

Stofnaam	Aflatoxine B1
Type methode	HPLC met broomderivatisering (zie ook onder 3)
Te onderzoeken in	Diervoeders
Minimum bepaalbaarheidsgrens	0.5µg/kg
Herhaalbaarheid	Zie tabel hieronder, die is overgenomen uit het ringtestrapport
Reproduceerbaarheid	
Categorie	-
Titel	'Method for the determination of aflatoxin B1 in Animal Feed ' Deze methode is gepubliceerd in het EC-rapport EUR 19058 EN (2000) 'Validation of an Analytical Method to Determine the Content of Aflatoxin in Animal Feed Stuff'

Opmerking: Deze methode is mogelijk minder geschikt voor het bepalen van het aflatoxine B1-gehalte in vetrijke producten

	Spiked 1.2 ng/g	Spiked 3.6 ng/g	Natural 0.5 ng/g 'b'	Natural 1 ng/g 'c'	Natural 5 ng/g 'd'
<b>MEAN of accepted results</b>	1.33	3.82	0.54	0.87	4.19
<b>Number or results</b>	21	21	21	21	21
<b>Number of outliers</b>	1	1	3	2	3
<b>Number of accepted results</b>	20	20	18	19	18
<b>R (ng/g)</b>	0.22	0.69	0.11	0.21	0.72
<b>Sr (ng/g)</b>	0.08	0.25	0.04	0.08	0.26
<b>RSD<sub>r</sub> (%)</b>	5.9	6.4	7.2	8.7	6.2
<b>Hor (ng/g)</b>	0.21	0.26	0.22	0.29	0.26
<b>R (ng/g)</b>	0.72	1.87	0.27	0.47	2.30
<b>SR (ng/g)</b>	0.26	0.67	0.10	0.17	0.82
<b>RSD<sub>r</sub> (%)</b>	19.4	17.5	17.9	19.4	19.6
<b>HORRAT ratio</b>	0.45	0.47	0.36	0.42	0.54

## METHOD FOR THE DETERMINATION OF AFLATOXIN B<sub>1</sub> IN ANIMAL FEED

### 1. SCOPE

This method (based on Agribiological Research, 44 (4), 289-298) can be applied to the determination of aflatoxin B<sub>1</sub> in animal feed. The limit of quantification of the method has been demonstrated to be better than 0.5 $\mu$ g/kg for aflatoxin B<sub>1</sub> for a signal to noise ratio of 6.

### CAUTION

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1. This method requires the use of solutions of aflatoxin B<sub>1</sub>. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the International Agency for Research on Cancer (WHO) [1].
  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 of aluminium foil.
  3. The use of non-acid 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. sulphuric 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).
  4. Decontamination procedures for laboratory wastes [1.2] were developed and validated by the International Agency for Research on Cancer (WHO).
  5. This method requires the use of inflammable liquids such as acetone, methanole and acetonitrile. Keep away from heat, sparks or open flames.
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### 2. NORMATIVE REFERENCES

This method incorporates dated or undated references and provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed at the end. For dated references, subsequent amendment to or revisions of any of these publications apply to this method only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

ISO 3696:1987: Water of analytical laboratory use – Specification and test methods  
ISO 5725:1986: Precision of test methods – Determination of repeatability and reproducibility for a standard test method by inter-laboratory test.

### 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, and a portion is applied on an immunoaffinity column containing antibodies specific to aflatoxin B<sub>1</sub>. The aflatoxin B<sub>1</sub> is removed from the immunoaffinity column with neat methanol, and then quantified by reverse-phase high performance liquid chromatography (RP-HPLC) with post column derivatisation (PCD) involving bromination. The PCD is achieved with either electrochemically generated bromine or with pyridinium hydrobromide perbromide (PBPB) followed by fluorescence detection.

### 4. REAGENTS

All reagents shall be of recognised analytical grade.

Unless otherwise stated, use water complying with grade 3 of ISO 3696.

