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Aflatoxin B_1 in compound feedstuffs containing citrus pulp. Procedures for screening and semi-quantitative determination

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Summary

Methods described in the literature for the detection and determination of aflatoxin B_1 in animal feedstuffs containing citrus pulp, seldom give satisfactory results. The method described in this paper consists of an extraction and clean-up section and a two-dimensional thin-layer chromatographic procedure.

The cleaned extract is first subjected to a (not earlier published) screening procedure on small thin-layer plates. The detection limit is 2 μg/kg. Then examination is carried out on normal plates, enabling visual semi-quantitative or densitometric determination. The detection limit is 1 μg/kg.

Introduction

It is commonly known that products intended for human consumption can be contaminated with aflatoxins. Aflatoxin B_1 is known to be the most carcinogenic compound. Lactating cows fed with fodder containing aflatoxin B_1 secrete in their milk the metabolite aflatoxin M_1. The carry-over is approximately 2–3 % (Purchase, 1972; Verhülsdonk et al., 1972). In view of its structural relationship with aflatoxin B_1 the appearance of aflatoxin M_1 in milk is undesirable (Canton et al., 1974). In order to minimize the risk of contamination of the milk with M_1 it is necessary to minimize the amount of aflatoxin B_1 in fodder.

As well as for contamination of milk with organochlorine pesticides the solution should be tracked at the source (Tuinstra, 1974), namely the animal feedstuff and its raw materials.

Starting 1 January 1976, maximum levels for aflatoxin B_1 in all sorts of animal feedstuff will be effected within the EEC (Anon., 1974). Though in literature many methods are described, most of them only concern one single substrate (groundnuts, soy-beans, cotton seed, etc.). For compound feedstuffs, however, the number of methods is limited (Pons et al., 1970; Romer, 1973). In these methods one-dimensional thin-layer chromatography (TLC) is involved to determine aflatoxin

with a detection limit of 5 μg/kg. When citrus products are present in the mixed food these methods do not give satisfactory results.

Though better results are obtained with the method described by Verhülsdonk & Schuller (1973), by applying two-dimensional TLC, the method of analysis described below is solving the problem of the citrus products interference, as well as having a detection limit for aflatoxin B_1 of 1 μg/kg of animal feed. By performance of TLC on smaller plates, here for the first time introduced as a screening test, the chromatography is speeded up considerably. The detection limit will then be 2 μg/kg.

Method of analysis

Principle

Aflatoxin B_1 is extracted from animal feed with chloroform and purified by liquid-liquid partitioning and column chromatography. The cleaned extract is analysed by means of TLC according to the antidiagonal technique (Beljaars et al., 1973). By comparing the intensity of the spot from the sample extract and those of known standards spotted on the same plate the amount of aflatoxin B_1 of the sample can be determined both visually and densitometrically. The detection limit is 1 μg/kg. TLC on small plates gives quick information on the presence of aflatoxin B_1.

Reagents

All reagents shall be analytical grade unless mentioned otherwise:
- aflatoxin B_1 (R.I.V.) standard solution in chloroform, 20 μg/ml, ampoule volume 2.5 ml;
- silica gel for column chromatography, Kieselgel 60 Merck[1], 0.063–0.20 mm;
- fluted filter paper (Schleicher und Schüll No 595 ½[1]), 24 cm;
- celite 545 (Johnson and Manfield[1]), washed in acid;
- fertig-Platte Kieselgel '60, Merck[1], 20 × 20 cm;
- fertig-Platte Alufolien Kieselgel, Merck[1], No 5553.

Apparatus, Glassware

Usual laboratory apparatus and glassware, and in particular:
- shaking machine or magnetic stirrer;
- chromatographic column, glass, 22 × 300 mm with Teflon (PTFE) stopcock, reservoir type, 250 ml;
- UV lamp for use at 360 nm wavelength, intensity juist sufficient to visualize a spot of 1 ng of aflatoxin B_1 at a distance of 10 cm;
- grinding and/or mixing apparatus; size of particles 1 mm.

Determination

Note: the determination shall be carried out under the exclusion of daylight.

[1] Specific trade marks and/or names are not mentioned for recommendation, but for identification.

Extraction
Add to 50.0 g of the ground and homogenized sample 25 g of Celite, 25 ml of distilled water and 250 ml of chloroform. Shake during 30 min in the shaking apparatus. Filter and collect 50 ml of the filtrate.

