II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DIRECTIVE 92/95/EEC

of 9 November 1992

amending the Annex to the Seventh Directive 76/372//EEC establishing Community methods of analysis for the official control of feedingstuffs

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 70/373/EEC of 20 July 1970 on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs (1), as last amended by the Acts of Accession of Spain and Portugal, and in particular Article 2 thereof,

Whereas the Seventh Commission Directive 76/372/EEC of 1 March 1976 establishing Community methods of analysis for the official control of feedingstuffs (2), as amended by Directive 81/680/EEC (3), prescribes the methods to be used for the determination of aflatoxin B_1 ;

Whereas there are grounds for adapting these methods in the light of advances in scientific and technical knowledge ; whereas it is advisable particularly to have available a method of controlling the very low limits for aflatoxin fixed by Council Directive 74/63/EEC of 17 December 1973 on the fixing of maximum permitted levels for undesirable substances and products in animal nutrition (4), as last amended by Directive 91/132/EEC (5) ;

Whereas it is also advisable to have available a method of determining aflatoxin B_1 in the presence of interfering substances, such as citrus pulp ;

Whereas the measures provided for in this Directive are in accordance with the opinion of the Standing Committee for Feedingstuffs,

HAS ADOPTED THIS DIRECTIVE :

Article 1

The Annex to Directive 76/372/EEC is amended in accordance with the Annex to this Directive.

Article 2

Member States shall bring into force not later than 1 October 1993 the laws, regulations and administrative provisions necessary to comply with the provisions of this Directive. They shall fortwith inform the Commission thereof.

When Member States adopt these provisions, they shall contain a reference to this Directive or shall be accompanied by such reference at the time of their official publication. The procedure for such reference shall be adopted by the Member States.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 9 November 1992.

For the Commission

Ray MAC SHARRY

Member of the Commission

(1) OJ No L 170, 3. 8. 1970, p. 2.
(2) OJ No L 102, 15. 4. 1976, p. 8.
(3) OJ No L 246, 29. 8. 1981, p. 32.
(4) OJ No L 38, 11. 2. 1974, p. 31.
(5) OJ No L 66, 13. 3. 1991, p. 16.

ANNEX

I. Under Part A 'One-dimensional thin layer chromatographic method' the text at point 1 'Purpose and scope' is replaced by the following text:

'1. **Purpose and scope**

The method makes it possible to determine the level of aflatoxin B_1 in raw materials and straight feedingstuffs. This method can not be applied in the presence of 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 B (high performance liquid chromatography).'

II. Under Part B 'Two-dimensional thin layer chromatographic method' the text is replaced by the following:

'B. DETERMINATION OF AFLATOXIN B_1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

1. **Purpose and scope**

This method is for the determination of aflatoxin B_1 in animal feedingstuffs including those containing citrus pulp. The lower limit of determination is 0,001 mg/kg (1 ppb).

2. **Principle**

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.

3. **Reagents**

3.1. Chloroform, stabilized with 0,5 to 1,0 % of ethanol, by mass. See observation 10.2.

3.2. Methanol, HPLC grade for preatation of 3.6.

3.3. Acetone.

3.4. Acetonitril, HPLC grade.

3.5. Eluting solvents: Prepare one day before use, or remove air in the solvents 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 acetonitril (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 10 µg/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:

$$\text{Concentration } (\mu g/ml) = \frac{312 \times A \times 1000}{22\,300} = 13{,}991 \times A$$

3.12.1. Aflatoxine 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 calibraltion solutions HPLC.

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

3.13.1. Calibration solution 4ng/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 400 µl 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 inet 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 repetitive 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 aflatoxins 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 sulphate, anhydrous, granular.

4. Apparatus

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 ml. 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 3µm 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 wavelenths. (For filter instrument : emission wavelength > 400 nm). Detection of at least 0,05 ng aflatoxin B_1 shall 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 1/4 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 250 µl (see 4.5).

4.21. 100 µl 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

5.1. *Preparation of the sample.*

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

5.2. *Test portion.*

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

5.3. *Extraction*

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 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 :