INTERNATIONAL STANDARD

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Animal feeding stuffs — Determination of aflatoxin B_1 content of mixed feeding stuffs — Method using high-performance liquid chromatography

Aliments des animaux — Détermination de la teneur en aflatoxine B_1 dans les aliments composés — Méthode par chromatographie liquide à haute performance

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.

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

Annexes A and B of this International Standard are for information only.

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Animal feeding stuffs — Determination of aflatoxin B_1 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 B_1 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 B_1 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 B_1, B_2, G_1 and G_2. 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, subseqent 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 C_{18} cartridge. The final separation and determination is achieved by high-performance liquid chromatography (HPLC) using a reverse-phase C_{18} column, followed by post-column derivatization with iodine or bromine, and fluorescence detection.

4 Reagents and materials

Use only reagents of recognized analytical grade.

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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, $c(H_2SO_4)$ = 18 mol/l, $\rho(H_2SO_4)$ = 1,84 g/ml.

4.3 Sulfuric acid, $c(H_2SO_4)$ = 2 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 B_1 content of about 5 µg/kg by combining samples of previous determinations with an aflatoxin B_1 content of about 5 µg/kg. Mix thoroughly.

The aflatoxin B_1 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 B_1 content, the standard deviation and the coefficient of variation should be calculated.

4.5 Acid-washed Celite® 545, or product of equivalent quality[2].

4.6 Florisil® Sep-Pak style cartridge, Waters No. 51960, or product of equivalent qualty[3].

4.7 C_{18} Sep-Pak style cartridge, Waters No. 51910, or product of equivalent quality[3].

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).

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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 C_{18} Sep-Pak style catridge 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, $c(HNO_3)$ = 14 mol/l, $\rho(HNO_3)$ = 1,40 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 µm 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 µm 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), ρ(active chlorine) = 100 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 B_1 standard material ($C_{17}H_{12}O_6$), 2,3,6*a*α,9*a*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 B_1 has to be soaked overnight in a hypochlorite solution (4.21), before cleaning, in order to remove traces of aflatoxin B_1.

4.24 Aflatoxin B_1 standard solution, ρ(aflatoxin B_1) ≈ 10 µg/ml.

Transfer the content of an ampoule containing aflatoxin B_1 (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 B_1 content of about 10 µg/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 B_1 stock standard solution.

Transfer quantitatively 2,5 ml of the aflatoxin B_1 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 B_1 calibration solutions for HPLC.

4.26.1 Calibration solution I, ρ(aflatoxin B_1) ≈ 4 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 B_1) 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, ρ(aflatoxin B_1) ≈ 3 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, ρ(aflatoxin B_1) ≈ 2 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, ρ(aflatoxin B_1) ≈ 1 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 B_1, B_2, G_1 and G_2 in 1 ml of chloroform with concentrations of approximately 1,0 µg/ml, 0,5 µg/ml, 1,0 µg/ml and 0,5 µg/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 B_1. 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® tip[4].

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.

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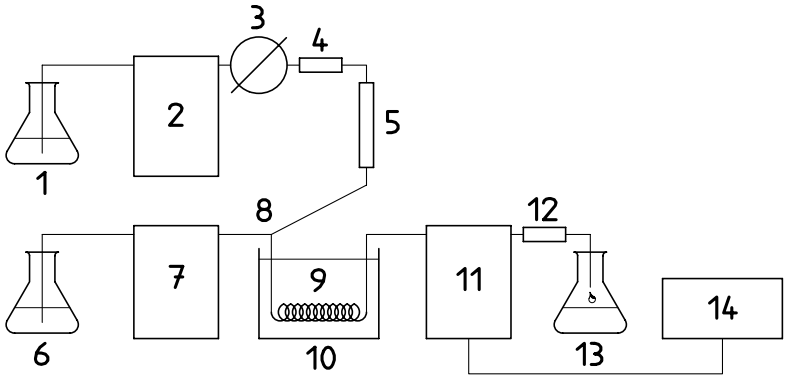
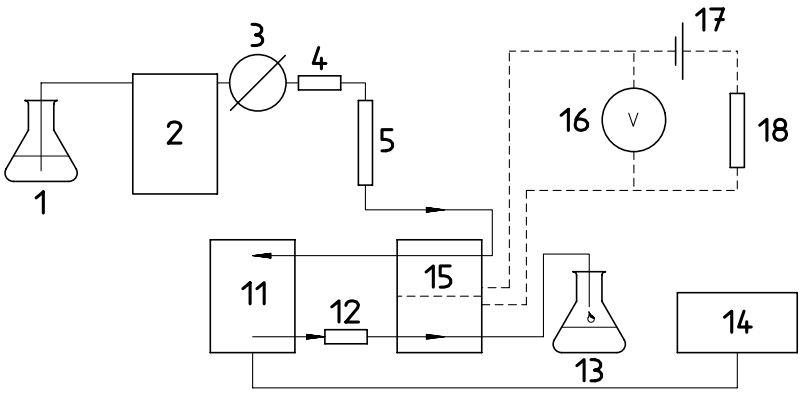
[4] Luer® is the trade-name of a commercially available product. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.



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.

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 B_1 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: C_{18} 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: C_{18} 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.

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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 B_1 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 B_1 standard solution

In cuvettes, determine the absorption spectrum of the aflatoxin B_1 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 B_1 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 B 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 B_1 on the C_{18} cartridge.

Add 1 ml of methanol (4.9), swirl the flask to dissolve aflatoxin B_1 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 C_{18} purification step (8.4.2).

8.4.2 C_{18} purification

8.4.2.1 Preparation of the column-cartridge assembly

Attach a stopcock (5.9) to the shorter stem of a C_{18} 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 B_1 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 B_1. 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.

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