

1. SCOPE

This procedure specifies a method for the determination of the aflatoxin B₁ content in compounded animal feed by high performance liquid chromatography (HPLC) and post-column derivatization with electrochemically generated bromine and fluorescence detection.

2. FIELD OF APPLICATION

This method has been applied so far to mixed feeding stuffs including those containing citruspulp. The lower limit of determination for aflatoxin B₁ is 1 µg/kg based upon the presence of extract components with R_t values close to aflatoxin B₁. The repeatability and reproducibility of a similar method (differing in post column derivatization with I₂ instead of Br₂) are 11% and 18% respectively at a level ranging from 7-14 µg/kg (9.2).

The method has been successfully used in practice in the period 1990-1997 with mobile phase solvent I (5.10.1). During this period 70 samples of BCR CRM 376 (certified mass fraction of aflatoxin B₁ : 9.3 ± 0.5 µg/kg) were analysed, leading to the following results for within-laboratory reproducibility:

AVG (Average)	: 9.75 µg aflatoxin B ₁ / kg
SD (Standard deviation)	: 1.19 µg aflatoxin B ₁ / kg
CV (Coefficient of variation)	: 12.2 %

See file "Kwaliteitsbeheersing SOP ARO 074" (see ARO-MIS CB\AMAP)

3. PRINCIPLE

Extraction of the test portion with chloroform, filtration, and purification of an aliquot portion over a Florisil cartridge, subsequently followed by a C₁₈ cartridge. Final separation and determination is achieved on a C₁₈ reversed phase HPLC column, followed by postcolumn derivatization with electrochemically generated bromine.

4. HAZARD

The method described requires the use of solutions of aflatoxins. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO) (9.3). Manipulations should be performed in a designated fume hood. Special precautions should be taken when toxins are in a dry form because of their electrostatic nature and resulting tendency to disperse in working areas. Swab accidental spills of toxin with 5% Sodium hypochlorite (NaOCl) bleach.

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 subdued 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).

5. REAGENTS

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.

- 5.1 Chloroform, stabilized with 0.5 to 1.0% of ethanol, by mass.
- 5.2 Methanol, (p.a. grade for preparation of 5.9.2 and HPLC cleanup)
- 5.3 Methanol, (HPLC grade for preparation of 5.10)
- 5.4 Acetone
- 5.5 Potassium bromide
- 5.6 Nitric acid (70% W/W)
- 5.7 Acetonitrile, HPLC grade
- 5.8 Acetic acid, HPLC grade
- 5.9 Eluting solvents.
 - Prepare 1 day before use, or remove (excess) air in the solvents ultrasonically.
- 5.9.1 Acetone/water (98+2) (V/V)
- 5.9.2 Water/methanol (80+20) (V/V)
- 5.9.3 Water/acetone (85+15) (V/V)
- 5.10 Mobile phase HPLC

Note: The composition of the mobile phase solvent may need to be adjusted, depending on the type of HPLC column used.

5.10.1 Mobile phase solvent I.

Dissolve 289 mg potassium bromide (5.5) in 1300 ml water, add 700 ml methanol (5.3), 400 ml acetonitrile (5.7) and 152 µl nitric acid (5.6). Mix solvent thoroughly and filter through a membrane filter (6.14).

5.10.2 Mobile phase solvent II (Alternative).

Another mobile phase solvent has been recently found appropriate. It is considered to be superior for the separation of aflatoxin B₁, aflatoxin B₂, aflatoxin G₁ and aflatoxin G₂.

Dissolve 289 mg potassium bromide (5.5) in 1650 ml water, add 465 ml methanol (5.3), 390 ml acetonitrile and 152 µl nitric acid (5.6). Mix solvent thoroughly and filter through a membrane filter (6.14).

