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SEVENTH COMMISSION DIRECTIVE
of 1 March 1976
establishing Community methods of analysis for the official control of feedingstuffs

(76/372/EEC)

(OJ L 102, 15.4.1976, p. 8)

Amended by:

		Official Journal		
		No	page	date
►**M1**	Commission Directive 81/680/EEC of 30 July 1981	L 246	32	29.8.1981
►**M2**	Commission Directive 92/95/EEC of 9 November 1992	L 327	54	13.11.1992
►**M3**	Commission Directive 94/14/EC of 29 March 1994	L 94	30	13.4.1994

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SEVENTH COMMISSION DIRECTIVE

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establishing Community methods of analysis for the official control of feedingstuffs

(76/372/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to the Council Directive of 20 July 1970 on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs (1), as last amended by the Act of Accession (2), and in particular Article 2 thereof,

Whereas that Directive requires that official control of feedingstuffs be carried out using Community methods of sampling and analysis for the purpose of checking compliance with requirements arising under the provisions laid down by law, regulation or administrative action concerning the quality and composition of feedingstuffs;

Whereas Commission Directives 71/250/EEC of 15 June 1971 (3), 71/393/EEC of 18 November 1971 (4), 72/199/EEC of 27 April 1972 (5), 73/46/EEC of 5 December 1972 (6), 74/203/EEC of 25 March 1974 (7) and 75/84/EEC of 20 December 1974 (8) have already established a number of Community methods of analysis; whereas the progress of work since then makes it advisable to adopt a seventh set of methods;

Whereas the measures provided for in this Directive are in accordance with the opinion of the Standing Committee for Feedingstuffs,

HAS ADOPTED THIS DIRECTIVE:

Article 1

The Member States shall require that analyses for official controls of feedingstuffs, as regards their content of aflatoxin B_1, be carried out in accordance with the methods described in the Annex to this Directive.

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Article 2

The Member States shall, not later than 1 October 1976, bring into force the laws, regulations or administrative provisions necessary to comply with this Directive. They shall forthwith notify the Commission thereof.

Article 3

This Directive is addressed to the Member States.

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(1) OJ No L 170, 3. 8. 1970, p. 2.
(2) OJ No L 73, 27. 3. 1972, p. 14.
(3) OJ No L 155, 12. 7. 1971, p. 13.
(4) OJ No L 279, 20. 12. 1971, p. 7.
(5) OJ No L 123, 29. 5. 1972, p. 6.
(6) OJ No L 83, 30. 3. 1973, p. 21.
(7) OJ No L 108, 22. 4. 1974, p. 7.
(8) OJ No L 32, 5. 2. 1975, p. 26.

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ANNEX

DETERMINATION OF AFLATOXIN B_1

A. ONE-DIMENSIONAL THIN LAYER CHROMATOGRAPHIC METHOD

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1. **Purpose and scope**

The method makes it possible to determine the level of aflatoxin B_1 in raw materials and straight feedingstuffs. This method can not be applied in the presence of citrus pulp. The lower limit of determination is 0,01 mg/kg (10 ppb).

In the presence of interfering substances it is necessary to repeat the analysis using method B (high performance liquid chromatography).

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2. **Principle**

The sample is subjected to extraction with chloroform. The extract is filtered, and an aliquot portion taken and purified by column chromatography on silica gel. The eluate is evaporated and the residue redissolved in a specific volume of chloroform or of a mixture of benzene and acetonitrile. An aliquot portion of this solution is subjected to thin-layer chromatography (TLC). The quantity of aflatoxin B_1 is determined under UV irradiation of the chromatogram, either visually or by fluorodensitometry, by comparison with known quantities of standard aflatoxin B_1. The identity of the aflatoxin B_1 extracted from the feedingstuff must be confirmed by the procedure indicated.

3. **Reagents**

NB: All the reagents must be of 'analytical reagent' quality unless otherwise stated.

3.1. Acetone.

3.2. Chloroform, stabilized with 0·5 to 1·0 % of 96 % ethanol (v/v).

3.3. N-hexane.

3.4. Methanol.

3.5. Anhydrous diethyl ether, free from peroxides.

3.6. Mixture of benzene and acetonitrile: 98/2 (v/v).

3.7. Mixture of chloroform (3.2) and methanol (3.4): 97/3 (v/v).

3.8. Silica gel, for column chromatography, particle size 0·05 to 0·20 mm.

3.9. Absorbent cotton wool, previously defatted with chloroform, or glass wool.

3.10. Sodium sulphate, anhydrous, granular.

3.11. Inert gas, e.g. nitrogen.

3.12. 1 N Hydrochloric acid.

3.13. 50 % (v/v) sulphuric acid.

3.14. Kieselguhr (hyflosupercel), washed in acid.

3.15. Silica gel G-HR or equivalent, for TLC.

3.16. Standard solution with about 0·1 µg of aflatoxin B_1 per ml in chloroform (3.2) or the benzene/acetonitrile mixture (3.6), prepared and checked as indicated in Section 7.

3.17. Standard solution for qualitative testing purposes containing about 0·1 µg of aflatoxin B_1 and B_2 per ml in chloroform (3.2) or the benzene/acetonitrile mixture (3.6). These concentrations are given as a guide. They must be adjusted so as to obtain the same intensity of fluorescence for both aflatoxins.

3.18. Developing solvents:

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3.18.1. Chloroform (3.2)/acetone (3.1): 9/1 (v/v), unsaturated tank;

3.18.2. Diethyl ether (3.5)/methanol (3.4)/water: 96/3/1 (v/v/v), unsaturated tank;

3.18.3. Diethyl ether (3.5)/methanol (3.4)/water: 94/4·5/1·5 (v/v/v), saturated tank;

3.18.4. Chloroform (3.2)/methanol (3.4): 94/6 (v/v), saturated tank;

3.18.5. Chloroform (3.2)/methanol (3.4): 97/3 (v/v), saturated tank.

4. **Apparatus**

4.1. Grinder-mixer.

4.2. Shaking apparatus or magnetic stirrer.

4.3. Fluted filter papers, Schleicher and Schüll No 588 or equivalent, diameter: 24 cm

4.4. Glass tube for chromatography (internal diameter: 22 mm, length: 300 mm), with a PTFE cock and a 250-ml reservoir.

4.5. Rotary vacuum evaporator, with a 500-ml round-bottom flask.

4.6. 500-ml conical flasks, with ground-glass stoppers.

