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Animal feeding stuffs — Determination of aflatoxin B_1

Aliments des animaux — Dosage de l'aflatoxine B_1

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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 2.

The main task of technical committees is to prepare International Standards. 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 document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 17375 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Animal feeding stuffs — Determination of aflatoxin B_1

1 Scope

This International Standard specifies a method for the determination of aflatoxin B_1 in animal feeding stuffs using high-performance liquid chromatography with post-column derivatization.

It is applicable to animal feeding stuffs with a fat content of up to 50 %.

The limit of quantification of this method has been demonstrated to be better than 0,5 µg/kg for aflatoxin B_1 for a signal-to-noise ratio of 6.

NOTE The method is based on that given in Reference [1].

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696:1987, *Water for analytical laboratory use — Specification and test methods*

3 Principle

A test portion is extracted with a solvent solution (acetone/water). The sample extract is filtered, diluted with water or phosphate-buffered saline to a specified solvent concentration. A test portion is applied on an immunoaffinity column (IAC) containing antibodies specific to aflatoxin B_1. The aflatoxin B_1 is removed from the IAC with neat methanol, and then quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) with post-column derivatization (PCD) involving bromination. The PCD is achieved with either electrochemically generated bromine or with pyridinium hydrobromide perbromide (PBPB) followed by fluorescence detection.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

WARNING — This method requires the use of toxic inflammable liquids such as acetone, methanol and acetonitrile. Avoid contact and keep away from heat, sparks or open flames.

NOTE Decontamination procedures for laboratory wastes [2],[3] have been developed and validated by the International Agency for Research on Cancer (WHO).

4.1 Water, complying with grade 3 in accordance with ISO 3696:1987.

4.2 Phosphate buffer saline (PBS), pH 7,4.

PBS may be prepared from potassium chloride (0,20 g), potassium dihydrogen phosphate (0,20 g), anhydrous disodium hydrogen phosphate (1,16 g) [or disodium hydrogen phosphate dodecahydrate (2,92 g)] and sodium chloride (8,00 g) added to 900 ml purified water. Adjust the pH to pH 7,4 (with 0,1 mol/l HCl or 0,1 mol/l NaOH as appropriate) and dilute the solution to 1,0 l.

Alternatively, commercially available phosphate-buffered saline tablets with equivalent properties may be used.

PBS is not microbiologically stable and should be prepared fresh at least once a week.

4.3 Pyridinium hydrobromide perbromide (PBPB, CAS: 39416-48-3).

This reagent is not required in the case of using electrochemically generated bromine.

4.4 Potassium bromide.

This reagent is not required in the case of using the PBPB reagent.

4.5 HPLC-grade acetonitrile.

4.6 HPLC-grade methanol.

4.7 Acetone, pure.

4.8 HPLC grade water, complying with grade 1 of ISO 3696:1987.

4.9 Extraction solvent, solution of acetone (4.7) and water (4.8) [85+15 (by volume)].

4.10 Nitric acid, $c(HNO_3)$ = 4 mol/l.

This reagent is not required in the case of using the PBPB reagent.

4.11 Immunoaffinity column (IAC)

The IAC should contain antibodies raised against aflatoxin B_1. The IAC should have a capacity of not less than 40 ng of aflatoxin B_1 and should give a recovery of not less than 80 % for aflatoxin B_1 when applied as a standard solution in acetone/water containing 0,25 ng of aflatoxin B_1.

4.12 HPLC mobile phase solvent A, for use with PBPB post column reagent only.

Use a solution of water (4.8)/acetonitrile (4.5)/methanol (4.6) [6+2+3 (by volume)]. The ratio of solvents may be adjusted to give optimum separation parameters.

4.13 HPLC mobile phase solvent B, for use with electrochemically generated bromine only.

Use a solution of water (4.8)/acetonitrile (4.5)/methanol (4.6) [6+2+3 (by volume)] containing 120 mg potassium bromide (4.4) and 350 µl nitric acid at 4 mol/l (4.10) per litre of mobile phase. The ratio of solvents may be adjusted to give optimum separation parameters.