#### 4.1 Phosphate buffer saline (PBS) [pH 7.4]

PBS can 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 x 12 H<sub>2</sub>O (2.92 g)] and sodium chloride (8.00 g) added to 900 mL purified water. After dissolution the pH must be adjusted to pH 7.4 (with 0.1 mol/L HCl or 0.1 mol/L NaOH as appropriate) and the solution made to 1.0 L.

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

#### 4.2 Pyridinium hydrobromide perbromide (PBPB) [CAS: 39416-48-3]

(This reagent is NOT required in the case of using a KOBRA cell ®).

#### 4.3 Potassium bromide

(This reagent is NOT required in the case of using the PBPB reagent).

#### 4.4 HPLC grade acetonitrile

#### 4.5 HPLC grade methanol

#### 4.6 Acetone (pure)

#### 4.7 HPLC grade water, complying with grade 1 of ISO 3696.

#### 4.8 Extraction solvent: acetone (4.6) – water solution [85 + 15 (v+v)].

#### 4.9 Nitric acid, $c(\text{HNO}_3) = 4 \text{ mol/L}$ .

(This reagent is NOT required in the case of using the PBPB reagent).

#### 4.10 Immunoaffinity column:

The affinity column should contain antibodies raised against aflatoxin B<sub>1</sub>. The column should have a capacity of not less than 40 ng of aflatoxin B<sub>1</sub> and should give a recovery of not less than 80% for aflatoxin B<sub>1</sub> when applied as a standard solution in acetone/water containing 0.25 ng of aflatoxin B<sub>1</sub>.

#### 4.11 HPLC mobile phase solvent (A):

For use with PBPB post column reagent only:

Water (4.7)/acetonitrile(4.4)/methanol(4.5)solution [ 6 + 2 + 3 (v+v+v)].

The ratio of solvents can be adjusted to guarantee best separation parameters.

#### 4.12 HPLC mobile phase solvent (B):

For use with electrochemically generated bromine only:

for use with electrochemically generated bromine: water(4.7)/acetonitrile(4.4)/methanol(4.5) solution [ 6 + 2 + 3 (v+v+v)] containing 120 mg potassium bromide (4.3) and 350  $\mu\text{L}$  nitric acid at 4 mol/L(4.9) per litre mobile phase. The ratio of solvents can be adjusted to guarantee best separation parameters.

*NOTE 1: The mobile phase solvent (4.11/4.12) should be degassed.*

#### 4.13 Post column reagent:

For use with PBPB post column reagent only:

Dissolve 25 mg PBPB (4.2) in 500 mL H<sub>2</sub>O. Solution can be used for up to four days if stored in a dark place at room temperature. This post column reagent is to be used combination with HPLC mobile phase solvent A (4.11) but not with HPLC mobile phase solvent B (4.12).

#### 4.14 Toluene/acetonitrile [98 + 2 (v+v)].

#### 4.15 Aflatoxin B<sub>1</sub> standard material

Aflatoxin B<sub>1</sub> standard in form of crystals or a dry film for analytical purposes.

#### 4.16 Calibrant stock solutions for HPLC:

Prepare a aflatoxin B<sub>1</sub> (4.15) stock solution containing 50.0 ng/mL in touene/acetonitrile (4.14).

#### **Option A (See: method description 6.3)**

Pipette from this solution the volumes as listed in Table I (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. To each flask, add 7 mL of methanol, let aflatoxins dissolve, then fill up to the mark with water, and shake well.

*NOTE 2: Bear in mind that methanol and water are subject to volume contraction when mixed.*

#### **Option B (See: method description 6.3)**

Pipette from this solution the volumes as listed in Table 1 (Option B) 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. To each flask, add approx. 10 ml of methanol, let aflatoxins dissolve, then fill up further with neat methanol (NOT with Methanol/Water) to the mark and shake well. Exactly 1 mL of this working calibrant is then transferred into an acid washed glass vial (see: caution item 3), evaporated to dryness according to option B under (chapter 6.3) and than re-dissolved in exactly the same volume that will be used to re-

dissolve the samples prior injection (section 6.3). Calculate the concentration of aflatoxin B<sub>1</sub> in the evaporated and re-dissolved solution in ng/mL. Use these concentration values for the calculation according section 6.6. In this case the calibration range will remain unchanged.

