Method for the determination of aflatoxin B_1 in compounded animal feed
(based on EC-collaborative study carried out in 1988)

1. Scope

This proposal specifies a method for the determination of the aflatoxin B_1 content, based on detection with high performance liquid chromatography (HPLC), with fluorescence detection.

2. Field of application

This method has been applied so far to mixed feeding stuffs containing citruspulp. The lower limit of determination for aflatoxin B_1 is 1 µg/kg.

3. Principle

Extraction of the test portion with chloroform, filtration, and purification of an aliquot portion over a Florisil cartridge, subsequently followed by a C_{18} cartridge. Final separation and determination is achieved on a C_{18} reversed phase HPLC column, followed by postcolumn derivatization with I_2 in water and fluorescence detection.

4. Reagents

Reference to a company and/or product is for the purpose of information and identification only and does not imply approval or recommendation of the product by the National Institute of Public health and Environmental Protection nor the Commission of the European Communities to the exclusion of others which may also be suitable.

All reagents shall be of recognized analytical quality.
The water used shall be distilled water or water of at least equivalent purity.
Solvents used for the HPLC mobile phase shall be of HPLC grade. Note: The use of normal analytical grade reagents for the HPLC mobile phase (in particular CH_3CN) may lead to erroneous results, by interference in the postcolumn reaction.

4.1 Chloroform, stabilized with 0.5 to 1.0% of ethanol, by mass.
4.2 Methanol
4.3 Acetone
4.4 Eluting solvents.
Prepare 1 day before use, or remove (excess) air in the solvents ultrasonically.
4.4.1 Acetone/water (98+2) (V/V)
4.4.2 Water/methanol (80+20) (V/V)
4.4.3 Water/acetone (85+15) (V/V)
4.5 Mobile phase HPLC

Note: The composition of the mobile phase solvent may need to be adjusted, depending on the type of HPLC column used.
Water/methanol/acetonitril (130+70+40) (V/V/V)

4.6 Iodine
4.7 Satured iodine solution in water. Add 2 g of iodine to 400 ml of water. Mix for at least 90 minutes and filter through a Millipore filter (5.16). Protect the satured solution from light to prevent photo-degradation.

4.8 Acid washed Celite 545 or equivalent

4.9 Florisil cartridge (Waters SEP-pak)

4.10 C_{18} cartridge (Waters SEP-pak)

4.11 Inert gas, for instance nitrogen

4.12 Ampoule containing aflatoxin B_1 calibrant solution, concentration 10 µg/ml, in 2.5 ml chloroform (RIVM, P.O. Box 1, 3720 BA Bilthoven, The Netherlands).

4.12.1 Aflatoxin B_1 stock solution.
Transfer the contents of the ampoule (4.12) to a 50 ml volumetric flask and adjust to the mark with chloroform. Store this stock solution (0.5 µg/ml) in a cool place (4°C) in the dark, well sealed and wrapped in aluminium foil.

4.13 Aflatoxin B_1 calibration solutions for HPLC.
Note: Use acid-washed glassware for the preparation of these solutions (See 5).

4.13.1 Calibration solution 4 ng/ml.
Allow the volumetric flask with stock solution (4.12.1) to warm up to roomtemperature in the aluminium foil (a few hours). Transfer 400 µl of the stock solution (200 ng) into a 50 ml volumetric flask, and evaporate the solution to dryness in a current of inert gas (4.11). Dissolve the residue obtained in ca 20 ml water-acetone (4.4.3), adjust to the mark with water-acetone (4.4.3) and mix well.

4.13.2 Calibration solution 3 ng/ml
Dilute 7,5 ml of the 4 ng/ml calibration solution (4.13.1) to 10 ml with water-acetone (4.4.3) and mix well.

4.13.3 Calibration solution 2 ng/ml
This solution is also to be used for repetitive injection during HPLC, and is futher referred to as reference standard.
Dilute 25 ml of the 4 ng/ml calibration solution (4.13.1) to 10 ml with water-acetone (4.4.3) and mix well.

