Methods for the determination of aflatoxine B$_1$ in feedingstuffs intended for dairy cattle.

Verzendlijst: directeur, sektorhoofden, directie VKA, afd. OCON (4x), afd. Normalisatie/Harmonisatie (Humme), Projektbeheer, Projektleider.
METHODS FOR THE DETERMINATION OF AFLATOXIN B₁ IN FEEDINGSTUFFS INTENDED FOR DAIRY CATTLE.

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This report is written on request of the "Committee of Experts on Methods of Analysis of Feedingstuffs" (Commission of the European Communities, Directorate-General for Agricultural VI B 3).
Introduction

Until the first of January 1984 within EC an aflatoxin B\textsubscript{1} tolerance of 20 µg/kg was in force for feedingstuffs intended for dairy cattle (Directory 74/73/EEG dd. 17-12-1973) including a method of analysis 76/372/EC dd. 1-3--1976).

This official method has some disadvantages.

a) The limit of detection, using two dimensional thin layer chromatography (TLC) is about 10 µg/kg.

b) When citrus pulp is used as ingredient the method can not be used. (Already in 1975 it was indicated in literature (1) that by changing the composition of the TLC eluents separation between aflatoxin B\textsubscript{1} and interfering compounds from citrus pulp could be improved and solve the problem.)

c) The method is time consuming.

From the first of January 1984 the aflatoxin B\textsubscript{1} tolerance became 10 µg/kg (74/63/EEG dd. 28-7-1983). In general it is desirable that a method of analysis has a limit of determination being 10-20% of the tolerance. In other words with a tolerance of 10 µg/kg a determination limit of 1-2 µg/kg should be realized meaning a detection limit of < 1 µg/kg.

As the method used up till now has a detection limit in the order of the new tolerance another method should be used.

During the 56th meeting of "the committee of experts on methods of analysis of feedingstuffs" (dd. 16-17 april 1984) it was decided that the several delegations should send methods of analysis to the RIKILT and when possible accompanied with "data". As no other methods were received the list of methods shows only the methods made available during the above mentioned meeting.

From the German delegation a more detailed method was received and some data (without relevant text) concerning ringtests.

In Annex 1-4 the methods are given; in Annex 5 the Dutch data and in Annex 6 the German data.

Below the principles of the four methods will be described.
Principals of proposed methods

a. Proposal of the Netherlands (2,3,4) (Annex 1 and 5).
- Aflatoxin B₁ is extracted with chloroform. For concentrated cattle feed an aliquot of 40 mg is cleaned with thin layer chromatography on silica coated alumina plates. The aflatoxin B₁ area is cut out of the plate and extracted determination in the extract of 16 mg is carried out on a reversed phase HPLC column with a fluorescence detector after post column derivatization at 60°C with an iodine water solution. Absolute limit of detection is about 0,02 ng.
- The method is applicable to the determination of aflatoxin B₁ in concentrated cattle feed. The limit of determination is 1 µg/kg. Recovery at 5 µg/kg is between 80-100%.
- For raw materials, without clean-up, the limit of determination is about 25 µg/kg. Recovery is better than 80%.

- Aflatoxin B₁ is extracted with chloroform. An aliquot of 24 gram is cleaned by a disposable sep-pak silica cartridge. Determination in the extract of 1 gram is carried out on a silica HPLC column with a fluorescence detector. Absolute limit of detection is about 0,4 ng.
- The method is applicable to the determination of aflatoxin B₁ in concentrated cattle feed. The limit of determination is about 0,4 µg/kg.

- Aflatoxin B₁ is extracted with chloroform. An aliquot of 10 gram is concentrated to dryness and solved in cyclohexane-ethylacetate (1/1 v/v) and cleaned with gelpermeation chromatography on bio beads SX3. Determination in the extract of 2.4 gram is carried out with high performance thin layer chromatography (HPTLC) and densitometric quantification.
- Absolute limit of detection is about 0,5 ng.
- The method is applicable to the determination of aflatoxin B₁ in concentrated cattle feed. The limit of determination is about 0,3 µg/kg.
- Aflatoxin B₁ is extracted with chloroform. An aliquot of 10 gram is cleaned on a silica column. Determination in the extract of 100 mg is carried out with a HPLC silica column with a fluorescence detector equipped with a packed flow-cell. Absolute limit of detection is about 0,05 ng.
- The method is applicable to the determination of aflatoxin B₁ in concentrated cattle feed. The limit of determination is 0.5 µg/kg.

