STATUTORY INSTRUMENTS

1994 No. 1610

AGRICULTURE

The Feedingstuffs (Sampling and Analysis) (Amendment) Regulations 1994

Made - - - -	*6th June 1994*
Laid before Parliament	*20th June 1994*
Coming into force - -	*11th July 1994*

The Minister of Agriculture, Fisheries and Food, the Secretary of State for Scotland and the Secretary of State for Wales, acting jointly, in exercise of the powers conferred on them by sections 66(1), 79(2) and 84 of the Agriculture Act 1970(**1**), and of all other powers enabling them in that behalf, after consultation as required by section 84(1) of the said Act with such persons or organisations as appear to them to represent the interests concerned, hereby make the following Regulations:

Title, commencement and interpretation

1.—(1) The Regulations may be cited as the Feedingstuffs (Sampling and Analysis) (Amendment) Regulations 1994 and shall come into force on 11th July 1994.

(2) In these Regulations "the principal Regulations" means the Feedingstuffs (Sampling and Analysis) Regulations 1982(**2**).

Amendment of the principal Regulations

2. The principal Regulations are hereby further amended in accordance with regulation 3 below.

3. In Schedule 2 (**METHODS OF ANALYSIS**)—

(a) in paragraph 3 (Methods of Analysis)—

(i) item 16 shall be deleted,

(ii) for item 26a there shall be substituted the following item:

"**26a.** Aflatoxin B_1 — one-dimensional thin layer chromatographic method", and

(**1**) 1970 c. 40. The Act was amended by the Agriculture Act 1970 Amendment Regulations 1982 (S.I.1982/980). Section 66(1) contains definitions of the expressions "the Ministers", "prescribed" and "regulations". The definition of "the Ministers" was amended by the Transfer of Functions (Wales) (No.1) Order 1978 (S.I. 1978/272), Schedule 5, paragraph 1.

(**2**) S.I. 1982/1144, amended by S.I. 1984/52 and S.I. 1985/1119.

(iii) for item 26b there shall be substituted the following item:

"**26b.** Aflatoxin B_1 — high performance liquid chromatographic method";

(b) for the provisions relating to method 4: Protein, there shall be substituted the provisions set out in Schedule 1 to these Regulations;

(c) for the provisions relating to method 9: Fibre, there shall be substituted the provisions set out in Schedule 2 to these Regulations;

(d) the provisions contained in method 16: Copper — Diethyldithiocarbamate spectrophotometric method, shall be deleted;

(e) in method 26a: Aflatoxin B_1 — for certain straight feedingstuffs—

(i) for the heading and paragraph 1 there shall be substituted the following provisions:

"26a. AFLATOXIN B_1 — ONE-DIMENSIONAL THIN LAYER CHROMATOGRAPHIC METHOD

Scope and field of application

1. This method is for the determination of aflatoxin B_1 in raw materials and straight feedingstuffs. This method is not applicable to raw materials and straight feedingstuffs containing citrus pulp. The lower limit of determination is 0.01 mg/kg (10 ppb). In the presence of interfering substances it is necessary to repeat the analysis using method 26b (high performance liquid chromatography).";

(ii) in paragraph 5.0 (Defatting) for the word "before" there shall be substituted the word "after";

(f) for the provisions relating to method 26b: Aflatoxin B_1 — for all other feedingstuffs, there shall be substituted the provisions set out in Schedule 3 to these Regulations.

In witness whereof the Official Seal of the Minister of Agriculture, Fisheries and Food is hereunto affixed on

L.S.

Gillian Shephard
26th May 1994. Minister of Agriculture, Fisheries and Food

Hector Monro
Parliamentary Under Secretary of State, Scottish
6th June 1994 Office

Signed by authority of the Secretary of State for Wales

Wyn Roberts
Minister of State, Welsh Office

31st May 1994

SCHEDULE 1 Regulation 3(b)

PROVISIONS TO BE SUBSTITUTED FOR METHOD 4
IN SCHEDULE 2 TO THE PRINCIPAL REGULATIONS

"4.

PROTEIN

1 Scope and Field of Application

This method is for the determination of the protein content of feedingstuffs on the basis of the nitrogen content, determined according to the Kjeldahl method.

2 Principle

2. The sample is digested by sulfuric acid in the presence of a catalyst. The acidic solution is made alkaline with sodium hydroxide solution. The ammonia is distilled and collected in a measured quantity of sulfuric acid, the excess of which is titrated with a standard solution of sodium hydroxide.

3 Reagents

3

3.1. Potassium sulfate.

3.2. Catalyst: copper (II) oxide CuO or copper (II) sulfate pentahydrate, $CuSO_4$ — $5H_2O$.

3.3. Granulated zinc.

3.4. Sulfuric acid, ρ_{20} = 1.34 g/ml.

3.5. Sulfuric acid c($\frac{1}{2}H_2SO_4$) = 0.5 mol/l.

3.6. Sulfuric acid c($\frac{1}{2}H_2SO_4$) = 0.1 mol/l.

3.7. Methyl red indicator; dissolve 300 mg of methyl red in 100 ml of ethanol, σ = 95-96% (v/v).

3.8. Sodium hydroxide solution (Technical grade may be used) β = 40 g/100 ml (m/v: 40%).

3.9. Sodium hydroxide solution c = 0.25 mol/l.

3.10. Sodium hydroxide solution c = 0.1 mol/l.

3.11. Granulated pumice stone, washed in hydrochloric acid and ignited.

3.12. Acetanilide (m.p. = 114deg;C, N = 10.36%).

3.13. Sucrose (nitrogen free).

4 Apparatus

4. Apparatus suitable for performing digestion, distillation and titration according to the Kjeldahl procedure.

5 Procedure

5

Digestion.

