II

(Acts whose publication is not obligatory)

COMMISSION

COMMISSION DIRECTIVE

of 20 December 1983

amending Directives 71/393/EEC, 72/199/EEC and 78/633/EEC establishing Community methods of analysis for the official control of feedingstuffs

(84/4/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 Commission Directives 71/393/EEC (2), 72/199/EEC (3) and 78/633/EEC (4) set out the methods of analysis for crude oils and fats, virginiamycin and zinc bacitracin ; whereas there is a need to replace these methods by methods which reflect advances in scientific and technical knowledge ;

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

In the Annex to Directive 71/393/EEC, part IV 'Determination of crude oils and fats' is replaced by Annex I to this Directive.

Article 2

In Annex II to Directive 72/199/EEC, part 5 'Determination of virginiamycin by diffusion in an agar medium' is replaced by Annex II to this Directive.

Article 3

In the Annex to Directive 78/633/EEC, part 1 'Determination of zinc bacitracin by diffusion in an agar medium' is replaced by Annex III to this Directive.

Article 4

Member States shall, by 1 June 1984 at the latest, bring into force the laws, regulations or administrative provisions necessary to comply with this Directive and shall forthwith inform the Commission thereof.

Article 5

This Directive is addressed to the Member States.

Done at Brussels, 20 December 1983.

For the Commission

Poul DALSAGER

Member of the Commission

(1) OJ No L 170, 3. 8. 1970, p. 2.
(2) OJ No L 279, 20. 12. 1971, p. 7.
(3) OJ No L 123, 29. 5. 1972, p. 6.
(4) OJ No L 206, 29. 7. 1978, p. 43.

ANNEX I

'4. DETERMINATION OF CRUDE OILS AND FATS

1. Purpose and scope

This method makes it possible to determine the content of crude oils and fats in feedingstuffs. It does not cover the analysis of the oil seeds and oleaginous fruit defined in Council Regulation 136/66/EEC of 22 September 1966.

Depending on the nature of the feedingstuff, either of the two methods described must be used.

1.1. *Method A*

Applicable to sraight feedingstuffs of plant origin, with the exception of those which are known to contain oils and fats which cannot be totally extracted with light petroleum without prior hydrolysis. Among these are glutens, yeasts, soya and potato proteins. This method is also applicable to compound feedingstuffs, with the exception of those which contain milk powder or from which oils and fats cannot be totally extracted with light petroleum without prior hydrolysis.

1.2. *Method B*

Applicable to straight feedingstuffs of animal origin as well as to feedingstuffs mentioned under point 1.1 as being exceptions for method A.

2. Principle

2.1. *Method A*

The sample is extracted with light petroleum. The solvent is distilled off and the residue dried and weighed.

2.2. *Method B*

The sample is treated under heating with hydrochloric acid. The mixture is cooled and filtered. The residue is washed and dried and submitted to the determination according to method A.

3. Reagents

3.1. Light petroleum, boiling range : 40 to 60 °C. The bromine value must be less than 1 and the residue of evaporation less than 2 mg/100 ml.

3.2. Sodium sulphate, anhydrous.

3.3. Hydrochloric acid 3N.

3.4. Filtration aid, e.g. Kieselgur, Hyflo-supercel.

4. Apparatus

4.1. Extraction apparatus. If fitted with a siphon (Soxhlet apparatus), the reflux rate should be such as to produce about 10 cycles per hour; if of the non-siphoning type, the reflux rate should be about 10 ml per minute.

4.2. Extraction thimbles, free of matter soluble in light petroleum and having a porosity consistent with the requirements of point 4.1.

4.3. Drying oven, either a vacuum oven set at 75 ± 3 °C or an air-oven set at 100 ± 3 °C.

5. Procedure

5.1. *Method A* (see point 8.1)

Weigh 5 g of the sample to the nearest 1 mg, transfer it to an extraction thimble (4.2) and cover with a fat-free wad of cotton wool.

Place the thimble in an extractor (4.1) and extract for six hours with light petroleum (3.1). Collect the light petroleum extract in a dry, weighed flask containing fragments of pumice stone (1).

Distil off the solvent. Dry the evaporation residue maintaining the flask for one and a half hours in the drying oven (4.3). Leave to cool in a dessiccator and weigh. Dry again for 30 minutes to ensure that the weight of the oils and fats remains constant (loss in weight between two successive weighings must be less than 1 mg).

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(1) Where the oil or fat has to undergo subsequent quality tests, replace the fragments of pumice stone by glass beads.

5.2. *Method B*

Weigh 2,5 g of the sample to the nearest 1 mg (see point 8.2), place in a 400 ml beaker or a 300 ml conical flask and add 100 ml of hydrochloric acid 3N (3.3) and fragments of pumice stone. Cover the beaker with a watch glass or fit the conical flask with a reflux condenser. Bring the mixture to a gentle boil over a low flame or a hot-plate and keep it there for one hour. Do not allow the product to stick to the sides of the container.

Cool and add a quantity of filtration aid (3.4) sufficient to prevent any loss of oil and fat during filtration. Filter through a moistened, fat-free, double filter paper. Wash the residue in cold water until a neutral filtrate is obtained. Check that the filtrate does not contain any oil or fats. Their presence indicates that the sample must be extracted with light petroleum, using method A, before hydrolysis.

