
**Animal feeding stuffs — Determination of
aflatoxin B₁ content of mixed feeding
stuffs — Method using high-performance
liquid chromatography**

*Aliments des animaux — Détermination de la teneur en aflatoxine B₁ dans
les aliments composés — Méthode par chromatographie liquide à haute
performance*



Foreword

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Animal feeding stuffs — Determination of aflatoxin B₁ content of mixed feeding stuffs — Method using high-performance liquid chromatography

1 Scope

This International Standard specifies a high-performance liquid chromatographic (HPLC) method for the determination of aflatoxin B₁ content of animal feeding stuffs including those containing citrus pulp.

The lower limit of determination is 1 µg/kg.

NOTE 1 This International Standard may be applicable for the determination of the aflatoxin B₁ content of a number of raw materials and straight feeding stuffs such as corn gluten, groundnut, palm kernel, copra, citrus pulp, tapioca, soya bean, rice bran, pollard, rape seed, niger seed and cotton seed (see references [1] and [2]). These materials were, however, not included in the collaborative testing of the method.

NOTE 2 This International Standard may also be applicable for the determination of the content of the sum of the aflatoxins B₁, B₂, G₁ and G₂. However, the method has not been validated for this parameter by collaborative testing.

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, this publication 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 IEC and ISO editions maintain registers of currently valid International Standards.

ISO 6498:1998, *Animal feeding stuffs — Preparation of test sample*.

3 Principle

The sample is extracted with chloroform. The extract is filtered and an aliquot portion is purified on a Florisil®¹⁾ cartridge and a C₁₈ cartridge. The final separation and determination is achieved by high-performance liquid chromatography (HPLC) using a reverse-phase C₁₈ column, followed by post-column derivatization with iodine or bromine, and fluorescence detection.

4 Reagents and materials

Use only reagents of recognized analytical grade.

1) Florisil® is the trade-name of a commercially available product. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

4.1 Water, demineralized or deionized, with resistivity of at least 10 MΩ·cm, or water of at least equivalent purity.

4.2 Concentrated sulfuric acid, $c(\text{H}_2\text{SO}_4) = 18 \text{ mol/l}$, $\rho(\text{H}_2\text{SO}_4) = 1,84 \text{ g/ml}$.

4.3 Sulfuric acid, $c(\text{H}_2\text{SO}_4) = 2 \text{ mol/l}$.

Carefully add 105 ml of concentrated sulfuric acid (4.2) to 895 ml of water and mix well. Avoid excessive heating of the solution.

4.4 Control sample.

Prepare a control sample of about 2 kg of compound feed with an aflatoxin B₁ content of about 5 µg/kg by combining samples of previous determinations with an aflatoxin B₁ content of about 5 µg/kg. Mix thoroughly.

The aflatoxin B₁ content of the control sample should be determined five times by two analysts following the procedure described in clause 8. From the results the mean aflatoxin B₁ content, the standard deviation and the coefficient of variation should be calculated.

4.5 Acid-washed Celite® 545, or product of equivalent quality²⁾.

4.6 Florisil® Sep-Pak style cartridge, Waters No. 51960, or product of equivalent quality³⁾.

4.7 C₁₈ Sep-Pak style cartridge, Waters No. 51910, or product of equivalent quality³⁾.

4.8 Acetone.

4.9 Methanol.

4.10 Acetonitrile.

4.11 Chloroform, stabilized with ethanol (mass fraction 0,5 % to 1,0 %).

WARNING: Chloroform is a toxic substance. Avoid inhalation of and exposure to chloroform. Work in a fumehood when handling the solvent and solutions thereof.

The adsorption characteristics of the Florisil® cartridge (4.6) may change if stabilizers other than ethanol are used. When chloroform as described is not available, the adsorption characteristics should be verified in accordance with clause 8.

4.12 Mixture of acetone and water, 98 + 2 (by volume).

Combine 980 ml of acetone (4.8) and 20 ml of water (4.1). Mix well.

4.13 Mixture of acetone and water, 15 + 85 (by volume).

Combine 150 ml of acetone (4.8) and 850 ml of water (4.1). Mix well.

4.14 Mixture of acetone and water, 5 + 95 (by volume).

2) Celite® is the trade-name of a commercially available product.

3) Florisil® is the trade-name of a commercially available product. Florisil® Sep-Pak style cartridge article number 51960 and C₁₈ Sep-Pak style cartridge article number 51910, from Waters Associates (Milwaukee, USA), are examples of suitable products available commercially.

This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

Combine 50 ml of acetone (4.8) and 950 ml of water (4.1). Mix well.

4.15 Mixture of methanol and water, 20 + 80 (by volume).

Combine 200 ml of methanol (4.9) and 800 ml of water (4.1). Mix well.

4.16 Concentrated nitric acid, $c(\text{HNO}_3) = 14 \text{ mol/l}$, $\rho(\text{HNO}_3) = 1,40 \text{ g/ml}$, for HPLC with bromine derivatization.

4.17 Potassium bromide (KBr), for HPLC with bromine derivatization.

4.18 Mobile phase for HPLC.

4.18.1 Mobile phase for HPLC with iodine derivatization.

Combine 120 ml of acetonitrile (4.10), 210 ml of methanol (4.9) and 390 ml of water (4.1) and mix. Filter the eluent through a $0,45 \mu\text{m}$ PTFE membrane filter using the solvent filtration system (5.1) and degas for 10 min in the ultrasonic bath (5.2) before use.

