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RAPID DETERMINATION OF AFLATOXIN B_1 IN DUTCH FEEDING STUFFS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND POST-COLUMN DERIVATIZATION

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SUMMARY

Concentrated feeding-stuff samples are extracted with chloroform. Clean-up of the extract is carried out by applying six samples in the middle of a thin-layer plate and developing it first with diethylether. After drying and cutting off a part of the plate, development is carried out in the opposite direction with a mixture of chloroform, acetone and water. The aflatoxin-containing part of the chromatogram is removed and extracted with a mixture of dichloromethane and acetone. In subsequent high-performance liquid chromatography (HPLC), the use of post-column derivatization with iodine in water results in a 50-fold increase in sensitivity, permitting determinations at the μg/kg level. Recovery is better than 80%, and the detection limit is better than 1 μg/kg. In the HPLC system described extracts from citrus pulp are eluted after aflatoxin B_1.

INTRODUCTION

The contamination of milk with aflatoxin M_1 is caused by contamination of cattle feed with aflatoxin B_1. Recently, tolerances for aflatoxin M_1 in milk were set at 0.05 μg/kg in Switzerland. In The Netherlands, an advisory group within the Ministry of Agriculture and Fisheries also recommended a tolerance of 0.05 μg/kg milk. The carry-over factor for aflatoxin B_1 from feed to milk is 1–2%[1–3]. Although, within the EEC, tolerances for aflatoxin B_1 in concentrated feed are set at 20 μg/kg, the proposed tolerance for aflatoxin M_1 in milk in combination with the carry-over factor allows a maximum aflatoxin B_1 value in feed of only about 5–10 μg/kg. Therefore, there is a need for methods of analysis with a limit of detection at 1 μg/kg or less, together with a high through out of samples. Methods have been published for several types of matrices with a nearly sufficiently low limit of detection[4–9], some times obtained by derivatization of aflatoxin B_1[10,11]. Clean-up procedures used are silica gel chromatography[4], Sep-Pak[5,9,10], gel permeation chromatography[7], Extrelut[8] and alumina column chromatography[11]. In our laboratory, experiments have been carried out, based on these publications. The methods were either laborious or inadequate for the required limit of detection. In thin-layer chromatography (TLC),

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some extracts were found to contain many compounds in the neighbourhood of aflatoxin B_1, often originating from the use of citrus pulp in the feed. Recently, a post-column derivatization reaction[12] was introduced in high-performance liquid chromatography (HPLC) of aflatoxins based on the work of Davis and Diener[13]. The treatment of aflatoxin B_1 with a saturated aqueous solution of iodine resulted in a 50-fold increase of fluorescence, compared to the original aflatoxin B_1 fluorescence in reversed-phase HPLC systems. As the mobility of aflatoxin B_1, like that of aflatoxin M_1[14], on thin-layer plated with dry diethyl ether as developer is low, this phenomenon is used as the first step in the double TLC clean-up procedure for the determination of aflatoxin B_1in feeding-stuff extracts.

EXPERIMENTAL

Materials

All reagents should be of such quality that under the given analytical conditions no interference with aflatoxin B_1 occurs.

Celite 545 is treated overnight with diluted (1:1) hydrochloric acid, filtered through Whatman No. 540 paper, washed with distilled water to neutrality and then dried at 110°C. Aflatoxin B_1 stock solution in chloroform (1 μg/ml) is diluted ten times in chloroform for TLC. For HPLC a standard curve is prepared with 0.2, 0.1, 0.05, 0.025, 0.01 and 0.005 μg aflatoxin B_1 per ml of methanol. The saturated iodine solution is prepared by adding 1 g of iodine to 200 ml of water, mixing for 15 min and then filtering through a Millipore filter (pore size 0.45 μm, Cat.No. HAWP 047 00).