4.7. TLC apparatus.

4.8. Glass plates for TLC, 200 × 200 mm, prepared as follows (the quantities indicated are sufficient to cover five plates). Put 30 g of silica gel G-HR (3.15) into a conical flask. Add 60 ml water, stopper and shake for a minute. Spread the suspension on the plates so as to obtain a uniform layer 0·25 mm thick. Leave to dry in the air and then store in a desiccator containing silica gel. At the time of use, activate the plates by keeping them in the oven at 110 ºC for one hour.

Ready-to-use plates are suitable if they give results similar to those obtained with the plates prepared as indicated above.

4.9. Long-wavelength (360 nm) UV lamp. The intensity of irradiation must make it possible for a spot of 1 ng of aflatoxin B_1 to be still clearly distinguished on a TLC plate at a distance of 10 cm from the lamp.

4.10. 10 ml graduated tubes with polyethylene stoppers.

4.11. UV spectrophotometer.

4.12. Fluorodensitometer (optional).

5. **Procedure**

5.1. *Preparation of the sample (see under 'Observations', Part C, point 1).*

Grind the sample so that the whole of it will pass through a sieve with a 1-mm mesh (in accordance with recommendation ISO R 565).

5.2. *Extraction*

Put 50 g of ground, homogenized sample into a 500-ml conical flask (4.6). Add 25 g of Kieselguhr (3.14), 25 ml of water and 250 ml of chloroform (3.2). Stopper the flask, shake or stir for 30 minutes with the apparatus (4.2) and filter through a fluted filter paper (4.3). Discard the first 10 ml of the filtrate and then collect 50 ml.

5.3. *Column clean-up*

Insert into the lower end of a chromatography tube (4.4) a cotton or glass wool plug (3.9), fill two-thirds of the tube with chloroform (3.2) and add 5 g of sodium sulphate (3.10).

Check that the upper surface of the sodium sulphate layer is flat, then add 10 g of silica gel (3.8) in small portions. Stir carefully after each addition to eliminate air bubbles. Leave to stand for 15 minutes and then carefully add 15 g of sodium sulphate (3.10). Let the liquid fall until it is just above the upper surface of the sodium sulphate layer.

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Mix the 50 ml of extract collected in 5.2 with 100 ml of n-hexane (3.3) and quantitatively transfer the mixture to the column. Let the liquid fall until it is just above the upper surface of the sodium sulphate layer. Discard this washing. Then add 100 ml of diethylether (3.5) and again allow it to fall to the upper surface of the sodium sulphate layer. During these operations see that the rate of flow is 8 to 12 ml per minute and that the column does not run dry. Discard the liquid that comes out. Then elute with 150 ml of the chloroform/methanol mixture (3.7) and collect the whole of the eluate.

Evaporate the latter almost to dryness at a temperature not exceeding 50 ºC and under a stream of inert gas (3.11) with the rotary evaporator (4.5). Quantitatively transfer the residue, using chloroform (3.2) or the benzene-acetonitrile mixture (3.6), to a 10 ml graduated tube (4.10). Concentrate the solution under a stream of inert gas (3.11) and then adjust the volume to 2 ml with chloroform (3.2) or the benzene/acetonitrile mixture (3.6).

5.4. *Thin-layer chromatography*

Spot on a TLC plate (4.8), 2 cm from the lower edge and at intervals of 2 cm, the volumes indicated below of the standard solution and the extract:

— 10, 15, 20, 30 and 40 µl of the standard aflatoxin B_1 solution (3.16);

— 10 µl of the extract obtained in 5.3 and, *superimposed on the same point*, 20 µl of the standard solution (3.16);

— 10 and 20 µl of the extract obtained in 5.3.

Develop the chromatogram in the dark with one of the developing solvents (3.18). The choice of the solvent must be made beforehand, by depositing 25 µl of the qualitative standard solution (3.17) on the plate and checking that, when developed, aflatoxin B_1 and B_2 are completely separated.

Let the solvents evaporate in the dark and then irradiate with UV light, placing the plate 10 cm from the lamp (4.9). The spots of aflatoxin B_1 give a blue fluorescence.

5.5. *Quantitative determinations*

Determine either visually or by fluorodensitometry as indicated below.

5.5.1. Visual measurements

Determine the quantity of aflatoxin B_1 in the extract by matching the fluorescence intensity of the extract spots with that of one of the standard solution spots. Interpolate if necessary. The fluorescence obtained by the superimposition of the extract on the standard solution must be more intense than that of the 10 µl of extract and there must not be more than one visible spot. If the fluorescence intensity given by the 10 µl of extract is greater than that of the 40 µl of standard solution, dilute the extract 10 or 100 times with chloroform (3.2) or the benzene/acetonitrile mixture (3.6) before subjecting it again to thin-layer chromatography.

5.5.2. Measurements by fluorodensitometry

Measure the fluorescence intensity of the aflatoxin B_1 spots with the fluorodensitometer (4.12) at an excitation wavelength of 365 nm and an emission wavelength of 443 nm. Determine the quantity of aflatoxin B_1 in the extract spots by comparison of their fluorescence intensities with that of the standard aflatoxin B_1 spots.

5.6. *Confirmation of the identity of aflatoxin B_1*

Confirm the identity of the aflatoxin B_1 in the extract by the processes indicated below.

5.6.1. Treatment with sulphuric acid

Spray sulphuric acid (3.13) on to the chromatogram obtained in 5.4. The fluorescence of the aflatoxin B_1 spots must turn from blue to yellow under UV irradiation.