In table I a few important parameters are summarized.

<table>
<thead>
<tr>
<th>Proposal of</th>
<th>Amount of material extracted (g)</th>
<th>cleaned (g)</th>
<th>applied (g)</th>
<th>Absolute detection (ng)</th>
<th>Limit of µg/kg determination</th>
</tr>
</thead>
<tbody>
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<td>25</td>
<td>0,040</td>
<td>0,016</td>
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<tr>
<td>U.K.</td>
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<td>0,4</td>
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<td>24</td>
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<tr>
<td>Danmark</td>
<td>50</td>
<td>10</td>
<td>0,100</td>
<td>0,05</td>
<td>0,5</td>
</tr>
</tbody>
</table>

Discussion: Advantages and drawbacks

Ad A Dutch proposal
Experience shows that for a good performance of the method considerable experience and skill is needed. The HPLC in combination with the post column derivatization requires careful adjustment of the different parameters. To achieve good repeatability the HPLC eluent and the derivatization reagent should be freshly made, flows should be checked regularly as well as the temperature of the water bath.
When carrying out the clean-up on TLC plates dry ethylether must be used to avoid elution of aflatoxin B₁. It is advised to dry the ether with metallic sodium and to check this part of the clean-up by controlling under UV light if aflatoxin B₁ stayed at the spot where it should be. In Annex 5 some data concerning reproducibility with in one lab is reported.

Ad B U.K. proposal
Experiments have shown that the capacity of sep-pak used for clean-up is rather limited and that separation between aflatoxin B₁ and compounds interfering with the HPLC method is rather poor.
The combination of a silica sep pak and a silica HPLC column seems questionable from a point of view of getting rid of interfering compounds.

Further experience with a great scale of feedingstuffs of different composition seems desirable, with special attention to citrus pulp. The use of silica HPLC columns has some drawbacks. The composition of the eluent is critical; small changes of the water content influences the activity of the column thus the separation and the sensitivity. The reproducibility from day to day with respect of retention time and sensitivity will be more difficult than with reverse phase columns. Striking is the high flow (2.5 ml/min) used for the HPLC separation as described in the proposed method. (See also further remarks.)

Ad C German proposal

Experiments at the RIKILT with bio-beads SX3 GPC columns in the system toluene-ethylacetate (1:1) showed that when analysing citrus pulp containing feedingstuffs the fraction containing aflatoxin B₁ also contained strong fluorescing compounds (from the citrus pulp). On the other hand prof. Ranfft informed us that with his system (cyclohexane-ethylacetate 1:1) no compounds from citrus pulp were detectable on the TLC.

Into our opinion in the German proposal it should therefore be necessary to describe how to optimize collecting the correct fraction. Also should be indicated how much of the chloroform extract is used for the GPC clean-up (and not "as much as possible").

For the TLC determination the extract is applied in 200 μl (2.4 gram). It is believed that this procedure brings problems e.g. flow out of the spot on the plate.

An improved description of the spotting technique is desirable. Though the GPC technique can be automated and a high throughput of samples can be anticipated the literature (6) indicates however that one technician can handle only 5 samples a day. Annex 6 indicates good reproducibility.
Ad D Danish proposal
As this proposal is derived from the original EC method (76/372/EC dd. 1-3-1978) which excludes the method as suitable for citrus pulp containing feedingstuffs and keeping in mind that HPLC separation on a silica column is not as good as a two dimensional TLC separation this proposal seems less suitable for routine analysis in a great variety of samples.
The use of a silica packed flow cell enhances the fluorescence of aflatoxin B₁ strongly. Experience however learned that in the time due to adsorbing materials, the cell shows an increased background signal influencing the gain in sensitivity in a negative way.
Problems mentioned with the reproducibility as indicated above for the UK method are also here appropriate.

Further remarks
From the UK delegation information was received that the proposed UK method should be ringtested in the coming year. They confirmed their experience with the Dutch method and were of the opinion that the Dutch method was more appreciated than the own method.
For the time being and in expectation of the results of the UK ring-test with the UK method they had no objections to exclude the UK method from further discussion.

