INTERNATIONAL STANDARD

ISO 6651

Third edition
2001-12-15

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

Reference number
ISO 6651:2001(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.ch
Web www.iso.ch

Printed in Switzerland

Contents

Page

1 Scope **1**
2 Normative reference **1**
3 Principle **1**
4 Reagents **2**
5 Apparatus **3**
6 Sampling **4**
7 Procedure **5**
8 Expression of results and calculations **11**
9 Interlaboratory tests **12**
10 Test report **12**

Annex

A Results of interlaboratory tests **13**

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).