5.6.2. Two-dimensional chromatography involving the formation of aflatoxin B_1-hemiacetal (aflatoxin B_{2a})

NB: The operations described below must be carried out following carefully the diagram in figure 3.

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5.6.2.1. Application of the solutions

Score two straight lines on the plate (4.8) parallel to two contiguous sides (6 cm in from each side) to limit migration of the solvent fronts. Spot the following solutions on the plate using capillary pipettes or microsyringes:

— *on point A:* a volume of purified extract of the sample, obtained in 5.3, containing about 2·5 nm of aflatoxin B_1;

— *on points B and C:* 25 µl of the standard solution (3.16).

5.6.2.2. Development

Develop the chromatogram in direction I, in the dark, using the developing solvent (3.18.1) (1 cm layer in an unsaturated tank) until the solvent front reaches the solvent limit line.

Remove the plate from the tank and allow to dry in the dark at ambient temperature for five minutes. Then spray hydrochloric acid (3.12) along a band 2·5 cm high, covering points A and B (indicated by the hatched area in figure 3) until it darkens, protecting the rest of the plate with a glass sheet. Allow to react for 10 minutes in the dark and dry with a stream of air at ambient temperature.

Next, develop the chromatogram in direction II, in the dark, using the developing solvent (3.18.1) (1 cm layer in an unsaturated tank) until the solvent front reaches the solvent limit line. Remove the plate from the tank and allow to dry at ambient temperature.

5.6.2.3. Interpretation of the chromatogram

Examine the chromatogram under UV light (4.9) and check for the following features.

(a) Appearance of a blue fluorescent spot of aflatoxin B_1 originating from the standard solution applied at C (migration in the direction I).

(b) Appearance of a blue fluorescent spot of unreacted (with the hydrochloric acid) aflatoxin B_1 and a more intense blue fluorescent spot of aflatoxin B_1-hemiacetal, both originating from the standard solution applied at B (migration in direction II).

(c) Appearance of spots matching those described in (b), originating from the sample extract applied at A. The position of these spots is defined first by the migration distance of the aflatoxin B_1 from point A in direction I (the same as that travelled by the standard applied at C), and then by the migration distances from there in direction II of the aflatoxin B_1-hemiacetal (same as those travelled by the standard applied at B). The fluorescence intensities of hemiacetal spots originating from the extract and from the standard applied at B should match.

6. Calculation of the results

6.1. *From the visual measurements*

The content in micrograms of aflatoxin B_1 per kg of sample (ppb) is given by the formula:

$$\frac{S \cdot Y \cdot V}{W \cdot X}$$

in which:

Y and X are respectively the volumes in microlitres of the standard solution of aflatoxin B_1 (3.16) and of the extract having a similar intensity of fluorescence;

S = concentration in micrograms of aflatoxin B_1 per ml in the standard solution (3.16);

V = final volume of the extract in microlitres, allowing for any dilution that was necessary;

W = weight in grammes of the sample corresponding to the volume of extract subjected to column clean-up.

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6.2. *From the fluorodensitometric measurements*

The content in micrograms of aflatoxin B_1 per kg of sample is given by the formula:

$$\frac{S \cdot V}{W \cdot Y}$$

in which:

Y = volume in microlitres of the extract spotted on the plate (10 or 20 µl);

S = quantity in nanograms of aflatoxin B_1 in the extract spot (proportional to the value of Y taken), deduced from the measurements;

V = final volume of the extract in microlitres, allowing for any dilution that was necessary;

W = weight in grammes of the sample corresponding to the volume of extract subjected to column clean-up.

7. **Preparation and testing of the standard solution (3.16)**

7.1. *Determination of the concentration of aflatoxinB_1*

Prepare a standard solution of aflatoxin B_1 in the chloroform (3.2) or the benzene/acetonitrile mixture (3.6) with a concentration of 8 to 10 µg/ml. Determine the absorption spectrum between 330 and 370 nm with the aid of the spectrophotometer (4.11).

Measure the optical density (A) at 363 nm in the case of the chloroform solution; or at 348 nm in the case of the solution in the benzene/acetonitrile mixture.

Calculate the concentration in micrograms of aflatoxin B_1 per ml of solution from the formulae below:

$\frac{312 \cdot A \cdot 1000}{20600}$ for the chloroform solution;

$\frac{312 \cdot A \cdot 1000}{19800}$ for the solution in the benzene/acetonitrile mixture.

Dilute as appropriate, away from day light, to obtain a working standard solution with a concentration of aflatoxin B_1 of about 0·1 µg/ml. If kept in a refrigerator at 4 ºC, this solution is stable for two weeks.

7.2. *Testing of chromatographic purity*

Spot on a plate (4.8) 5 µl of the standard solution of aflatoxin B_1 containing 8 to 10 µg/ml (7.1). Develop the chromatogram as indicated in 5.4. In UV light the chromatogram should show only one spot and no fluorescence must be perceptible in the original deposit zone.

8. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample by the same analyst should not exceed:

— 25 % related to the highest result for contents of aflatoxin B_1 from 10 and up to 20 µg/kg;

— 5 µg, in absolute value, for contents greater than 20 and up to 50 µg/kg;

— 10 % related to the highest value for contents above 50 µg/kg.

9. **Reproducibility**

See under ‘Observations’, Part C, point 2.

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B. DETERMINATION OF AFLATOXIN B_1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

1. **Purpose and scope**

This method is for the determination of aflatoxin B_1 in animal feedingstuffs including those containing citrus pulp. The lower limit of determination is 0,001 mg/kg (1 ppb).

2. **Principle**

The sample is extracted with chloroform. The extract is filtered and an aliquot portion is purified on a Florisil cartridge followed by a C_{18} cartridge. The final separation and determination is achieved by high performance liquid chromatography (HPLC) using a reversed phase C_{18} column, followed by post-column derivatization with iodine in water, and fluorescence detection.

Note: mycotoxins are extremely toxic sustances. Manipulations should be performed in a designated fume cupboard. Special precautions should be taken when toxins are in a dry form because of their electrostatic nature and resulting tendency to disperse in working areas.