Recommendations
It seems advisable to focus attention to only two methods e.g. the Dutch and German proposal.
To gather information on repeatability and reproducibility a preliminary interlaboratory study should be carried out.
May be it is advisable to look out for collaborating with the mycotoxin group of the BCR.
Literature


The determination of aflatoxine B₁ in feedingstuffs and raw materials with HPLC and post column derivatization.

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1. Purpose and scope
The method is applicable to the determination of aflatoxin B₁ in concentrated cattle feed up to 1 µg/kg. Recovery at 5 µg/kg is between 80-100%. For raw materials the method is applicable, without clean-up up to 25 µg/kg. Recovery is better than 80%.

2. Principle
Aflatoxin B₁ is extracted with chloroform. For concentrated cattle feed an aliquot is cleaned with thin layer chromatography on silicagel coated alumina plates. The aflatoxin B₁ area is cut out of the plate and extracted. For raw materials an aliquot is just evaporated to dryness and solved in methanol. Final separation and determination is carried out on a reverse phase column. Post column derivatization at 60°C is carried out with a iodine water solution (1,2).

3. Reagents
The reagents should be of such a quality that under the given analytical circumstances no interference occurs. This should be tested.

3.1 Celite 545. (Johns Mansville) cat.no. 53999.

3.2 Chloroform, stabilized with 0,5-1% ethanol.

3.3 Aceton.
5.2 Extraction
Take a 25 g test sample and introduce it in the 250 ml glass flask. Add 12.5 g celite, 12.5 ml water and 125 ml chloroform. Close the flask with the teflon lined rubber and screwcap and shake 30 min. Filter over a fold filter and collect for cattle feed 5.0 ml in a calibrated vial. Evaporate at 50°C under N₂ stream to dryness. Dissolve the extract in 500 μl chloroform (using a vortex mixer) and use extract for clean-up with thin layer chromatography.
(For raw materials after filtering collect 0.5 ml in a calibrated vial and continue as described under 5.4.)
Evaporate at 50°C under N₂ stream to dryness. Dissolve the extract in 1 ml methanol (using a vortex mixer) and proceed as described under 5.3.

5.3 Clean up with thin layer chromatography.

5.3.1 Spotting
Draw with a pencil, according to fig. 2, lines on the plate. Spot at A, B and C 1 ng aflatoxin B₁ (≈ 10 μl of 0.1 μg/ml).
Spot at S 20 μl of six different sample extracts (5.1).

5.3.2 Development of the plate
Develop with diethylether (remark 1) in a saturated tank in the first direction until the solvent front reaches the top of the plate (≈ 45 min). Remove the plate from the tank, evaporate the ether in the dark at room-temperature during 15 min. and cut the plate as indicated in fig. 2 (remark 2).
Develop the plate in an unsaturated tank with a mixture of chloroform: acetone:water = 88:12:0.2 (v/v/v) in the opposite direction of the first development until the front reaches the top of the plate (≈ 30 min.). Remove the plate from the tank and evaporate the solvents at room temperature in the dark.
Examine the plate under UV-light to localize the blue spots of the aflatoxin B₁ standards. Mark these spots by drawing .5 cm above and below the aflatoxin B₁ standard a small line (see fig. 3).
Draw a line with a pencil connecting the three marks above and below the aflatoxin B₁ standard. Cut for every one of the six samples, the parts from the plate. Each part is put into a separate test tube. Add 4 ml of dichloromethane/aceton (6/4 v/v), mix with vortex mixer. Filter the dichloromethane/aceton mixture over glasswool in a second test tube (see remark 3). Rinse the first test tube with resp. 2 and 1 ml dichloromethane/aceton mixture and combine, after filtering, the three portions. Evaporate at 40°C the combined portions under a nitrogen stream and re-dissolve the residue in 50 μl methanol. Use again the Vortex mixer.

5.4 HPLC

5.4.1 Preparation of standard graph
Prepare a standard curve by injecting 20 μl of aflatoxin B₁ solution containing resp.: 0.05; 0.1; 0.2; 0.4; 0.8 and 1.6 ng B₁. Plotting the injected quantities against the area of the obtained aflatoxin B₁ responses should result in a straight line.

