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RAPID AND SIMPLE DETERMINATION OF AFLATOXIN M_1 IN MILK IN THE LOW PARTS PER 10^{12} RANGE

R. GAUCH, U. LEUENBERGER and E. BAUMGARTNER
Kantonales Laboratorium, P.O. Box, CH-3000 Bern 9 (Switzerland)
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SUMMARY

A method for extracting aflatoxin M_1 from milk is proposed in which the use of disposable Extrelut clean-up columns simplifies the analysis considerably in comparison with existing methods. The quantitative determination is based on one-dimensional thin-layer chromatography and fluorescence densitometric measurement. The detection limit is 5 ppt (parts per 10^{12}) in milk and the recovery is $78 \pm 4\%$ at a level of 50 ppt.

INTRODUCTION

It is well known that a variety of nut products can be contaminated with highly carcinogenic aflatoxins, the metabolites of several fungi such as *Aspergillus flavus* and *A. parasiticus*.

Ground peanuts and extracted ground nut meal from oil refineries are mainly used for mixing with milk cattle feed. Approximately 1% or even more of the original aflatoxin B_1 content in the diet is excreted as aflatoxin M_1 in the milk[1–3]. This metabolite has almost a similar carcinogenity to that of aflatoxin B_1 towards animals[4–6] and hence its presence in milk is undesirable.

For this reason, the use of ground nut meal, contaminated with aflatoxins for milk cattle has been prohibited in Switzerland since August 1977. In spite of this regulation, in the winter of 1978–79 some samples of raw milk could be found that contained aflatoxin M_1. Considering a proposed acceptable risk of one fatality per 10^6 years, Schlatter[5] calculated a maximal daily intake of about 1–10 ng per person, and based on a daily consumption of 1 l of milk or equivalent amounts of milk products per person, a detection limit of at least 5–10 ppt* is needed. As a consequence, the Swiss food control organisations were confronted with the problem of analysing hundreds of milk samples in order to check individual milk producers. The methods cited in the literature were either not sensitive enough or were too tedious [two-dimensional thin-layer chromatography (TLC), column chromatography

* Throughout this article the American trillion (10^{12}) is meant.

and high-performance liquid chromatography (HPLC) with an extended clean-up][7–10].

In this paper, we describe a simple and rapid procedure for the determination of aflatoxin M_1 contents in milk with a detection limit of 5 ppt. The clean-up is based on a method introduced successfully for the analysis of the mycotoxin patulin in fruit juices and preservatives and saccharin in various foods[11,12]. In addition, a conventional clean-up procedure with a detection limit of 5 ppt is described.

EXPERIMENTAL

Principle

The raw milk proteins are precipitated with concentrated hydrochloric acid before dilution with methanol. The coagulation is accelerated by heating the mixture. The filtrate is pipetted directly on to an Extrelut clean-up column and aflatoxin M_1 is eluted with a mixture of dichloromethane and toluene. This eluate is washed with aqueous sodium hydroxide and hydrochloric acid prior to TLC. The silica gel plate with the spotted standards and samples is developed first with chloroform–acetone (9:1) and subsequently in the same direction with dichloromethane–acetone (6:4). Densitometric quantification is effected by fluorescence (365 nm excitation and 420 nm emission).

Apparatus and reagents

Extrelut pre-packed columns and Extrelut refill pack were obtained from Merck (Darmstadt, G.F.R.). For the densitometric measurements a chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.) connected to an SP 4000 integrator (Spectra Physics, Santa Clara, Calif., U.S.A.) was used. TLC plates (Kieselgel 60) were purchased from Merck and aflatoxin M_1 from Bio-Science Products (Reussbühl, Lucerne, Switzerland). Chloroform and acetone were distilled in glass, whereas dichloromethane, sodium sulphate and hydrochloric acid were used as received from Merck.

Preparation of standard solution

The preparation and determination of an aflatoxin M_1 standard solution were carried out according to the AOAC[13], and the identity of aflatoxin M_1 was confirmed by mass spectrometry. The standard had to be diluted with chloroform to 77 pg/μl for TLC analysis with the Extrelut column and to 125 pg/μl for conventional clean-up.

