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Animal feeding stuffs — Semi-quantitative determination of aflatoxin B_1 — Thin-layer chromatographic methods

Aliments des animaux — Dosage semi-quantitatif de l'aflatoxine B_1 — Méthodes par chromatographie sur couche mince

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 6651 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

This third edition cancels and replaces the second edition (ISO 6651:1987), of which it constitutes a minor revision.

Annex A of this International Standard is for information only.

Animal feeding stuffs — Semi-quantitative determination of aflatoxin B_1 — Thin-layer chromatographic methods

1 Scope

1.1 This International Standard specifies two methods for the determination of aflatoxin B_1 in animal feeding stuffs. These methods can only be used for semi-quantitative determinations.

1.2 **Method A** is applicable to the following simple feeding stuffs:

— oilseeds and oilseed residues, and in particular groundnut, copra, linseed, soya, babassu palm;

— manioc meal;

— maize germ expeller;

— cereals and cereal products;

— pea meal;

— potato pulp and flour.

In the presence of substances interfering with the determination by method A, it is recommended that the determination be carried out in accordance with method B.

1.3 **Method B** is applicable to mixed feeding stuffs and to simple feeding stuffs not mentioned in 1.2.

This method is not applicable to feeding stuffs containing citrus pulp.

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the normative document indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6498, *Animal feeding stuffs — Preparation of test samples*

3 Principle

A test portion is extracted with chloroform then filtration. An aliquot portion is purified on a silica gel column.

The eluate is evaporated and the residue is dissolved in a specified volume of chloroform or a mixture of benzene and acetonitrile.

Thin-layer chromatography, one-dimensional for method A and two-dimensional for method B, is carried out on an aliquot portion of this solution.

The aflatoxin B_1 content is determined either visually or by fluorodensitometry, by examination of the chromatogram under ultraviolet light and comparison with known quantities of standard aflatoxin B_1 applied to the same plate as the test portion extract.

The identify of aflatoxin B_1 is confirmed by formation of the hemiacetal derivative.

4 Reagents

Use only reagents of recognized analytical quality, and distilled or deonized water or water of at least equivalent purity.

4.1 Chloroform, stabilized with 0,5 % to 1,0 % of 96 % (volume fraction) ethanol.

4.2 *n*-Hexane.

4.3 Diethyl ether, anhydrous, free from peroxides.

4.4 Benzene/acetonitrile, (98 + 2) mixture.

Mix 98 volumes of benzene with 2 volumes of acetonitrile.

4.5 Chloroform/methanol, (97 + 3) mixture.

Mix 97 volumes of chloroform with 3 volumes of methanol.

4.6 Developing solvents.

The solvents should be used in covered tanks. When saturated tanks are specified, this is achieved by lining the tanks with absorbent paper and allowing the interiors to become saturated with solvent vapour.

4.6.1 Chloroform/acetone, (90 + 10) mixture.

Mix 90 volumes of chloroform with 10 volumes of acetone, in an unsaturated tank.

4.6.2 Diethyl ether/methanol/water, (96 + 3 + 1) mixture.

Mix 96 volumes of diethyl ether, 3 volumes of methanol and 1 volume of water, in an unsaturated tank.

4.6.3 Diethyl ether/methanol/water, (94 + 4,5 + 1,5) mixture.

Mix 94 volumes of diethyl ether with 4,5 volumes of methanol and 1,5 volumes of water, in a saturated tank.

4.6.4 Chloroform/methanol, (94 + 6) mixture.

Mix 94 volumes of chloroform with 6 volumes of methanol, in a saturated tank.

4.6.5 Chloroform/methanol, (97 + 3) mixture.

Mix 97 volumes of chloroform with 3 volumes of methanol, in a saturated tank.

4.7 Silica gel, for column chromatography, of particle size 0,05 mm to 0,20 mm.

4.8 Silica gel, G-HR or equivalent, for thin-layer chromatography.

4.9 Diatomaceous earth (Hyflosupercel), acid-washed.

4.10 Sodium sulfate, anhydrous granules.

4.11 Trifluoroacetic acid.

4.12 Inert gas, for example nitrogen.

4.13 Sulfuric acid, 50 % solution (volume fraction).

4.14 Aflatoxin B_1, standard solution containing about 0,1 μg of aflatoxin B_1 per millilitre, in the chloroform (4.1) or in the benzene/acetonitrile mixture (4.4).