5.1. Weigh 1 g of the sample to the nearest 0.001 g and transfer the sample to the flask of the digestion apparatus. Add 15 g of potassium sulfate (3.1.), an appropriate quantity of catalyst (3.2.) (0.3 to 0.4 g of copper (II) oxide or 0.9 to 1.2 g of copper (II) sulfate pentahydrate), 25 ml of sulfuric acid (3.4.) and a few granules of pumice stone (3.11.) and mix. Heat the flask moderately at first, swirling from time to time if necessary until the mass has carbonized and the foam has disappeared; then heat more intensively until the liquid is boiling steadil y. Heating is adequate if the boiling acid condenses on the wall of the flask. Prevent the sides from becoming overheated and organic particles from sticking to them. When the solution becomes clear and light green continue to boil for another two hours, then leave to cool.

Distillation.

5.2. Add carefully enough water to ensure complete dissolution of the sulfates. Allow to cool and then add a few granules of zinc (3.3.).

Place in the collecting flask of the distillation apparatus an exactly measured quantity of 25 ml of sulfuric acid (3.5.) or (3.6.) depending on the presumed nitrogen content. Add a few drops of methyl red indicator (3.7.).

Connect the digestion flask to the condenser of the distillation apparatus and immerse the end of the condenser in the liquid contained in the collecting flask to a depth of at least 1 cm (see observation 8.3.). Slowly pour 100 ml of sodium hydroxide solution (3.8.) into the digestion flask without loss of ammonia (see observation 8.1.).

Heat the flask until the ammonia has distilled over.

Titration.

5.3. Titrate the excess sulfuric acid in the collecting flask with sodium hydroxide solution (3.9.) or (3.10.) depending on the concentration of the sulfuric acid used, until the end point is reached.

Blank test.

5.4. To confirm that the reagents are free from nitrogen, carry out a blank test (digestion, distillation and titration) using 1 g of sucrose (3.13.) in place of the sample.

6 Calculation of results

6. The content of protein is calculated according to the following formula:

$$\frac{(V_0 - V_1) \times c \times 0.014 \times 100 \times 6.25}{m}$$

Where,

V_0 = Volume (ml) of NaOH (3.9. or 3.10.)

V_1 = Volume (ml) of NaOH (3.9. or 3.10.) used in the sample titration.

c = Concentration (mol/l) of sodiumm hydroxide (3.9 or 3.10).

m = Mass (g) of sample.

7 Verification of the method

7

Repeatability.

7.1. The difference between the results of two parallel determinations carried out on the same sample must not exceed:

0.2% in absolute value, for protein contents of less than 20%;

1.0% relative to the higher value, for protein contents from 20% to 40%;

0.4% in absolute value, for protein contents of more than 40%.

Accuracy.

7.2. Carry out the analysis (digestion, distillation and titration) on 1.5 to 2.0 g of acetanilide (3.12.) in the presence of 1 g of sucrose (3.13.); 1 g acetanilide consumes 14.80 ml of sulfuric acid (3.5.). Recovery must be at least 99%.

8 Observations

8

8.1. Apparatus may be of the manual, semi-automatic or automatic type. If the apparatus requires transference between the digestion and distillation steps, this transfer must be carried out without loss. If the flask of the distillation apparatus is not fitted with a dropping funnel, add the sodium hydroxide immediately before connecting the flask to the condenser, pouring the liquid slowly down the side.

8.2. If the digest solidifies, recommence the determination using a larger amount of sulfuric acid (3.4.) than that specified above.

8.3. For products with a low nitrogen content, the volume of sulfuric acid (3.6.) to be placed in the collecting flask may be reduced, if necessary, to 10 or 15 ml and made up to 25 ml with water.”

SCHEDULE 2 Regulation 3(c)

PROVISIONS TO BE SUBSTITUTED FOR METHOD 9
IN SCHEDULE 2 TO THE PRINCIPAL REGULATIONS

“9.

FIBRE

1 Scope and Field of Application

This method is for the determination of the content in feedingstuffs of fat-free organic substances which are insoluble in acid and alkaline media and are conventionally described as fibre.

2 Principle

2. The sample, defatted where necessary, is treated successively with boiling solutions of sulfuric acid and potassium hydroxide of specified concentrations. The residue is separated by filtration on a sintered-glass filter washed, dried, weighed and ashed within a range of 475 to 500° C. The loss of weight resulting from ashing corresponds to the fibre present in the test sample.

