NINTH COMMISSION DIRECTIVE

of 31 July 1981

establishing Community methods of analysis for the official control of feedingstuffs

(81/715/EEC)

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 Act of Accession of Greece, and in particular Article 2 thereof,

Whereas that Directive requires that official control of feedingstuffs for the purpose of checking compliance with requirements under the provisions laid down by law, regulation or administrative action concerning the quality and composition of feedingstuffs be carried out using Community methods of sampling and analysis;

Whereas Commission Directives 71/250/EEC (2), 71/393/EEC (3), 72/199/EEC (4), 73/46/EEC (5), 74/203/EEC (6), 75/84/EEC (7), 76/372/EEC (8) and 78/633/EEC (9), as last amended by the Directive of 30 July 1981, have already established a number of Community methods of analysis; whereas the progress of work since then makes it advisable to adopt a ninth set of methods;

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

Member States shall require that analyses for official controls of feedingstuffs, as regards their content of avoparcin and monensin sodium, be carried out in accordance with the methods described in the Annex.

Article 2

Member States shall bring into force the laws, regulations or administrative provisions necessary to comply with this Directive on 1 December 1981 and shall forthwith inform the Community thereof.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 31 July 1981.

For the Commission
The President
Gaston THORN

(1) OJ No L 170, 3. 8. 1970, p. 2.
(2) OJ No L 155, 12. 7. 1971, p. 13.
(3) OJ No L 279, 20. 12. 1971, p. 7.
(4) OJ No L 123, 29. 5. 1972, p. 6.
(5) OJ No L 83, 30. 3. 1973, p. 21.
(6) OJ No L 108, 22. 4. 1974, p. 7.
(7) OJ No L 32, 5. 2. 1975, p. 26.
(8) OJ No L 102, 15. 4. 1976, p. 8.
(9) OJ No L 206, 29. 7. 1978, p. 43.

ANNEX

1. DETERMINATION OF AVOPARCIN BY DIFFUSION IN AN AGAR MEDIUM

1. PURPOSE AND SCOPE

The method is for the determination of avoparcin in feedingstuffs and premixes. The lower limit of determination is 2 mg/kg (2 ppm). The presence of polyether antibiotics may interfere in the determination.

2. PRINCIPLE

The sample is extracted with a mixture of acetone/water/hydrochloric acid. The antibiotic activity of the extract is determined by measuring the diffusion of avoparcin in an agar medium inoculated with Bacillus subtilis. Diffusion is shown by the formation of zones of inhibition of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration over the range of antibiotic concentrations employed.

3. MICRO-ORGANISM: BACILLUS SUBTILIS ATCC 6633 (NCIB 8054)

3.1. **Maintenance of stock culture**

Inoculate tubes containing slopes of culture medium (4.1) with Bacillus subtilis and incubate overnight at 30 °C. Store the culture in a refrigerator at about 4 °C. Reinoculate every month.

3.2. **Preparation of the spore suspension** [1]

Harvest the growth from a recently prepared agar slope (3.1) by means of 2 to 3 ml of sterile water. Use this suspension to inoculate 300 ml of culture medium (4.1) contained in a Roux flask and incubate for three to five days at 30 °C. Harvest the growth in 15 ml of ethanol (4.2) after having checked sporulation under the microscope, and mix well. This suspension may be kept for at least five months at about 4 °C.

4. CULTURE MEDIA AND REAGENTS

4.1. **Culture medium** [2]

Peptone	6·0 g
Tryptone	4·0 g
Yeast extract	3·0 g
Meat extract	1·5 g
Glucose	1·0 g
Agar	15·0 g
Water	1 000 ml

pH 6·5 (after sterilization).

4.2. Ethanol 20 % (v/v): dilute 200 ml of ethanol with 800 ml of water.

4.3. Hydrochloric acid, d: 1·18 to 1·19.

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(1) Other methods may be used provided that it has been established that they give similar spore suspensions.

(2) Any commercial culture medium of similar composition and giving the same results may be used.

4.4. Sodium hydroxide, 2 M solution.

4.5. Phosphate buffer, 0·1 M:

Potassium dihydrogen phosphate, KH_2PO_4:13·6 g.

Water to 1 000 ml.

Adjust pH to 4·5.

4.6. Mixture of acetone/water/hydrochloric acid (4.3): 65/32·5/2·5 (v/v/v).

4.7. Standard substance: avoparcin sulphate of known activity.

5. STANDARD SOLUTIONS

Dissolve an accurately weighed quantity of approximately 10 mg of the standard substance (4.7) in phosphate buffer (4.5) and dilute with this buffer to give a stock solution containing 100 µg avoparcin per millilitre. Stored in a stoppered flask at 4 °C, this solution is stable for up to seven days.