The mobile phase solvents (4.12 and 4.13) should be degassed.

4.14 Post-column reagent, for use with PBPB post column reagent only.

Dissolve 25 mg of PBPB (4.3) in 500 ml of water. This solution may be used for up to 4 days if stored in a dark place at room temperature. This post-column reagent shall be used only in combination with HPLC mobile phase solvent A (4.12) but not with HPLC mobile phase solvent B (4.13).

NOTE Post column reagent is only stable for 3 days.

4.15 Toluene/acetonitrile, 98 + 2 (by volume).

4.16 Aflatoxin B_1 standard material, in form of crystals or a dry film for analytical purposes.

WARNING 1 — This method requires the use of solutions of aflatoxin B_1. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO) [2].

WARNING 2 — Aflatoxins are subject to light degradation. Protect analytical work adequately from the daylight, and keep aflatoxin standard solutions protected from light by using amber vials or aluminium foil.

4.17 Calibration stock solutions for HPLC

4.17.1 General stock solution

Prepare an aflatoxin B_1 (4.16) stock solution containing 10,0 µg/ml in toluene/acetonitrile (4.15).

NOTE The toluene/acetonitrile stock solution is stable for at least one year provided it is stored in acid-washed glassware and kept for storage at –18 °C in the dark. If stock solutions are used up in a much shorter period (maximum of 3 months), methanol (4.6) might be used as an alternative. Note that methanolic solutions are more sensitive to an alkaline ambient of the glass surface and to day light than toluene/acetonitrile solutions.

Wrap the flasks tightly in aluminium foil and store them at less than 4 °C. To determine the exact concentration of aflatoxins in this stock solution, record the absorption curve between a wavelength of 330 nm and 370 nm in 1 cm quartz glass cells (5.21) in a spectrometer (5.20), with the stock solution solvent in the reference cell. Calculate the mass concentration of each aflatoxin, c_a, in micrograms per millilitre, using Equation (1):

$$c_a = A_{max} \times \frac{M_a \times 100}{\varepsilon_a \times d} \quad (1)$$

where

A_{max} is the absorbance determined at the maximum of the absorption curve;

M_a is the molar mass of aflatoxin B_1, in grams per mole (312 g/mol);

ε_a is the molar absorptivity of aflatoxin B_1, in square metres per mole (1930 m^2/mol for toluene/acetonitrile and 2150 m^2/mol for methanol solutions);

d is the optical path length of the cell, in centimetres.

4.17.2 Calibration stock solution

Prepare an aflatoxin B_1 (4.16) calibration solution containing 50,0 ng/ml in either toluene/acetonitrile (4.15) or methanol (4.6) from the stock solution (4.17.1).

4.17.3 Option A (see 6.3)

Pipette from the calibration stock solution (4.17.2) the volumes as listed in Table 1 (Option A) into a set of 20 ml calibrated volumetric flasks. Evaporate the toluene/acetonitrile solution just to dryness under a stream of nitrogen at room temperature. If methanol is used in the preparation of the stock solution, evaporation is not required. To each flask, add 7 ml of methanol. Allow the aflatoxins to dissolve, then dilute to the mark with water, and shake well.

NOTE Remember that methanol and water are subject to volume contraction when mixed.

4.17.4 **Option B** (see 6.3)

Pipette from the calibration stock solution (4.17.2) the volumes as listed in Table 1 (Option B) into a set of at least 5 different 20 ml volumetric flasks. Evaporate the toluene/acetonitrile solution just to dryness under a stream of nitrogen at room temperature. If methanol is used in preparation of the stock solution, evaporation is not required. To each flask, add approximately 10 ml of methanol. Allow the aflatoxins to dissolve, then dilute further with neat methanol (not with methanol/water) to the mark and shake well. Then transfer exactly 1 ml of this calibration working solution to an acid-washed glass vial (see Warning in 5.7), evaporated to dryness according to Option B (6.3.3) and than redissolved in exactly the same volume that will be used to redissolve the samples prior to injection (6.3). Calculate the concentration of aflatoxin B_1 in the evaporated and redissolved solution in nanograms per millilitre. Use these concentration values for the calculation according to 6.6. In this case the calibration range will remain unchanged.