- 5.11 Acid washed Celite 545 or equivalent
- 5.12 Florisil cartridge (Waters SEP-pak)
- 5.13 C₁₈ cartridge (Waters SEP-pak)
- 5.14 Inert gas, e.g. nitrogen
- 5.15 Ampoule containing aflatoxin B₁ calibrant solution, concentration 10 µg/ml, in 2.5 ml chloroform (RIVM, P.O. Box 1, 3720 BA Bilthoven, The Netherlands).
Aflatoxin B₁ stock solution:
Transfer the contents of the ampoule (5.15) 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 (approximately 4°C) in the dark, well sealed and wrapped in aluminium foil. This solution is stable for at least 4 months.
- 5.16 Aflatoxin B₁ calibration solutions for HPLC.
These solutions are stable for at least one month if stored in the dark in well-sealed containers.

Note Use acid-washed glassware for preparation of these solutions (See 6).

5.16.1 Calibration solution 2 ng/ml.

Allow the volumetric flask with stock solution (5.15) to warm up to room temperature in the aluminium foil (a few hours). Transfer 400 µl of the stock solution (200 ng) into a 100 ml volumetric flask, and evaporate the solution to dryness in a current of inert gas (5.14). Dissolve the residue obtained in ca 20 ml water-acetone (5.9.3), adjust to the mark with water-acetone (5.9.3) and mix well. This solution is also referred to as the reference standard to be used for repetitive injection during HPLC (7.5).

5.16.2 Calibration solution 1.5 ng/ml

Dilute 750 µl of the 2 ng/ml calibration solution (5.16.1) with 250 µl water-acetone (5.9.3) in a 1 ml vial, and mix well.

5.16.3 Calibration solution 1 ng/ml

Dilute 500 µl of the 2 ng/ml calibration solution (5.16.1) with 500 µl water-acetone (5.9.3) in a 1 ml vial, and mix well.

5.16.4 Calibration solution 0.5 ng/ml

Dilute 250 µl of the 2 ng/ml calibration solution (5.16.1) with 750 µl water-acetone (5.9.3) in a 1 ml vial, and mix well.

5.17 Ampoule containing mixture of aflatoxins B₁, B₂, G₁, G₂, 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). Chromatographic test solution:
Transfer the contents of the ampoule into a glass-stoppered test-tube or screw-capped vial. Transfer 20 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 N₂ and redissolve into 10 ml water-acetone (5.9.3).

Note The use of normal analytical grade reagents for the HPLC mobile phase (in particular acetonitrile) may lead to erroneous results, by interference in the postcolumn reaction.

6. APPARATUS

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 company or product by the National Institute of Public Health and the Environment.

- 6.1 Grinder/mixer
- 6.2 Sieve of aperture size 1.0 mm
- 6.3 Mechanical shaker
- 6.4 Balance
- 6.5 Rotary vacuum evaporator, equipped with a 150 ml round bottomed flask.
- 6.6 High performance liquid chromatograph, injector with loop of 250 µl or ³ 500 µl.
- 6.7 HPLC analytical column: 3 mm or 5 mm. Column dimensions of 10 cm x 4.6 mm (3 µ) and 15 cm x 4.6 mm (5 µ) have found to be appropriate in practice.
- 6.8 KOBRA-cell (Lamers & Pleuger; VU Amsterdam). See remarks (8.2).
- 6.9 DC-supply in series with the KOBRA-cell, capable of providing a constant current of ca. 100 µA.
- 6.10 Reaction tube; Teflon. Dimensions of 120 mm x 0.25 mm ? have found to be appropriate in combination with 5 µm and 3 µm HPLC columns.
- 6.11 Fluorescence detector, with excitation at 365 nm and emission at 435 nm wavelengths. (For filter instrument: emission wavelength > 400 nm). Detection of at least 0.05 ng B₁ shall be possible. (Signal/noise >10, e.g. Jasco 820-FP or 821-FP with spectral band width for excitation: 18 nm, and for emission: 18 nm; time constant approx. 1.5 sec).