3. **Reagents**

3.1. Chloroform, stabilized with 0,5 to 1,0 % of ethanol, by mass. See observation 10.2.

3.2. Methanol, HPLC grade for preatation of 3.6.

3.3. Acetone.

3.4. Acetonitril, HPLC grade.

3.5. Eluting solvents: Prepare one day before use, or remove air in the solvents ultrasonically.

3.5.1. Mixture of acetone (3.3) and water, 98 + 2 (v+v).

3.5.2. Mixture of water and methanol (3.2), 80 + 20 (v+v).

3.5.3. Mixture of water and acetone (3.3), 85 + 15 (v+v).

3.6. Mobile phase for HPLC

Mixture of water, methanol (3.2) and acetonitril (3.4), 130 + 70 + 40 (v+v+v).

NB The composition of the mobile phase solvent may need to be adjusted, depending on the characteristics of the HPLC column used.

3.7. Saturated iodine solution: add 2 g of iodine to 400 ml of water. Mix for at least 90 min and filter through a membrane filter (4.15). Protect the saturated solution from light to prevent photodegradation.

3.8. Acid washed Celite 545, or equivalent.

3.9. Florisil cartridge (Waters SEP-PAK), or equivalent.

3.10. C_{18} cartridge (Waters SEP-PAK), or equivalent.

3.11. Inert gas e.g. nitrogen.

3.12. Aflatoxin B_1 standard solution in chloroform, concentration 10 µg/ml. Check the concentration of the solution as follows: determine the absorption spectrum of the solution between 330 and 370 nm by means of the spectrophotometer (4.23). Measure the absorbance (A) at the maximum near 363 nm. Calculate the concentration of aflatoxin B_1 in micrograms per millilitre of solution from the formula:

$$\text{Concentration}(\text{èmg/ml}) = \frac{312 \times A \times 1000}{22\,300} = 13,991 \times A$$

3.12.1. Aflatoxine B_1 stock standard solution in chloroform.

Transfer quantitatively 2,5 ml of the aflatoxin B_1 standard solution (3.12) to a 50 ml volumetric flask and adjust to the mark with

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chloroform (3.1). Store this solution in a cool place (4 ºC) in the dark, well sealed and wrapped in aluminium foil.

3.13. Aflatoxin B_1 calibraltion solutions HPLC.

NB Use alcid-washed glassware for preparation of these solutions (see 4, Apparatus).

3.13.1. Calibration solution 4ng/ml.

Allow the volumetric flask with stock standard solution (3.12.1) to warm up to room temperature in the aluminium foil (a few hours). Transfer 400 µl of the stock standard solution (200 ng aflatoxin B_1) into a 50 ml volumetric flask, and evaporate the solution to dryness in a current of inet gas (3.11).

Dissolve the residue obtained in approximately 20 ml of water/acetone mixture (3.5.3), make up to the mark with the water/acetone mixture and mix well.

3.13.2. Calibration solution 3 ng/ml.

Transfer quantitatively 7,5 ml of the calibration solution (3.13.1) into a 10 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3), and mix well.

3.13.3. Calibration solution, 2 ng/ml.

Transfer quantitatively 25 ml of the calibration solution (3.13.1) to a 50 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3) and mix well.

This solution is also referred to as the reference standard, to be used for repetitive injection during HPLC (5.5).

3.13.4. Calibration solution 1 ng/ml.

Transfer quantitatively 2,5 ml of the calibration solution (3.13.1) to a 10 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3) and mix well.

3.14. Ampoule containing a mixture of aflatoxins B_1, B_2, G_1 and G_2 concentrations approximately 1, 0,5, 1 and 0,5 µg/ml respectively, in 1 ml chloroform.

3.14.1. Chromatographic test solution.

Transfer the content of the ampoule (3.14) into a glass-stoppered test-tube or screw-capped vial. Transfer 40 µl of this solution into a glass-stoppered test-tube (acid-rinsed) (4.22). Evaporate the chloroform in a stream of inert gas (3.11) and redissolve into 10 ml of the water/acetone mixture (3.5.3).

3.15. Reagents for confirmatory test (6).

3.15.1. Sodium chloride saturated solution.

3.15.2. Sodium sulphate, anhydrous, granular.

4. **Apparatus**

Caution: Use of non acid-washed glassware for aqueous aflatoxin solutions may cause losses. Particular care should be taken with new glassware and disposable glassware such as autosampler vials and Pasteur pipettes. Therefore laboratory glassware coming into contact with aqueous solutions of aflatoxins should be soaked in dilute acid (e.g. sulfuric acid = 2 mol/l) for several hours, then rinsed well with distilled water to remove all traces of acid (e.g. three times, check with pH-paper). In practice, this treatment is necessary for the round bottomed flask (4.4), the volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly vials for autosamplers), and Pasteur pipettes, if these are used to transfer calibration solutions or extracts.

4.1. Grinder-mixer.

4.2. Sieve of aperture size 1,0 mm, (ISO R 565).

4.3. Mechanical shaker.

4.4. Rotary vacuum evaporator, equipped with a 150 to 250 ml round bottomed flask.

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4.5. High performance liquid chromatograph, injector with a loop suitable for the injection of 250 ml. See the manufacturers instructions for partial or complete loop filling.

4.6. HPLC analytical column: 3 µm or 5 µm C_{18} packing.

4.7. Pulse-free pump for delivery of the iodine post-column reagent.

4.8. Valco zero dead volume Tee, stainless steel (1/16″ × 0,75 mm).

4.9. Spiral reaction coil; Teflon or stainless steel. Dimensions of 3 000 × 0,5 mm to 5 000 × 0,5 mm have been found to be appropriate in combination with 5 µm or 3µm HPLC columns.

4.10. Thermostatically controlled water-bath adjusted to 60 ºC, capable of temperature regulation to better than 0,1 ºC.

4.11. Fluorescence detector, with excitation at 365 nm and emission at 435 nm wavelenths. (For filter instrument: emission wavelength > 400 nm). Detection of at least 0,05 ng aflatoxin B_1 shall be possible. Some back pressure may be advisable (e.g. restrictor, Teflon or stainless steel coil connected to the outlet of the detector), to suppress air bubbles in the flow-cell.