Table I. Preparation of working calibrant solutions

Option	Option A		Option B	
	Aliquot stock solution in (μL)	Concentration in (ng AfB1/mL)	Aliquot stock solution in (μL)	Concentration in (ng AfB1/mL)
1	20	0.050	100	0.250
2	45	0.113	225	0.563
3	70	0.175	350	0.875
4	95	0.238	475	1.188
5	120	0.300	600	1.500
6	145	0.363	725	1.813
7	170	0.425	850	2.125
8	195	0.488	975	2.438
9	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**, Ø 24 cm, prefolded (e.g. Whatman 2 V)

5.3 **Erlenmayer flask** with screw top or glass stopper.

5.4 **Glass microfiber filter paper**, Ø 5 cm.

5.5 **Reservoir**, 75 mL with luer tip connector for immunoaffinity column (IAC).

5.6 **Hand pump**, 20 mL syringe with luer lock or rubber stopper for IAC.

5.7 **Volumetric glassware**, 5 mL, 10 mL and 20 mL.

(shall have an accuracy of at least 0.5%)

5.8 **HPLC pump**, suitable for flow rate at  $1.000 \pm 0.005$  mL/minute.

5.9 **Injection system**. Capable for total loop injection. (A valve with a loop of at least 100  $\mu$ l is recommended).

*NOTE 3: It must 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<sub>1</sub> (concentration equivalent to a contamination level of 1 ng/g) results in a value of max 10%. This data must be reported.*

5.10 **RP-HPLC column**, e.g. LC-18 or ODS-2

(Optional but recommended: pre-column).

5.11 **Post column derivatisation system with PBPB** (alternative to 5.12)

Second HPLC pulse-less pump, zero-dead volume T-piece, reaction tubing min. 45 cm x 0.5 mm internal diameter PTFE (the reaction time must be at least 4 seconds before detection).

5.12 **System for derivatisation with electrochemically generated bromine**, (e.g. KOBRA cell) the device must be installed according to the manufactures instructions.

*NOTE 5: In order to confirm the aflatoxin B<sub>1</sub> the HPLC column has to be disconnected from the bromination device and must 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 remaining bromine in the cell membrane of the device).*

5.13 **Fluorescence detector**, with a wavelength of  $\lambda = 360$  nm excitation filter and a wavelength  $> 420$  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  $\mu\text{m}$ ).

*NOTE 6: Prior to usage it has to be verified that no aflatoxin losses occur during filtration (recovery testing) since there is a possibility that various filter materials can retain aflatoxin B<sub>1</sub>.*

5.15 **Single marked pipettes**, 1 mL, 2 mL, 5 mL and 10 mL capacity.

5.16 **Analytical balance**, capable of weighing to 0.1 mg.

5.17 **Laboratory balance**, capable of weighing to 0.01 g.

5.18 **Calibrated microliter syringe(s) or microliter pipette(s)**  
(20  $\mu\text{L}$  – 500  $\mu\text{L}$ ).

5.19 **Evaporator** (optional; only needed for option B, sector 6.3).

## 6. PROCEDURES

### 6.1 Conditioning of immunoaffinity columns:

Immunoaffinity columns (4.10) should be at room temperature prior to conditioning. For conditioning follow the instructions of the manufacturer. If not stated different, apply 10 mL of PBS (4.0) on the top of the column and let it pass at a speed of 2-3 mL/min through the column (gravity). Make sure that a small portion (0.5 mL) of the PBS remains on the column until the sample solution is applied.