3 Reagents

3

3.1. Sulfuric acid, ($c(½H_2SO_4)$ = 0.13 mol/l).

3.2. Anti-foaming agent (e.g. n—octanol).

3.3. Filter aid (Celite 545 or equivalent), heated at 500° C for four hours (8.6.).

3.4. Acetone.

3.5. Light petroleum, boiling-range 40 to 60° C.

3.6. Hydrochloric acid, (c = 0.5 mol/l).

3.7. Potassium hydroxide solution, (c equals; 0.23 mol/l).

4 Apparatus

4

4.1. Heating unit for digestion with sulfuric acid or potassium hydroxide solution, equipped with a support for the filter crucible (4.2.) and provided with an outlet tube with a tap to the liquid outlet and vacuum, possibly with compressed air. Before use each day preheat the unit with boiling water for five minutes.

4.2. Glass filter crucible with fused sintered-glass filter plate (pore size 40-90µm). Before first use, heat to 500° C for a few minutes and cool (8.6.).

4.3. Cylinder of at least 270 ml with a reflux condenser, suitable for boiling.

4.4. Drying oven with thermostat, to a temperature of 130 ± 5° C.

4.5. Muffle furnace with thermostat, to a temperature in the range 475 to 500° C.

4.6. Extraction unit consisting of a support plate for the filter crucible (4.2.) and with a discharge pipe with a tap to the vacuum and liquid outlet.

4.7. Connecting rings to assemble the heating unit (4.1.), crucible (4.2.) and cylinder (4.3.) and to connect the cold extraction unit (4.6.) and crucible.

5 Procedure

5. Weigh out to the nearest 0.001 g, 1 g of the prepared sample and place in the crucible (4.2.), (see observations 8.1., 8.2. and 8.3.) and add 1 g of filter aid (3.3.).

Assemble the heating unit (4.1.) and the filter crucible (4.2.), then attach the cylinder (4.3.) to the crucible. Pour 150 ml of boiling sulfuric acid (3.1.) into the assembled cylinder and crucible and if necessary add a few drops of anti-foaming agent (3.2.).

Bring the liquid to the boil within 52 minutes and boil vigorously for exactly 30 minutes.

Open the tap to the discharge pipe (4.1.) and, under vacuum, filter the sulfuric acid through the filter crucible and wash the residue with three consecutive 30 ml portions of boiling water, ensuring that the residue is filtered dry after each washing.

Close the outlet tap and pour 150 ml of boiling potassium hydroxide solution (3.7.) into the assembled cylinder and crucible and add a few drops of anti-foaming agent (3.2.). Bring the liquid to boiling point within 5 ± 2 minutes and boil vigorously for exactly 30 minutes. Filter and repeat the washing procedure used for the sulfuric acid step.

After the final washing and drying, disconnect the crucible and its contents and reconnect it to the cold extraction unit (4.6.). Apply the vacuum and wash the residue in the crucible with three consecutive 25 ml portions of acetone (3.4.) ensuring that the residue is filtered dry after each washing.

Dry the crucible to constant weight in the oven at 130° C. After each drying cool in the desiccator and weigh rapidly. Place the crucible in a muffle furnace and ash to constant weight at 475C to 500° C for at least 30 minutes.

After each heating cool first in the furnace and then in the desiccator before weighing.

Carry out a blank test without the sample. Loss of weight resulting from ashing must not exceed 4 mg.

6 Calculation of results

6. The fibre content as a percentage of the sample is given by the expression:

$$\frac{(b - c) \times 100}{a}$$

where

a = mass of sample in g;

b = loss of mass after ashing during the determination, in g;

c = loss of mass after ashing during the blank test, in g.

7 Repeatability

7. The difference between two parallel determinations carried out on the same sample must not exceed:

— 0.3 in absolute value for fibre contents lower than 10%,

— 3% relative to the higher result, for fibre contents equal to or greater than 10%.

8 Observations

8

8.1. Feedingstuffs containing more than 10% fat must be defatted prior to analysis with light -petroleum (3.5.). Connect the filter crucible (4.2.) and its contents to the cold extraction unit (4.6.) and apply vacuum and wash the residue with three consecutive 30 ml portions of light petroleum, ensuring that the residue is dry. Connect the crucible and its contents to the heating unit (4.1.) and continue as described under 5.

8.2. Feedingstuffs containing more than 10% fat after extraction with light petroleum (3.5.) must be defatted as shown in 8.1. and then defatted once more after boiling with acid.

After boiling with acid and the subsequent washing connect the crucible and its contents to the cold extraction unit (4.6.) and wash three times with 30 ml acetone followed by three further

washings with 30 ml portions of light petroleum. Filter under vacuum until dry and continue the analysis as described under 5, beginning with potassium hydroxide treatment.

8.3. If the feedingstuffs contain over 5% of carbonates, expressed as calcium carbonate, connect the crucible (4.2.) with the weighed sample to the heating unit (4.1.). Wash the sample three times with 30 ml hydrochloric acid (3.6.). After each addition let the sample stand for about one minute before filtering. Wash once with 30 ml water and then continue as described under 5.