5.4.2 Determination
Inject 20 μl of the sample extract (5.3) into the HPLC system. Compare retention times for identification purpose. Calculate the aflatoxin B₁ content from the standard curve.

5.5 Remark
1. Drying the ether with metallic sodium is essential because in the presence of water aflatoxin B₁ will elute in the first direction.
2. After development of the thin layer plate in the first direction, before cutting the plate, the spot of aflatoxin B₁ should be localized.
3. It is of great importance that no pieces of silicagel enters the extract (if so adsorption resulting in a bad recovery will occur). The use of a pipet tip (e.g. Finn tip no. 62) is recommended.
Literature
DETERMINATION OF AFLATOXIN B₁

1. **Purpose and Scope**

   The method makes it possible to determine the level of aflatoxin B₁ in all animal feeding stuffs. The lower limit of determination is 0.4 μg/kg (0.4 ppb).

2. **Principle**

   The sample is extracted with a mixture of chloroform and water. The extract is then purified by using a disposable Sep-Pak/cartidge. Aflatoxin B₁ is determined by HPLC. Confirmation by conversion to aflatoxin B₂a is described.

3. **Reagents**

   NB: All the reagents must be of analytical reagent quality.
   CAUTION: Chloroform is a possible carcinogen.

   3.1 Chloroform
   3.2 Celite 545 filter aid
   3.3 Aflatoxin B₁ standard (5 μg B₁/ml)
   3.4 Toluene
   3.5 Methanol
   3.6 Chloroform - methanol mixture: Mix 97 ml chloroform with 3 ml of methanol.
   3.7 Mobile phase for HPLC - Water-saturated toluene-ethyl acetate - formic acid (85+25+5)
   3.8 Toluene - acetonitrile (98+2)
   3.9 Trifluoroacetic acid

4. **Apparatus**

   4.1 Wrist-action shaker
   4.2 Grinder-mixer
   4.3 Conical flasks, fitted with polyethylene stoppers
4.4 Filter papers GF/A or equivalent, 15 cm diameter
4.5 Rotary evaporator, with a 500 ml round-bottom flask
4.6 Suitable liquid chromatograph
4.7 HPLC columns - stainless steel 250 x 4.6 mm id, packed with 5 μm Partisil 5 or 5 μm Spherisorb ODS
4.8 Sep-Pak silica disposable cartridge

5. **Procedure**

5.1 **Preparation of the sample**
Grind the sample so that the whole of it will pass through a sieve with a 1 mm mesh.

5.2 **Extraction**
Weigh 50 g of finely ground feed into a 500 ml conical flask (4.3) and add 250 ml of chloroform (3.1) followed by 25 ml of distilled water. Stopper the flask and shake (4.1) for 30 minutes. Filter the extract through 10 g Celite 545 filter aid (3.2) in a folded glass fibre filter paper (4.4). Collect two 50 ml portions of filtrate. Add 10 μl of aflatoxin standard (3.3) to one portion. Evaporate both portions to near dryness using the rotary evaporator (4.5)

5.3 **Sep-Pak clean-up**
Add extract to cartridge by using two 0.5 ml portions of toluene (3.4). Wash with 10 ml toluene. Elute aflatoxin B₁ with 10 ml of chloroform-methanol (3.6) (fraction II). Evaporate fraction II to near dryness and transfer to a small vial with chloroform (3.1).

5.4 **High performance liquid chromatography**
Set the fluorescence detector (4.6) to excitation 365 nm (15 nm slit), emission 425 nm (20 nm slit). Stabilise the system at a flow rate of 2.5 ml/min. of mobile phase (3.7). Dilute the aflatoxin standard solution (3.3) with toluene/acetonitrile (3.8) to give a solution containing 0.2 μg B₁/ml. Inject 50 μl and
adjust the sensitivity of the fluorescence detector (4.6) to give approx. 60-80% FSD. Evaporate fraction II to dryness, dissolve in 0.5 ml toluene/acetonitrile (3.8) and inject 50 μl. Compare peak heights obtained from the sample and the spiked extracts to calculate the concentration of aflatoxin B₁ in the sample.