Sample extraction and preparation

To 50 ml of fluid milk *ca.* 0.4 ml of 25% hydrochloric acid was added in order to adjust the pH to 4.6 $\pm$ 0.05. The suspension was mixed with a volume of 15 ml of methanol, heated for 15 min at 50° in a stoppered flask and then filtered through a folded filter-paper (Schleicher & Schuell, Feldbach, Switzerland; No. 595 1/2, diameter 15 cm). The first 10 ml of the filtrate were poured back onto the filter. A 20 ml volume of the cooled filtrate was pipetted on to the Extrelut column and allowed to infiltrate for 15 min.

The toxin-containing fraction was eluted with 200 ml of dichloromethane

toluene (4:1) for 60–80 min. The eluate was washed twice with 15 ml of 0.01 *N* aqueous sodium hydroxide and subsequently once with 15 ml of 0.1 *N* hydrochloric acid. This procedure cleaned up the extract considerably but had to be completed within 10 min, as aflatoxin M_1 is not stable in alkaline media. The organic layer was dried with 8 g of anhydrous sodium sulphate, filtered through cotton-wool and evaporated to dryness on a rotary evaporator *in vacuo* at 60°. The dry residue was transferred quantitatively with 2 ml of chloroform into a small vial and the solvent removed with nitrogen.

Thin-layer chromatography

Three standards and up to five samples were spotted as lines of length 8 mm on one TLC plate (distance between samples, 14 mm; distance to edges, 20 mm). A 10-μl volume of standard solution, corresponding to 50 ppt of aflatoxin M_1, was applied with a 10-μl syringe, whereas the sample vials were rinsed with three 40-μl portions of chloroform and spotted with a 50-μl syringe.

The plate was developed first with chloroform–acetone (9:1) in a paper-lined tank for *ca.* 40 min, then dried with a warm air blower for 5 min. The second development in the same direction was performed with dichloromethane–acetone (6:4) in a paper-lined tank for *ca.* 35 min.

Quantitative densitometry

The operating conditions of the Zeiss densitometer were as follows. The beam path arrangement was light source (mercury lamp), filter (365 nm) (excitation), sample, monochromator (420 nm) (emission), slit width 0.6 mm, photomultiplier. The scanning speed of the TLC stage and the paper speed were 50 mm/min. The calibration graph was linear at concentrations above 100 ppt.

Confirmatory procedures

In order to confirm the presence of aflatoxin M_1 in a sample, it was necessary to form derivatives, as proposed elsewhere[14–16]. These experiments were adapted in order to confirm the presence of concentrations above 50 ppt.

The spots of the presumed aflatoxin M_1 and two standards were scraped off, extracted with 2 ml of chloroform–methanol (1:1) directly into small vials and dried with nitrogen. The residues of one standard and the sample were dissolved in 100 μl of acetic anhydride–pyridine (7:3), allowed to stand for 30 min at room temperature and evaporated to dryness with nitrogen. The residues were again spotted on a new silica gel plate as described under *Thin-layer chromatography*, together with the untreated standard. The R_F value of the acetylated aflatoxin M_1 was *ca.* 0.55.

In a similar way the trifluoroacetic acid (TFA) derivative could be formed, with the following exceptions. The dry residues desorbed from the presumed aflatoxin M_1 location and two standards were spotted on a new TLC plate. A 5-μl volume of TFA was superposed on one standard and the sample. This procedure was repeated after 5 min and the TLC plate, covered with a glass plate, was heated for 20 min in an oven at 100°.

The unreacted TFA was removed with a blower for 5 min without a glass cover. The chromatographic system used to develop the plate was as described under

Thin-layer chromatography. The R_F value of untreated aflatoxin M_1 was *ca.* 0.3 and that of the TFA derivative was *ca.* 0.15.

In addition to the two confirmatory TLC tests on derivatives, an HPLC system can be used to prove the identity of aflatoxin M_1. The toxin-containing TLC spot was desorbed as described above and the dry residue was carefully dissolved in 40 μl of methanol and then diluted with 20 μl of water. A 40-μl volume from these 60 μl were injected and chromatographed under the following conditions (according to Zimmerli[17]): reversed-phase column (Merck, RP-8, 5 μm), length 30 cm, solvent acetonitrile–water (1:3), ambient temperature, flow-rate 0.6 ml/min (*ca.* 2400 p.s.i.). A fluorimetric detector (360 nm excitation and cut-off filter 415 nm, Wratten 2A, Kodak) was used. Under these conditions aflatoxin M_1 contents of 50 ppt and above could be confirmed.