Table 1 — Preparation of calibration working solutions

Working standard	Option A		Option B	
	Calibration stock solution	Concentration of aflatoxin B_1	Calibration stock solution	Concentration of aflatoxin B_1
	µl	ng/ml	µl	ng/ml
1	20	0,050	100	0,250
2	70	0,175	350	0,875
3	120	0,300	600	1,500
4	170	0,425	850	2,125
5	220	0,550	1100	2,750

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 **Vertical or horizontal shaker**, **adjustable**.

5.2 **Filter paper**, of diameter 24 cm, prefolded (e.g. cellulose for fine precipitates).

5.3 **Erlenmeyer flask**, with screw top or glass stopper.

5.4 **Glass microfibre filter paper**, of diameter 5 cm (e.g. 1,6 µm retention).

5.5 **Reservoir**, 75 ml with Luer tip connector for IAC.

5.6 **Hand pump**, 20 ml syringe with Luer lock or rubber stopper for IAC.

5.7 **Volumetric flasks**, of 5 ml, 10 ml and 20 ml capacity, with an accuracy of at least 0,5 %.

WARNING — The use of non acid-washed glassware (e.g. vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. Special attention should be taken with new glassware. Thus, before use, soak the glassware in dilute acid (e.g. sulfuric acid, 2 mol/l) for several hours, then rinse extensively with distilled water to remove all traces of acid (this can be checked by using a pH paper).

5.8 **HPLC pump**, suitable for flow rate at (1,000 ± 0,005) ml/min.

5.9 Injection system.

Suitable for total loop injection (a valve with a loop of at least 100 µl is recommended). It shall be guaranteed that the relative standard deviation (RSD) of the integrator signal for a multiple injection (n = 10) of a standard solution of aflatoxin B_1 (concentration equivalent to a contamination level of 1 µg/kg) results in a maximum value of 10 %. These data shall be reported.

5.10 RP-HPLC column, e.g. LC-18 or ODS-2, with optional but recommended pre-column.

5.11 Post-column derivatization system with PBPB (alternative to 5.12), comprising.

— second HPLC pulse-less pump,

— zero-dead volume T-piece, and

— reaction tubing with minimum internal diameter of 45 cm × 0,5 mm, of PTFE.

The reaction time shall be at least 4 s before detection.

5.12 System for HPLC post-column derivatization with electrochemically generated bromine.

The device shall be installed according to the manufacturer's instructions. In order to confirm the aflatoxin B_1 content, the HPLC column shall be disconnected from the bromination device and shall be connected directly to the fluorescence detector.

Switching-off the electrical current with the bromination device still in line is not recommended due to the possibility of bromine remaining in the cell membrane of the device.

5.13 Fluorescence detector, with a 360 nm excitation filter and a > 420 nm cut-off emission filter, or equivalent.

Recommended settings for adjustable detectors are Ex = 365 nm, Em = 435 nm, BW = 18 nm.

5.14 Disposable filter unit (0,45 µm), prior to usage verify that no aflatoxin losses occur during filtration (recovery testing) since there is a possibility that various filter materials can retain aflatoxin B_1.

5.15 One-mark pipettes, of 1 ml, 2 ml, 5 ml and 10 ml capacity.

5.16 Analytical balance, capable of weighing to the nearest 0,1 mg.

5.17 Laboratory balance, capable of weighing to the nearest 0,01 g.

5.18 Calibrated microlitre syringe(s) or microlitre pipette(s), 20 µl to 500 µl.

5.19 Evaporator, optional, only needed for Option B (6.3.3).

5.20 Spectrophotometer.

5.21 Quartz glass cell, 1 cm optical path length.