S.I. No. 370/1993 - European Communities (Feedingstuffs) (Methods of Analysis) (Amendment) Regulations, 1993.

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EUROPEAN COMMUNITIES (FEEDINGSTUFFS) (METHODS OF ANALYSIS) (AMENDMENT) REGULATIONS, 1993.

I, JOE WALSH, Minister for Agriculture, Food and Forestry, in exercise of the powers conferred on me by Section 3 of the European Communities Act, 1972 (No. 27 of 1972), and for the purposes of giving effect to Commission Directive 92/95/EEC of 9 November, 1992[(1)], hereby make the following Regulations:

1. (1) These Regulations may be cited as the European Communities (Feedingstuffs) (Methods of Analysis) (Amendment) Regulations, 1993.

(2) The European Communities (Feedingstuffs) (Methods of Analysis) Regulations 1978 to 1985, and these Regulations may be cited together as the European Communities (Feedingstuffs) (Methods of Analysis) Regulations, 1978 to 1993.

2. The European Communities (Feedingstuffs) (Methods of Analysis) Regulations, 1978 (S.I. No. 250 of 1978) is hereby amended by the substitution of the following paragraph for paragraph 23 of Part II:

"23 DETERMINATION OF AFLATOXIN B_1

(A) ONE DIMENSIONAL THIN LAYER CHROMATOGRAPHIC METHOD

1. *Purpose and Scope*

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. In the presence of interfering substances it is necessary to repeat the analysis using method B (High Performance Liquid Chromatography). The lower limit of determination is 0.01 mg/kg (10 ppb).

2. *Principle*

The sample is subjected to extraction with chloroform. The extract is filtered, and an aliquot portion taken and purified by column chromatography on silica gel. The eluate is evaporated and the residue redissolved in a specific volume of chloroform or of a mixture of benzene and acetonitrile. An aliquot portion of this solution is subjected to thin-layer chromatography (TLC). The quantity of aflatoxin B_1 is determined under UV irradiation of the chromatogram, either visually or by flourodensitometry, by comparison with known quantities of standard aflatoxin B_1. The identity of the aflatoxin B_1 extracted from the feedingstuff must be confirmed by the procedure indicated.

[1]O.J. No. L327 13-11-1992. p.54.

3. *Reagents*

3.1 Acetone.

3.2 Chloroform, stabilized with 0.5 — 1.0% of 96% ethanol (v/v).

3.3 N-hexane.

3.4 Methanol.

3.5 Anhydrous diethyl ether, free from peroxides.

3.6 Mixture of benzene and acetonitrile: 98/2 (v/v).

3.7 Mixture of chloroform (3.2) and methanol (3.4): 97/3 (v/v).

3.8 Silica gel, for column chromatography, particle size 0.05—0.20 mm.

3.9 Absorbent cotton wool, previously defatted with chloroform, or glass wool.

3.10 Sodium sulphate, anydrous, granular.

3.11 Inert gas, e.g. nitrogen.

3.12 Hydrochloric acid solution, 1 N.

3.13 Sulphuric acid solution, 50% (v/v).

3.14 Kieselguhr (hyflosupercel), washed in acid.

3.15 Silica gel G-HR or equivalent, for TLC.

3.16 Standard solution with about 0.1 µg of aflatoxin B_1 per ml in chloroform (3.2) or the benzene/acetonitrile mixture (3.6), prepared and checked as indicated in Section 7.

3.17 Standard solution for qualitative testing purposes containing about 0.1 µg of aflatoxin B_1 and B_2 per ml in chloroform (3.2) or the benzene/acetonitrile mixture (3.6). These concentrations are given as a guide. They must be adjusted so as to obtain the same intensity of fluorescence for both aflatoxins.