Conventional clean-up

For confirmatory purposes we adapted a clean-up procedure based on liquid–liquid partition and silica gel column purification at a detection limit of the required 5 ppt level. The proteins in 50 ml of milk were precipitated with 130 ml of methanol for 15 min at room temperature. A 90-ml volume of the filtrate, diluted with 60 ml of aqueous sodium chloride, was defatted with 70 ml of light petroleum and then extracted with 150 ml of chloroform. The organic layer was washed twice with 15 ml 0.01 *N* aqueous sodium hydroxide and once with 15 ml 0.1 *N* hydrochloric acid. These steps had to be executed rapidly: the loss of aflatoxin M_1 was shown to be *ca.* 10% within 15 min with 0.1 *N* aqueous sodium hydroxide. The chloroform solution was evaporated *in vacuo* at 40° after drying with anhydrous sodium sulphate and re-dissolved in 2.5 ml of dichloromethane–acetone (1:1). A silica gel column (Kieselgel 60 reinst, 0.063–0.200 mm, Merck) was prepared by filling a glass tube with a slurry of 4 g of silica gel in 30 ml of dichloromethane–acetone (1:1). The extract solution was pipetted carefully on the sedimented column layer. The aflatoxin M_1 fraction was eluted with 25 ml of the same organic solvent mixture. The eluate was evaporated to dryness and then treated as described under *Thin-layer chromatography*.

RESULTS AND DISCUSSION

The recovery of milk samples fortified with 50 ppt of aflatoxin M_1 was 78 ± 4% with Extrelut clean-up and 90 ± 3% with the conventional procedure.

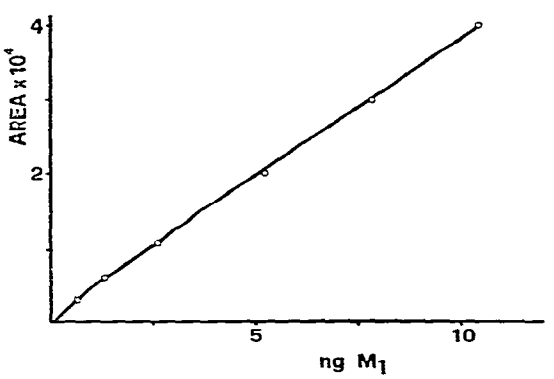

Fig. 1. Calibration graph for aflatoxin M_1.

