COMMISSION

TENTH COMMISSION DIRECTIVE

of 25 July 1984

establishing Community methods of analysis for the official control of feedingstuffs

(84/425/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], 73/46/EEC [3], 74/203/EEC [4], 75/84/EEC [5], 76/372/EEC [6], as last amended by Directive 81/680/EEC [7], Directives 71/393/EEC [8], 72/199/EEC [9], 78/633/EEC [10], as last amended by Directive 84/4/EEC [11], and Directive 81/715/EEC [12] have already established a number of Community methods of analysis ; whereas the progress of work since then makes it advisable to adopt a new method ;

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 spiramycin be carried out in accordance with the method described in the Annex.

Article 2

Member States shall bring into force the laws, regulations or administrative provisions necessary to comply with this Directive by 30 June 1985 at the latest and shall forthwith inform the Community thereof.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 25 July 1984.

For the Commission

Poul DALSAGER

Member of the Commission

(1) OJ No L 170, 3. 8. 1970, p. 2.
(2) OJ No L 155, 12. 7. 1971, p. 13.
(3) OJ No L 83, 30. 3. 1973, p. 21.
(4) OJ No L 108, 22. 4. 1974, p. 7.
(5) OJ No L 32, 5. 2. 1975, p. 26.
(6) OJ No L 102, 15. 4. 1976, p. 8.
(7) OJ No L 246, 29. 8. 1981, p. 32.
(8) OJ No L 279, 20. 12. 1971, p. 7.
(9) OJ No L 123, 29. 5. 1972, p. 6.
(10) OJ No L 206, 29. 7. 1978, p. 43.
(11) OJ No L 15, 18. 1. 1984, p. 28.
(12) OJ No L 257, 10. 9. 1981, p. 38.

ANNEX

DETERMINATION OF SPIRAMYCIN BY DIFFUSION IN AN AGAR MEDIUM

1. Purpose and scope

The method is for the determination of spiramycin in feedingstuffs and premixes. The lower limit of determination is 1 mg/kg (1 ppm) (1)

2. Principle

The sample is extracted with a mixture of methanol/phosphate-bicarbonate buffer at pH 8. The extract is decanted or centrifuged and diluted. Its antibiotic activity is determined by measuring the diffusion of spiramycin in an agar medium inoculated with *Micrococcus luteus*. 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 : *Micrococcus luteus* ATCC 9341 (NCTC 8340, NCIB 8553)

3.1. *Maintenance of stock culture*

Inoculate tubes containing slopes of culture medium (4.1) with *Micrococcus luteus* and incubate for 24 hours at 30 °C. Store the culture in a refrigerator at about 4 °C. Reinoculate every two weeks.

3.2. *Preparation of the bacterial suspension* (a)

Harvest the growth from a recently prepared agar slope (3.1) with 2 to 3 ml of sodium chloride solution (4.3). Use this suspension to inoculate 250 ml of culture medium (4.1) contained in a Roux flask and incubate for 18 to 20 hours at 30 °C. Harvest the growth in 25 ml of sodium chloride solution (4.3) and mix. Dilute the suspension to 1/10 with sodium chloride solution (4.3). The light transmission of the suspension must be about 75 %, measured at 650 nm in a 1 cm cell against sodium chloride solution (4.3). This suspension may be kept for one week at about 4 °C.

4. Culture media and reagents

4.1. *Culture medium* (b)

Meat peptone	6,0 g
Tryptone	4,0 g
Yeast extract	3,0 g
Meat extract	1,5 g
Glucose	1,0 g
Agar	10,0 to 20,0 g
Water	1 000 ml

pH 6,5 to 6,6 (after sterilization).

4.2. *Assay medium* (b)

Tryptone	5,0 g
Yeast extract	4,0 g
Meat extract	3,0 g
Agar	10,0 to 20,0 g
Water	1 000 ml

pH 8,0 (after sterilization).

(1) 1 mg spiramycin base is equivalent to 3 200 international units (IU).
(a) Other methods may be used provided that it has been established that they give similar bacterial suspensions.
(b) Any commercial culture medium of similar composition and giving the same results may be used.

4.3. *Sodium chloride solution 0,8 % (w/v)*

Dissolve 8 g of sodium chloride in water and dilute to 1 000 ml ; sterilize.

4.4. *Phosphate-bicarbonate buffer, pH 8,0*

Dipotassium hydrogen phosphate K_2HPO_4	16,7 g
Potassium dihydrogen phosphate KH_2PO_4	0,5 g
Sodium hydrogen carbonate $NaHCO_3$	20,0 g
Water to	1 000 ml

4.5. *Mixture of methanol phosphate-bicarbonate buffer* (4.4)

50/50 (v/v).

4.6 *Standard substance*

Spiramycin of known activity (in IU).

5. Standard solutions

Dissolve an accurately weighed quantity of the standard substance (4.6) in the mixture (4.5) and dilute with the same mixture to give a stock solution containing 1 000 IU spiramycin per millilitre. Stored in a stoppered flask at 4 °C this solution is stable for up to five days.