Apparatus

For TLC Kieselgel 60 plates (Merck No. 5553), 20 × 20 cm, were used. For HPLC, the following components were assembled (see Fig. 1); column, 100 × 4.6 mm, Cp^{tm} Microsphere C_{18} (Chrompack, The Netherlands); eluent, acetonitrile–water (3:7), flow-rate, 0.5 ml/min; sample volume, 20 μl; detector, Waters 420 fluorescence detector, excitation at 360 nm, emission at > 420 nm. A Valco zero dead-volume Tee, SS 1/16 in. × 0.75 mm, was used to combine the saturated solution

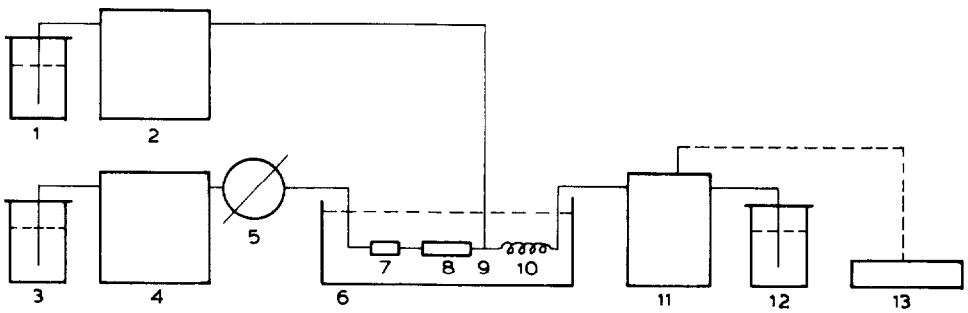

Fig. 1. Diagram of the HPLC system for the determination of aflatoxin B_1 by post-column derivatization. 1 = Reagents; 2, 4 = pump; 3 = mobile phase; 5 = injection valve; 6 = thermostatically controlled bath; 7 = guard column; 8 = analytical column; 9 = T-joint; 10 = reaction coil; 11 = fluorescence detector; 12 = waste; 13 = two-pen recorder.

of iodine in water (flow-rate 0.4 ml/min) with the column effluent. Both the column and PTFE reaction coil (3000 × 0.5 mm) were kept at 60°C.

Procedure

The determination should be carried out with the exclusion of daylight.

Extraction. Weigh 25 g test sample in a 250-ml glass flask. Add 12.5 g of Celite, 12.5 ml of water and 125 ml of chloroform. Close the flask with a PTFE-lined screw-cap and shake for 30 min. Filter the chloroform extract through Whatman No. 5 paper and collect 5.0 ml in a calibrated vial. Evaporate this to dryness at 50°C under a stream of nitrogen. Dissolve the extract in 200 µl of chloroform with the aid of a Vortex mixer.

Clean-up by TLC. Draw lines on the plate with a pencil, according to Fig. 2. Spot 1 ng aflatoxin B_1 at A, B and C. Spot 40 µl of six different sample extracts at S. Develop the plate with dry diethyl ether in a vapour-saturated tank in the first direction until the solvent front reaches the top of the plate (approximately 45 min). Remove the plate from the tank, allow the ether to evaporate in the dark at room temperature for 15 min and cut the plate as indicated in Fig. 2. Develop the plate in an unsaturated tank with a mixture of chloroform–acetone–water (88:12:0.2, v/v/v) in a direction opposite to the first development until the front reaches the top of the plate (approximately 30 min). Remove the plate from the tank, and allow the solvents to evaporate at room temperature in the dark.