8.4. If an apparatus in the form of a stand is used (several crucibles attached to the same heating unit) no two individual determinations on the same sample for analysis may be carried out in the same series.

8.5. If after boiling it is difficult to filter the acidic and basic solutions, use compressed air through the discharge pipe of the heating unit and then continue filtering.

8.6. The temperature for ashing should not be higher than 500° C in order to extend the lifetime of the glass filter crucibles. Care must be taken to avoid excessive thermal shock during heating and cooling cycles. Repeat the determination if filtration is not completed within 10 minutes."

SCHEDULE 3

Regulation 3(f)

PROVISIONS TO BE SUBSTITUTED FOR METHOD 26b IN SCHEDULE 2 TO THE PRINCIPAL REGULATIONS

"26b.

AFLATOXIN B_1 — HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

1 Scope and Field of Application

This method is for the determination of aflatoxin B_1 in feedingstuffs whether or not containing citrus pulp. The lower limit of determination is 0.001 mg/kg (1 ppb).

2 Principle

2. The sample is extracted with chloroform. The extract is filtered and an aliquot portion is purified on a Florisil cartridge followed by a C_{18}cartridge. The final separation and determination is achieved by high performance liquid chromatography (HPLC) using a reversed phase C_{18}column, followed by post-column derivatization with iodine in water, and fluorescence detection.

Note:

mycotoxins are extremely toxic sustances. Manipulations should be performed in a designated fume cupboard. 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, with the danger of ingestion. Care should be taken to decontaminate all equipment after use.

3 Reagents

3

3.1. Chloroform, stabilized with 0.5 to 1.0% of ethanol, by mass. See observation 10.2.

3.2. Methanol, HPLC grade.

3.3. Acetone.

3.4. Acetonitrile, HPLC grade.

3.5. Eluting solvents: Prepare one day before use, remove air ultrasonically.

3.5.1. Mixture of acetone (3.3.) and water, 98 + 2 (v + v).

3.5.2. Mixture of water and methanol (3.2.), 80 + 20 (v + v).

3.5.3. Mixture of water and acetone (3.3.), 85 + 15 (v + v).

3.6. Mobile phase for HPLC.

Mixture of water, methanol (3.2.) and acetonitrile (3.4.), 130 + 70 + 40 (v + v + v).

NB The composition of the mobile phase solvent may need to be adjusted, depending on the characteristics of the HPLC column used.

3.7. Saturated iodine solution: add 2 g of iodine to 400 ml of water. Mix for at least 90 min and filter through a membrane filter (4.15.). Protect the saturated solution from light to prevent photodegradation.

3.8. Acid washed Celite 545, or equivalent.

3.9. Florisil cartridge (Waters SEP—PAK), or equivalent.

3.10. C_{18} cartridge (Waters SEP—PAK), or equivalent.

3.11. Inert gas, e.g. nitrogen.

3.12. Aflatoxin B_1 standard solution in chloroform, concentration 10ng/ml. Check the concentration of the solution as follows: determine the absorption spectrum of the solution between 330 and 370 nm by means of the spectrophotometer (4.23.). Measure the absorbance (A) at the maximum, near 363 nm. Calculate the concentration of aflatoxin B_1 in micrograms per millilitre of solution from the formula:

$$\text{Concentration } (\mu g/ml) = \frac{312 \times A \times 1,000}{22,300} = 13.991 \times A.$$

3.12.1. Aflatoxin B_1 stock standard solution in chloroform.

Transfer quantitatively 2.5 ml of the aflatoxin B_1 standard solution (3.12.) to a 50 ml volumetric flask and adjust to the mark with chloroform (3.1.). Store this solution in a cool place (4° C) in the dark, well sealed and wrapped in aluminium foil.

3.13. Aflatoxin B_1 calibration solutions for HPLC.

NB Use acid-washed glassware for preparation of these solutions (see 4, Apparatus).

3.13.1. Calibration solution 4 ng/ml.

Allow the volumetric flask with stock standard solution (3.12.1.) to warm up to room temperature in the aluminium foil (a few hours). Transfer 400µl of the stock standard solution (200 ng aflatoxin B_1) into a 50 ml volumetric flask, and evaporate the solution to dryness in a current of inert gas (3.11.).

Dissolve the residue obtained in approximately 20 ml of water/acetone mixture (3.5.3.), make up to the mark with the water/acetone mixture and mix well.

3.13.2. Calibration solution 3 ng/ml.

Transfer quantitatively 7.5 ml of the calibration solution (3.13.1.) into a 10 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3.), and mix well.

3.13.3. Calibration solution, 2 ng/ml.

Transfer quantitatively 25 ml of the calibration solution (3.13.1.) to a 50 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3.) and mix well.

This solution is also referred to as the reference standard, to be used for repeated injection during HPLC (5.5.).

3.13.4. Calibration solution 1 ng/ml.

Transfer quantitatively 2.5 ml of the calibration solution (3.13.1.) to a 10 ml volumetric flask, make up to the mark with the water/acetone mixture (3.5.3.) and mix well.

3.14. Ampoule containing a mixture of alflatoxins B_1, B_2, G_1 and G_2, concentrations approximately 1, 0.5, 1 and 0.5 µg/ml respectively, in 1 ml chloroform.

