
**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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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.

International Standard ISO 14718 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 10, *Animal feeding stuffs*.

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Printed in Switzerland

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).