4.12. Strip chart recorder.

4.13. Electronic integrator (optional).

4.14. Fluted filter paper diameter: 24 cm, Macherey-Nagel 617 1/4 or equivalent.

4.15. Membrane filter with a pore size of 0,45 µm, Millipore HAWP 04700 or equivalent.

4.16. 500 ml glass stoppered conical flask.

4.17. Glass column (internal diameter approximately 1 cm, length approximately 30 cm) equipped with a Luer tip.

4.18. Luer chloroform-resistant stopcock (e.g. Bio-rad 7328017, Analytichem A1 6078, J.T. Baker 4514 or equivalent).

4.19. Chemically resistant syringe, 10 ml Luer connector.

4.20. Syringe suitable for HPLC injection of 250 µl (see 4.5).

4.21. 100 µl microsyringe for preparation of calibration solutions (check that the accuracy is within 2 % by weighing).

4.22. 10 ml glass stoppered calibrated tubes.

4.23. Spectrophotometer, suitable for making measurements in the UV region of the spectrum.

4.24. Equipment for confirmatory test (6).

4.24.1. Acid-rinsed 100 ml separating funnel with Teflon stopcock.

4.24.2. Heating block, 40 to 50 ºC.

5. **Procedure**

5.1. *Preparation of the sample.*

Grind the sample so that it passes through the sieve (4.2).

5.2. *Test portion.*

Weigh 50 g of the prepared test sample into the conical flask (4.16).

5.3. *Extraction*

Add 25 g of Celite (3.8), 250 ml of chloroform (3.1) and 25 ml of water to the test portion (5.2). Stopper the flask, and shake for 30 minutes on a mechanical shaker (4.3). Filter through a fluted filter paper (4.14). Collect 50 ml of the filtrate. If necessary, take an aliquot of the filtrate and dilute to 50 ml with chloroform so that the concentration of aflatoxin B_1 is not greater than 4 ng/ml.

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5.4. *Clean-up (the procedure should be carried out without significant interruptions).*

Caution:

— protect the laboratory, where the analyses are done, adequately from daylight. This can be achieved effectively by using:

(i) UV absorbing foil on the windows in combination with subdued light (no direct sunlight);

(ii) Curtains or blinds in combination with artificial light (fluorescent tubes are acceptable);

— Aflatoxin containing solutions must be protected from light as much as possible (keep in the dark, use aluminium foil).

5.4.1. Florisil SEP-PAK purification

5.4.1.1. Preparation of the column-cartridge assembly

Attach a stopcock (4.18) to the shorter stem of a Florisil cartridge (3.9) (see figure 1). Wash the cartridge and remove aid by taking 10 ml chloroform (3.1) and passing 8 ml via the stopcock rapidly through the cartridge using a syringe (4.19). Attach the longer stem of the cartridge to a glass column (4.17) and pass the reamining 2 ml chloroform through the cartridge into the column. Close the stopcock. Remove the syringe.

5.4.1.2. Purification

Add the filtrate collected in 5.3 to the column-cartridge assembly and drain by gravity. Rinse with 5 ml of chloroform (3.1), followed by 20 ml of methanol (3.2). Discard the eluates. During these operations, ensure that the column-cartridge assembly does not run dry.

Elute aflatoxin B_1 with 40 ml of the acetone-water mixture (3.5.1) and collect the whole of the eluate in the round bottomed flask of the rotary evaporator (4.4). Concentrate the eluate on the rotary evaporator at 40 ºC to 50 ºC until no more acetone is distilled. (*NB* approximately 0,5 ml of liquid remains in the flask at this point. Experiments have shown that further evaporation is not harmful and that when 0,5 ml of liquid remains, there is then no significant amount of acetone. Residues of acetone might lead to losses of aflatoxin B_1 on the C_{18} cartridge) Add 1 ml of methanol (3.2), swirl the flask to dissolve aflatoxin B_1 on the sides of the flask, add 4 ml water, and mix. Disconnect and discard the cartridge. Rinse the glass column with water and retain for the C_{18} purification step.

5.4.2. C_{18} SEP-PAK purification

5.4.2.1. Preparation of the column-cartridge assembly.

Attach a stopcock (4.18) to the shorter stem of a C_{18}-cartridge (3.10) (see figure 1). Prime the cartridge and remove any air by passing 10 ml methanol (3.2) via the stopcock rapidly through the cartridge with a syringe (4.19) (Air bubbles in the cartridge are visible as light spots on the otherwise greyish background). Take 10 ml water, and pass 8 ml through the cartridge (Avoid introduction of air into the cartridge, when switching from methanol to water). Attach the longer stem of the cartridge to a glass column (4.17) and pass the remaining 2 ml water through the cartridge in the column. Close the stopcock. Remove the syringe.

5.4.2.2. Purification

Transfer the extract collected in 5.4.1.2 quantitatively to the glass column (4.17), rinsing the flask twice with 5 ml water/methanol mixture (3.5.2) and drain by gravity. During these operations, ensure that the column-cartridge assembly does not run dry. (When air bubbles develop in the constriction near the cartridge, stop the flow and tap the top of the glass column, to remove the air bubbles. Then continue). Elute with 25 ml water/methanol mixture. Discard the eluate. Elute the aflatoxin B_1 with 50 ml water/acetone mixture (3.5.3), and collect the whole of the eluate in a 50 ml volumetric flask. Make up to the mark with water and mix: the resulting test solution is used for chromatography (5.5).

Caution: Filtration of the final extract prior to HPLC is normally not necessary. If considered necessary, cellulose filters are not to be used, because they may lead to losses of aflatoxin B_1. Teflon filters are acceptable.

▼**M2**

5.5. *High performance liquid chromatography*

(See Figure 2 for setting-up of the equipment). Allow sufficient time for conditioning and stabilizing the instruments.