3.18 Developing solvents:

3.18.1 Chloroform (3.2)/acetone (3.1): 9/1 (v/v), unsaturated tank;

3.18.2 Diethyl ether (3.5)/methanol (3.4)/water: 96/3/1 (v/v/v), unsaturated tank;

3.18.3 Diethyl ether (3.5)/methanol (3.4)/water: 94/4.5/1.5 (v/v/v), saturated tank;

3.18.4 Chloroform (3.2)/methanol (3.4): 94/6 (v/v), saturated tank;

3.18.5 Chloroform (3.2)/methanol (3.4): 97/3 (v/v), saturated tank.

4. *Apparatus*

4.1 Grinder-mixer.

4.2 Shaking apparatus or magnetic stirrer.

4.3 Fluted filter papers, Schleicher and Schull No. 588 or equivalent, diameter: 24 cm.

4.4 Glass tube for chromatography (internal diameter: 22 mm approximately, length: 300 mm approximately), with a PTFE cock and a suitable reservoir.

4.5 Rotary vacuum evaporator, with a 500 ml round-bottom flask.

4.6 500 ml conical flasks, with ground-glass stoppers.

4.7 TLC apparatus.

4.8 Glass plates for TLC, 200 x 200 mm, prepared as follows (the quantities indicated are sufficient to cover five plates). Put 30 g of silica gel G-HR (3.15) into a conical flask. Add 60 ml water, stopper and shake for a minute. Spread the suspension on the plates so as to obtain a uniform layer 0.25 mm thick. Leave to dry in the air and then store in a desiccator containing silica gel. At the time of use, activate the plates by keeping them in the oven at 110°C for 1 hour.

Ready-to-use plates are suitable if they give results similar to those obtained with the plates prepared as indicated above.

4.9 Long-wavelength (360 nm) UV lamp. The intensity of irradiation must make it possible for a spot of 1 ng of aflatoxin B_1 to be still clearly distinguished on a TLC plate at a distance of 10 cm from the lamp.

4.10 10 ml graduated tubes with polyethylene stoppers.

4.11 UV spectrophotometer.

4.12 Fluorodensitometer (optional).

5. *Procedure*

5.1 *Preparation of the sample (see under "Observations", Point 10)*

Grind the sample so that the whole of it will pass through a sieve with a 1 mm mesh (in accordance with recommendation ISO R 565).

5.2 *Extraction*

Put 50 g of ground, mixed sample into a conical flask (4.6). Add 25 g of Kieselguhr (3.14), 25 ml of water and 250 ml of chloroform (3.2). Stopper the flask, shake or stir for 30 minutes with the apparatus (4.2) and filter through a fluted filter paper (4.3). Discard the first 10 ml of the filtrate and then collect 50 ml.

5.3 *Column clean up*

Insert into the lower end of a chromatography tube (4.4) a cotton or glass wool plug (3.9), fill two-thirds of the tube with chloroform (3.2) and add 5 g of sodium sulphate (3.10).

Check that the upper surface of the sodium sulphate layer is flat, then add 10 g of silica gel (3.8) in small portions. Stir carefully after each addition to eliminate air bubbles. Leave to stand for 15 minutes and then carefully add 15 g of sodium sulphate (3.10). Let the liquid fall until it is just above the upper surface of the sodium sulphate layer.

Mix the 50 ml of extract collected in 5.2 with 100 ml of n-hexane (3.3) and quantitatively transfer the mixture to the column. Let the liquid fall until it is just above the upper surface of the sodium sulphate layer. Discard this washing. Then add 100 ml of diethylether (3.5) and again allow it to fall to the upper surface of the sodium sulphate layer. Discard this washing. Then add 100 ml of diethylether (3.5) and again allow it to fall to the upper surface of the sodium sulphate layer. During these operations see that the rate of flow is 8—12 ml per minute and that the column does not run dry. Discard the liquid that comes out. Then elute with 150 ml of the chloroform/methanol mixture (3.7) and collect the whole of the eluate. Evaporate the latter almost to dryness at a temperature not exceeding 50°C under a stream of inert gas (3.11) with the rotary evaporator (4.5). Quantitatively transfer the residue, using chloroform (3.2) or the benzeneacetonitrile mixture (3.6), to a 10 ml graduated tube (4.10). Concentrate the solution under a stream of inert gas (3.11) and then adjust the volume to 2 ml with chloroform (3.2) or the benzene/acetonitrile mixture (3.6).

5.4 *Thin-layer chromatography*