5.5 Confirmation of the identity of aflatoxin B₁
a) Change the fluorescence excitation wavelength to 330 nm and sequentially inject 50 μl of a solution containing 0.2 μg B₁/ml followed by 50 μl of sample extract. Check that the peak height ratio 365/330 nm obtained with the sample extract agrees to within 10% of that obtained with the standard solution.

b) Evaporate the extract to dryness, add 50 μl of trifluoro acetic acid (3.9) and examine again by HPLC. Aflatoxin B₁ is thus converted to aflatoxin B₂a which has a longer retention time than aflatoxin B₁ under the HPLC conditions described.

6. Observation
This method has been adapted from the multimycotoxin screening procedure described by M V Howell and P W Taylor in J. Assoc. Off. Anal. Chem., 1981, 64 No 6 1356.
Bestimmung von Aflatoxin B₁ mit GPC-Reinigung

1. Zweck und Anwendungsbereich
Die Methode erlaubt die Bestimmung von Aflatoxin B₁ in Futtermitteln, die Citrustrester enthalten. Sie ist auch für citrustresterfreie Futtermittel geeignet. Die untere Grenze der Bestimmbarkeit beträgt 3 mg/kg.

2. Prinzip

3. Reagentien
3.1 Chloroform, p.a., phosgen- und säurefrei
3.2 Cyclohexan, p.a.
3.3 Essigsäureethylester, p.a.
3.4 Aceton, p.a.
3.5 Ameisensäure, p.a., 100 %ig
3.6 Toluol, p.a.
3.7 Celite 545
3.8 Mischung aus Cyclohexan (3.2) und Essigsäureethylester (3.3) 1 : 1 (V/V)
3.9 Laufmittel 1 : Mischung aus Chloroform (3.1) und Aceton (3.4) 9 : 1 (V/V)
3.10 Lauflmittel 2 : Mischung aus Toluol (3.6), Essigsäureethylster (3.3) und Ameisensäure (3.5) 5 : 4 : 1 (V/V/V/)

4. Geräte

4.1 Mahlgerät (Mahlfeinheit mindestens 0,5 mm)
4.2 Schüttelmaschine
4.3 Faltenfilter (Macherey-Nagel 614 1/4 oder gleichwertige Qualität)
4.4 Chromatographierohr aus Glas für Gelpermeation, Länge der Gelpermeationsfüllung: ca. 35 cm, innerer Durchmesser ca. 2,5 cm
   Füllung: Biobeads S-X III oder gleichwertiges Material
4.5 Vakuum-Rotationsverdampfer mit 500-ml-Rundkolben
4.6 500-ml-Erlenmeyerkolben oder Rundkolben mit Schliff
4.7 Ausrüstung für Dünnenschichtchromatographie
4.8 Dünnenschichtplatte (Fertigplatten für Dünnenschichtchromatographie 20 x 20 cm, Fa. Baker · Si 250 oder gleichwertiges Material
4.9 UV-Analysenlampe, Bereich 365 nm
4.10 Betrachtungsgehäuse zur Aufnahme der UV-Analysenlampe sowie der DC-Platten
4.11 Fluorodensitometer mit Schreiber bzw. Integrator
4.12 Membranfilter 0,45 μm (regenerierte Zellulose)
4.13 10-ml-Meßkolben

5. Ausführung

5.1 Vorbereitung der Probe
   Die Probe wird gemahlen, so daß sie vollständig durch ein 0,5-mm-Sieb hindurchgeht

5.2 Extraktion
   50,0 g der gemahlenen und homogenisierten Probe werden in einen 500-ml-Erlenmeyerkolben oder Rundkolben (4.6) eingewogen, man fügt 25 g Celite 545 (3.7) hinzu und vermischt mit einem Glasstab;
dann gibt man 25 ml Wasser hinzu, vermischt nochmals mit dem Glasstab und fügt schließlich 250 ml Chloroform (3.1) hinzu. Der Kolben wird verschlossen und 60 Min. mit der Schüttelmaschine (4.2) geschüttelt. Anschließend wird durch ein Faltenfilter (4.3) filtriert. Die ersten 10 ml des Filtrats werden verworfen, der Rest wird aufgefangen.