### 6.2 Extraction:

Weigh, to the nearest 0.1 g, approximately 50 g of the test portion into a 500 mL Erlenmeyer flask with screw top or glass stopper. Add 250 mL acetone/water extraction-solvent (4.8). Shake intensively by hand for the first 15 s – 30 s and then for 30 min. with a shaker (5.1).

Filter the extract using prefolded paper (5.2). Pipette 5.0 mL of the clear filtrate into a 500 mL calibrated volumetric flask and fill up to the mark with PBS or water.<sup>1</sup> If the solution is not clear, re-filter through a glass fibre and apply exactly 50 mL of the clear filtrate on a reservoir that is placed on a conditioned immunoaffinity column. (If the solution is clear the diluted solution can be directly applied on the immunoaffinity column). Apply the solution on the column as described in section 6.3.

### 6.3 Immunoaffinity clean up:

*NOTE 7: Methods for conditioning, loading, washing and eluting vary slightly between immunoaffinity column manufacturers and the specific instructions supplied with the columns should be followed precisely. In general, procedures involving sample extraction with methanol/water, filtration or centrifugation, possible sample dilution with PBS or water, loading under pressure onto (possibly pre-washed) column, washing of column with distilled water and elution of aflatoxin B<sub>1</sub> with methanol or acetonitrile.*

Pass the filtrate through the column at a flow of approximately 1 drop/s (approx. 3 mL/min) (gravity). Do not exceed a flow rate of 5 mL/min. Wash the column with approx. 20 mL of water (4.7) – applied in two portions of approx. 10 mL/min. and dry by applying a light vacuum for 5 s – 10 s or passing air through the immunoaffinity column by means of a syringe for 10 s.

Elute the aflatoxin B<sub>1</sub> in a two step procedure:

- Apply 0.50 mL methanol on the column and let it pass through by gravity. Collect the elute in a calibrated volumetric flask of 5 mL (5.7).
- Wait for 1 minute and apply a second portion of 1.25 mL methanol. Collect most of the applied elution solvent by pressing air through, after most has passed through by gravity.

#### **Option A (recommended):**

*NOTE 8: This option is recommended, but requires an appropriate fluorescence detector*

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<sup>1</sup> The dilution solvent – PBS or water – must be selected according the specifications of the IAC manufacture. If not stated differently, the dilution must be made with PBS.

*and injection system (see also NOTE 3 in section 5.9). Option B only applies if the detector signal is insufficient for the analysis according to option A.*

Collect the eluate in a calibrated volumetric flask of 5 mL (5.7). Fill flask to the mark with water and shake well (final volume according to section 6.6). If the solution is clear it can be used directly for HPLC analysis. If the solution is not clear, pass it through a disposal filter unit (0.45 µm) (5.14) prior HPLC injection (see note 6, section 5.14). The injection by total loop mode guarantees maximum accuracy. It is recommended (depending on the injection system, e.g. syringe or autosampler) to take a sample volume of 3 times the injection loop size and to inject at least 2/3 of this volume into the valve, to ensure that the middle fraction remains in the injection loop. Thus, the loop is rinsed with the injection solvent while enough solvent remains in the valve.

**Option B (only if applicable):**

If the detector signal is not sufficient, to guarantee the required RSD (see note 3, section 5.9) an additional evaporation step may be included to meet the required RSD.

Collect the aflatoxin containing methanol eluate from the IAC in an acid washed glass vial (see caution item 3). Evaporate the eluate to dryness under a gentle stream of nitrogen at 40 °C. Re-dissolve the aflatoxin in an aqueous methanol solution (35% MeOH). Use **exactly the same volume** for the evaporated sample residues as you will use for the evaporated calibrants (see chapter 4.17). The volume for re-dissolving (final volume according to section 6.6) will depend on the size of your injection loop. Use the total loop mode for injection as described in option A.