NOTE The composition of the mobile phase solvent may need adjustment depending on the characteristics of the HPLC column used.

4.18.2 Mobile phase for HPLC with bromine derivatization.

Combine 400 ml of acetonitrile (4.10), 700 ml of methanol (4.9) and 1 300 ml of water (4.1) and mix. Add to the mixture 286 mg of potassium bromide (4.17) and 152 μl of concentrated nitric acid (4.16). Mix well and degas with a stream of inert gas for 15 min.

4.19 Saturated iodine solution for HPLC with iodine derivatization.

Add 2 g of iodine to 400 ml of water. Mix for at least 90 min and filter through a $0,45 \mu\text{m}$ PTFE membrane filter (see 5.1). Prepare the solution fresh on the day of use.

Protect the saturated solution from light to prevent photodegradation.

4.20 Sodium hypochlorite solution (household quality), $\rho(\text{active chlorine}) = 100 \text{ g/l}$.

4.21 Sodium hypochlorite solution, volume fraction 1 %.

Dilute 10 ml of sodium hypochlorite solution (4.20) with 990 ml of water-acetone mixture (4.14).

4.22 Inert gas, e.g. nitrogen.

4.23 Aflatoxin B₁ standard material ($\text{C}_{17}\text{H}_{12}\text{O}_6$), 2,3,6 α ,9 α -tetrahydro-4-methoxycyclopenta[c]furo[3',2':4,5]-furo[2,3-*h*][1]benzopyran-1,11-dione; Chemical Abstracts Service Registry (CAS) number 1162-65-8.

WARNINGS

1 Mycotoxins are extremely toxic substances. Perform all manipulations in a designated fume cupboard. Take special precautions when toxins are in a dry form because of their electrostatic nature and resulting tendency to disperse in working areas.

2 Aflatoxins are sensitive to UV radiation. Therefore, conduct all operations in the absence of sunlight or artificial white light. Provide sufficient, but not excessive, illumination with tungsten filament lamps. Low-energy lamps and fluorescent tubes may be used, but the use of amber glassware (vials, volumetric flasks) is recommended.

3 Glassware that has been in contact with solutions of aflatoxin B₁ has to be soaked overnight in a hypochlorite solution (4.21), before cleaning, in order to remove traces of aflatoxin B₁.

4.24 Aflatoxin B₁ standard solution, $\rho(\text{aflatoxin B}_1) \approx 10 \mu\text{g/ml}$.

Transfer the content of an ampoule containing aflatoxin B₁ (4.23) to a flask and dissolve in chloroform (4.11). Transfer the solution to a convenient size volumetric flask and dilute to the mark with chloroform so as to obtain a solution with an aflatoxin B₁ content of about 10 $\mu\text{g/ml}$. Mix.

Transfer the solution to amber vials or an airtight screw-cap bottle and store in a cool place (4 °C) in the dark, well sealed and wrapped in aluminium foil.

4.25 Aflatoxin B₁ stock standard solution.

Transfer quantitatively 2,5 ml of the aflatoxin B₁ standard solution (4.24) to a 50 ml volumetric flask and dilute to the mark with chloroform (4.11).

Transfer the solution to amber vials or an airtight screw-cap bottle and store in a cool place (4 °C) in the dark, well sealed and wrapped in aluminium foil.

4.26 Aflatoxin B₁ calibration solutions for HPLC.**4.26.1 Calibration solution I, $\rho(\text{aflatoxin B}_1) \approx 4 \text{ ng/ml}$.**

Allow the volumetric flask with stock standard solution (4.25) to reach room temperature in the aluminium foil (a few hours).

Transfer 400 μl of the stock standard solution (equivalent to about 200 ng of aflatoxin B₁) to an acid-washed 50 ml volumetric flask, and evaporate the solution to dryness in a stream of inert gas (4.22). Dissolve the residue in 20 ml of the water-acetone mixture (4.13). Dilute to the mark with the water-acetone mixture and mix well.

4.26.2 Calibration solution II, $\rho(\text{aflatoxin B}_1) \approx 3 \text{ ng/ml}$.

Transfer quantitatively 7,5 ml of the calibration solution I (4.26.1) to an acid-washed 10 ml volumetric flask. Dilute to the mark with the water-acetone mixture (4.13) and mix well.

4.26.3 Reference calibration solution, $\rho(\text{aflatoxin B}_1) \approx 2 \text{ ng/ml}$.

Transfer quantitatively 25 ml of the calibration solution I (4.26.1) to an acid-washed 50 ml volumetric flask. Dilute to the mark with the water-acetone mixture (4.13) and mix well.

This solution is used for repetitive injection during HPLC (8.5).

4.26.4 Calibration solution III, $\rho(\text{aflatoxin B}_1) \approx 1 \text{ ng/ml}$.

Transfer quantitatively 2,5 ml of the calibration solution I (4.26.1) to an acid-washed 10 ml volumetric flask. Dilute to the mark with the water-acetone mixture (4.13) and mix well.

4.27 Chromatographic test solution.

Prepare an ampoule containing a mixture of aflatoxins B₁, B₂, G₁ and G₂ in 1 ml of chloroform with concentrations of approximately 1,0 $\mu\text{g/ml}$, 0,5 $\mu\text{g/ml}$, 1,0 $\mu\text{g/ml}$ and 0,5 $\mu\text{g/ml}$ respectively.