4.13.4 Calibration solution 1 ng/ml
Dilute 2,50 ml of the 4 ng/ml calibration solution (4.13.1) to 10 ml water-acetone (4.4.3) in a 1 ml vial, and mix well.

4.14 Ampoule containing mixture of aflatoxins B_1, B_2, G_1, G_2, concentrations approximately 1, 0.5, 1 and 0.5 µg/ml respectively, in 1 ml chloroform (RIVM, P.O. Box 1, 3720 BA Bilthoven, The Netherlands).

4.14.1 Chromatographic test solution
Transfer the contents of the ampoule into a glass-stoppered test-tube or screw-capped vial. Transfer 40 µl of this solution into a 10 ml measuring flask or a glass-stoppered test-tube (acid rinsed). Evaporate the chloroform in a stream of N_2 and redissolve into 10 ml water-acetone (4.4.3).

5. Apparatus

Caution: Use of non-acid washed glassware for aqueous aflatoxine solutions may cause loss of aflatoxins. Particular care should be taken with new glassware and pasteur pipettes. Therfore laboratory glassware coming into contact with aqueous solutions of aflatoxins should be soaked in diluted acid (e.g. Sulphuric acid, 2 mol/l) for several hours, then rinsed well with distilled water to remove all traces of acid (e.g. three times, check with pH-paper). In practice, this treatment is nesessary for the round bottemed

flask (5.5), the volumetric flasks/measuring cylinders (5.17), (4.13), vials or tubes used for calibration solutions and final extracts (particularly vials for autosamplers), and pasteur pipettes if these are used to transfer calibration solutions or extracts.

5.1 Grinder/mixer
5.2 Sieve of aperture size 1.0 mm
5.3 Mechanical shaker
5.4 Balance
5.5 Rotary vacuum evaporator, equipped with a 150 ml round bottomed flask.
5.6 High performance liquid chromatograph, injector with loop of 250 µl or = 500 µl.
5.7 HPLC analytical column: 3 µ or 5 µ.
5.8 Pulse-free pump for delivery of the iodine post-column reagent, e.g. HPLC- or purpose-built post column pump. Peristaltic pump may work satisfactorily, if sufficient pressure is delivered, and if number of revolutions is high enough to prevent pulsation.
5.9 Valco zero volume Tee ss 1/16" x 0,75 mm.
5.10 Spiral reaction coil; Teflon or stainless steel. Dimensions of 3000 x 0,5 mm to 5000 x 0,5 mm have found to be appropriate in combination with 5 µ and 3 µ HPLC columns.
5.11 Thermostatically controlled water bath adjusted to 60ºC, capable of temperature regulation to better than 0.1ºC.
5.12 Fluorescence detector, capable of providing ca. 365 nm excitation and ca. 435 nm emission wavelengths. (For filter instrument: emission wavelength > 400 nm). Detection of at least 0.05 ng B_1 shall be possible.
Some back pressure may be advisable (e.g. restrictor or teflon/stainless steel coil connected to the outlet of the detector), to suppress air bubbles in the flow-cell.
5.13 Strip chart recorder
5.14 Electronic integrator (optional)
5.15 Fluted filter paper. MN 617 1/4 ? 24 cm or equivalent.
5.16 Millipore filter with a pore size of 0.45 µm (e.g. H.A.W.P. 04700)
5.17 50 ml glass stoppered volumetric flasks or measuring cylinders
5.18 500 ml glass stoppered conical flask
5.19 Glass column (internal diameter ca. 1.0 cm, length ca. 30 cm) equipped with a Luer tip.
5.20 Luer nylon chloroform-resistant stopcock (e.g. Bio-Rad 7328017, Analytichem AI 6078, J.T. Baker 4514).
5.21 Chemically resistant syringe, 10 ml Luer
5.22 HPLC injection syringe of 250 µl for loop ? 500 µl, or 1 ml for loop of 250 µl.
5.23 100 µl microsyringe (check that the accuracy is within 2% by weighing)
5.24 10.0 ml glass stoppered calibrated tubes

6. Procedure

6.1 Preparation of the sample.
Grind the sample so that the whole of it will pass through the sieve (5.2.).

6.2 Test portion:
Weigh 50 g of the prepared test sample into the conical flask (5.18).

6.3 Extraction:
Add 25 g of Celite (4.8), 250 ml of chloroform (4.1) and 25 ml of water. Stopper the flask, and shake for 30 min. on a mechanical shaker (5.3). Filter through a fluted filter paper (5.15). Collect 50 ml of the filtrate.