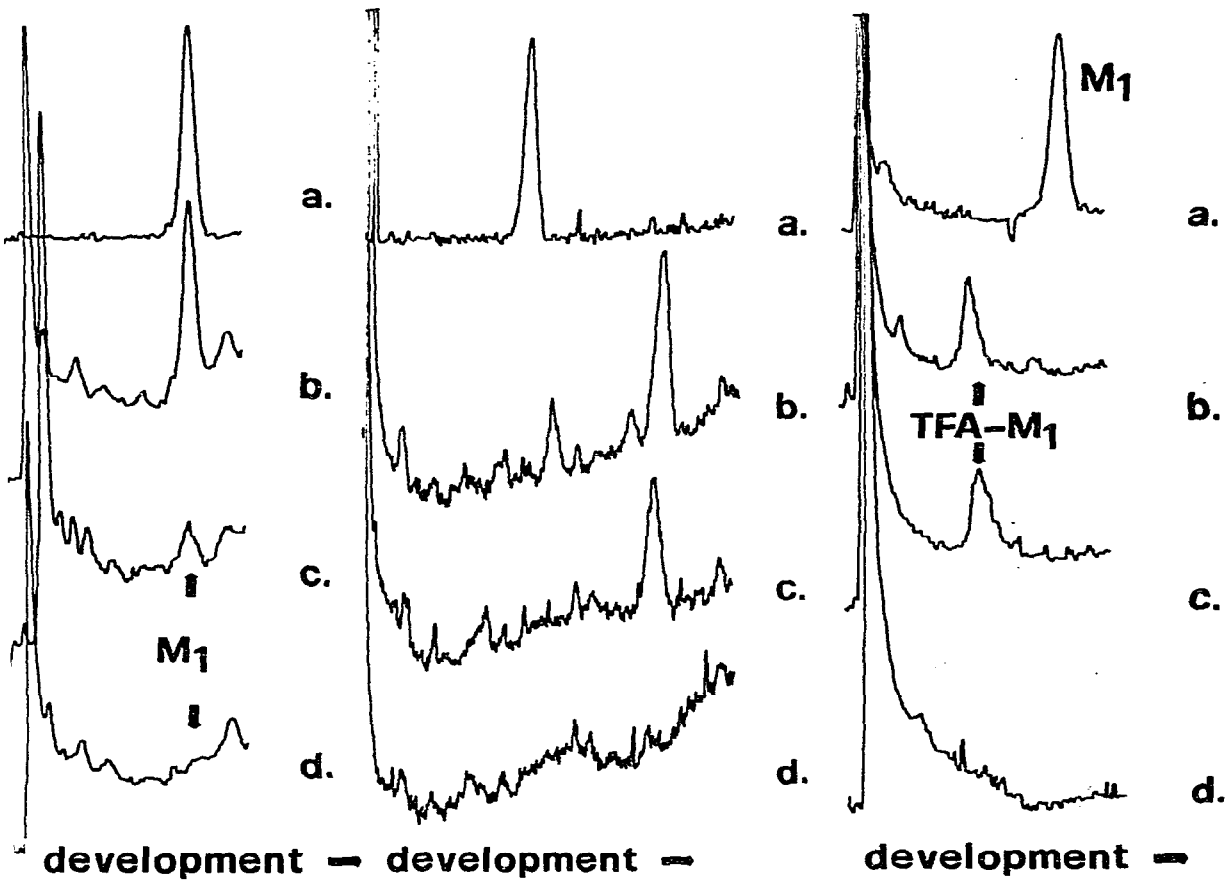

Fig. 2. TLC of raw milk extract. (a) Standard, corresponding to 50 ppt of toxin (= 770 pg); (b) fortified with 50 ppt of toxin; (c) fortified with 5 ppt of toxin; (d) not fortified.

Fig. 3. TLC of acetate derivative of aflatoxin M_1. (a) Untreated toxin; (b) standard; (c) milk extract fortified with 50 ppt of toxin; (d) milk extract without toxin.

Fig. 4. TLC of TFA derivative of aflatoxin M_1. (a) Untreated toxin; (b) standard; (c) milk extract fortified with 50 ppt of toxin; (d) milk extract without toxin.

The detection limit was 5 ppt for both clean-up methods. The calibration graph is linear at concentrations above 100 ppt (1.5 ng of aflatoxin M_1 per spot) and slightly convex below 100 ppt, as illustrated in Fig. 1. The Extrelut extraction allows convenient serial investigations and is at least twice as rapid as methods mentioned in the literature. Fig. 2 shows typical chromatograms of milk extracts and Figs. 3 and 4 illustrate confirmatory derivatizations of aflatoxin M_1 with TFA and acetic anhydride.

The proposed HPLC system is only useful for confirmatory purposes above 50 ppt, as shown in Fig. 5. The milk extract cleaned up with Extrelut is also suitable for direct HPLC, but with a considerable loss of detection. Only *ca.* 50 ppt of toxin can be seen, owing to interferences. The ease and speed of the Extrelut clean-up prior to TLC makes this method more convenient than an extended clean-up combined with HPLC.

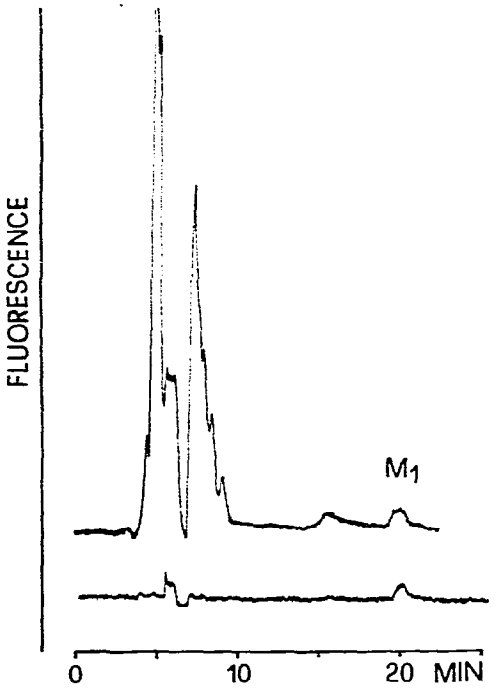

Fig. 5. Confirmation of positive TLC result with HPLC (aflatoxin M_1 zone scraped off the TLC plate). (a) Milk extract fortified with 50 ppt of toxin; (b) standard corresponding to 50 ppt of toxin. Milk extracts without toxin show no peak at the relevant location on the chromatogram.