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

S_8	1	IU/ml
S_4	0,5	IU/ml
S_2	0,25	IU/ml
S_1	0,125	IU/ml

6. Preparation of the extract and assay solutions

6.1. *Extraction*

Weigh out a quantity of sample of 20,0 g in the case of feedingstuffs, of 1,0 to 20,0 g for premixes. Add 100 ml of the mixture (4.5) and shake for 30 minutes. Centrifuge or decant and dilute the supernatant solution with the mixture (4.5) to obtain an expected spiramycin in content of 1 IU/ml (= U_8).

For expected levels of spiramycin lower than 2,5 mg/kg of feedingstuff the extraction must be carried out as follows. Weigh out a quantity of sample of 20,0 g. Add 100 ml of the mixture (4.5) and shake for 30 minutes. Centrifuge for a few minutes, take 50 ml of the supernatant solution and evaporate to about 4 ml under reduced pressure in a rotary evaporator at a temperature not exceeding 40 °C. Dilute the residue with the mixture (4.5) to obtain an expected spiramycin content of 1 IU/ml (= U_8).

6.2. *Assay solutions*

From solution U_8 prepare solutions U_4 (expected content : 0,5 IU/ml), U_2 (expected content : 0,25 IU/ml) and U_1 (expected content : 0,125 IU/ml) by means of successive dilution (1 + 1) with the mixture (4.5).

7. Assay procedure

7.1. *Inoculation of the assay medium*

Inoculate the assay medium (4.2) with the bacterial suspension (3.2) at about 50 °C . By preliminary trials on plates with assay medium (4.2) determine the quantity of bacterial suspension required to give the largest and clearest zones of inhibition with the various concentrations of spiramycin.

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 (4.2), 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 evalution of 32 zones of inhibition.

7.3. *Incubation*

Incubate the plates for 16 to 18 hours at 30 ± 2 °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 lowest level (SL) using the formula:

$$\text{(a) } SL = \frac{7\,s_1 + 4\,s_2 + s_4 - 2\,s_8}{10}$$

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

$$\text{(b) } SH = \frac{7\,s_8 + 4\,s_4 + s_2 - 2\,s_1}{10}$$

Similarly calculate the 'best fit' points for the extract lowest level (UL) and the extract highest level (UH) by substituting u_1, u_2, u_4 and u_8 for s_1, s_2, s_4 and s_8 in the above formulae (1).

Record the calculated SL and SH values on the same graph paper and join them to give the 'best fit' line for the standard solution. Similarly record UL and UH and join them to give the 'best fit' line for the extract.

In the absence of any interference the lines should be parallel. For practical purposes the lines can be considered parallel if the values (SH — SL) and (UH — UL) do not differ by more than 10 % from their mean value.

If the lines are found to be non-parallel either u_1 and s_1 or u_8 and s_8 may be discarded and SL, SH, UL and UH calculated, using the alternative formulae, to give alternative 'best fit' lines:

$$\text{(a') } SL = \frac{5\,s_1 + 2\,s_2 - s_4}{6} \quad \text{or} \quad \frac{5\,s_2 + 2\,s_4 - s_8}{6}$$

$$\text{(b') } SH = \frac{5\,s_4 + 2\,s_2 - s_1}{6} \quad \text{or} \quad \frac{5\,s_8 + 2\,s_4 - s_2}{6}$$

and similarly for UL and UH. The same criteria of parallelism should be satisfied. The fact that the result has been calculated from three levels should be noted on the final report.

(1) The small letters 's' and 'u' refer to the diameters of the zones of inhibition.

When the lines are considered as being parallel, calculate the logarithm of the relative activity (log A) by means of one of the following formulae, depending upon whether three or four levels have been used for the assessment of parallelism.

For four levels

$$\text{(c) Log A} = \frac{(u_1 + u_2 + u_4 + u_8 - s_1 - s_2 - s_4 - s_8) \times 0{,}602}{u_4 + u_8 + s_4 + s_8 - u_1 - u_2 - s_1 - s_2}$$

For three levels

$$\text{(d) Log A} = \frac{(u_1 + u_2 + u_4 - s_1 - s_2 - s_4) \times 0{,}401}{u_4 + s_4 - u_1 - s_1}$$

or

$$\text{(d') Log A} = \frac{(u_2 + u_4 + u_8 - s_2 - s_4 - s_8) \times 0{,}401}{u_8 + s_8 - u_2 - s_2}$$

Activity of sample extract = activity of relevant standard × A

$$(U_8 = S_8 \times A)$$

If the relative activity is found to be outside the range of 0,5 to 2,0, then repeat the assay making appropriate adjustments to the extract concentrations or, if this is not possible, to the standard solutions. When the relative activity cannot be brought into the required range, any result obtained must be considered as approximate and this should be noted on the final report.

When the lines are considered as not being parallel, repeat the determination. If parallelism is still not achieved, the determination must be considered as unsatisfactory.

Express the result in milligrams of spiramycin base per kilogram of feedingstuff.

9. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample by the same analyst should not exceed:

— 2 mg/kg, in absolute value, for contents of spiramycin base up to 10 mg/kg,
— 20 % related to the highest value for contents of 10 to 25 mg/kg,
— 5 mg/kg, in absolute value, for contents of 25 to 50 mg/kg,
— 10 % related to the highest value for contents above 50 mg/kg.