WARNING — Aflatoxins are highly carcinogenic and must be handled with great care.

Prepare and check the solution as follows.

4.14.1 Preparation of stock solution and determination of concentration

Prepare a solution of aflatoxin B_1 in the chloroform (4.1) or the benzene/acetonitrile mixture (4.4) such that the concentration is between 8 μg/ml and 10 μg/ml. Determine the absorption spectrum between 330 nm and 370 nm by means of the spectrometer (5.9).

Measure the absorbance (A) at 363 nm in the case of the chloroform solution, or at 348 nm in the case of the benzene/acetronitrile mixture solution.

Calculate the concentration of aflatoxin B_1, in micrograms per millilitre of solution, from the formulae:

a) for the chloroform solution

$$\frac{312 \times A \times 1\,000}{22\,300}$$

b) for the solution in the benzene/acetonitrile mixture

$$\frac{312 \times A \times 1\,000}{19\,800}$$

4.14.2 Dilution

Dilute the stock solution (4.14.1), as appropriate, away from daylight, to obtain a standard solution with a concentration of aflatoxin B_1 of about 0,1 μg/ml.

If kept in a refrigerator at 4 °C, this solution is stable for 2 weeks.

4.14.3 Testing of chromatographic purity of the solution

Onto a plate (5.7), apply a spot of 5 μl of the standard aflatoxin B_1 solution of concentration 8 μg/ml to 10 μg/ml (4.14.1). Develop the chromatogram as indicated in 7.5.1. Under ultraviolet light, the chromatogram shall show only one spot and no fluorescence shall be perceptible in the original deposition zone.

4.15 Aflatoxin B_1 and B_2 (see the warning in 4.14), solutions for qualitative testing, containing about 0,1 μg of aflatoxin B_1 and B_2 per millilitre, in the chloroform (4.1) or in the benzene/acetonitrile mixture (4.4).

These concentrations are given as a guide. They shall be adjusted so as to obtain the same intensity of fluorescence for both aflatoxins (see 7.5.1).

5 Apparatus

Usual laboratory equipment and, in particular, the following.

5.1 Grinder/mixer.

5.2 Sieve, of aperture size 1,0 mm.

For details, see ISO 565[1)].

5.3 Shaking apparatus or **magnetic stirrer**.

5.4 Chromatographic tubes, made of glass (internal diameter 22 mm, length 300 mm), with a polytetrafluoroethylene tap and a 250 ml reservoir, plugged at the bottom end with cotton or glass wool.

5.5 Rotary vacuum evaporator, with a 500 ml round-bottomed flask.

5.6 Apparatus for thin-layer chromatography (TLC), i.e. that necessary for the preparation of the plates (5.7) and application of spots (capillary pipettes or microsyringes), a developing tank, and spraying apparatus for applying the sulfuric acid (4.13) to the plates.

5.7 Glass TLC plates, 200 mm × 200 mm, prepared as follows (the quantities indicated are sufficient to cover five plates).

Place 30 g of the silica gel (4.8) in a conical flask, add 60 ml of water, stopper and shake for 1 min. 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 an oven at 110 °C for 1 h.

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

5.8 Long-wavelength (360 nm**) ultraviolet lamp**.

The intensity of irradiation shall make it possible for a spot of 1,0 ng of aflatoxin B_1 to be clearly distinguished on a TLC plate at a distance of 10 cm from the lamp.

WARNING — Ultraviolet light is dangerous to the eyes. Protective goggles shall be worn.

5.9 Spectrometer, suitable for making measurements in the ultraviolet region of the spectrum.

5.10 Fluorodensitometer (optional).

5.11 Fluted filter paper.

5.12 Graduated tube, of capacity 10,0 ml, with a polyethylene stopper.

5.13 Conical flask, of capacity 500 ml, with a ground glass stopper.

5.14 Pipette, of capacity 50 ml.

5.15 Analytical balance.

6 Sampling

Take the laboratory sample from the material to be sampled in accordance with the International Standard for the material concerned unless sampling for the determination of aflatoxin is excluded from its field of application. If no appropriate International Standard exists, agreement shall be reached between the parties concerned, taking into account the characteristics of the material being sampled.