3.14.1. Chromatographic test solution.

Transfer the content of the ampoule (3.14.) into a glass-stoppered test-tube or screw-capped vial. Transfer 40 µl of this solution into a glass-stoppered test-tube (acid-rinsed) (4.22.). Evaporate the chloroform in a stream of inert gas (3.11.) and redissolve into 10 ml of the water/acetone mixture (3.5.3.).

3.15. Reagents for confirmatory test (6).

3.15.1. Sodium chloride saturated solution.

3.15.2. Sodium sulfate, anhydrous, granular.

3.16. Light petroleum, boiling range 40 to 60° C.

4 Apparatus

4. *Caution:* Use of non acid-washed glassware for aqueous aflatoxin solutions may cause losses. 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. sulfuric 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 round bottomed flask (4.4.), the volumetric flasks, measuring cylinders, 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.

4.1. Grinder-mixer.

4.2. Sieve of aperture size 1.0 mm, (ISO R 565).

4.3. Mechanical shaker.

4.4. Rotary vacuum evaporator, equipped with a 150 to 250 ml round bottomed flask.

4.5. High performance liquid chromatograph, injector with a loop suitable for the injection of 250 µl. See the manufacturers instructions for partial or complete loop-filling.

4.6. HPLC analytical column: 3 µm or 5 µm C_{18}packing.

4.7. Pulse-free pump for delivery of the iodine post-column reagent.

4.8. Valco zero dead volume Tee, stainless steel (1/16" × 0.75 mm).

4.9. Spiral reaction coil; Teflon or stainless steel. Dimensions of 3,000 × 0.5 mm to 5,000 × 0.5 mm have been found to be appropriate in combination with 5 µm or 3 µm HPLC columns.

4.10. Thermostatically controlled water-bath adjusted to 60° C, capable of temperature regulation to better than 0.1° C.

4.11. 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 aflatoxin B_1 should be possible. Some back pressure may be advisable (e.g. restrictor, Teflon or stainless steel coil connected to the outlet of the detector) to suppress air bubbles in the flow-cell.

4.12. Strip chart recorder.

4.13. Electronic integrator (optional).

4.14. Fluted filter paper, diameter 24 cm, Macherey-Nagel 617 ¼ or equivalent.

4.15. Membrane filter with a pore size of 0.45 µm, Millipore HAWP 04700 or equivalent.

4.16. 500 ml glass-stoppered conical flask.

4.17. Glass column (internal diameter approximately 1 cm, length approximately 30 cm) equipped with a Luer tip.

4.18. Luer chloroform-resistant stopcock (e.g. Bio-rad 7328017, Analytichem A1 6078, J.T. Baker 4514 or equivalent).

4.19. Chemically resistant syringe, 10 ml Luer connector.

4.20. Syringe suitable for HPLC injection of 250µl (see 4.5.).

4.21. 100µl microsyringe for preparation of calibration solutions (check that the accuracy is within 2% by weighing).

4.22. 10 ml glass-stoppered calibrated tubes.

4.23. Spectrophotometer, suitable for making measurements in the UV region of the spectrum.

4.24. Equipment for confirmatory test (6).

4.24.1. Acid-rinsed 100 ml separating funnel with Teflon stopcock.

4.24.2. Heating block, 40 to 50° C.

5 Procedure

5

Defatting.

5.0. Samples containing more than 5% oil or fat must be defatted with light petroleum (3.16.) after the material is ground and sieved (5.1.). In such cases the analytical results may be expressed in terms of the weight of the non-defatted sample.

Preparation of the sample.

5.1. Grind the sample so that it passes through the sieve (4.2.).

Test portion.

5.2. Weigh 50 g of the prepared test sample into the conical flask (4.16.).

Extraction.

5.3 Add 25 g of Celite (3.8.), 250 ml of chloroform (3.1.) and 25 ml of water to the test portion (5.2.). Stopper the flask, and shake for 30 minutes on a mechanical shaker (4.3.). Filter through a fluted filter paper (4.14.). Collect 50 ml of the filtrate. If necessary, take an aliquot portion of the filtrate and dilute to 50 ml with chloroform so that the concentration of aflatoxin B_1 is not greater than 4 ng/ml.

5.4. *Clean-up* (the procedure should be carried out without significant interruptions).

Caution:

— protect the laboratory, where the analyses are done, adequately from daylight. This can be achieved effectively by using:

  (i) UV absorbing foil on the windows in combination with subdued light (no direct sunlight);

  (ii) Curtains or blinds in combination with artificial light (fluorescent tubes are acceptable);

— solutions containing aflatoxin must be protected from light as much as possible (keep in the dark, use aluminium foil).

5.4.1. Florisil SEP—PAK purification.

5.4.1.1. Preparation of the column-cartridge assembly.

Attach a stopcock (4.18.) to the shorter stem of a Florisil cartridge (3.9.) (see Figure 1). Wash the cartridge and remove air by taking 10 ml of chloroform (3.1.) and passing 8 ml via the stopcock rapidly through the cartridge using a syringe (4.19.). Attach the longer stem of the cartridge to a glass column (4.17.) and pass the remaining 2 ml of chloroform through the cartridge into the column. Close the stopcock. Remove the syringe.