5.3 Gelpermeationschromatographische Reinigung

Auf dem Markt werden sowohl automatische Geräte als auch Fertigsäulen zur gelpermeationschromatographischen Reinigung angeboten.

Von dem nach 5.2 gewonnenen Extrakt wird ein möglichst großer aliquoter Anteil am Rotationsverdampfer (4.5) zur Trockene eingedampft. Der Trockenrückstand wird mit der Mischung Cyclohexan/Essigsäureethylester (3.8) in Lösung gebracht und quantitativ in einen 10-ml-Meßkolben übergeführt; mit der Mischung (3.8) wird zur Marke aufgefüllt. Man filtriert durch ein Membranfilter (4.12) und gibt von diesem Filtrat 5 ml entweder mittels eines Probengebers bzw. mittels einer Spritze auf die GPC-Säule (4.4). Mit dem Laufmittel Cyclohexan/Essigsäureethylester (3.8) wird bei einer Durchflussgeschwindigkeit von 5 ml/Min. eluiert. Die Fraktion der ersten 32 Min. (ca. 160 ml) werden als Vorlauf verworfen. Während der nächsten 12 Min. (ca. 60 ml) wird eine Fraktion, die das gesamte Aflatoxin B$_1$ enthält, gesammelt. 20 Min. (entsprechend 100 ml) werden für das Nachwaschen angesetzt. Diese aufgeführten Zahlen sind als Richtdaten zu werten. Sie müssen für jede Säule mittels eines Eichstandards überprüft werden.

Die durch Gelpermeationschromatographie gewonnene aflatoxinhaltige Fraktion wird am Rotationsverdampfer (4.5) zur Trockene eingedampft und anschließend mit 2,0 ml Chloroform (3.1) aufgenommen.
5.4 Zweidimensionale Dünnschichtchromatographie
Hier wird genau so verfahren wie in der Amtlichen Methode B unter Punkt 5.4 angegeben (s.a. Methodenbuch III, Kap. 16.1.2), es sind jedoch die Laufmittel

Chloroform/Aceton (3.9) in der ersten Laufrichtung

und

Toluol/Essigsäureethylester/Ameisensäure (3.10)
in der zweiten Laufrichtung
zu benutzen.

Je nach Aflatoxin-Gehalt können bis zu 200 µl des nach 5.3 gewonnenen Extraktes auf die DC-Platte aufgetragen werden.

Die Platte muß sowohl nach der ersten Entwicklung als auch nach der zweiten Entwicklung (vor der Fluoreszenzmessung) völlig lösungsmittelfrei sein (ggf. Vakuumtrockenschrank bei Zimmertemperatur benutzen).

5.5 Quantitative Auswertung der DC-Platten
Die Auswertung erfolgt fluorodensitometrische Messung, wobei Standardflecke mit bekannten Aflatoxin B₁-Mengen auf derselben Platte als Maßgröße zugrunde zu legen sind. Unabhängig hiervon empfiehlt es sich, auf einer anderen Platte der gleichen Charge eine entsprechende Menge an Aflatoxin B₁ als Standard zweidimensional dünnschichtchromatographisch zu untersuchen und die Fluoreszenzintensität des zweidimensional entwickelten Aflatoxin B₁-Fleckes zu messen. Erfahrungsgemäß sind die Unterschiede bei den unter 4.8 genannten DC-Platten gleicher Charge so gering, daß die Fluoreszenzintensität nach zweidimensionaler Chromatographie mit derjenigen nach eindimensionaler Chromatographie übereinstimmt (Lösungsmittelfreiheit der Platten vorausgesetzt).

Determination of Aflatoxin $B_1$ by HPLC.

B.T. Viuf, Statens Foderstofkontrol

1. **Purpose and scope.**

   The method makes it possible to determine aflatoxin $B_1$ in feedingstuffs. The lower limit of determination is 1 $\mu$g/kg.

2. **Principle.**

   Instead of thin layer chromatography add: The amount of aflatoxin is determined by HPLC-chromatography and measurement of fluorescence in a packed flow-cell.

3. **Reagents.**

   Add.

   3.19 HPLC-solvent. Prepare from water-saturated methylenechloride, cyclohexane, acetonitrile (100+30+4) a mixture containing about 0.5% absolute ethanol. The amount of ethanol can be varied to obtain optimum resolution of aflatoxin $B_1$. All reagents must be HPLC-grade.