1) ISO 565, *Test sieves — Metal wire cloth, perforated metal plate and electroformed sheet — Nominal sizes of openings*.

7 Procedure

7.1 Preparation of test sample

7.1.1 If the sample contains more than 5 % of fat, it shall be defatted with light petroleum before grinding.

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

7.1.2 Grind the laboratory sample so that is completely passes through the sieve (5.2). Mix thoroughly. See ISO 6498.

7.2 Test portion

Weigh, to the nearest 0,01 g, 50 g of the prepared test sample into the conical flask (5.13).

7.3 Extraction

Add to the test portion (7.2) 25 g of the diatomaceous earth (4.9), 25 ml of water, and 250 ml of the chloroform (4.1) accurately measured from a measuring cylinder. Stopper the flask, and shake or stir for 30 min using the shaking apparatus (5.3). Filter through the fluted filter paper (5.11), taking care to discard the first 10 ml of the filtrate, and subsequently collect at least 50 ml of the filtrate.

7.4 Column clean-up

7.4.1 Preparation of the column

Fill two-thirds of the chromatographic tube (5.4) with the chloroform (4.1) and add 5 g of the sodium sulfate (4.10). Check that the upper surface of the sodium sulfate layer is flat, then add 10 g, in small portions, of the silica gel (4.7). Stir carefully after each addition to eliminate air bubbles. Leave to stand for 15 min and then carefully add 10 g of the sodium sulfate (4.10). Open the tap and allow the liquid to flow until it is just above the upper surface of the sodium sulfate layer. Close the tap.

7.4.2 Purification

Transfer, by means of the pipette (5.14), 50 ml of the filtrate collected in 7.3 to a 250 ml conical flask, and add 100 ml of the *n*-hexane (4.2). Mix and quantitatively transfer the mixture to the column, rinsing the flask with the *n*-hexane. Open the tap and allow the liquid to flow at a rate of 8 ml/min to 12 ml/min until it is level with the upper surface of the sodium sulfate layer. Close the tap. Discard the liquid collected and pour 100 ml of the diethyl ether (4.3) into the column. Again open the tap and allow the liquid to flow until it is level with the upper surface of the sodium sulfate layer. During these operations, ensure that the column does not run dry.

Elute with 150 ml of the chloroform/methanol mixture (4.5) and collect the whole of the eluate in the 500 ml flask of the rotary evaporator (5.5). Evaporate to dryness on the rotary evaporator, preferably under a stream of inert gas (4.12), at a temperature not exceeding 50 °C, and under reduced pressure.

If a rotary evaporator is not available, add a boiling aid and evaporate almost to dryness on a water bath.

Quantitatively transfer the residue, using the chloroform (4.1) or the benzene/acetonitrile mixture (4.4), to the 10 ml graduated tube (5.12). Again evaporate the solution, for example on a water bath, preferably under a stream of inert gas (4.12), and adjust the volume to 2,0 ml with the chloroform (4.1) or the benzene/acetonitrile mixture (4.4).

7.5 Thin-layer chromatography

7.5.1 Method A — One-dimensional thin-layer chromatography

7.5.1.1 Choice of solvent

The choice of solvent (4.6.1, 4.6.2, 4.6.3, 4.6.4 or 4.6.5) shall be made beforehand to ensure that aflatoxins B_1 and B_2 are completely separated when the plate is developed, which depends on the batch of plates in use.

Place 25 µl of the qualitative solution (4.15) on the prepared plates (5.7) (one plate for each solvent to be checked).

Follow the procedure in 7.5.1.2 for development, evaporation and irradiation.

Two distinct spots are produced by a suitable solvent.

7.5.1.2 Procedure

Onto a TLC plate (5.7), and using a capillary pipette or microsyringe, apply 20 mm from the lower edge, and at intervals of 20 mm, the volumes indicated below of the standard aflatoxin B_1 solution and the extract:

— 10 µl, 15 µl, 20 µl, 30 µl and 40 µl of the standard aflatoxin B_1 solution (4.14);

— 10 µl of the extract obtained in 7.4.2 and, superimposed on the same point, 20 µl of the standard aflatoxin B_1 solution (4.14);

— 10 µl and 20 µl of the extract obtained in 7.4.2.