5.4.1.2. Purification.

Add the filtrate collected in 5.3. to the column-cartridge assembly and drain by gravity. Rinse with 5 ml of chloroform (3.1.), followed by 20 ml of methanol (3.2.). 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 (3.5.1.) and collect the whole of the eluate in the round bottomed flask of the rotary evaporator (4.4.). Concentrate the eluate on the rotary evaporator at 40° C to 50° C until no more acetone is distilled. (*NB* approximately 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 then no significant amount of acetone remaining. Residues of acetone might lead to losses of aflatoxin B_1 on the C_{18} cartridge). Add 1 ml of methanol (3.2.), 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 the C_{18} purification step.

5.4.2. C_{18} SEP—PAK purification.

5.4.2.1. Preparation of the column-cartridge assembly.~

Attach a stopcock (4.18.) to the shorter stem of a C_{18}-cartridge (3.10.) (see Figure 1). Prime the cartridge and remove any air by passing 10 ml methanol (3.2.) via the stopcock rapidly through the cartridge with a syringe (4.19.). (Air bubbles in the cartridge are visible as light spots on

the otherwise greyish background). Take 10 ml of water, and pass 8 ml 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 (4.17.) and pass the remaining 2 ml of water through the cartridge in the column. Close the stopcock. Remove the syringe.

5.4.2.2. Purification.

Transfer the extract collected in 5.4.1.2. quantitatively to the glass column (4.17.), rinsing the flask twice with 5 ml water/methanol mixture (3.5.2.) and drain by gravity. During these operations, ensure that the column-cartridge assembly does not run dry. (When 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 water/methanol mixture. Discard the eluate. Elute the aflatoxin B_1 with 50 ml water/acetone mixture (3.5.3.), and collect the whole of the eluate in a 50 ml volumetric flask. Make up to the mark with water and mix: the resulting test solution is used for chromatography (5.5.).

Caution: Filtration of the 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.

High performance liquid chromatography.

5.5. (See Figure 2 for setting-up of the equipment). Allow sufficient time for conditioning and stabilizing the instruments.

Note 1:

The flow-rates given for the mobile phase and the post-column reagent are indicative only. They may need to be adjusted depending on the characteristics of the HPLC column.

Note 2:

The detector response to aflatoxin B_1 depends on the temperature, therefore compensation should be made for drift (see Figure 3). By injecting a fixed amount of aflatoxin B_1 reference standard (3.13.3.) 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, 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 the1in the sample extracts are determined directly by reference to the adjacent standards.

5.5.1. HPLC pump settings.

Set the HPLC pump (4.5.) to give a flow of 0.5 or 0.3 ml/min for a 5 μm or a 3 μm HPLC column (4.6.) respectively, using the mobile phase (3.6.).

5.5.2. Post-column pump settings.

Set the pump (4.7.) to give a flow of 0.2 to 0.4 ml/min of the iodine-saturated water solution (3.7.). As a rough guide: Flows of approximately 0.4 or 0.2 ml/min are advisable in combination with flows of 0.5 and 0.3 ml/min of the mobile phase (3.6.) respectively.

5.5.3. Fluorescence detector.

Set the fluorescence detector (4.11.) to exc. = 365 nm and em = 435 nm (filter instrument, > 400 nm). Adjust the detector attenuator to obtain approximately 80% full scale deflection of the recorder pen for 1 ng of aflatoxin B_1.

5.5.4. Injector.

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

5.5.5. Check of chromatographic separation.

Inject the chromatographic test solution (3.14.1.). Valleys should be less than 5% of the sum of peak heights of the adjacent peaks.

5.5.6. Check of the stability of the system.

Before each series of analyses, repeatedly inject the reference standard (3.13.3.), until stable peak areas are achieved (*NB* Peak responses for aflatoxin B_1 between consecutive injections should not differ by more than 6%). Proceed without delay with the check of linearity (5.5.7.).

5.5.7. Check of linearity.

Inject the aflatoxin B_1 calibration solutions (3.13.1. to 3.13.4.). For every third injection use the reference standard (3.13.3.), for correction of drift in response (*NB* Peak responses for this reference standard must not differ by more than 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 twice the standard error of the Y-estimate. Values found must not differ by more than 3% from the nominal values. If these requirements are fulfilled, continue without delay. If not, identify and correct the source of the problem before continuing.

5.5.8. Injection of sample extracts.

Inject the purified sample extracts (5.4.2.2.). After every two sample extracts repeat the injection of the reference standard (3.13.3.) according to the following sequence: reference standard, extract, extract, reference standard, extract, extract, reference standard etc.

6 Confirmatory test

6

Further treatment of the extract (5.4.2.2.).

6.1. Add 5 ml sodium chloride solution (3.15.1.) to the final extract obtained at 5.4.2.2. Extract three times each with 2 ml of chloroform (3.1.) for one minute, in the separate funnel (4.24.1.). Pour the combined chloroform extracts over approximately 1 g sodium sulfate (3.15.2.) into a 10 ml test tube. A small funnel (diameter: 4 cm) can be used with a piece of cottonwool in the constriction, covered with a approximately 1 g sodium sulfate.