Transfer the contents of the ampoule to a glass-stoppered test tube or screw-capped vial. Transfer 40 μl of this solution to an acid-washed glass-stoppered test tube (5.4). Evaporate the chloroform in a stream of inert gas (4.22) and dissolve the residue into 10 ml of the water-acetone mixture (4.13).

5 Apparatus

Before use, laboratory glassware coming into contact with aqueous solutions of aflatoxins shall be soaked in sulfuric acid (4.3) for several hours, then rinsed well (e.g. three times) with water to remove all traces of acid. Check the absence of acid with pH paper.

In practice, this treatment is necessary for the round-bottomed flask of the rotary evaporator (5.12), the volumetric flasks, measuring cylinders, vials or tubes used for calibration solutions and final extracts (particularly autosamplers vials), and Pasteur pipettes, if these are used to transfer calibration solutions or extracts.

NOTE Laboratory glassware coming into contact with aqueous solutions of aflatoxins has to be soaked in dilute acid because the use of non-acid-washed glassware may cause losses of aflatoxin B₁. Particular care should be taken with new glassware and disposable glassware such as autosampler vials and Pasteur pipettes.

Usual laboratory apparatus and, in particular, the following.

5.1 Solvent filtration system, suitable for PTFE membrane filters with a pore size of 0,45 µm.

5.2 Ultrasonic bath.

5.3 Microsyringe, of capacity 100 µl, for preparation of calibration solutions.

Check by weighing that the inaccuracy does not exceed 2 % of the mass.

5.4 Glass-stoppered calibrated tubes, of capacity 10 ml.

5.5 Spectrometer, suitable for measurements in the UV region of the spectrum, provided with quartz cuvettes of optical path length 10 mm ± 0,1 mm.

5.6 Conical flask, of capacity 500 ml, made of borosilicate glass, with a wide neck and a glass stopper or a screw cap fitted with a PTFE liner.

5.7 Mechanical shaker, horizontal rotation or reciprocating, with frequency 250 min⁻¹ to 300 min⁻¹.

5.8 Fluted filter paper, of diameter 24 cm.

5.9 Luer® chloroform-resistant threeway stopcock ⁴⁾.

5.10 Chemically resistant syringe, 10 ml, with Luer® connector ⁴⁾.

5.11 Glass column, with internal diameter 10 mm to 15 mm, length about 30 cm to 50 cm, equipped with a Luer® tip⁴⁾.

NOTE When a glass column of internal diameter about 10 mm and length about 30 cm is used, it is advisable to use a plastics reservoir (chemically resistant syringe barrel) of at least 70 ml capacity.

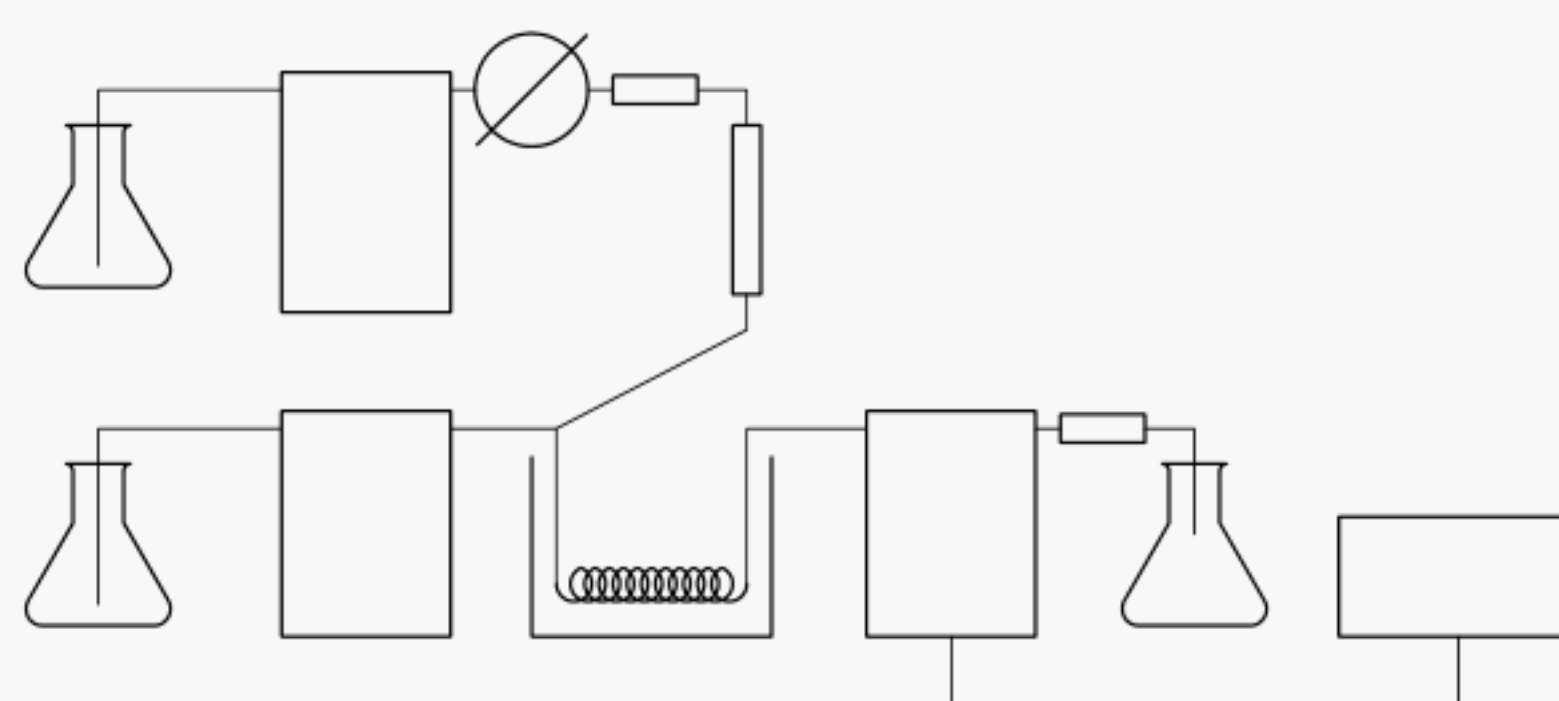
5.12 Rotary vacuum evaporator, equipped with a 150 ml to 250 ml round-bottomed flask.