Develop the chromatogram in the dark using the developing solvent chosen (see 7.5.1.1).

Remove the plate from the tank, allow the solvents to evaporate from the plate in the dark and then examine under ultraviolet light, placing the plate 10 cm from the lamp (5.8). The spots of aflatoxin B_1 show a blue fluorescence.

7.5.2 Method B — Two-dimensional thin-layer chromatography

7.5.2.1 Application of the solutions (see Figure 1)

Trace two straight lines on a plate (5.7) parallel to two contiguous sides (50 mm and 60 mm from each side respectively), to establish the limit of migration of the solvent fronts. Apply the following solutions to the plate using capillary pipettes or microsyringes:

— at point A: 20 µl of the purified sample extract obtained in 7.4.2;

— at point B: 20 µl of the standard aflatoxin B_1 solution (4.14);

— at point C: 10 µl of the standard aflatoxin B_1 solution (4.14);

— at point D: 20 µl of the standard aflatoxin B_1 solution (4.14);

— at point E: 40 µl of the standard aflatoxin B_1 solution (4.14).

Dry in a slow stream of air or inert gas (4.12). The spots obtained shall have a diameter of about 5 mm.

7.5.2.2 Development (see Figure 1)

Develop the chromatogram in direction I, in the dark, using the developing solvent (4.6.3) (1 cm layer in a saturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry, in the dark, at ambient temperature for at least 15 min.

Dimensions in millimetres

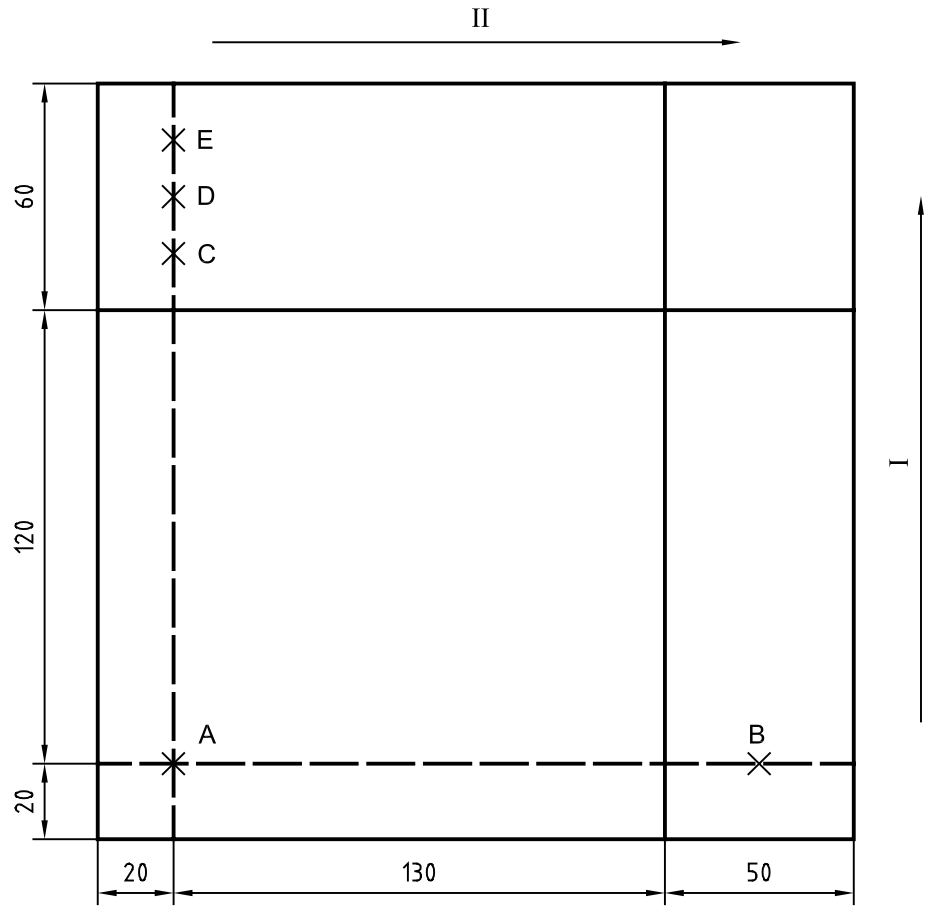

Figure 1 — Application of solutions

Then develop the chromatogram in direction II, in the dark, using the developing solvent (4.6.1) (1 cm layer in an unsaturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry, in the dark, at ambient temperature.