Note 1:

The flow-rates given for the mobile phase and the post-column reagent are indicative only. They may need to be adjusted depending on the characteristics of the HPLC column.

Note 2:

The detector response to aflatoxin B_1 depends on the temperature, therefore compensation should be made for drift (see Figure 3). By injecting a fixed amoung of aflatoxin B_1 reference standard (3.13.3) at regular intervals (i.e. every third injection), the aflatoxin B_1 peak values between these reference standards can be corrected using the mean response, provided that the difference between responses of consecutive reference standards is very small (< 10 %). Therefore injections must be made without interruptions. If interruption is necessary, the last injection before interruption and the first injection after interruption must be the reference standard (3.13.3). Because the calibration curve is linear and passes through the origin, the amounts of aflatoxin B_1 in the sample extracts are determined directly by reference to the adjacent standards.

5.5.1. HPLC pump settings

Set the HPLC pump (4.5) to give a flow of 0,5 or 0,3 ml/min for a 5 μm or a 3 μm HPLC column (4.6) respectively using the mobile phase (3.6).

5.5.2. Post-column pump settings

Set the pump (4.7) to give a flow of 0,2 to 0,4 ml/min of the iodine-saturated water solution (3.7). As a rough guide: Flows of approximately 0,4 or 0,2 ml/min are advisable in combination with flows of 0,5 and 0,3 ml/min of the mobile phase (3.6) respectively.

5.5.3. Fluorescence detector

Set the fluorescence detector (4,11) to exc. = 365 nm and em = 435 nm (filter instrument; > 400 nm). Adjust the detector attenuator to obtain approximately 80 % full scale deflection of the recorder pen for 1 ng of aflatoxin B_1.

5.5.4. Injector

For all solutions, inject 250 μl amounts following the instructions of the manufacturer of the injector.

5.5.5. Check of chromatographic separation

Inject the chromatographic test solution (3.14.1). Valleys should be less than 5 % of the sum of peak heights of the adjacent peaks.

5.5.6. Check of the stability of the system

Before each series of analyses, respectively inject the reference standard (3.13.3), until stable peak areas are achieved (*NB* Peak responses for aflatoxin B_1 between consecutive injections should not differ by more than 6 %). Proceed without delay with the check of linearity (5.5.7).

5.5.7. Check of linearity

Inject the aflatoxin B_1 calibration solutions (3.13.1 to 3.13.4). Every third injection use the reference standard (3.13.3), for correction of drift in response (*NB* Peak responses for this reference standard must not differ by more than 10 % in 90 minutes). Correct for drift according to the formula in 7. The calibration graph should be linear and pass through the origin, within twice standard error of Y-estimate. Values found must not differ by more than 3 % from the nominal values. If these requirements are fulfilled, continue without delay. If not, identify and correct the sources of the problem before continuing.

5.5.8. Injection of sample extracts

Inject the purified sample extracts (5.4.2.2). After every two sample extracts repeat the injection of the reference standard (3.13.3) according to the following sequence: reference standard, extract, extract, reference standard, extract, extract, reference standard etc.

▼M2

6. **Confirmatory test**

6.1. *Further treatment of the extract (5.4.2.2)*

Add 5 ml sodium chloride solution (3.15.1) to the final extract obtained at 5.4.2.2.. Extract three times each with 2 ml chloroform (3.1) for one minute, in the separating funnel (4.24.1). Pour the combined chloroform extracts over approximately 1 g sodium sulphate (3.15.2) into a 10 ml test tube. A small funnel (diameter: 4 cm) can be used with a piece of cottonwool in the constriction, covered with a approximately 1 g sodium sulphate.

Wash the sodium sulphate layer with a few ml of chloroform and collect the washing in the same test tube. Evaporate the chloroform extract to dryness in the same test-tube using the heating block (4.24.2) and redissolve in 1 ml of chloroform.

6.2. *Preparation of derivative and thin layer chromatography:*

See Annex to Council Directive 76/372/EEC method A, point 5.6.2.

7. **Calculation of results**

Calculate the aflatoxin B_1 content (µm/kg) present in the sample, using the formula:

$$\text{aflatoxin } B_1 \text{ content in èmg/kg} = \frac{m \times V_{ext}}{V_m \times M \times \frac{V_f}{V_c}}$$

where:

m = amount of aflatoxin B_1 in ng represented by the B_1 peak of the sample, calculated as follows:

$$m = \frac{P(\text{sample})}{P(st_1) + P(st_2)} \times 2\ r(st)$$

P(sample) = peak area of aflatoxin B_1 for the sample

$P(st_1)$ = peak area of aflatoxin B_1 resulting from the preceeding injection of reference standard (3.13.3)

$P(st_2)$ = peak area of aflatoxin B_1 resulting from the following injection of reference standard (3.13.3)

r(st) = injected amount of aflatoxin B_1 in the reference standard (3.13.3) in ng

V_m = volume of the injected sample extract in ml

V_{ext} = final volume of sample extract in ml, allowing for any dilution that was made (5.3)

M = mass of sample in g

V_f = volume of filtrate transferred to Florisil cartridge (5.4.1.2) in ml

V_c = volume of chloroform, used for the extraction of the sample in ml

If the procedure is followed as in this protocol, the formula reduces to:

aflatoxin B_1 content in µg/kg = 20 × m.

7.1. Calculations of the results may also be done by peak height measurement.

8. **Repeatability:**

see under 10.1

9. **Reproducibility:**

see under 10.1

▼M2

10. **Observations**

10.1. *Precision*

A collaborative study [1], carried out at the international level on mixed feeding stuffs gave the results for repeatability and reproducibility indicated in Table 1. The term repeatability (r) used here is defined as the largest ratio which is not significant at the 95 % probability level for comparison of two readings of the same sample in the same laboratory under similar conditions. The term reproducibility (R) is similarly defined for comparing two different laboratories. In accordance to ISO 3534 — 1977, 2.35 [2] and Commission Decision 89/610/EEC [3] r and R are also given in Table 1 in terms of coefficients of variation.

