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THIRD COMMISSION DIRECTIVE

of 27 April 1972

establishing Community methods of analysis for the official control of feedingstuffs

(72/199/EEC)

(OJ L 123, 29.5.1972, p. 6)

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► <u>M1</u> Commission Directive 81/680/EEC of 30 July 1981	L 246	32	29.8.1981
► <u>M2</u> Commission Directive 84/4/EEC of 20 December 1983	L 15	28	18.1.1984
► <u>M3</u> Commission Directive 93/28/EEC of 4 June 1993	L 179	8	22.7.1993
► <u>M4</u> Commission Directive 98/54/EC of 16 July 1998	L 208	49	24.7.1998
► <u>M5</u> Commission Directive 1999/79/EC of 27 July 1999	L 209	23	7.8.1999

Corrected by:

► **C1** Corrigendum, OJ L 320, 27.11.1980, p. 43 (72/199/EEC)

▼B**THIRD COMMISSION DIRECTIVE****of 27 April 1972****establishing Community methods of analysis for the official control of feedingstuffs**

(72/199/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community;

Having regard to the Council Directive of 20 July 1970 ⁽¹⁾ on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs, and in particular Article 2 thereof;

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

Whereas Commission Directives No 71/250/EEC ⁽²⁾ of 15 June 1971 and No 71/393/EEC ⁽³⁾ of 18 November 1971 have already established a number of Community methods of analysis; whereas, in view of progress made in subsequent work, a third set of methods should be adopted;

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 Member States shall require that analyses for official controls of feedingstuffs as regards their levels of starch, crude protein, crude protein which can be dissolved by pepsin and hydrochloric acid, of free and total gossypol and as regards pepsin activity be carried out using the methods described in Annex I to this Directive.

▼M1▼B*Article 2*

The Member States shall require that analyses for official controls of feedingstuffs, for the purpose ►M4 ————— ◀ for determining the levels of ►M4 ————— ◀ tylosin, and virginiamycin in feedingstuffs, be carried out using the methods described in Annex II to this Directive.

▼M1▼B*Article 3*

The Member States shall, not later than 1 July 1973, bring into force the laws, regulations or administrative provisions necessary to comply with this Directive. They shall forthwith inform the Commission thereof.

⁽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.

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Article 4

This Directive is addressed to the Member States.

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ANNEX I

▼M5**1. DETERMINATION OF STARCH**

POLARIMETRIC METHOD

1. Purpose and scope

This method makes it possible to determine the levels of starch and of high molecular weight starch degradation products in feedingstuffs for the purpose of checking compliance with Commission Directive 86/174/EEC and Council Directive 96/25/EC.

2. Principle

The method comprises two determinations. In the first, the sample is treated when hot with dilute hydrochloric acid. After clarification and filtration the optical rotation of the solution is measured by polarimetry.

In the second, the sample is extracted with 40 % ethanol. After acidifying the filtrate with hydrochloric acid, clarifying and filtering, the optical rotation is measured as in the first determination.

The difference between the two measurements, multiplied by a known factor, gives the starch content of the sample.

3. Reagents

3.1. 25 % (w/w) hydrochloric acid, d: 1,126 g/ml.

3.2. 1,128 % (w/v) hydrochloric acid.

The concentration must be checked by titration using a sodium hydroxide solution 0,1 mol/litre in the presence of 0,1 % (w/v) methyl red in 94 % (v/v) ethanol. 10 ml = 30,94 ml of NaOH 0,1 mol/lite.

3.3. Carrez solution I: dissolve 21,9 g of zinc acetate $Zn(CH_3COO)_2 \cdot 2H_2O$ and 3 g of glacial acetic acid in water. Make up to 100 ml with water.

3.4. Carrez solution II: dissolve 10,6 g of potassium ferrocyanide $[K_4(Fe(CN)_6)] \cdot 3H_2O$ in water. Make up to 100 ml with water.

3.5. 40 % (v/v) ethanol, d: 0,948 g/ml at 20 °C.

4. Apparatus

4.1. 250 ml erlenmeyer flask with standard ground-glass joint and with reflux condenser.

