in milk. The following simple and rapid procedure has been developed.

ASSAY PROCEDURE

Extraction from liquid milk

Mix 25 ml of milk with 25 ml of acetone and 50 g of acid-washed Celite. Transfer the mass obtained into a chromatographic column (600 × 45 mm). Elute the column with 200 ml of chloroform. Collect this fraction and evaporate to dryness. Dissolve the residue in 50 ml of methanol and 40 ml of n-pentane. Transfer the solution quantitatively into a separatory funnel containing 75 ml of water and a few grammes of sodium chloride. Shake and draw off the water phase into a second separatory funnel and extract again with 50 ml of n-pentane. Discard the pentane phase and extract the water phase with four 25 ml portions of chloroform. Evaporate the combined extracts to dryness and dissolve the residue in 100 µl of chloroform.

Extraction from milk powder

Mix 10 g of milk powder with 10 g of acid-washed Celite and 4 g of glass beads in a conical flask. Add 100 ml of chloroform and 5 ml of water and shake mechanically for 30 min. Filter through a fluted filter and collect 50 ml of the filtrate. Evaporate this solution to dryness and continue the procedure as described for liquid milk. However dissolve the final residue into 200 µl instead of 100 µl of chloroform.

Thin-layer chromatography

Besides the efficiency of the extraction procedure, the satisfactory estimation of aflatoxin M_1 depends to a large extent upon successful thin-layer chromatographic separation of aflatoxin M_1 from a number of interfering substances which are very close to each other and to aflatoxin M_1. In extracts obtained with the procedure described above substances interfering with the estimation can be expected. Instead of trying to eliminate the interfering substances completely from the extracts, attempts were made to improve the separation of aflatoxin M_1 by thin-layer chromatography. A two-dimensional technique came up to expectations.

Spot with a Hamilton* microsyringe on a ready-made silica gel G-HR

* Mention of a company name in this publication does not constitute a guarantee or warranty of the company's product by the National Institute of Public Health and does not imply its approval by the Institute to the exclusion of other products that may be also suitable.

coated glass plate (Macherey and Nagel) (20 × 20 cm) a spot, at 2.5 cm above the lower edge and 2.5 cm from the left edge, of 20 µl of the prepared extract, corresponding to 5 ml of liquid milk or 0.5 g of freeze-dried milk. Spot 25 µl of an aflatoxin M_1 standard (conc. 0.1 µg ml^{-1}) at 2.5 cm from the left edge and 2.5 cm below the upper edge. This standard serves only for the comparison of the R_f values. Spot for the second direction two 25 µl spots of the aflatoxin M_1 standard on an imaginary line 2.5 cm above the lower edge of the plate and 2.5 and 4.5 cm respectively from the right edge. These standards are used for calculation of the aflatoxin M_1 content. Mark lines as solvent stops in the layer of the t.l.c. plate 7 cm from the right edge and 6 cm below the upper edge. Develop the plate first in diethyl ether–methanol–water (94:4.5:1.5) in a saturated tank and after drying for 30 minutes develop the plate in the second direction in chloroform–acetone–methanol (90:10:2) in an unsaturated tank.

Estimation of aflatoxin

It is well known that the accuracy and precision of aflatoxin measurements have been greatly improved by the use of more sensitive fluorodensitometric measurements of aflatoxin directly on silica gel coated plates.

In all our experiments aflatoxin M_1 was quantitatively measured by reflectance fluorodensitometry using a Zeiss spectrofluorodensitometer equipped with a Servogor recorder type RE 511 and a Kipp integrator type BC 1. The standard aflatoxin M_1 used was made by ourselves. The purity and stability of the standard was checked by the procedure of Rodricks[21].

EXPERIMENTAL

All liquid milk and milk powder samples analysed in this study were naturally contaminated. The experiments carried out in this study can be summarized as follows.

Linearity of response

An essentially linear relationship between emitted fluorescence of aflatoxin M_1 and concentration over a reasonable concentration range is a desirable feature for analysis (*Table 1*).

Table 1. Linearity of area *v.* concentration for aflatoxin M_1 after one-dimensional chromatography

Amount spotted (ng)	Integrator area counts	Coefficient of variation	Number of experiments
0.5	12.5	6.0	6
1.0	24.5	5.0	6
2.5	66.9	4.5	12
5.0	125.0	4.5	8
10.0	240.0	2.5	4

The linear relationship between fluorescence and concentration is at least fulfilled over the range 0.25 to 10 × 10^{-3} µg per spot. This relationship also exists for two-dimensionally developed spots (*Table 2*).