5.13 General HPLC system.

See Figures 1 and 2 for a diagrammatic representation of the HPLC system for derivatization with iodine and bromine respectively.

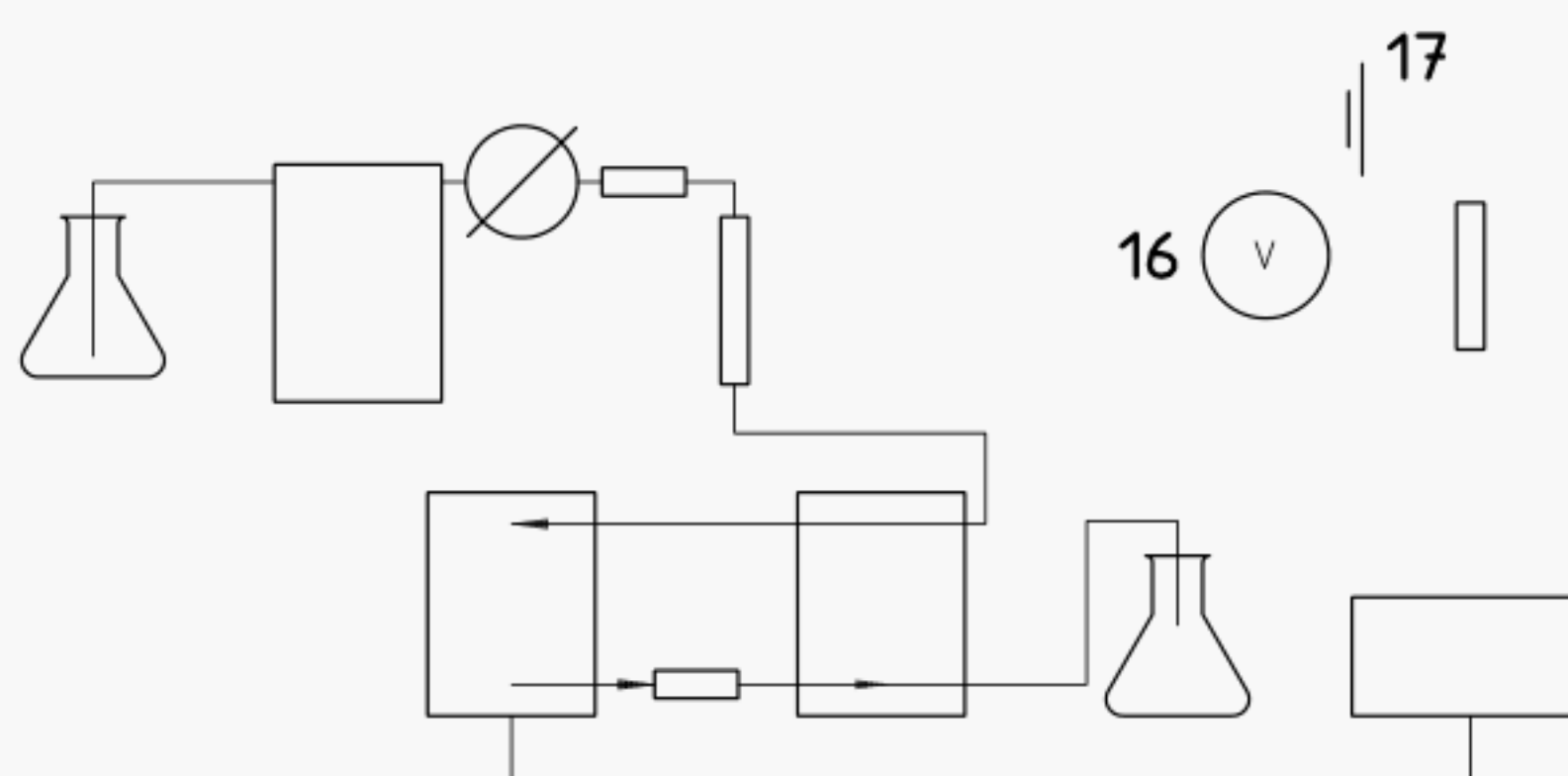
5.13.1 Pump, pulse free, capable of maintaining a volume flow rate of 0,1 ml/min to 1,0 ml/min.