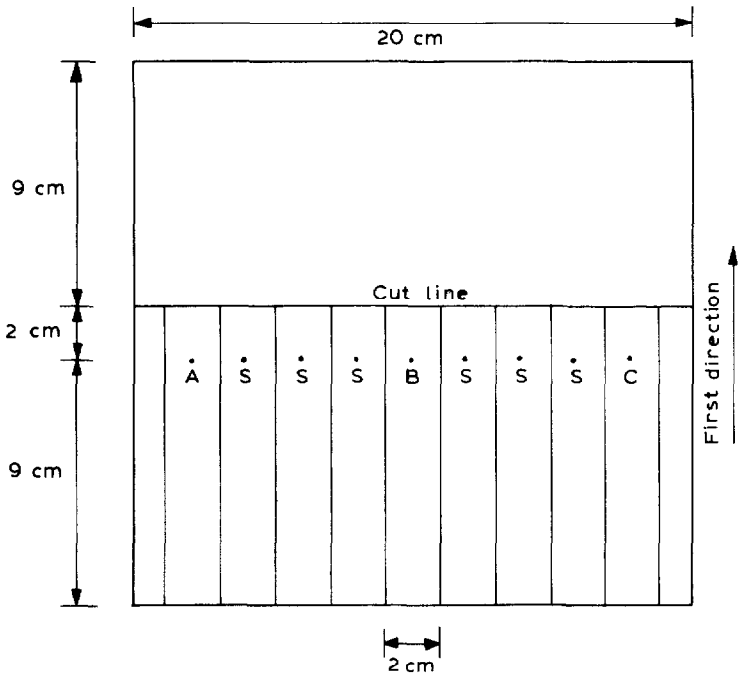

Fig. 2. Spotting of sample extracts (S) and standard solutions (A, B and C) for clean-up by TLC.

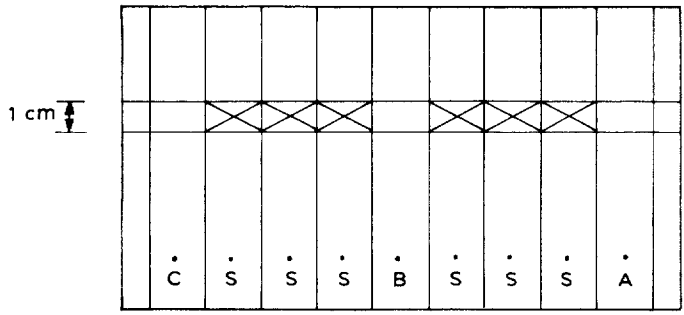

Fig. 3. TLC results after second development. Marking of aflatoxin B_1 spots according to standards (A, B and C).

mately 30 min). Remove the plate from the tank, and allow the solvents to evaporate at room temperature in the dark.

Examine the plate under UV light (366 nm) to locate the blue spots of the aflatoxin B_1 standards. Mark these spots by drawing a line 0.5 cm above and below the aflatoxin B_1 standard (see Fig. 3). Remove these zones of the six samples from the plate and elute them in a test-tube by shaking for 15 min with 4 ml dichloromethane–acetone (6:4, v/v). Filter the dichloromethane–acetone mixture through a Millipore filter* (Cat. No. FHLPO 1300) into a second test-tube. Rinse the first test-tube with 2 ml and then 1 ml of the dichloromethane actone mixture and combine the filtrates. Evaporate them at 40°C under a stream of nitrogen and redissolve the extract in 50 μl methanol, again with the aid of a Vortex mixer.

HPLC determination. Prepare a standard curve by injecting 20 μl of aflatoxin B_1 solution containing 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 ng, respectively. Plotting the injected quantities against the area of the aflatoxin B_1 peaks should result in a straight line. Inject 20 μl of the sample extract into the HPLC system. Compare the retention times for identification purposes. Calculate the aflatoxin B_1 content from the standard curve.

RESULTS AND DISCUSSION

As the dimensions of our analytical column deviate from the one used by Thorpe *et al.*[12], some experiments had to be carried out to find optimum separation and reaction times. Under the conditions given the reaction was completely less than 1 min. With a peak width at the baseline of 1.0 min, about 0.05 ng aflatoxin B_1 are detectable, an improvement over the absolute detection limit without post-column derivatization by a factor of about 50. Recovery in the TLC clean-up and elution from the TLC plate was determined with standards, ranging from 0.3 to 2 ng, spotted on the plate, eluted and injected in the HPLC system. From Table I it is clear that losses are small, the mean recovery being 88 + 5% with sufficient linearity.