6.4 Clean-up (the clean-up procedure should be carried out without significant interruptions).
Caution:
- Protect the laboratory, where the analyses are done, adequately from daylight. This can be effectively achieved by using:
1) UV absorbing foil on the windows in combination with sundued light (no direct sunlight!)
2) Curtains or blinds in combination with artificial light (fluorescent tubes are acceptable).
- Aflatoxin containing solutions must be protected from light as much as possible. (Keep in dark, use Al foil).

6.4.1 Florisil SEP-pak purification

6.4.1.1 Preparation of the column-cartridge assembly.
Attach a stopcock (5.20) to the shorter stem of a Florisil cartridge (4.9) (see fig. 1). Wash the cartridge and remove air by taking 10 ml chloroform and passing 8 ml chloroform via the stopcock rapidly through the cartridge with a syringe (5.21). Attach the longer stem of the cartridge to a glass column (5.19) and pass the remaining 2 ml chloroform through the cartridge into the column. Close the stopcock. Remove the syringe.

6.4.1.2 Purification
Add the filtrate collected in 6.3 to the column-cartridge assembly and drain by gravity. Rinse with 5 ml of chloroform (4.1), followed by 20 ml of methanol (4.2). Discard the eluates. During these operations, ensure that the column-cartridge assembly does not run dry.
Elute aflatoxin B_1 with 40 ml of the acetone/water mixture (4.4.1) and collect the whole of the eluate in the (150 ml) round bottomed flask of the rotary evaporator (5.5). Concentrate the eluate on the rotary evaporator (40°- 50°C) until acetone stops being distilled.
(Note: Ca 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 might lead to losses of aflatoxin B_1 on the C18 cartridge). Add 1 ml of methanol (4.2), swirl the flask to dissolve aflatoxin B_1 on the sides of the flask, add 4 ml water and mix. Disconnect and discard the cartridge. Rinse the glass column with water and retain for C_{18} purification step.

6.4.2 C_{18} SEP-pak purification

6.4.2.1 Preparation of the column-cartridge assembly.
Attach a stopcock (5.20) to the shorter stem of a C_{18} cartridge (4.10) (see fig. 1).
Prime the cartridge and remove any air by rapidly passing 10 ml methanol (4.2) via the stopcock through the cartridge with a syringe (5.21) (Airbubbles in the cartridge are visible as light spots in the otherwise greyish background).

Take 10 ml water, and pass 8 ml through the cartridge (Avoid introduction of air in the cartridge, when switching from methanol to water).
Attach the longer stem of the cartridge to a glass column and pass the remaining 2 ml water through the cartridge in the column. Close the stopcock. Remove the syringe.

6.4.2.2 Purification.

Transfer the extract collected in 6.4.1.2 quantitatively to the column, rinsing the flask twice with 5 ml water/methanol mixture (4.4.2) and drain by gravity. During these operations, ensure that the column-cartridge assembly does not run dry. (When airbubbles develop in the constriction near the cartridge, stop the flow and tap the top of the glass column, to remove the airbubbles. Then continue).
Elute with 25 ml water/methanol mixture. Discard the eluates.
Elute with 50 ml water/methanol mixture. Discard the eluates. Elute aflatoxine B_1 with 50 ml water/acetone mixture (4.4.3), and collect the whole of the eluate in a volumetric flask or measuring cylinder (5.17). Adjust the volume to 50 ml with water and mix: the resulting test solution is used for chromatography (6.5).