Table 2. Linearity of area *v.* concentration for aflatoxin M_1 after two-dimensional chromatography

Amount spotted (ng)	Integrator area counts	Coefficient of variation	Number of experiments
0.5	13.8	6.5	8
2.5	68.1	5.0	8

Influence of developing solvent

The fluorescence of aflatoxin M_1 adsorbed on silica gel does not vary with the developing solvent used as in the case with aflatoxin B_1 [22] (*Table 3*).

Table 3. Fluorescence of aflatoxin M_1 after separation by two developing systems

Amount spotted (ng)	Chloroform–acetone–methanol 90:10:2	Diethyl ether–methanol–water 94:4.5:1.5
	Integrator area counts	Integrator area counts
0.5	12.0 ± 6.0*	12.2 ± 6.0*
1.0	22.0 ± 5.0*	21.5 ± 6.0*
2.5	63.0 ± 5.0*	61.6 ± 4.0*

* Coefficient of variation.

Reproducibility of two-dimensional chromatography

All the results mentioned below are calculated using the standards of aflatoxin M_1 developed in the second direction (chloroform–acetone–methanol) as reference.

To establish the reproducibility of the two-dimensional technique in comparison with the one-dimensional method five extracts of a milk sample from a cow fed with 0.08 mg aflatoxin B_1 per day were analysed in duplicate (sample 1). The same was done for a milk sample from a cow fed with 1.2 mg aflatoxin B_1 per day (sample 2). The coefficient of variation was calculated from differences between duplicates.

From the results (*Tables 4* and *5*) it seems that the two-dimensional method

Table 4. Reproducibility of the one- and two-dimensional t.l.c. methods for aflatoxin M_1 in liquid milk

	Aflatoxin M_1 content ($\mu g\, l^{-1}$) Sample 1	Sample 2
One-dimensional	Immeasurable	0.77 ± 10.0%
Two-dimensional	0.107 ± 9.0%	0.68 ± 3.0%

is at least as reproducible as the one-dimensional method for both liquid and freeze-dried milk. In one further example where one liquid milk extract from a milk sample containing 0.11 μg aflatoxin M_1 per litre was estimated two-dimensionally in tenfold, a coefficient of variation of the same order was obtained.

Table 5. Reproducibility of the one- and two-dimensional t.l.c. methods for aflatoxin M_1 in freeze-dried milk

	Aflatoxin M_1 content ($\mu g\, l^{-1}$)	
	Sample 1	Sample 2
One-dimensional	0.77 ± 6.0%	5.27 ± 8.5%
Two-dimensional	0.57 ± 4.0%	4.87 ± 8.0%

In the case of very low concentrations of aflatoxin M_1, the presence of non-aflatoxin spots in the chromatogram background on the one-dimensionally developed plate prevents the exact measurement of the aflatoxin. The lower value obtained with the two-dimensional method can be explained by the presence of non-aflatoxin spots, in the chromatogram background of the one-dimensionally developed plate, which may have increased the densitometer reading of the aflatoxin M_1 spot.

Accuracy of two-dimensional chromatography

To establish the accuracy of the two-dimensional technique (in comparison with the one-dimensional technique) aflatoxin M_1 was added to an extract of milk at 0.4 ppb level and to an extract of freeze-dried milk at 4.0 ppb level. Each extract was analysed in tenfold both one- and two-dimensionally. The results, summarized in *Table 6*, demonstrate a somewhat better accuracy of the two-dimensional method.

Table 6. Recovery of aflatoxin M_1 added to an extract of liquid milk and freeze-dried milk

	One-dimensional	Two-dimensional
Milk	Immeasurable	0.11
Milk + 0.4 ppb	0.54	0.50
Recovery	—	98%
Freeze-dried milk	3.97	4.09
Freeze-dried milk + 4.0 ppb	7.42	7.84
Recovery	86%	94%

ACKNOWLEDGEMENT

The authors are highly indebted to Mr B. J. van Gansewinkel for his skilfull and zealous technical assistance and to Ir. G. Kingma, director and

Ir. F. J. Olieman from the Cie. NV Mengvoeder UT-Delfia Maarssen, the Netherlands, for the help in obtaining the milk samples from their experimental farm 'Groot Kantwijk' at Vreeland.

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