- 6.12 Electronic integrator.
- 6.13 Fluted filter paper. MN 617 1/4 ? 24 cm or equivalent.
- 6.14 Membrane filter with a pore size of 0.45 µm (e.g. Schleicher & Schuell Ref.-No.410212).
- 6.15 Glass Filter Holder, ? 47 mm. Borosilicate glass funnel and base with coarse-frit glass support for filter; anodized aluminium spring clamp; Neoprene stopper (e.g. Millipore XX10 047 00).
- 6.16 Glass stoppered volumetric flasks 50 ml or measuring cylinders.
- 6.17 Glass stoppered conical flask 500 ml.
- 6.18 Glass column (internal diameter ca. 1.0 cm, length ca. 30 cm) equipped with a Luer tip.
- 6.19 Luer nylon chloroform-resistant stopcock (e.g. Bio-Rad 7328017, Analytichem AI 6078, Baker 4514).
- 6.20 Chemically resistant syringe, 10 ml Luer tip.
- 6.21 HPLC injection syringe of 250 µl for loop ? 500 µl, or 1 ml for loop of 250 µl.
- 6.22 Microsyringe 100 µl (check that the accuracy is within 2% by weighing)

Caution: Use of non-acid washed glassware for aqueous aflatoxin solutions may cause loss of aflatoxins. Particular care should be taken with new glassware and disposable glassware such as autosampler vials and Pasteur pipettes. Therefore laboratory glassware coming into contact with aqueous solutions of aflatoxins should be soaked in dilute 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 necessary for the volumetric flasks (6.16), the vials or tubes used for calibration and the Pasteur pipettes, if these are used to transfer calibration solutions or extracts. It is not necessary for roundbottomed flasks and measuring cylinders, provided they undergo a washing procedure, which includes an acid rinsing step. The latter is common practice in the laboratory washing machine at ARO.

7. PROCEDURE

7.1 Preparation of the sample:

Grind the sample with a grinder/mixer (6.1). If desired the particle size can be checked by passing it through the sieve (6.2.).

7.2 Test portion:

Weigh 50 g of the prepared test sample into the conical flask (6.17.).

7.3 Extraction:

Add 25 g of Celite (5.11), 250 ml of chloroform (5.1) and 25 ml of water. Stopper the flask, and shake for 30 min. on a mechanical shaker (6.3). Filter through a fluted filter paper (6.13). Collect 50 ml of the filtrate.

7.4 Clean-up (the clean-up procedure should be carried out without significant interruptions).

7.4.1 Florisil SEP-pak purification

7.4.1.1 Preparation of the column-cartridge assembly.

Attach a stopcock (6.19) to the shorter stem of a Florisil cartridge (5.12) (see figure 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 (6.20).

Attach the longer stem of the cartridge to a glass column (6.18) and pass the remaining 2 ml chloroform through the cartridge into the column.

Close the stopcock. Remove the syringe.

7.4.1.2 Purification

Add the filtrate collected in 7.3 to the column-cartridge assembly and drain by gravity. Rinse with 5 ml of chloroform (5.1), followed by 20 ml of methanol (5.2).

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

Elute aflatoxin B₁ with 40 ml of the acetone/water mixture (5.9.1) and collect the whole of the eluate in the (150 ml) round bottomed flask of the rotary evaporator (6.5).

Concentrate the eluate on the rotary evaporator (at 40°- 50°C) until acetone stops being distilled.

Note Ca 0.5 ml of liquid remains in the flask at this point. Add 1 ml of methanol (5.2), swirl the flask to dissolve aflatoxin B₁ 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 C₁₈ purification step.

7.4.2 C₁₈ SEP-pak purification

7.4.2.1 Preparation of the column-cartridge assembly.

Attach a stopcock (6.19) to the shorter stem of a C₁₈ cartridge (5.13) (see figure 1).

Prime the cartridge and remove any air by rapidly passing 10 ml methanol (5.2) via the stopcock through the cartridge with a syringe (6.20) (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.

7.4.2.2 Purification.

Transfer the extract collected in 7.4.1.2 quantitatively to the column, rinsing the flask twice with 5 ml water/methanol mixture (5.9.2) 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 aflatoxin B₁ with 50 ml water/acetone mixture (5.9.3), and collect the whole of the eluate in a volumetric flask or glass-stoppered measuring cylinder (6.16). Adjust the volume to 50 ml with water and mix: the resulting test solution is used for chromatography (7.5).

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 B₁. Teflon filters are acceptable.