Table 1

Repeatability (r) and reproducibility (R) expressed as ratios and corresponding coefficients of variation

(15 laboratories)

Level	r	R	CV_r (*)	CV_R
(µg/kg)			(%)	(%)
8 & 14	1,4	1,7	11	18

(*) CV = coefficient of variation

10.2. *Stabilization of chloroform (3.1)*

The adsorption characteristics of the florisil cartridge may be changed if stabilizers other than ethanol are used. This should be verified in accordance with 10.3 when the chloroform described is not available.

10.3. *Accuracy*

The correct applications of the method shall be verified by making replicate measurements on certified reference materials. If these are not available, the performance of the method should be verified by recovery experiments made on the fortified blank samples. The deviation of the mean from the actual value, expressed as a percentage of the actual value, shall not lie outside the limits −20 to +10 %.

(1) Egmond, H.P. van, Heisterkamp, S.H. and Paulsch, W.E. (1991). *Food Additives and Contaminants* 8, 17-29.
(2) ISO 3534-1977.
(3) OJ No L 351, 2. 12. 1989, p. 39.

▼M2

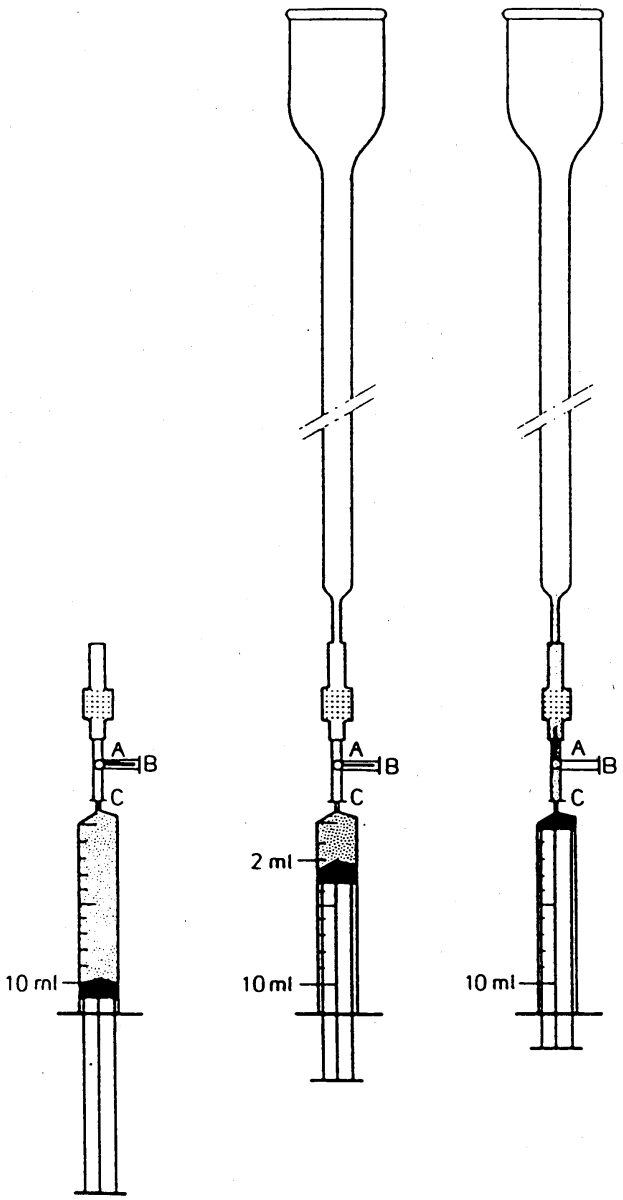
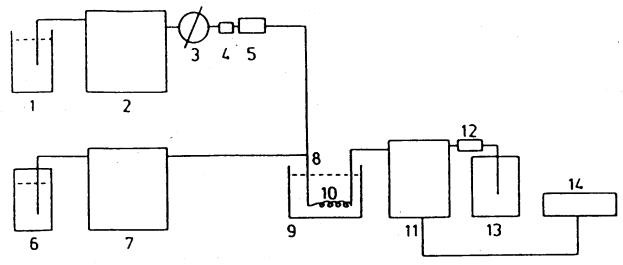


Figure 1: Column-cartridge assembly



1. Mobile phase
2. Pump
3. Injection valve
4. Guard column
5. HPLC analytical column
6. Saturated iodine solution
7. Reagent pump
8. T-joint
9. Thermostatically controlled bath
10. Spiral reaction coil
11. Fluorescence detector
12. Restrictor
13. Waste
14. Strip chart recorder/integrator

Figure 2: Flow diagram of the LC system with iodine post-column derivatization

▼M2

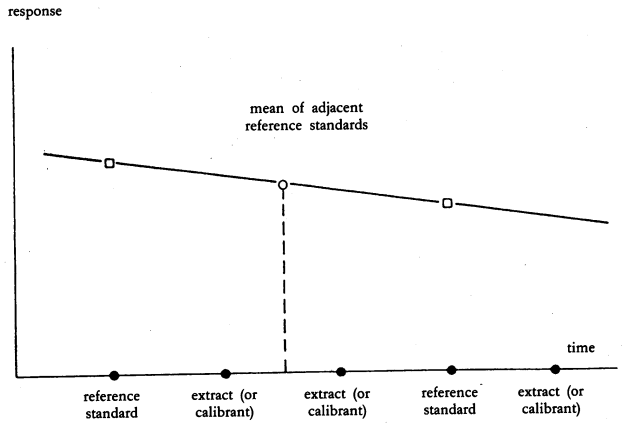


Figure 3: Compensation for drift in aflatoxin B_1 response by injecting reference standard (3.13.3) at regular intervals

▼M3

C. OBSERVATIONS CONCERNING METHODS A AND B

1. **Defatting**

Samples containing more than 5 % fats must be defatted with light petroleum (bp 40 to 60 ºC) after the preparations indicated in 5.1.

In such cases, the analytical results must be expressed in terms of the weight of the non-defatted sample.