In the method, filtration through a Millipore filter is prescribed.Heavy losses occur when a silica gel particles remain in the final filtrate. The process of scraping

* A Finntip plugged with glass-wool may be used instead.

TABLE I

COMPARISON OF PEAK AREA, OBTAINED BY HPLC, OF STANDARD AFLATOXIN B_1 ELUTED FROM THE TLC PLATE

Amount of B_1 (ng)	*Peak area (cm^2) after TLC and HPLC*	*Peak area (cm^2) after HPLC*	*Recovery (%)*
0.3	2.56	2.91	88.0
0.5	4.83	5.82	83.0
1.0	9.51	10.21	93.2
2.0	19.12	21.50	88.9
Correlation coefficient	0.99982	0.99861	

of the silica gel off the plate and eluting it is time-consuming and may lead to contamination of the filtrate with small silica particles.

In Fig. 4 two very intense peaks are seen with longer retention times than aflatoxin B_1. They are due to compounds extracted from citrus pulp. When citrus pulp is not an ingredient from the cattle feed, aflatoxin B_1 is the last compound eluted from the HPLC system. In case of a simple matrix, *e.g.*, yellow corn meal, the extract can be injected without the TLC clean-up. The detection limit is about 1 μg/kg. For peanut meal, where the official tolerance is 1 mg/kg, 50 μg/kg are easily detectable without clean-up.

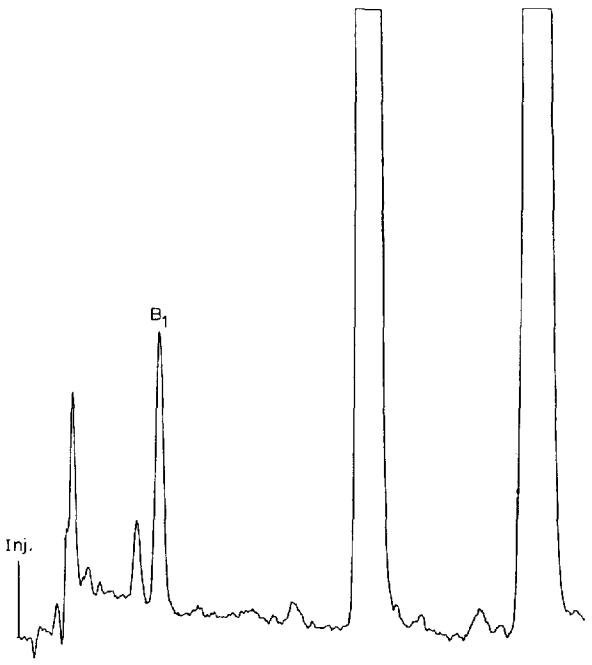

Fig. 4. Chromatogram of 0.08 g of cattle feed, containing citrus pulp, with an aflatoxin B_1 content of 6 μg/kg. Retention time: aflatoxin B_1, 7.5 min. For conditions see text.

As previously described[12], aflatoxin B_1, B_2, G_1 and G_2 can be separated by changing the eluent composition (acetonitrile–water, 15:85). This, of course affects the limit of detection and the time of analysis. In a survey program 61 samples have been analyzed up to now by the above method, showing a range < 1–60 μg/kg and median 9 μg/kg concentration of aflatoxin B_1 in feed. Most of the feed samples, if not all, show some response at the retention time of aflatoxin B_1, in agreement with the GPC results[7]. Analysis of grass, however, showed no response.

CONCLUSION

The use of post-column derivatization for the determination of aflatoxin B_1 results in a 50-fold increase in the fluorescence of aflatoxin B_1. In combination with an adequate TLC clean-up procedure at least 20 samples of concentrated cattle feed can be processed by one analyst in a day. The limit of detection is about 1 μg/kg and the recovery about 85%.

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