7.4.3 Optional dilution of the extract

Dilute the final extract if necessary, so that the concentration of aflatoxin B₁ is within the linear range of the calibration curve.

7.5 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 stabilise.

Note 1 The flow-rate given for the HPLC solvent is indicative only. They may need to be adjusted depending on the type and size of the HPLC column.

Note 2 The detector response to aflatoxin B₁ depends on the temperature, therefore compensation should be made for drift (see figure 3). By injecting a fixed amount of aflatoxin B₁ reference standard (5.16.3) at regular intervals (i.e. every third injection), the aflatoxin B₁ 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. (5.16.3.) Because the calibration curve is linear and passes through the origin, the amounts of aflatoxin B₁ in sample extracts are determined directly by reference to the adjacent standards.

7.5.1 HPLC pump settings

Set the HPLC pump (6.6) to give a flow of 0.5 or 0.3 ml/min for a 5 µm or 3 µm HPLC column (6.7) respectively: use mobile phase (5.10).

7.5.2 DC-powersupply

Turn the DC-powersupply (6.9) on.

7.5.3 Fluorescence detector

Set the fluorescence detector (6.11) to $\lambda_{\text{exc.}}$ 365 nm and $\lambda_{\text{em.}}$ 435 nm. (For filter instrument: emission wavelength >400 nm).

7.5.4 Injector

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

7.5.5 "Check of chromatographic separation". (optional, can be applied if doubt exists about the quality of chromatographic separation).

Inject a chromatographic test solution (5.17.1). Valleys should be less than 5% of the sum of peakheights of the adjacent peaks.

7.5.6 Check of stability of the system

Before each series of analyses, repetitively inject the reference standard (5.16.3), until stable peak areas are achieved (N.B. Peak responses for aflatoxin B₁ between consecutive injections should differ by less than 6%). Proceed without delay with check of linearity.

7.5.7 Check of linearity

Inject the aflatoxin B₁ calibration solutions (5.16.1-5.16.4).

Every third injection use the reference standard (5.16.3), for correction of drift in response.

Note Peak responses for the reference standard should differ by less than 10% in 90 minutes.

Calculate the amount aflatoxin B₁ found, according to the formula (2) in 8.1.

Plot the amount aflatoxin B₁ found (Y) against the injected amount of aflatoxin B₁. Calculate the regression line. The calibration graph should be linear and pass through the origin, within 2x standard error of Y-estimate. Values found should differ by less than 3% from the nominal values. If the requirements are fulfilled, continue without delay. If not, identify and correct the sources of the problems before continuation.

7.5.8 Injection of sample extracts.

Inject the purified sample extracts (7.4.2.2). After every two sample extracts repeat injection of the reference standard (5.16.3) in the following sequence: ref. standard, extract, extract, ref. standard, extract, extract, ref. standard etc.

8. CALCULATION OF RESULTS

8.1 Formula:

Calculate the aflatoxin B₁ content (μg/kg) present in the sample, using the formula:

$$\text{aflatoxin B}_1 \text{ content (in } \mu\text{g/kg}) = \frac{m \times V_{\text{ex}}}{V_m \times M \times \frac{V_f}{V_e}} \quad (1)$$

where m = amount of aflatoxin B₁ in ng represented by the aflatoxin B₁ peak of the sample calculated as follows:

$$m = \frac{P(\text{sample})}{P(\text{st}_1) + P(\text{st}_2)} \cdot 2i \quad (2)$$

where:

p (sample) = peak area of aflatoxin B₁ in sample

p (st₁) = peak area of aflatoxin B₁ in preceding reference standard (5.16.3) (fig. 3)

p(st₂) = peak area of aflatoxin B₁ in next-following reference standard (5.16.3) (fig. 3).

i = injected amount of reference standard (5.16.3) in ng.

V_m = volume of injected sample extract in ml

V_{ex} = final volume of sample extract in ml, allowing for any dilution that was made (7.4.3)

M = mass of sample in g

V_f = volume of filtrate transferred to Florisil cartridge (7.4.1.2.) in ml

V_e = amount of chloroform, used for the extraction of the sample, in ml

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

$$\text{aflatoxin B1 content (in } \mu\text{g/kg}) = 20 m \quad (3)$$

8.2 Remark

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

Examples of disturbances which may occur with the KOBRA cell are given in the file "Kwaliteitsbeheersing SOP ARO 074" (see ARO-MIS CB\AMAP).