⁴⁾ Luer® is the trade-name of a commercially available product. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

**Key**

1	HPLC mobile phase	10	Reaction coil
2	HPLC pump	11	Fluorescence detector
3	Injector	12	Restrictor
4	Guard column	13	Waste
5	Analytical column	14	Recorder/integrator
6	Saturated iodine solution	15	Derivatization cell (KOBRA®)
7	Reagent pump	16	Tension meter
8	Tee joint	17	Power supply, 10 V d.c.
9	Water bath (60 °C)	18	Resistor, 100 kΩ

Figure 1 — Diagrammatic representation of the HPLC system for derivatization with iodine



NOTE See Figure 1 for Key.

Figure 2 — Diagrammatic representation of the HPLC system for derivatization with bromine

5.13.2 Injection system, with loop suitable for the injection of 250 µl.

5.13.3 Fluorescence detector, with excitation at a wavelength of 365 nm and emission at wavelength of 435 nm (for filter instruments: emission wavelength > 400 nm). Detection of at least 0,05 ng aflatoxin B₁ shall be possible. Some back pressure may be advisable [e.g. by applying a restrictor or a coil of stainless steel or polytetrafluorethylene (PTFE) connected to the outlet of the detector] to suppress air bubbles in the flow cell.

5.13.4 Recorder.

5.13.5 Guard column: C₁₈ packing, particle size 37 µm to 50 µm, length 10 mm to 20 mm, internal diameter 3,9 mm; or a guard column of equivalent quality.

5.13.6 Analytical column: C₁₈ packing, particle size 3 µm or 5 µm, length 200 mm, internal diameter 3,0 mm; or an analytical column of equivalent quality.

5.13.7 Electronic integrator (optional).

5.14 HPLC system for HPLC with iodine derivatization.

5.14.1 Pump, pulse free, for delivery of the iodine post-column reagent.

5.14.2 Zero dead volume Tee, stainless steel, 1,59 mm × 0,75 mm.

5.14.3 Spiral reaction coil, polytetrafluorethylene (PTFE) or stainless steel.

Dimensions of 3 000 mm × 0,5 mm to 5 000 mm × 0,5 mm have been found to be appropriate in combination with 5 µm or 3 µm HPLC columns.

5.14.4 Thermostatically controlled water bath or solid-state heating device, adjusted to 60 °C, capable of temperature regulation to the nearest 0,1 °C.

5.15 HPLC system for HPLC with bromine derivatization.

5.15.1 Electrochemical derivatization cell: Kok's Bromine Apparatus (KOBRA®⁵⁾).

5.15.2 Power supply, 0 V to 20 V d.c.

5.15.3 Tension meter, range 0 V to 10 V d.c., impedance > 50 kΩ.

5.15.4 Resistor, 100 kΩ.

5.16 Syringe, suitable for HPLC injection of 250 µl.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [7].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

⁵⁾ KOBRA® is the trade-name of a commercially available apparatus. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent apparatus may be used if it can be shown to lead to the same results.

7 Preparation of the test sample

Prepare the test sample in accordance with ISO 6498.

Grind the laboratory sample (usually 500 g) so that it passes completely through a sieve with 1 mm apertures. Mix thoroughly.

8 Procedure

8.1 General

To each series, add a spiked blank sample with an aflatoxin B₁ content of 10 µg/kg and a certified reference material or a control sample (4.4). Addition of a blank sample to each series is strongly recommended to check for contamination from the glassware.

The results shall comply with the criteria in clause 10.

8.2 Determination of the absorption spectrum of the aflatoxin B₁ standard solution

In cuvettes, determine the absorption spectrum of the aflatoxin B₁ standard solution (4.24) between wavelengths of 330 nm and 370 nm by means of the spectrometer (5.5), using chloroform as blank. Measure the absorbance (*A*) at the maximum near a wavelength of 363 nm.

8.3 Extraction

Weigh, to the nearest 0,1 g, 50,0 g of the prepared test sample (see clause 7) into the conical flask (5.6). Consecutively add 25 g of Celite® (4.5), 250 ml of chloroform (4.11) and 25 ml of water. Stopper the flask, swirl and release the pressure. Restopper the flask and shake it for 30 min on the mechanical shaker (5.7).

NOTE In order to reduce the use of chloroform, one-half of the specified quantities may be used; i.e. 25,0 g of the prepared test sample (see clause 7), 12,5 g of Celite® (4.5), 125 ml of chloroform (4.11) and 12,5 ml of water.

Filter through a fluted filter paper (5.8). If filtration is proceeding slowly, cover the funnel in order to prevent evaporation of chloroform. Collect 50 ml of the filtrate (*V_s*).

If necessary, take an aliquot portion of the filtrate and dilute to 50 ml (*V_f*) with chloroform so that the aflatoxin B₁ content does not exceed 4 ng/ml.

Use the filtrate for sample clean-up in accordance with 8.4.

8.4 Clean-up

Carry out the procedure without significant interruptions.

8.4.1 Florisil® purification

8.4.1.1 Preparation of the column-cartridge assembly

Attach a stopcock (5.9) to the shorter stem of a Florisil® cartridge (4.6). Wash the cartridge and remove air by taking 10 ml of chloroform (4.11) and passing 8 ml of it via the stopcock rapidly through the cartridge using a syringe (5.10).

Attach the longer stem of the cartridge to a glass column (5.11) and pass the remaining 2 ml of chloroform through the cartridge into the column. Close the stopcock. Remove the syringe.