7.5.2.3 Interpretation of the chromatogram (see Figure 2)

Examine the chromatogram under ultraviolet light by placing the plate 10 cm from the lamp (5.8). Locate the position of the blue fluorescent spots B, C, D and E of the aflatoxin B_1 from the standard solution and trace two imaginary lines passing through these spots and at right angles to the directions of development. The point P of intersection of these lines is the location at which to expect the aflatoxin B_1 spot originating from the test portion extract applied at point A (see Figure 1). However, the actual location of the aflatoxin B_1 spot may be at a point Q at the intersection of two imaginary straight lines forming an angle of about 100° between them and passing through points B and C respectively.

Dimensions in millimetres

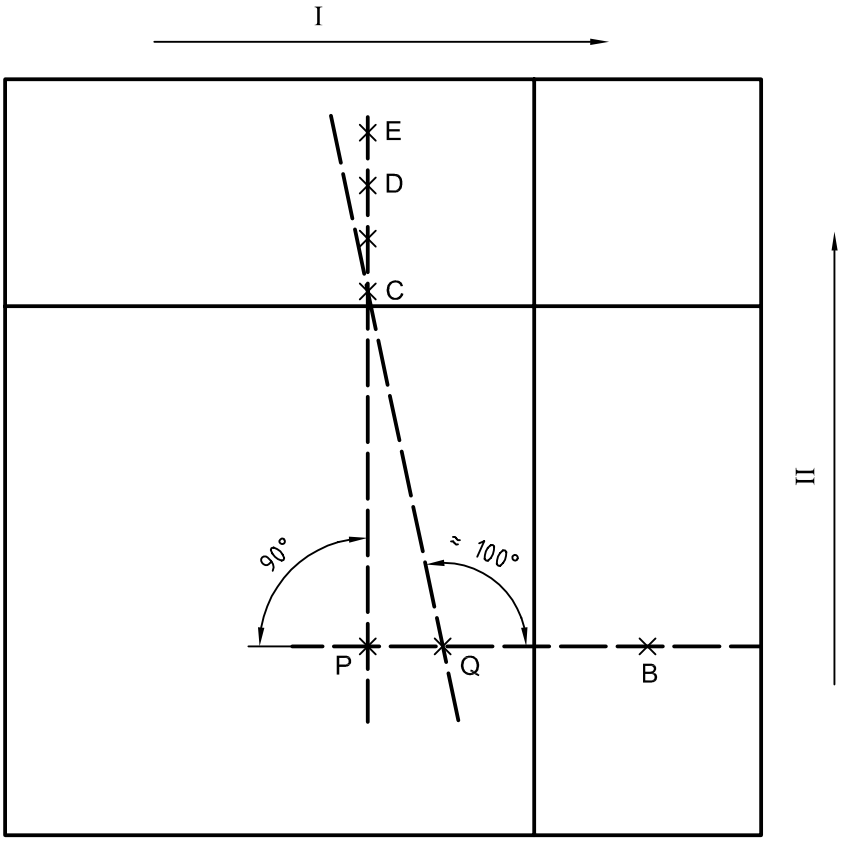

Figure 2 — Interpretation of the chromatogram

7.5.2.4 Supplementary chromatography

Trace on a new plate (5.7) two straight lines parallel to two contiguous sides, as indicated in Figure 1, and apply, at point A, 20 µl of the purified test portion extract obtained in 7.4.2 and, superimposed on it, 20 µl of the standard aflatoxin B_1 solution (4.14). Develop as indicated in 7.5.2.2. Examine the chromatogram under ultraviolet light and check that

— the aflatoxin B_1 spots from the extract and the standard solution are superimposed, and

— the fluorescence of this spot is more intense than that of the aflatoxin B_1 spot developed at point Q on the first plate.