Wash the sodium sulfate layer with a few ml of chloroform and collect the washing in the same test tube. Evaporate the chloroform extract to dryness in the same test-tube using the heating block (4.24.2.) and redissolve in 1 ml of chloroform.

Preparation of derivative and thin layer chromatography.

6.2. See Annex to Council Directive 76/372/EEC(**3**) method A, point 5.6.2.

7 Calculation of results

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

(**3**) OJ No. L 102, 15.4.1976, p. 8.

$$\text{aflatoxin } B_1 \text{ content in } \mu g/kg = \frac{m \times V_{ext}}{V_m \times M \times \frac{V_f}{V_c}}$$

where:

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

$$m = \frac{P(sample)}{P(st_1) + P(st_2)} \times 2r(st)$$

P(sample) = peak area of aflatoxin B_1 for the sample

$P(st_1)$ = peak area of aflatoxin B_1 resulting from the preceding injection of reference standard (3.13.3.).

$P(st_2)$ = peak area of aflatoxin B_1 resulting from the following injection of reference standard (3.13.3.).

r(st) = injected amount of aflatoxin B_1 in the reference standard (3.13.3.) in ng.

V_m = volume of the injected sample extract in ml.

V_{cxt} = final volume of the sample extract in ml, allowing for any dilution that was made (5.3.).

M = mass of sample in g.

V_f = volume of filtrate transferred to the Florisil cartridge (5.4.1.2.) in ml.

V_c = volume of chloroform, used for the extraction of the sample in ml.

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

aflatoxin B_1 content in µg/kg = 20 × m.

7.1. Calculations of the results may also be done by peak height measurement.

8 Repeatability

8. see under 10.1.

9 Reproducibility

9. see under 10.1.

10 Observations

10

Precision.

10.1. A collaborative study(**4**), carried out at the international level on mixed feedingstuffs gave the results for repeatability and reproducability indicated in Table 1. The term repeatability (r) used here is defined as te largest ratio which is not significant at the 95% probability level for comparison of two readings of the same sample in the same laboratory under similar conditions. The term reproducability (R) is similarly defined for comparing

(**4**) Egmond, H.P. van, Heisterkamp, S.H. and Paulsch, W.E. (1991). Food Additives and Contaminants 8, 17-29.

two different laboratories. In accordance with ISO 3534 — 1977, 2.35(**5**) and Commission Decision, 89/610/EEC(**6**) r and R are also given in Table 1 in terms of coefficients of variation.

Table 1

Repeatability (r) and reproducibility (R) expressed as ratios and corresponding coefficients of variation (15 laboratories)

Level	r	R	CV_r (1)	CV_R
(µg/kg)			(%)	(%)
8 & 14	1.4	1.7	11	18

(1) CV = coefficient of variation.

Stabilization of chloroform (3.1.).

10.2. The adsorption characteristics of the Florisil cartridge may be changed if stabilizers other than ethanol are used. This should be verified in accordance with 10.3. when the chloroform described is not available.

Accuracy.

10.3. The correct application of the method shall be verified by making replicate measurements on certified reference materials. If these are not available, the performance of the method should be verified by recovery experiments made on the fortified blank samples. The deviation of the mean from the actual value, expressed as a percentage of the actual value, shall not lie outside the limits − 20 to + 10%.

(**5**) ISO 3534-1977.
(**6**) OJ No. L 351, 2.12.1989, p. 39.