Caution: Filtering of final extract prior to HPLC is normally not necessary. If considered necessary, cellulose filters are not to be used, because they may lead to losses of aflatoxin B_1. Teflon filters are acceptable.

6.5 High performance liquid chromatography:

(See figure 2 for setting-up of the equipment). Allow sufficient time for the instruments before use to warm up and stabilize before use.

Note 1: The flow-rates given for the HPLC solvent and the post-column reagent are indicative only. They may need to be adjusted depending on the type and size of the HPLC column.

Note 2: The peak responses of aflatoxins depend on the temperature, therefore compensation should be made for drift (see figure 3). By injecting a fixed amount of aflatoxin B_1 standard (reference standard 4.13.3) at regular intervals (i.e. every third injection), the aflatoxin B_1 peak values between these reference standards can be corrected using the mean response of these reference standards, provided that the difference between responses of consecutive reference standards is very small (< 10%). Therefore injections must be made without interruptions. If interruption is necessary, the last injection before interruption and the first injection after interruption must be the reference standard.
Because the calibration curve is linear and passes through the origin, the amounts of aflatoxin B_1 in sample extracts are determined directly with the help of the adjacent standards.

6.5.1 HPLC pump settings

Set the HPLC pump (5.6) to give a flow of 0.5 or 0.3 ml/min for a 5 µ or 3 µ HPLC column (5.7) respectively: use mobile phase (4.5).

6.5.2 Post column pump settings
Set the pump (5.8) to give a flow of 0.2-0.4 ml/min of the iodine-satured water solution (4.7). As a rough guide: Flows of ca 0.4 or 0.2 ml/min are advised in combination with flows of 0.5 and 0.3 ml/min of the mobile phase respectively.

6.5.3 Fluorescence detector
Set the fluorescence detector (5.12) to $\lambda_{exc.}$ = 365 nm and $\lambda_{em.}$ = 435 nm. (filter instrument; >400 nm). Adjust the detector attenuator to obtain ca. 80% full scale deflection of the recorder pen for 1 ng aflatoxin B_1.

6.5.4 Injector
For all solutions, inject 250 µl amounts following the instructions of the manufacturer of the injector.

6.5.5 Check of chromatographic separation
Inject a chromatographic test solution (4.14.1). Valleys should be less than 5% of the sum of peakheights of the adjacent peaks.

6.5.6 Check of stability of the system
Before each series of analyses, repetitively inject the reference standard (4.13.3), until stable peak areas are achieved (Note: Peak responses for aflatoxin B_1 between consecutive injections should differ by less then 6%). Proceed without delay with check of linearity.

6.5.7 Check of linearity
Inject the aflatoxin B_1 calibration solutions (4.13.1-4.13.4). Inject every third injection the reference standard (4.13.3), for correction of drift in response. (Note: Peak responses for the reference standard should differ by less then 10% in 90 minutes).
Correct for drift according to the formula in 7. The calibration graph should be linear and pass through the origin, within 2x standard errror of Y-estimate. Values found should differ by less then 3% from the nominal values. If the requirements are fullfilled, continue without delay. If not, identify and correct the source of the problems before continuation.

6.5.8 Injection of sample extracts.
Inject the sample extracts (6.4.2.2). After every two sample extracts repeat injection of the reference standard (4.13.3): Ref. standard, extract, extract, ref. standard, extract, extract, ref. standard etc.

8. Calculation

Calculate the aflatoxin B_1 content (µg/kg) present in the sample, using the formula:

aflatoxine B_1 content (in µg/kg) = $\dfrac{m \times V_{ext}}{V_m \times M \times \dfrac{V_f}{V_e}}$

where m = amount of aflatoxin B_1 in ng represented by the B_1 peak of the sample, calculated as follows:

$$m = \frac{p\ (sample)}{p\ (st_1) + p\ (st_2)} \ .\ 2i$$

p (sample) = peak area of aflatoxin B_1 in sample
p (st_1) = peak area of aflatoxin B_1 in preceding reference standard (4.13.3)
p(st_2) = peak area of aflatoxin B_1 in next-following reference standard (4.13.3).
i = injected amount of reference standard (4.13.3) in ng.
V_m = volume of injected sample extract in mls
V_{ex} = final volume of sample extract in mls
M = mass of sample in g
V_f = volume of filtrate transferred to Florisil cartridge (6.4.1.2.) in mls
V_e = amount of chloroform, used for the extraction of the sample, in mls