Separatory funnel clean-up
Evaporate the filtrate (see previous section) to complete dryness in a rotatory evaporator at 45 °C. Transfer the residue quantitatively to a separatory funnel by using 40 ml n-pentane, then 50 ml of methanol. Shake the methanol-pentane fraction with 75 ml of water containing 5 g NaCl. Discard the pentane fraction. Shake the methanol/water layer again with 40 ml of n-pentane. Discard the pentane fraction. Shake the aqueous methanol layer three times with 25-ml portions of chloroform. The collected chloroform fractions shall be evaporated to dryness in a rotatory evaporator under reduced pressure. Dissolve the residue in 50 ml of chloroform.

Column clean-up
– Fix a plug of glass wool at the bottom of the chromatographic column. Fill the column for 2/3 with chloroform and add 5 g of anhydrous Na_2SO_4 and then add 10 g of silica gel in small portions, stirring after each portion to avoid the eventual presence of air bubbles. Allow the silica gel to settle during 15 min, then carefully add 15 g of anhydrous Na_2SO_4 and drain the chloroform to just above the Na_2SO_4 layer.

– Add to the 50 ml chloroform residue (see previous section), 150 ml of n-hexane and transfer the mixture to the column. Eluate at 8–12 ml/min. Then eluate with 100 ml of diethyl ether. Ensure the column is not made dry. Eliminate these two fractions and finally eluate with 150 ml chloroform/methanol mixture (97:3 v/v). Collect this fraction, containing the aflatoxin B_1.
– Concentrate the chloroform/methanol fraction to almost dryness in a rotatory evaporator at 45 °C under reduced pressure. Transfer the residue quantitatively to a conical tube by means of a pasteur pipette using as little chloroform as possible. Evaporate the solvent completely under a constant stream of nitrogen at about

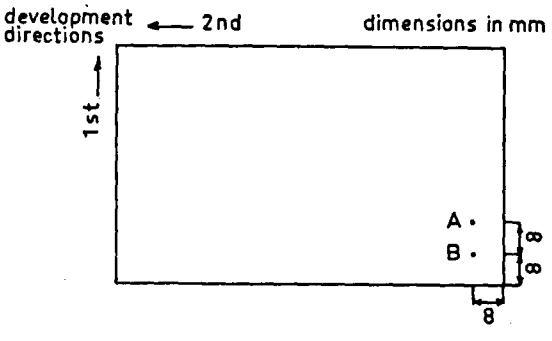

Fig. 1. Spotting of sample extract at A and standard solution at B. (See section 'Screening procedure'.)

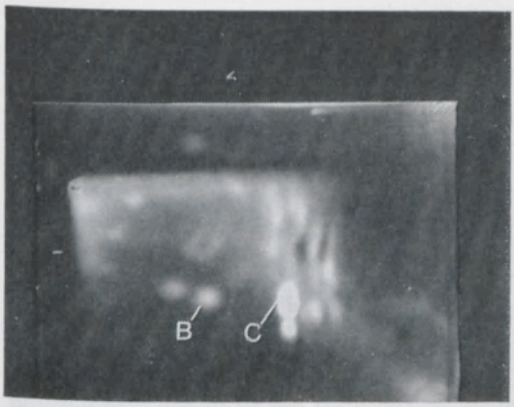

Fig. 2. Extract from contaminated bovine animal feeding-stuff. Applied 10 μl of extract ($\approx$ 0,5 g of the product). C = citrus pulp interference; B = aflatoxin B_1 from sample.

50 °C. Add with a micro-syringe 200 μl of chloroform to this residue, mix well and use this solution for the chromatographic analysis.

Thin-layer chromatography

Screening procedure

Cut into pieces of 5 × 6½ cm a DC Alufolien Kieselgel Merck. Apply 10 μl of the sample extract at A, according to Fig. 1 and develop first along the short axis until the solvent mixture chloroform/aceton 9:1 v/v reaches the upper edge of the plate. Use an unequilibrated tank (development time 10–15 min).

Dry during about 15 min at room temperature and apply 10 μl (corresponding with 1 ng) of the diluted standard aflatoxin B_1 solution at B, then develop along its long axis until the mixture toluene/ethylacetate/formic acid 5:4:1 v/v/v reaches about 1 cm from the upper edge of the plate. Use an unequilibrated tank (development time also 10–15 min). Allow the plate to dry at room temperature for 10 min, then dry the plate with a hair dryer until the excess of formic acid has disappeared.

Examine the plate under UV light. The detection limit is 2 μg/kg. (See Fig. 2).

This screening procedure is helpful to establish the dilution factor for the semi-quantitative determination.

Semi-quantitative procedure

Draw pencil lines on the plates as indicated in Fig. 3 and spot at A, B, C, D, E, F the following solutions in accordance with Fig. 3:

A	20 μl sample extract ($\approx$1 g of sample)
B	10 μl standard solution (= 1 ng aflatoxin B_1)
C	20 μl standard solution (= 2 ng aflatoxin B_1)
D	40 μl standard solution (= 4 ng aflatoxin B_1)
E and F	25 μl standard solution (= 2.5 ng aflatoxin B_1).