7.6 Determination

7.6.1 Visual measurement

7.6.1.1 Method A

Determine the quantity of aflatoxin B_1 in the extract by comparing the intensity of fluorescence of the extract spots with that of the standard solution spots. Interpolate if necessary.

The fluorescence obtained by the superimposition of the extract on the standard solution shall be more intense than that of the 10 µl of extract and shall be perceptible as only one spot. If the intensity of fluorescence 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 the chloroform (4.1) or with the benzene/acetonitrile mixture (4.4) before repeating thin-layer chromatography.

7.6.1.2 Method B

Determined the quantity of aflatoxin B_1 in the extract by comparing the intensity of the extract spot with that of spots C, D and E from the standard solution. Interpolate if necessary.

If the intensity of fluorescence given by the 20 µl of extract is greater than that of the 40 µl of standard solution, dilute the extract 10 or 100 times with the chloroform (4.1) or with the benzene/acetonitrile mixture (4.4) before repeating thin-layer chromatography.

7.6.2 Measurement of fluorodensitometry

Measure the intensity of fluorescence of the aflatoxin B_1 spots with the fluorodensitometer (5.10) at an excitation wavelength of 365 nm and an emission wavelength of 443 nm.

Determine, in the case of method A, the quantity of aflatoxin B_1 in the extract spots by comparison with the intensity of fluorescence of the spots from the standard solution and, in the case of method B, the quantity of aflatoxin B_1 in the extract spot by comparison with the intensity of fluorescence of spots C, D and E from the standard solution.

7.7 Confirmation of the identity of aflatoxin B_1

7.7.1 General

Confirm the identity of the aflatoxin B_1 in the extract by the presumptive test with sulfuric acid (see 7.7.2) and, if the result of this test is positive, by the actual confirmation test (7.7.3). If the result of the presumptive test with sulfuric acid is negative, there is no need to proceed with the actual confirmation since, in this case, no aflatoxin B_1 is present.

7.7.2 Presumptive test with sulfuric acid

Spray the sulfuric acid (4.13) onto the chromatogram obtained in 7.5.1 or 7.5.2. The fluorescence of the aflatoxin B_1 spots shall turn from blue to yellow under ultraviolet light.

7.7.3 Confirmation test

7.7.3.1 Formation of aflatoxin B_1-hemiacetal (aflatoxin B_{2a})

In the case of simple and only slightly pigmented feeds, use the one-dimensional thin-layer chromatographic method described in 7.7.3.2. In the case of simple pigmented feeds, mixed feeds, or in cases of doubt, use the two-dimensional thin-layer chromatographic method described in 7.7.3.3.

7.7.3.2 One-dimensional thin-layer chromatography

Trace a straight line on a plate (5.7) to divide it into two equal parts. Apply to each part, 20 mm from the lower edge and at intervals of 15 mm, the volumes indicated below of the standard aflatoxin B_1 solution and the extract:

— 25 µl of the standard aflatoxin B_1 solution (4.14);

— a volume of the extract obtained in 7.4.2 containing approximately 2,5 ng of aflatoxin B_1;

— 25 µl of the standard aflatoxin B_1 solution (4.14) and, superimposed on it, a volume of the extract obtained in 7.4.2 containing approximately 2,5 ng of aflatoxin B_1.

Apply to one of the two halves of the plate, superimposed on the spots previously applied, 1 µl to 2 µl of the trifluoroacetic acid (4.11). Dry in a stream of air at ambient temperature.

Develop the chromatogram, in the dark, using one of the developing solvents (4.6). The choice of the solvent shall be made beforehand. The solvent system shall ensure that the aflatoxin B_1-hemiacetal (aflatoxin B_{2a}) is clearly separated from interfering substances. The solvent front shall travel about 120 mm.

Allow the solvents to evaporate in the dark, and then spray sulfuric acid (4.13) onto the part of the plate not previously treated with the trifluoroacetic acid. Examine the plate under ultraviolet light.

The identify of aflatoxin B_1 is confirmed if

a) the R_f value of the aflatoxin B_1 derivative originating from the extract corresponds with that from the standard solution;

b) the aflatoxin B_1 derivative originating from the standard solution, superimposed on the extract, has a fluorescence more intense than the aflatoxin B_1 derivative originating from the extract.