5.1. **For premixes**

From this stock solution prepare by successive dilution with buffer (4.5) the following solutions:

S_8 4·0 µg/ml

S_4 2·0 µg/ml

S_2 1·0 µg/ml

S_1 0·5 µg/ml

5.2: **For feedingstuffs**

From the stock solution prepare by successive dilution with buffer (4.5) the following solutions:

S_8 2·0 µg/ml

S_4 1·0 µg/ml

S_2 0·5 µg/ml

S_1 0·25 µg/ml

6. PREPARATION OF THE EXTRACT AND ASSAY SOLUTIONS

6.1. **Premixes**

Weigh, to the nearest 10 mg, sufficient sample to contain 10 to 100 mg avoparcin. Transfer to a 100 ml graduated flask with 60 ml of the mixture (4.6) and shake for 15 minutes on a mechanical shaker. Check the pH and adjust to pH 2, if necessary, with hydrochloric acid (4.3). Make up to volume with the mixture (4.6) and mix well. Filter a portion through suitable filter paper (e.g. Whatman No 1), discarding the first 5 ml of the filtrate. Take an aliquot and adjust the pH to 4·5 with sodium hydroxide solution (4.4). Dilute this solution with buffer (4.5) to obtain an expected avoparcin concentration of 4 µg/ml (= U_8).

From this solution prepare solutions U_4 (expected content: 2 µg/ml), U_2 (expected content: 1 µg/ml) and U_1 (expected content: 0·5 µg/ml) by means of successive dilution (1 + 1) with buffer (4.5).

6.2. **Feedingstuffs**

Weigh out a quantity of sample of 50 g and shake 100 ml of mixture (4.6) for 30 minutes on a mechanical shaker. Clarify the extract by centrifugation (using stoppered centrifuge tubes), take an aliquot of the clarified extract (see table below) and adjust the pH to 4·5 with sodium hydroxide solution (4.4). Dilute this aliquot with buffer (4.5) to provide U_8 (see table below).

From this solution prepare solutions U_4 (expected content: 1 µg/ml), U_2 (expected content: 0·5 µg/ml) and U_1 (expected content: 0·25 µg/ml) by means of successive dilution (1 + 1) with buffer (4.5).

Presumed level of avoparcin (mg/kg)	5	7·5	10	15	20	40
Weight of sample (g (± 0·1 g))	50	50	50	50	50	50
Volume of mixture (4.6) (ml)	100	100	100	100	100	100
Volume of clarified extract (ml)	20	15	20	15	20	10
Final volume (ml): U_8	25	25	50	50	100	100
Expected U_8 concentration (µg/ml)	2	approx. 2	2	approx. 2	2	2

7. ASSAY PROCEDURE

7.1. **Inoculation of the assay medium**

Inoculate the assay medium (4.1) with the spore suspension (3.2) at 50 to 60 °C. By preliminary trials on plates with assay medium (4.1) determine the quantity of spore suspension required to give the largest and clearest zones of inhibition with the various concentrations of avoparcin.

7.2. **Preparation of the plates**

Diffusion through agar is carried out in plates with the four concentrations of the standard solution (S_8, S_4, S_2, S_1) and the four concentrations of the assay solution (U_8, U_4, U_2, U_1). These four concentrations of extract and standard must necessarily be placed in each plate. To this effect, select plates big enough to allow at least eight holes with a diameter of 10 to 13 mm and not less than 30 mm between centres to be made in the agar medium. The test may be carried out on plates consisting of a sheet of glass with a faced aluminium or plastic ring placed on top, 200 mm in diameter and 20 mm high.

Pour into the plates a quantity of the medium (4.1), inoculated as in 7.1, to give a layer about 2 mm thick (60 ml for a plate of 200 mm diameter). Allow to set in a level position, bore the holes and place in them exactly measured volumes of assay and standard solutions (between 0·10 and 0·15 ml per hole, according to the diameter). Apply each concentration at least four times so that each determination is subject to an evaluation of 32 zones of inhibition.

7.3. **Incubation**

Incubate the plates for 16 to 18 hours at 30 °C.

8. EVALUATION

Measure the diameter of the zones of inhibition to the nearest 0·1 mm. Record the mean measurements for each concentration on semi-logarithmic graph paper showing the logarithm of the concentrations in relation to the diameters of the zones of inhibition. Plot the 'best fit' lines of both the standard solution and the extract, for example as below.

Determine the 'best fit' point for the standard highest level (SL) using the formula:

$$(a)\ SL = \frac{7\,S_1 + 4\,S_2 + S_4 - 2\,S_8}{10}$$