During the application, dry at spotting position with a stream of nitrogen, taking care of the spots having the same diameter of max. 5 mm.

The development of the chromatogram

Develop the plate in the first direction in a mixture of chloroform/aceton (9:1 v/v) in an unsaturated jar, to the pencil line (about 1½ h). Take the plate out of the jar and allow to evaporate in the dark during 30 min at room temperature. Then turn the plate 90 degrees and develop, also in an unsaturated jar, in the second direction in a mixture of toluene/ethylacetate/formic acid (5:4:1 v/v/v) to the pencil line (about 2 h).

Interpretation of the chromatogram

Allow the plate to dry at room temperature for 10 min, then dry the plate with a hair dryer until the excess of formic acid has disappeared, before interpretation of the chromatogram.

Examine the chromatogram at a distance of some 10 cm from the UV lamp at

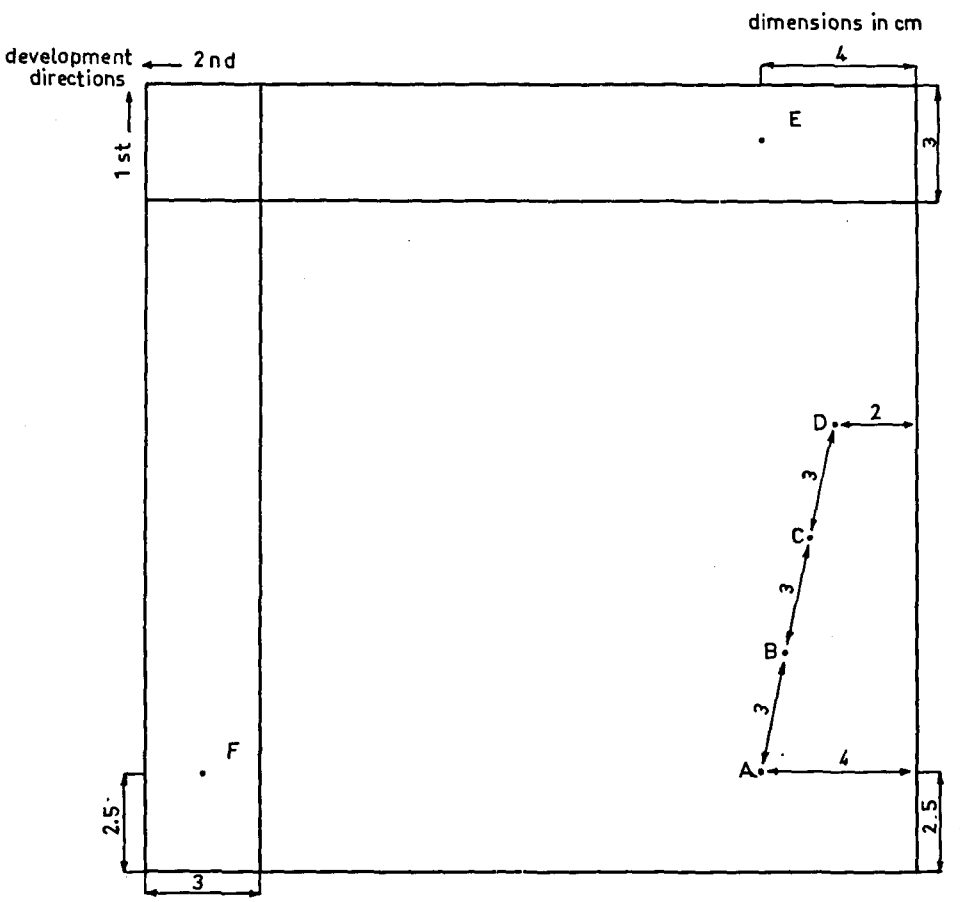

Fig. 3. Spotting of sample extracts at A and standard solutions at B, C, D, E, F. (See section 'Semi-quantitative procedure'.)