   3.20 Prepare in chloroform standardsolution containing 0.2, 0.1, and 0.05 $\mu$g/ml aflatoxin $B_1$ according to point 7.

4. **Apparatus**

   4.13 High pressure liquid chromatograph. Apparatus equipped with injektor, normal phase column (Spherisorb S 5 W 5pm or equivalent, 100 x 4.6 mm), fluorescence detector equipped with a flowcell packed with lichrosorb 60 silica gel (30 $\mu$m), emission 365 nm and excitation 425 nm. Use a flow rate 1.0 ml/min.

5. **Procedure**

   Add instead of 5.4 and 5.5: New 5.4

   Separation and detection.

   Prepare a standard curve by injecting 20 $\mu$l of aflatoxin $B_1$ standards containing resp.: 0.2, 0.1, and 0.05 $\mu$g/ml. Inject 20 $\mu$l of the sample extract dissolved in chloroform (5.3). Calculate the aflatoxin $B_1$ content from the standard curve.
RUN 654
AREA %
RT AREA TYPE AR/HT AREA %
8.22 9856100 PB 0.444 150.000

70 654
APR/15/83 14:53:37

8.22 8.36
STOP

RUN 656
AREA %
RT AREA TYPE AR/HT AREA %
8.36 7201900 BB 0.418 92.370
11.61 4429300 BB 0.567 57.122

8.69 8.68
STOP

RUN 97
SEP/16/82 11:48:00

5.20 324820 PV 0.329 1.401
6.64 3770900 YY 0.885 17.191
8.34 1.0475E+07 VB 0.661 47.256
10.92 5822300 BB 0.726 25.543
12.87 1.7824E+07 BB 0.821 13.015
In the first month's of 1984 five samples of cattle feed intended for dairy were five times at random on several days analysed with the HPLC method in order to estimate the reproducibility. The analyses were carried out by a technician on blind samples. In table I the results are given.

<table>
<thead>
<tr>
<th>Mean content of five analysis (HPLC) µg/kg</th>
<th>V.C. (R) %</th>
<th>Range µg/kg</th>
</tr>
</thead>
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<td>93</td>
<td>0,5-3,4</td>
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<tr>
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<td>32</td>
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<tr>
<td>11,1</td>
<td>10</td>
<td>9,6-12,3</td>
</tr>
</tbody>
</table>

At a level of 10 µg/kg a realistic coefficient of variation of less than 20% can be achieved.
### 1st collaborative study

<table>
<thead>
<tr>
<th>lab N°</th>
<th>aflatoxin B₁ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>2,5</td>
</tr>
<tr>
<td>2</td>
<td>2,0</td>
</tr>
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<td>2,4</td>
</tr>
<tr>
<td>4</td>
<td>9,0 x)</td>
</tr>
<tr>
<td>5</td>
<td>2,5</td>
</tr>
<tr>
<td>6</td>
<td>2,9</td>
</tr>
<tr>
<td>7</td>
<td>3,0</td>
</tr>
<tr>
<td>8</td>
<td>24,0 x)</td>
</tr>
<tr>
<td>9</td>
<td>2,0</td>
</tr>
<tr>
<td>10</td>
<td>2,0</td>
</tr>
<tr>
<td>11</td>
<td>1,7</td>
</tr>
</tbody>
</table>

N = 9  
\( \bar{X} = 2,3 \) ppb  
\( s = 0,44 \) ppb  
\( V (S\%) = 18,9 \% \)

### 2nd collaborative study

<table>
<thead>
<tr>
<th>lab N°</th>
<th>aflatoxin B₁ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6,9</td>
</tr>
<tr>
<td>2</td>
<td>7,7</td>
</tr>
<tr>
<td>3</td>
<td>7,5</td>
</tr>
<tr>
<td>4</td>
<td>6,6</td>
</tr>
<tr>
<td>5</td>
<td>6,3</td>
</tr>
</tbody>
</table>

N = 5  
\( \bar{X} = 7,0 \) ppb  
\( s = 0,59 \) ppb  
\( V (S\%) = 8,5 \% \)