8.4.1.2 Purification

Add the filtrate (V_s or V_f) collected in step 8.3 to the column-cartridge assembly and drain by gravity. Rinse with 5 ml of chloroform (4.11), followed by 20 ml of methanol (4.9). Discard the eluates.

During these operations, ensure that the column-cartridge assembly does not run dry.

Elute aflatoxin B₁ with 50 ml of the acetone-water mixture (4.12) and collect the eluate in the round-bottomed flask of the rotary evaporator (5.12).

NOTE 1 The quality of Florisil® varies per batch. Depending on this quality, 50 ml of acetone-water mixture (4.12) may not be sufficient for elution. If so, the use of 60 ml to 70 ml of the acetone-water mixture (4.12) is recommended.

Concentrate the eluate on the rotary evaporator at a temperature of between 40 °C and 50 °C until no more acetone is distilled.

NOTE 2 About 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 no significant amount of acetone. Residues of acetone may lead to losses of aflatoxin B₁ on the C₁₈ cartridge.

Add 1 ml of methanol (4.9), swirl the flask to dissolve aflatoxin B₁ on the sides of the flask, add 4 ml of water and mix. Disconnect and discard the cartridge. Rinse the glass column with water and retain for the C₁₈ purification step (8.4.2).

8.4.2 C₁₈ purification

8.4.2.1 Preparation of the column-cartridge assembly

Attach a stopcock (5.9) to the shorter stem of a C₁₈ cartridge (4.7). Prime the cartridge and remove any air by passing 10 ml of methanol (4.9) via the stopcock rapidly through the cartridge with a syringe (5.10). Air bubbles in the cartridge are visible as light spots on the otherwise greyish background. Take 10 ml of water and pass 8 ml of it 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 (5.11) and pass the remaining 2 ml of water through the cartridge in the column. Close the stopcock. Remove the syringe.

8.4.2.2 Purification

Transfer the extract obtained in 8.4.1.2 quantitatively to the glass column (5.11), rinsing the flask twice with 5 ml of the water-methanol mixture (4.15) and drain by gravity.

During these operations, ensure that the column-cartridge assembly does not run dry. If 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 of the water-methanol mixture (4.15). Discard the eluate. Elute the aflatoxin B₁ with 25 ml of the water-acetone mixture (4.13) and collect the eluate in a 50 ml volumetric flask. Dilute to the mark with water and mix. Use the resulting solution for chromatography (8.5).

NOTE Filtration of the final extract prior to HPLC is normally not necessary. If considered necessary, cellulose filters should not be used because they may lead to losses of aflatoxin B₁. PTFE filters are acceptable.

8.5 High-performance liquid chromatography

8.5.1 General

Allow sufficient time for conditioning and stabilizing the apparatus.

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

The detector response to aflatoxin B₁ depends on the temperature, therefore compensation should be made for drift.

By injecting a fixed amount of reference calibration solution (4.26.3) at regular intervals (e.g. every third injection), the aflatoxin B₁ peak values between the results for the reference calibration solution can be corrected for using the mean response, provided that the difference between responses of consecutive measurements of reference calibration solution is very small (< 10 %). Therefore injections shall be made without interruptions wherever possible. If interruption is necessary, the last injection before interruption and the first injection after interruption shall be the reference calibration solution (4.26.3).

Because the calibration curve is linear and passes through the origin, the mass of aflatoxin B₁ in the sample extracts is determined directly by reference to the adjacent results for the reference calibration solution.

8.5.2 HPLC pump settings

Set the pump (5.13.1) to give a volume flow rate of the mobile phase (4.18) of 0,5 ml/min or 0,3 ml/min for a 5 µm or a 3 µm HPLC column (5.13.6) respectively.

If iodine derivatization is used, proceed in accordance with 8.5.3.

If bromine derivatization is used, proceed in accordance with 8.5.4.

8.5.3 Post-column pump settings for HPLC with iodine derivatization

Set the pump (5.14.1) to give a volume flow rate of the saturated iodine solution (4.19) of between 0,2 ml/min and 0,4 ml/min. As a rough guide, volume flow rates of about 0,4 ml/min or 0,2 ml/min are advised in combination with volume flow rates of the mobile phase (4.18) of 0,5 ml/min and 0,3 ml/min respectively.

8.5.4 Fluorescence detector

Set the fluorescence detector (5.13.3) to an excitation wavelength of 365 nm and an emission wavelength of 435 nm (filter instrument: > 400 nm). Adjust the detector attenuator to obtain about 80 % full-scale deflection for 1 ng of aflatoxin B₁.

8.5.5 Injector

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

8.5.6 Check of chromatographic separation

Inject the chromatographic test solution (4.27). Valleys should be less than 5 % of the sum of the peak heights of the adjacent peaks.

8.5.7 Check of the stability of the system

Before each series of analyses, repetitively inject the reference calibration solution (4.26.3) until stable peak heights are achieved. Peak responses for aflatoxin B₁ between consecutive injections should not differ by more than 6 %. Proceed without delay with the check of linearity (8.5.8).

8.5.8 Check of linearity

Inject the aflatoxin B₁ calibration solutions (4.26.1 to 4.26.4). Every third injection use the reference calibration solution (4.26.3) for correction of drift in response. Peak responses for this reference calibration solution shall not differ by more than 10 % in 90 min. Correct for drift with the formula given in 9.3.

The calibration graph shall be linear and pass through the origin within twice the standard error of the estimate of the response. Values found shall 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 any problem before continuing.