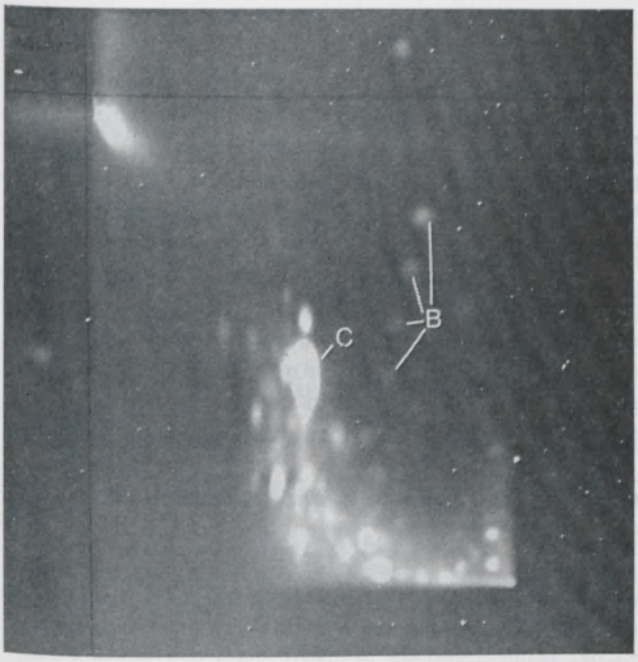

Fig. 4. Extract from the same contaminated feedstuff as Fig. 5. Determination as described in the text.

360 nm. Aflatoxin B_1 spots have a blue fluorescense. Draw an imaginary line straight on the second direction through the standard spot originating from E and another line straight on the first direction through the standard spot from F.

The area where the aflatoxin B_1 spot of the sample extract is to be expected is situated around the intersection of these two imaginary lines. When the imaginary intersection area is free from background interference, the sample extract is well separated in both directions. Locate the three standard aflatoxin B_1 spots originating from B, C and D. Draw and imaginary line through these three spots in the direction of the area where the aflatoxin B_1 spot of the sample is to be expected.

Aflatoxin B_1 is present in the sample when four blue fluorescing spots are observed on this imaginary line (compare Fig. 4).

Calculation

Calculate the quantity of aflatoxin B_1 in the sample by means of visual estimation or densitometrical measurement of the fluorescense intensities of the three standard B_1 spots and the intensity of the B_1 spot of the sample.

Confirmative test

When positive results are obtained from interpretation of the chromatogram, it is desirable to carry out a confirmation test (Verhülsdonk et al., 1974).

Results

With the method described above a number of compound feedstuffs for cattle, breeder's and store pigs, for poultry, rabits, caviae and mice, totally 115 samples, most of them bovine animal foods were analysed.

The detection limit of 1 μg/kg, mentioned in the introduction, could be reached for all types of animal feed.

Only in six of the samples analysed the TLC showed slight interferences not originating from citrus pulp, in the direct neighbourhood of the aflatoxin B_1. The interferences from citrus pulp, which prohibited the determination of aflatoxin B_1 in other methods of analysis, did not occur (see Figs 4 and 5).

With the densitometric technique, it was found that formic acid has a negative influence on the fluorescense of aflatoxin B_1 on the thin layer, and a positive one on that of aflatoxin B_2. These effects were repressed by drying the plate in a hot air flow.

The results of the 115 samples analysed have been reported earlier (Tuinstra et al., 1974).

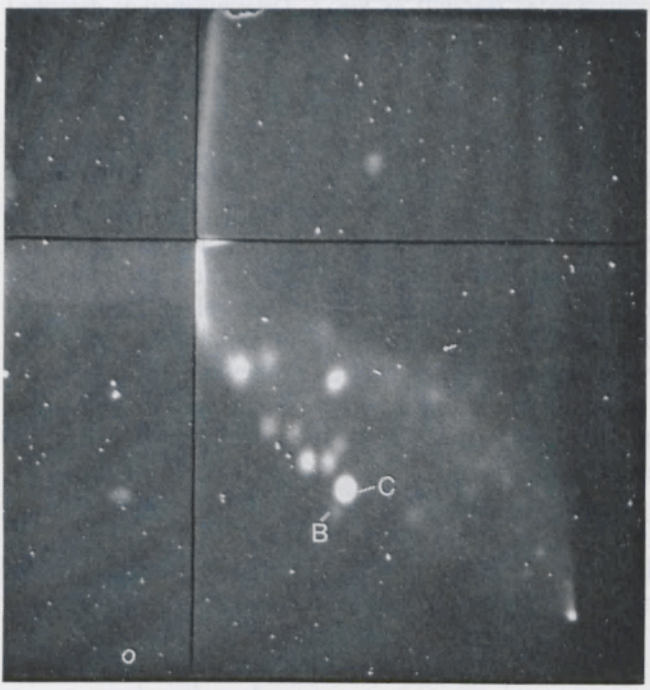

Fig. 5. Extract from contaminated animal feeding-stuff. Determination according to Verhülsdonk & Schuller (1973). Aflatoxin B_1 (B) has just been separated from citrus pulp interference (C).

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