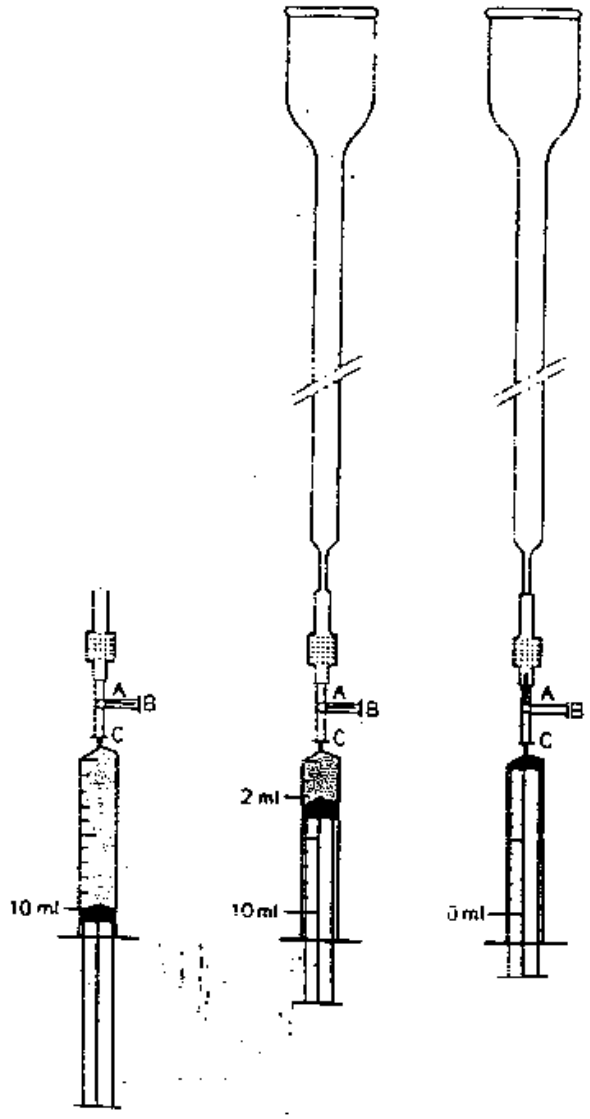
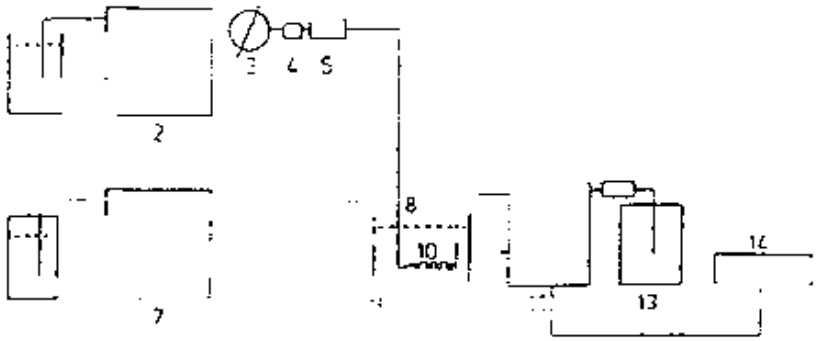


Figure 1: Column-cartridge assembly.



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

Figure 2: Flow diagram of the HPLC system with iodine post-column derivatization

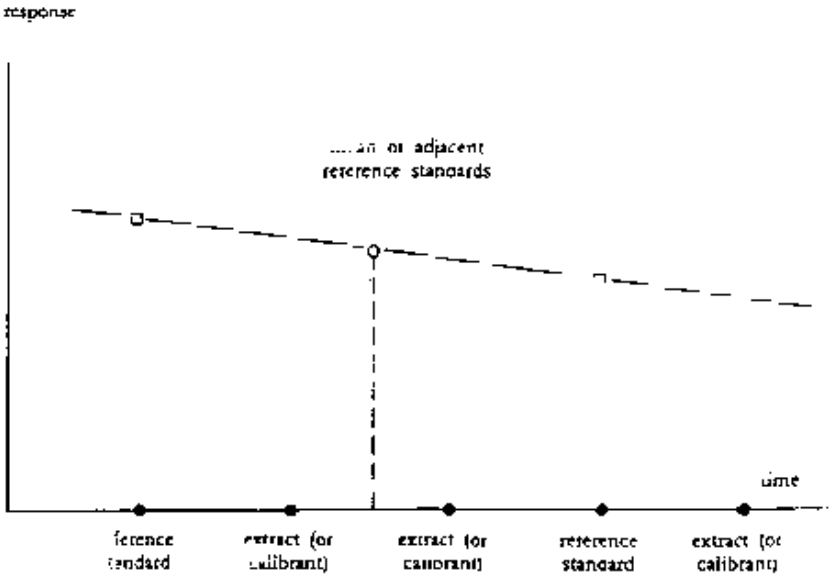


Figure 3: Compensation for drift in aflatoxin B_1 response by injecting reference standard (3.13.3.) at regular intervals"

EXPLANATORY NOTE

(This note is not part of the Regulations)

These Regulations further amend the Feedingstuffs (Sampling and Analysis) Regulations 1982 ("the principal Regulations"). Regulation 3 amends Schedule 2 to the principal Regulations, by modifying a method relating to Aflatoxin B_1, replacing methods relating to Aflatoxin B_1, protein and fibre and by deleting a method relating to Copper—Diethyldithiocarbamate.

These Regulations implement the following Community legislation:

(a) Commission Directive 92/89/EEC (OJNo. L 344, 26.11.92, p. 35), amending Fourth Commission Directive 73/46/EEC (OJ No. L 83, 30.3.73, p. 21) establishing Community methods of analysis for the official control of feedingstuffs;

(b) Commission Directive 92/95/EEC (OJ No. L 327, 13.11.92, p. 54), amending Seventh Commission Directive 76/372/EEC (OJ No. L 102, 15.4.76, p. 8) establishing Community methods of analysis for the official control of feedingstuffs.

(c) Commission Directive 93/28/EEC (OJ No. L 179, 22.7.93, p. 8), amending Third Commission Directive 72/119/EEC (OJ No. L 123, 29.5.72, p. 6) establishing Community methods of analysis for the official control of feedingstuffs;

(d) Commission Directive 94/14/EC (OJ No. L 94, 13.4.94, p. 30), amending Seventh Commission Directive 76/372/EEC (OJ No L 102, 15.4.76, p. 8) establishing Community methods of analysis for the official control of feedingstuffs.

A compliance cost assessment relating to these Regulations has been placed in the libraries of both Houses of Parliament.