8.5.9 Injection of sample extracts

Consecutively inject the reference calibration solution (4.26.3), the blank feed extract, the spiked blank feed extract, the reference calibration solution (4.26.3), the extract of a certified reference material or the control sample (4.4) and again the reference calibration solution (4.26.3).

Inject the purified sample extracts (8.4.2.2). After every two sample extracts, repeat the injection of the reference calibration solution (4.26.3). When the series contains more than 10 samples, the last injections should be the aflatoxin B₁ calibration solutions (4.26.1 to 4.26.4).

9 Calculation of results

9.1 Calculation of the aflatoxin B₁ content of the aflatoxin B₁ standard solution

Calculate the aflatoxin B₁ content of the aflatoxin B₁ standard solution (4.24) using the equation:

$$\rho = \frac{M \times A}{d \times \kappa}$$

where

ρ is the aflatoxin B₁ content of the aflatoxin B₁ standard solution (4.24), in mg/ml;

M is the molar mass of aflatoxin B₁, in g/mol ($M = 312$ g/mol);

A is the absorbance measured in 8.2, corrected for the blank;

d is the optical path length of the cuvette, in cm ($d = 1$ cm);

κ is the molar absorption coefficient of aflatoxin B₁ in chloroform at 363 nm, in l·mol⁻¹·cm⁻¹
($\kappa = 22\,300$ l·mol⁻¹·cm⁻¹).

9.2 Calculation of the mass of aflatoxin B₁ in the injected reference calibration solution

Calculate the mass of aflatoxin B₁ in the injected reference calibration solution (4.26.3) using the equation:

$$m_c = f \times \rho \times V_{ic}$$

where

m_c is the mass of aflatoxin B₁ in the reference calibration solution (4.26.3) injected, in ng;

f is the dilution and units correction factor, in ng/mg ($f = 200$ ng/mg);

ρ is the aflatoxin B₁ content of the aflatoxin B₁ standard solution (4.24), calculated in 9.1, in mg/ml;

V_{ic} is the volume of reference calibration solution (4.26.3) injected, in ml ($V_{ic} = 0,25$ ml).

9.3 Calculation of the mass of aflatoxin B₁ in the test solution

Calculate the mass of aflatoxin B₁ in the test solution using the equation:

$$m_a = \frac{A_s \times 2m_c}{A_{c1} + A_{c2}}$$

where

m_a is the mass of aflatoxin B₁ in the test solution, in ng;

A_s is the peak area corresponding to the aflatoxin B₁ in the test solution, in area units;

m_c is the mass of aflatoxin B₁ in the reference calibration solution (4.26.3) injected, calculated in 9.2, in ng;

A_{c1} is the peak area corresponding to aflatoxin B₁ resulting from the preceding injection of reference calibration solution (4.26.3), in area units;

A_{c2} is the peak area corresponding to aflatoxin B₁ resulting from the subsequent injection of reference calibration solution (4.26.3), in area units.

NOTE The peak height (in length units) may be used instead of the peak area.

9.4 Calculation of the aflatoxin B₁ content of the sample

Calculate the aflatoxin B₁ content of the test sample by the equation:

$$w_a = \frac{m_a \times V_s \times V_c}{m_s \times V_{is} \times V_f}$$

where

w_a is the aflatoxin B₁ content of the test sample, in µg/kg;

m_a is the mass of aflatoxin B₁ in the test solution, in ng;

V_s is the volume of the undiluted sample extract obtained in 8.3 used in the subsequent procedure, in ml ($V_s = 50$ ml if it is not necessary to dilute the filtrate obtained in 8.3);

m_s is the mass of the test sample, in g ($m_s = 50,0$ g);

V_{is} is the volume of sample extract injected (8.5.8), in ml ($V_{is} = 0,25$ ml);

V_f is the volume of filtrate used for the clean-up (8.4), in ml ($V_f = 50$ ml);

V_c is the volume of chloroform used for extraction of the sample (see 8.3), in ml ($V_c = 250$ ml).

If $m_s = 50,0$ g, $V_s = 50$ ml, $V_{is} = 0,25$ ml, $V_f = 50$ ml and $V_c = 250$ ml, the equation becomes:

$$w_a = 20 \text{ g}^{-1} \times m_a$$

10 Accuracy

10.1 Systematic error

Verify the correct application of the method by making replicate measurements on certified reference materials or control samples. If these are not available, verify the performance of the method by recovery experiments made on 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 of -20 % to +10 %.

10.2 Precision

10.2.1 Interlaboratory test

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

Annex B gives a summary of statistical results of interlaboratory tests of the equivalence of iodine and bromine derivatization.

10.2.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the repeatability limit r derived from the equation:

$$r = 0,37 \mu\text{g/kg} + 0,19\bar{w}_a \quad (w_a \leq 15 \mu\text{g/kg})$$

where

\bar{w}_a is the mean of the two test results, in $\mu\text{g/kg}$;

r is the repeatability limit, in $\mu\text{g/kg}$.

10.2.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limit R derived from the equation:

$$R = 0,67 \mu\text{g/kg} + 0,33\bar{w}_a \quad (w_a \leq 15 \mu\text{g/kg})$$

where

\bar{w}_a is the mean of the two test results, in $\mu\text{g/kg}$;

R is the reproducibility limit, in $\mu\text{g/kg}$.