2. **Reproducibility of the results for Method A**

The reproducibility of the results, i.e. the variation between the results obtained by two or more laboratories on the same sample has been estimated at:

± 50 % of the mean value for mean values of aflatoxin B, from 10 and up to 20 µg/kg;

± 10 µg/kg on the mean value for mean values greater than 20 and up to 50 µg/kg;

± 20 % of the mean value for mean values above 50 µg/kg.

▼B

Appendix to Annex

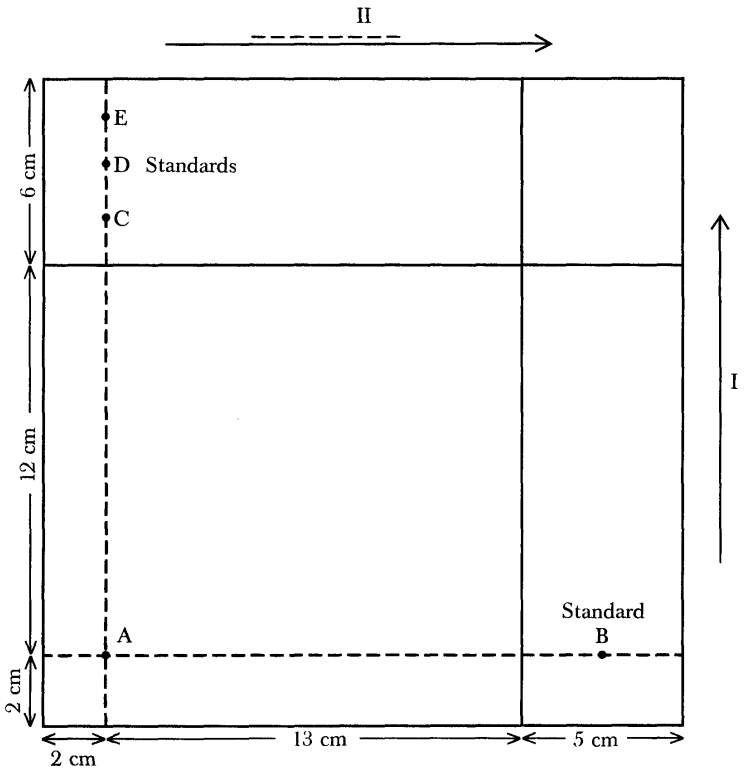


Figure 1

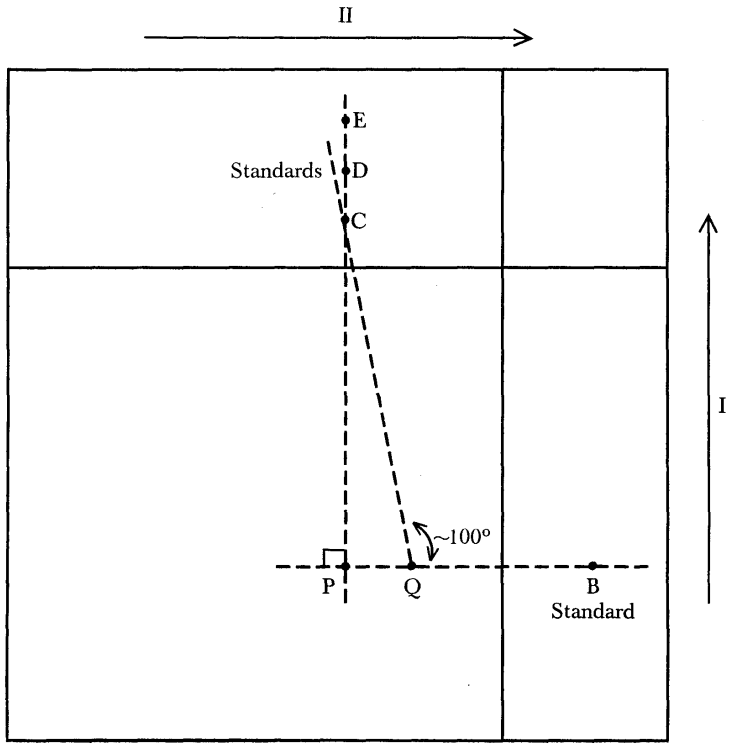


Figure 2

▼B

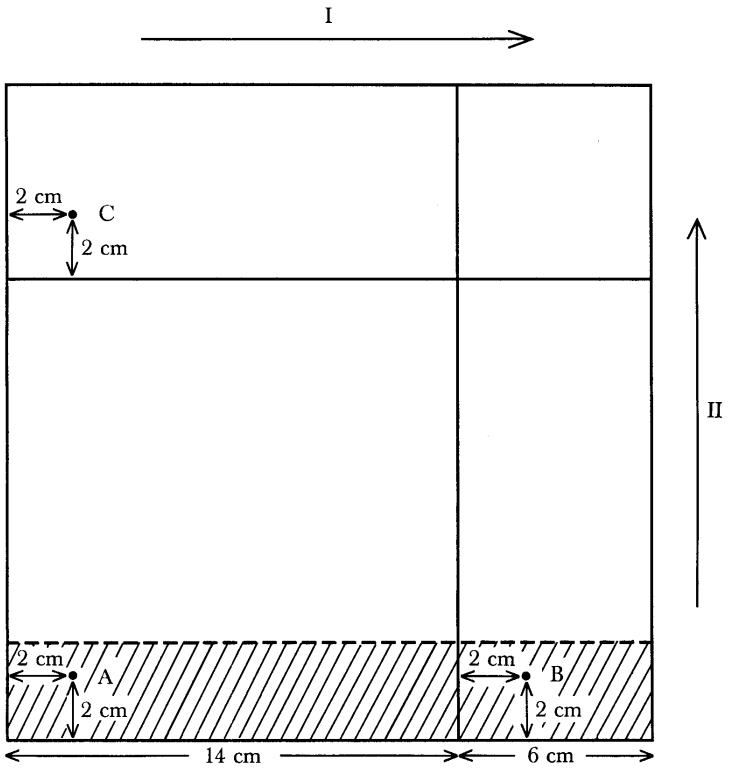


Figure 3