The sodium hydroxide extraction step prior to chromatography yielded considerably cleaner extracts and most fluorescent interferents could easily be removed. Experiments to transfer this step into the Extrelut column by incorporating a small basic layer in the column failed, probably because of the labile nature of aflatoxin M_1. One of the major problems in developing this method was the precipitation of the proteins without serious losses of the toxin. Experiments using only hydrochloric acid or trichloroacetic acid led to considerable losses, and so far only the procedure described here has yielded acceptable recoveries using an Extrelut column.

The excitation wavelength of 420 nm is not the optimum for the detection of aflatoxin M_1 but gave the best relationship between matrix and toxin signals.

More than 200 milk samples have been checked with the Extrelut clean-up procedure. It was shown that positive samples (50–280 ppt) could be traced to feed containing ground-nut meal contaminated with aflatoxin B_1.

Powdered milk can be analysed in a similar way. The milk powder is dissolved in an appropriate amount of water, as indicated on the package, prior to the protein sedimentation, which needs less concentrated hydrochloric acid (only *ca.* 0.2 ml instead of the 0.4 ml required for fluid milk) in order to obtain the optimal pH of 4.6*. The following steps are as for fluid milk. Samples with low contents of aflatoxin M_1 (5–10 ppt) should preferably be chromatographed twice with the second solvent mixture.

* For fluid milk as well as for powdered milk it is essential to adjust the pH with a glass electrode to 4.6 ± 0.05 in order to obtain a quantitative precipitation of the proteins.

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REFERENCES

1 F. Kiermeier, *Z. Lebensm.-Unters.-Forsch.*, 163 (1977) 171.
2 R. Sieber and B. Blanc, *Mitt. Geb. Lebensm. Hyg.*, 69 (1978) 477.
3 F. Kiermeier, in Ch. Schlatter (Editor), *Symposium on Health Risks by Aflatoxins 1978*, Institute of Toxicology, Schwerzenbach, Switzerland, 1979, p. 309.
4 G. N. Wogan, *Food Cosmet. Toxicol.*, 12 (1974) 381.
5 Ch. Schlatter, in Ch. Schlatter (Editor), *Symposium on Health Risks by Aflatoxins 1978*, Institute of Toxicology, Schwerzenbach, Switzerland, 1979, p. 51.
6 W. Mücke, *Ernährungsrundschau*, 25 (1978) 9.
7 F. Kiermeier and G. Weiss, *Z. Lebensm.-Unters.-Forsch.*, 160 (1976) 337.
8 W. A. Pons, A. F. Cucullu and L. S. Lee, *J. Ass. Offic. Anal. Chem*, 56 (1973) 1431.
9 P. L. Schuller, C. A. H. Verhülsdonk and W. E. Paulsch, *J. Int. Union Pure Appl. Chem.*, 35 (1973) 291.
10 L. G. M. Th. Tuinstra and J. M. Bronsgeest, *J. Chromatogr.*, 111 (1975) 448.
11 U. Leuenberger, R. Gauch and E. Baumgartner, *J. Chromatogr.*, 161 (1978) 303.
12 U. Leuenberger, R. Gauch and E. Baumgartner, *J. Chromatogr.*, 173 (1979) 343.
13 W. Horwitz (Editor), *Official Methods of Analysis of the Association of Official Analytical Chemists*, Association of Official Analytical Chemists, Washington, D.C., 1975, Ch. 26, par. 26.008*ff*, p. 3.
14 M. W. Trucksess, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 722.
15 M. E. Stack, A. E. Pohland, J. G. Dantzman and S. Nesheim, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 313.
16 H. P. van Egmond, W. E. Paulsch and P. L. Schuller, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 809.
17 B. Zimmerli, in Ch. Schlatter (Editor), *Symposium on Health Risks by Aflatoxins 1978*, Institute of Toxicology, Schwerzenbach, Switzerland, 1979, p. 196.