9. REFERENCES

9.1 SOP no. ARO/070;

Method for the determination of aflatoxin B₁ in compounded animal feed (basis of the EC-collaborative study carried out in 1988)

9.2 Egmond, H.P. van, Heisterkamp, S.H. and Paulsch, W.E. (1991).

Collaborative study - aflatoxin B₁ in animal feeding stuffs.

Food Addit. Cont. 8, 17-29 (ARO doc 7019)

9.3 Castegnaro, M., Hunt, D.C., Sansone, E.B., Schuller, P.L., Siriwardana, M.G., Telling, G.M., Egmond, H.P. van, Walker, E.A. (1981)

Laboratory decontamination and destruction of aflatoxins B₁, B₂, G₁, G₂ in laboratory wastes

IARC Scientific Publications 37 (ARO doc 7121)

10. FIGURES

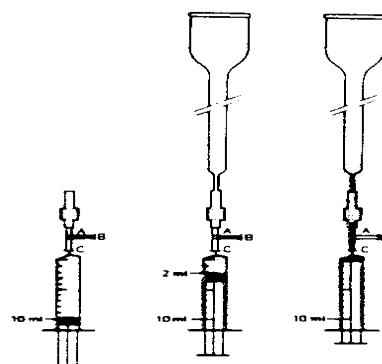


Figure 1: Column-cartridge assembly

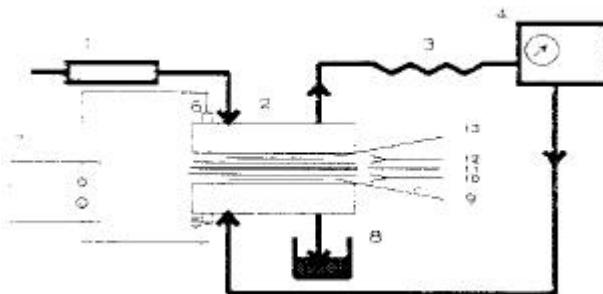
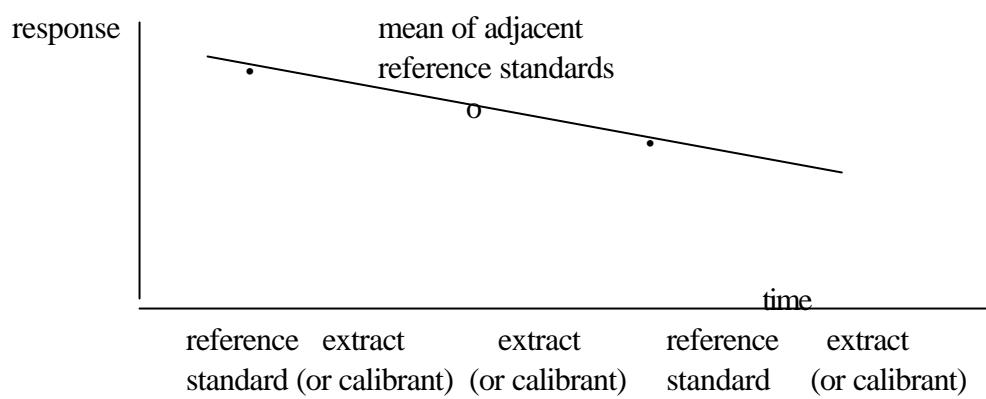


Figure 2: Flow diagram of the LC system with electrochemically generated bromine for postcolumn derivatization.

1. HPLC analytical column	8. Waste
2. KOBRA-cell	9. Counter electrode
3. Reaction coil	10. Spacers
4. Fluorescence detector	11. Membrane
5. Black connection	12. Spacers
6. Red connection	13. Working electrode
7. DC-powersupply	

Figure 3: Compensation for drift in aflatoxin B₁ response by injecting reference standard at regular intervals.