11 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, with reference to this International Standard;
- 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 obtained; or
- if the repeatability has been checked, the final quoted result obtained.

Annex A (informative)

Results of interlaboratory test

The precision of the method was established by an interlaboratory test carried out in accordance with ISO 5725-2:1994 [6]. In this test, 22 laboratories from 11 European countries participated. The study involved six unknown samples, consisting of blind duplicate samples of compounded feeding stuffs with target aflatoxin B₁ contents of < 2 µg/kg, 8 µg/kg and 14 µg/kg.

Table A.1 — Statistical results of the interlaboratory test of the method applying derivatization with iodine

Parameter	Sample ^a		
	1	2	3
Number of laboratories retained after eliminating outliers	15	15	15
Mean aflatoxin B ₁ content, µg/kg	0,8	7,0	12,6
Repeatability standard deviation (s_r), µg/kg	0,20	0,44	1,20
Repeatability coefficient of variation, %	25,0	6,3	9,5
Repeatability limit, r ($r = 2,8 \times s_r$), µg/kg	0,55	1,24	3,37
Reproducibility standard deviation, s_R , µg/kg	0,34	1,02	1,80
Reproducibility coefficient of variation, %	42,5	14,6	14,3
Reproducibility limit, R ($R = 2,8 \times s_R$), µg/kg	0,95	2,86	5,05
a) Sample 1: blank; target aflatoxin B ₁ content < 2 µg/kg; composition: 10 % barley, 20 % manioc, 15 % citrus pulp, 20 % "Hi-pro" soya, 10 % liquid molasses, 20 % pelleted cattle feed, 1,7 % fat, 1,5 % dicalcium phosphate and 0,7 % salt. Sample 2: target aflatoxin B ₁ content of 8 µg/kg; other components as sample 1. Sample 3: target aflatoxin B ₁ content of 14 µg/kg; other components as sample 1.			

NOTE In the report on the interlaboratory test (see reference [3]), values of r and R are mentioned (see Table A.2) which deviate from those in Table A.1. The differences result from calculations having been performed on the natural logarithms of the measured results and from the application of deviating definitions of r and R . In Table A.2 r is the largest ratio of two readings of the same sample in the same laboratory under similar conditions which is not yet significant at the 95 % probability level. R is similarly defined for comparing two different laboratories, always using single determinations.

Table A.2 — Summary of the published statistical results of the interlaboratory test

Aflatoxin B ₁ content µg/kg	r µg/kg	R µg/kg	Repeatability coefficient of variation %	Reproducibility coefficient of variation %
8 and 14	1,4	1,7	11	18

Annex B (informative)

Equivalence of iodine and bromine derivatization

The equivalence of the iodine and bromine derivatization can be deduced from published data (see references [3] and [4]). Furthermore, equivalence is apparent from data collected by KDLL (Kwaliteitsdienst Landbouwkundige Laboratoria: Quality Service for Agricultural laboratories, The Netherlands) in the period between October 1992 and July 1994. In this period a number of interlaboratory tests were performed in which both derivatization methods were tested for a number of different compound and straight feeding stuffs with aflatoxin B₁ contents between 2 µg/kg and 90 µg/kg. The results of these interlaboratory tests are included in Table B.1.

Table B.1 — Statistical results of interlaboratory tests of the equivalence of iodine and bromine derivatization

Sample	Iodine derivatization ^a			Bromine derivatization ^a			Significance ^b
	\bar{w}_a	n	s	\bar{w}_a	n	s	
Cattle feed (pelleted)	2,1	4	0,4	2,1	8	0,3	n.s.
	2,2	4	0,3	2,2	8	0,3	n.s.
	2,2	6	0,5	1,9	11	0,3	n.s.
	2,3	6	0,5	2,0	11	0,4	n.s.
	4,5	3	0,2	4,8	8	0,4	n.s.
	4,7	3	0,2	4,7	8	0,5	n.s.
	5,0	6	0,8	5,4	11	0,9	n.s.
	5,1	6	0,7	5,4	11	0,8	n.s.
	8,3	5	1,6	9,0	10	1,0	n.s.
	9,6	5	1,1	10,1	10	1,7	n.s.
	10,8	6	1,6	9,5	9	1,6	n.s.
	12,1	6	1,4	11,8	9	1,8	n.s.
Corn gluten	16,3	6	2,3	16,4	11	2,0	n.s.
	17,6	6	2,4	18,9	11	1,5	n.s.
Copra	19,7	6	4,4	17,7	8	2,7	n.s.
	20,2	6	3,1	18,6	8	2,3	n.s.
	27,9	4	3,4	29,3	8	1,6	n.s.
	29,0	4	3,1	33,2	8	1,7	$p < -0,01$
Palm kernel and groundnut	38,1	6	7,4	35,3	11	5,3	n.s.
	53,7	6	10,7	48,7	11	7,3	n.s.
Groundnut	82,0	4	4,1	84,4	7	7,1	n.s.
	83,0	4	9,9	86,0	7	10,7	n.s.
Groundnut and copra	80,0	4	5,5	86,1	9	7,3	n.s.
	85,5	4	9,2	94,1	9	9,3	n.s.
^{a)} \bar{w}_a is the mean aflatoxin B ₁ content, in µg/kg; n is the number of results; s is the standard deviation. ^{b)} n.s. means not significant in Student's <i>t</i> -test, iodine tested against bromine.							

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