Since fluorescent spots from the extract, having the same R_f value as the aflatoxin B_1-hemiacetal, might lead to a false positive interpretation of the chromatogram, their presence should be checked on the part of the plate treated with sulfuric acid.

In cases of doubt, confirmation by two-dimensional thin-layer chromatography (7.7.3.3) shall be used.

7.7.3.3 Two-dimensional thin-layer chromatography (see Figure 3)

7.7.3.3.1 Application of the solutions

Trace two straight lines on a plate (5.7), parallel to two contiguous sides (60 mm from each side), to establish the limit of migration of the solvent fronts. Apply the following solutions to the plate using capillary pipettes or microsyringes:

— at point A: a volume of purified extract from the sample, obtained in 7.4.2, containing about 2,5 ng of aflatoxin B_1 and a drop (1 µl to 2 µl) of the trifluoroacetic acid (4.11);

— at points B and C: 25 µl of the standard aflatoxin B_1 solution (4.14) and a drop of the trifluoroacetic acid (4.11).

Dry in a stream of air at ambient temperature.

7.7.3.3.2 Development

Develop the chromatogram in direction I (see Figure 3), in the dark, using the developing solvent (4.6.2) (1 cm layer in an unsaturated tank) until the solvent front reaches the limite line. Remove the plate from the tank and allow to dry, in the dark, at ambient temperature for 5 min.

Then develop the chromatogram in direction II, in the dark, using the developing solvent (4.6.1) (1 cm layer in an unsaturated tank) until the solvent front reaches the limit line. Remove the plate from the tank and allow to dry at ambient temperature.

7.7.3.3.3 Interpretation of the chromatogram

Examine the chromatogram under ultraviolet light from the lamp (5.8) and check for the following features.

a) Appearance of a blue fluorescent spot of aflatoxin B_1-hemiacetal, and sometimes a weak blue fluorescent spot of aflatoxin B_1 which has not reacted with the trifluoroacetic acid, originating from the standard solution applied at point C (migration in direction I) and from the standard solution applied at point B (migration in direction II).

b) Appearance of spots similar to those described in a), originating from the sample extract applied at point A. The position of these spots is defined by those originating from the standard solution applied at points B and C. The intensities of fluorescence of the aflatoxin B_1-hemiacetal spots originating from the extract and from the standard solution applied at points B and C should be comparable.