Place the double filter paper containing the residue on a watch glass and dry for one and a half hours in the oven at 100 ± 3 °C.

Place the double filter paper containing the dry residue in an extraction thimble (4.2) and cover with a fat-free wad of cotton wool. Place the thimble in an extractor (4.1) and proceed as indicated in the second and third paragraphs of point 5.1.

6. **Expression of result**

Express the weight of the residue as a percentage of the sample.

7. **Repeatability**

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

— 0,2 %, in absolute value, for contents of crude oils and fats lower than 5 %,

— 4,0 % related to the highest result for contents of 5 to 10 %,

— 0,4 % in absolute value, for contents above 10 %.

8. **Observations**

8.1. For products with a high content of oils and fats, which are difficult to crush or unsuitable for drawing a homogeneous reduced test sample, proceed as follows.

Weigh 20 g of the sample to the nearest 1 mg and mix with 10 g or more of anhydrous sodium sulphate (3.2). Extract with light petroleum (3.1) as indicated in point 5.1. Make up the extract obtained to 500 ml with light petroleum (3.1) and homogenize. Take 50 ml of the solution and place in a small, dry, weighed flask containing fragments of pumice stone (1). Distil off the solvent, dry and proceed as indicated in the last paragraph of point 5.1.

Eliminate the solvent from the extraction residue left in the thimble, crush the residue to a fineness of 1 mm, return it to the extraction thimble (do not add sodium sulphate) and proceed as indicated in the second and third paragraphs of point 5.1.

Calculate the content of oils and fats as a percentage of the sample by using the following formula :

$$(10\ a + b) \times 5$$

where :

a = mass in grams of the residue after the first extraction (aliquot part of the extract),

b = mass in grams of the residue after the second extraction.

8.2. For products low in oils and fats the test sample may be increased to 5 g.'

(1) Where the oil or fat has to undergo subsequent quality tests, replace the fragments of pumice stone by glass beads.

ANNEX II

'5. Determination of virginiamycin

— by diffusion in an agar medium —

1. **Purpose and scope**

The method is for the determination of virginiamycin in feedingstuffs and premixes. The lower limit of determination is 2 mg/kg (2 ppm) (1).

2. **Principle**

The sample is extracted with a methanolic solution of Tween 80. The extract is decanted or centrifuged and diluted. Its antibiotic activity is determined by measuring the diffusion of virginiamycin 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 cloride 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 and assay 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 (after sterilization).

4.2. *Phosphate buffer, pH 6*

Potassium hydrogen phosphate, K_2HPO_4	2,0 g
Potassium dihydrogen phosphate, KH_2PO_4	8,0 g
Water to	1 000 ml

4.3. Sodium chloride solution 0,8 % (w/v) : dissolve 8 g sodium chloride in water and dilute to 1 000 ml ; sterilize.

4.4. Methanol.

4.5. Mixture of phosphate buffer (4.2)/methanol (4.4) : 80/20 (v/v).

4.6. Tween 80 methanolic solution 0,5 % (w/v) : dissolve 5 g Tween 80 in methanol (4.4) and dilute with methanol to 1 000 ml.

4.7. Standard substance : virginiamycin of known activity.

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(1) 1 mg virginiamycin is equivalent to 1 000 UK units.

(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.

5. **Standard solutions**

Dissolve an accurately weighed quantity of the standard substance (4.7) in methanol (4.4) and dilute with methanol (4.4) to give a stock solution containing 1 000 µg virginiamycin per ml.

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	µg/ml
s_4	0,5	µg/ml
s_2	0,25	µg/ml
s_1	0,125	µg/ml

6. **Preparation of the extract and assay solutions**

6.1. *Extraction*

6.1.1. Products with a virginiamycin content up to 100 mg/kg

Weigh out a quantity of sample of 50 g, add 200 ml of solution (4.6) and shake for 30 minutes. Leave to settle or centrifuge, take 20 ml of the supernatant solution and evaporate to about 5 ml in a rotary evaporator at a temperature not exceeding 40 °C. Dilute the residue with the mixture (4.5) to obtain an expected virginiamycin content of 1 µg/ml (= u_8).

6.1.2. Products with a virginiamycin content greater than 100 mg/kg

Weigh out a quantity of sample not exceeding 10,0 g and containing between 1 and 50 mg virginiamycin, add 100 ml of solution (4.6) and shake for 30 minutes. Leave to settle or centrifuge, then dilute the supernatant solution with the mixture (4.5) to obtain an expected virginiamycin content of 1 µg/ml (= u_8).

6.2. *Assay solutions*

From solution u_8 prepare solutions u_4 (expected content : 0,5 µg/ml), u_2 (expected content : 0,25 µg/ml) and u_1 (expected content : 0,125 µg/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 (4.1) with the bacterial suspension (3.2) at about 50 °C. By preliminary trials on plates with the medium (4.1) determine the quantity of bacterial suspension required to give the largest and clearest zones of inhibition with the various concentrations of virginiamycin.

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 and s_1) and the four concentrations of the assay solution (u_8, u_4, u_2 and 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 point 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 ± 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 :