**6.4 Post column derivatisation:**

When using PBPB, mount the mixing T-piece and reaction tubing mentioned under 5.11, and then operate using the following parameters:

- flow rate: 1.00 mL/min (mobile phase 4.11)
- 0.30 mL/min (reagent 4.13)

When using electrochemically generated bromine (KOBRA cell) follow the instructions for the installation of the cell as supplied by the manufacturers and operate using the following parameters:

- flow rate: 1.00 mL/min (mobile phase 4.12)

- current: 100  $\mu$ A

### 6.5 Calibration curve:

Calibration curve should be prepared using the working calibration solutions described (4.16). These solutions cover the range of 0.5  $\mu$ g/kg – 5.5  $\mu$ g/kg for aflatoxin B<sub>1</sub>. Make the calibration curve prior to analysis according to Table 1 and check the plot for linearity.

*NOTE 8: In case that the content of aflatoxin B<sub>1</sub> in the sample will be outside of the calibration range, an appropriate calibration curves must be prepared. Alternatively the injection solution for HPLC analysis can be diluted to an aflatoxin B<sub>1</sub> content appropriate for the established calibration curve.*

### 6.6 Calculation:

Plot the signal as x-axis (height or area) against the concentration of aflatoxin B<sub>1</sub> [ng/mL] as y-axis – from the calibrants (section 6.5) into a table and calculate the calibration curve using linear regression<sup>2</sup>. Use the resulting function:

$$(y = ax + b)$$

to calculate the concentration of aflatoxin B<sub>1</sub> in the measured solution.

Calculation of the aflatoxin concentration from the calibration curve (function) obtained by linear regression:

W	= sample material taken for analysis [g] – (50 g)
Solvent	= solvent taken for extraction [mL] – (250 mL)
Aliquot <sub>Ex</sub>	= aliquot taken from extract [mL] – (5 mL)
Dilution	= volume achieved after dilution with PBS (water) [mL] (100 mL)
Aliquot <sub>IAC</sub>	= aliquot taken for the immunoaffinity clean-up [mL] – (50 mL)
Elution	= final volume achieved after elution from IAC [mL]
C <sub>smp</sub>	= concentration of aflatoxin calculated from the lin. regression [ng/mL]
Conta.	= contamination of sample material with aflatoxin B <sub>1</sub> [ $\mu$ g/kg]
signal <sub>smp</sub>	= area of height of aflatoxin peak obtained from the measured solution

$$C_{smp} \text{ [ng/mL]} = a \times \text{signal}_{smp} \text{ [units]} + b$$

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<sup>2</sup> Linear regression should be performed using a scientific calculator or a statistical program.

$$\text{Conta.} = \frac{C_{\text{smp}} \times \text{Solvent} \times \text{Elution} \times \text{Dilution} \quad [\text{ng} \times \text{mL} \times \text{mL} \times \text{mL}]}{W \times \text{AliquotEX} \times \text{AliquotIAC} \quad [\text{mL} \times \text{g} \times \text{mL} \times \text{mL}]}$$

$$\text{Conta.} = C_{\text{smp}} \times 100 \times \text{Elution} / W$$

Bear in mind, that for sample- and standard solutions the same volume must be injected to comply with the formula.

### 6.7 Spiking procedures for recovery determination:

For the determination of the recovery the spiking procedure has to be carried out using a methanol stock solution of aflatoxin B<sub>1</sub>. The spiking level must be within the calibration range (preferably mean value). Take care that not more than 2 mL of the spiking are added (solution must have an adequate concentration of aflatoxin B<sub>1</sub>) and that the subsequent evaporation takes place in the dark and should last 0.5 – 2 hours.

## 7. STATISTICAL EVALUATION

To be included after completion of the laboratory intercomparison.

## 8. TEST REPORT

The test report shall contain at least the following data:

- all information necessary for the identification of the sample;
- all information necessary for the identification of the calibrant;
- the results and the units in which the results have been expressed;
- date of test;
- any particular points observed in the course of the test;
- **Any operation not specified in this method or regarded as optional which might have affected the results.**

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