Dimensions in millimetres

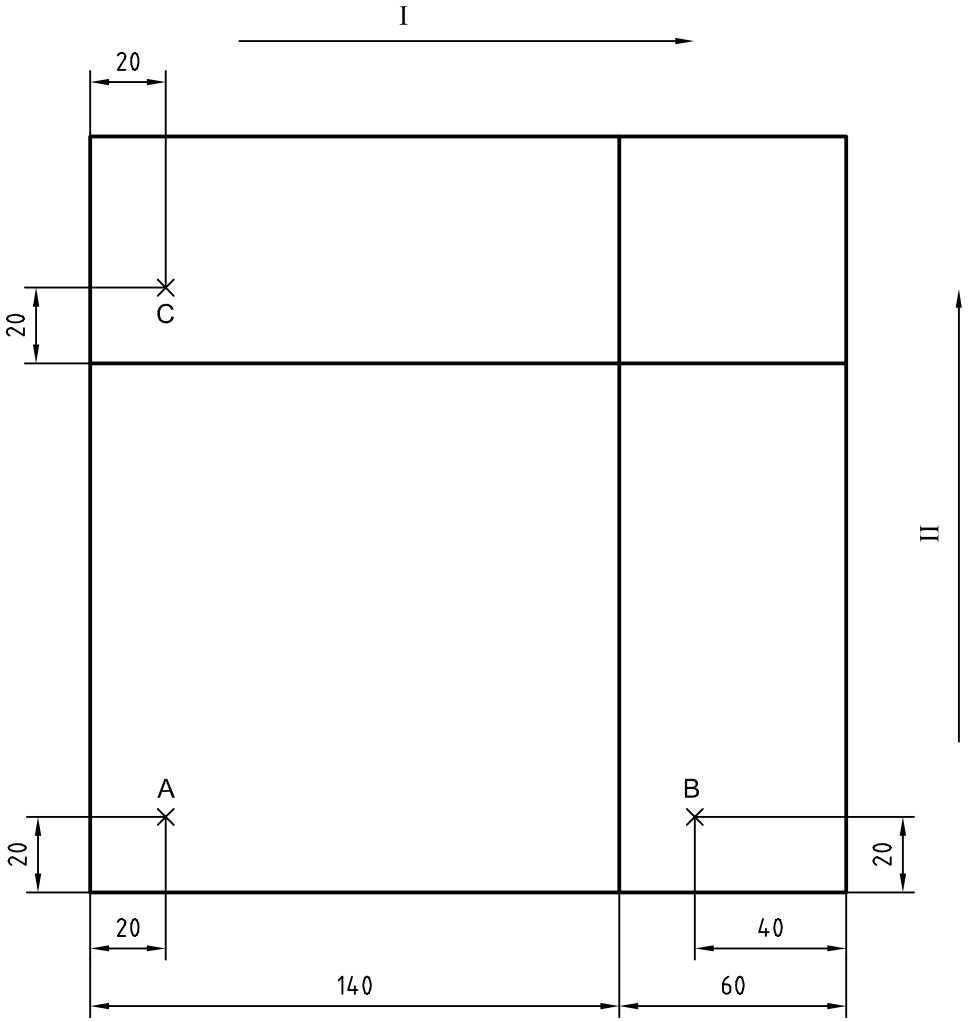

Figure 3 — Confirmation test

7.8 Number of determinations

Carry out two determinations on the same test sample.

8 Expression of results and calculations

8.1 Visual measurements

The aflatoxin B_1 content, expressed in micrograms per kilogram of sample, is equal to

$$\frac{C \cdot V_1 \cdot V_3}{m \cdot V_2}$$

where

C is the concentration, in micrograms of aflatoxin B_1 per millilitre of the standard solution (4.14) (approximately 0,1 μg/ml);

m is the mass, in grams, of the test portion corresponding to the volume of extract subjected to column clean-up (10,0 g);

V_1 is the final volume of the extract, in microlitres, taking into account any dilution that was necessary;

V_2 and V_3 are, respectively, the volumes, in microlitres, of the extract and of the standard aflatoxin B_1 solution (4.14), applied to the plate, having similar intensities of fluorescence.

8.2 Fluorodensitometric measurements

The aflatoxin B_1 content, expressed in micrograms per kilogram of sample, is equal to

$$\frac{m_1 \cdot V_1}{m \cdot V_2}$$

where

m is the mass, in grams, of the test portion corresponding to the volume of extract subjected to column clean-up (10,0 g);

m_1 is the mass, in nanograms, of aflatoxin B_1 in the extract spot (taking into account the volume V_2), deduced from the measurements;

V_1 is the final volume of the extract, in microlitres, taking into account any dilution that was necessary;

V_2 is the volume, in microlitres, of extract applied to the plate (10 µl or 20 µl).

9 Interlaboratory tests

Details of interlaboratory tests on the precision of the method are summarized in annex A. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used (A or B), with reference to this International Standard;

— the method of determination (visual or fluorodensitometric);

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the test result(s) obtained.

Annex A
(informative)

Results of interlaboratory tests

Three interlaboratory tests, two of which were carried out at the international level (Nos. 1 and 2), on mixed feeding stuffs (method B) gave the results indicated in Table A.1.

The 11 laboratories participating in trial 2 also analysed the sample by method A, its composition being suitable, and obtained results very similar to those when using method B, by visual or fluorodensitometric measurement.

Table A.1

Parameter	Test		
	1	2	3
Number of laboratories	23	11	13
Mean, μg/kg	162,7	25,4	13,4
Standard deviation of repeatability (s_r), μg/kg	16,9	2,7	1,7
Coefficient of variation of repeatability, %	10	11	13
Repeatability limit (2,83 s_r), μg/kg	47,8	7,6	4,8
Standard deviation of repeatability (s_R), μg/kg	45,2	6,8	4,0
Coefficient of variation of reproducibility, %	28	27	30
Reproducibility limit (2,83 s_R), μg/kg	128,0	19,2	11,3

ICS 65.120

Price based on 13 pages