If the procedure is followed as in this protocol, the formula reduces to:

aflatoxin B1 content (in µg/kg) = 20 m

8. Remarks

8.1 Calculation of results may also be done by peak height measurement.

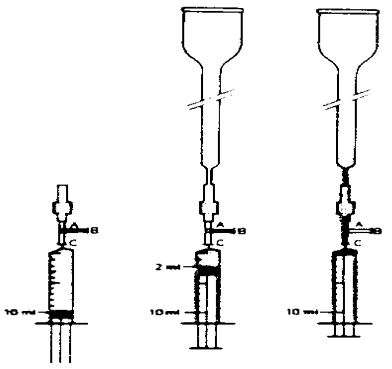

Figure 1: Column-cartridge assembly

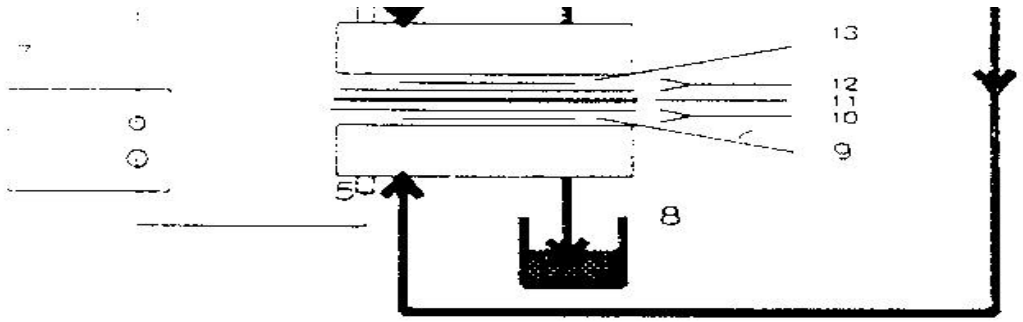

Figure 2: Flow diagram of the LC system with iodine postcolumn derivatization.

1. Mobile phase
2. Pump
3. Injection valve
4. Guard column
5. HPLC analytical column
6. Satured iodine solution
7. Reagent pump
8. T-joint
9. Thermostatically controled bath
10. Spiral reaction coil
11. Fluorescence detector
12. Restrictor
13. Waste
14. Strip chart recorder/integrator

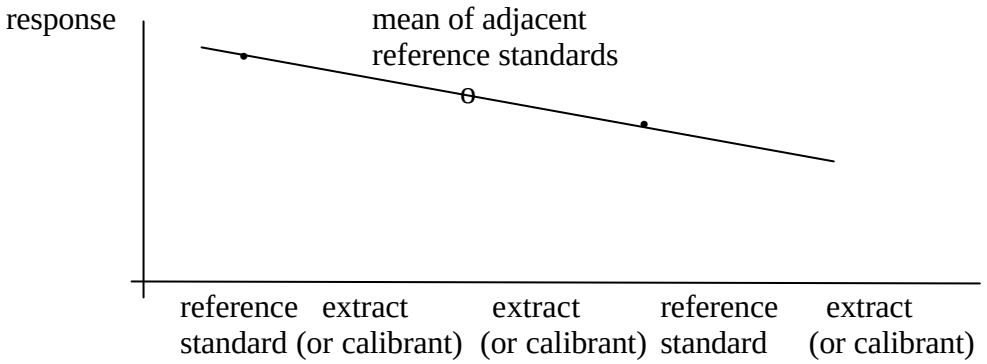

Figure 3: Compensation for drift in aflatoxin B_1 response by injecting reference standard at regular intervals.