4.2. Polarimeter or saccharimeter.

5. Procedure**5.1. Preparation of the sample**

Crush the sample until it is fine enough for all of it to pass through a 0,5 mm round-meshed sieve.

5.2. Determination of the total optical rotation (P or S) (see observation 7.1)

Weigh 2,5 g of the crushed sample to the nearest mg and place in a 100 ml graduated flask. Add 25 ml of hydrochloric acid (3.2), shake to obtain even distribution of the test sample and add a further 25 ml of hydrochloric acid (3.2). Immerse the flask in a boiling water bath shaking vigorously and steadily for the first three minutes to prevent the formation of agglomerates. The quantity of water in the water bath must be sufficient for the bath to remain at boiling point when the flask is introduced into it. The flask must not be taken out of the bath whilst being shaken. After exactly 15 minutes, remove from the bath, add 30 ml of cold water and cool immediately to 20 °C.

Add 5 ml of Carrez solution I (3.3) and shake for one minute. Then add 5 ml of Carrez solution II (3.4) and shake again for one minute. Make up to volume with water, mix and filter. If the filtrate is not perfectly clear (which is rare), repeat the determination using a larger quantity of Carrez solutions I and II, for example 10 ml.

▼ **M5**

Measure the optical rotation of the solution in a 200 mm tube with the polarimeter or saccharimeter.

5.3. *Determination of the optical rotation (P' or S') of substances soluble in 40 % ethanol*

Weigh 5 g of the sample to the nearest mg, place in a 100 ml graduated flask and add about 80 ml of ethanol (3.5) (see observation 7.2). Leave the flask to stand for 1 hour at room temperature; during this time, shake vigorously on six occasions so that the test sample is thoroughly mixed with the ethanol. Make up to volume with ethanol (3.5), mix and filter. Pipette 50 ml of the filtrate (= 2,5 g of the sample) into a 250 ml erlenmeyer flask, add 2,1 ml of hydrochloric acid (3.1) and shake vigorously. Fit a reflux condenser to the erlenmeyer flask and immerse the latter in a boiling water bath. After exactly 15 minutes, remove the erlenmeyer flask from the bath, transfer the contents to a 100 ml graduated flask, rinsing with a little cold water, and cool to 20 °C. Clarify using Carrez solutions I (3.3) and II (3.4), make up to volume with water, mix, filter and measure the optical rotation as indicated in the second and third paragraphs of 5.2.

6. Calculation of results

The starch content (%) is calculated as following:

6.1. *Measurement by polarimeter*

$$\text{Starch content (\%)} = \frac{2000 (P - P')}{[\alpha]_D^{20^\circ}}$$

P = total optical rotation in angle degrees

P' = optical rotation in angle degrees of the substances soluble in 40 % (V/V) ethanol

$[\alpha]_D^{20^\circ}$ = specific optical rotation of pure starch. The numerical values conventionally accepted for factor are the following

- + 185.9°: rice starch
- + 185.4°: potato starch
- + 184.6°: maize starch
- + 182.7°: wheat starch
- + 181.5°: barley starch
- + 181.3°: oat starch
- + 184.0°: other types of starch and starch mixtures in compound feedingstuffs

6.2. *Measurement by saccharimeter*

$$\text{Starch content (\%)} = \frac{2000}{[\alpha]_D^{20^\circ}} \times \frac{(2N \times 0,665) \times (S - S')}{100} - \frac{26,6 N \times (S - S')}{[\alpha]_D^{20^\circ}}$$

S = total optical rotation in saccharimeter degrees

S' = optical rotation in saccharimeter degrees of the substances soluble in 40 % (V/V) ethanol

N = weight (g) of saccharose in 100 ml of water yielding an optical rotation of 100 saccharimeter degrees when measured using a 200 mm tube

16,29 g for the French saccharimeters

26,00 g for the German saccharimeters

20,00 g for mixed saccharimeters.

$[\alpha]_D^{20^\circ}$ = specific optical rotation of pure starch (see 6.1)

6.3. *Repeatability*

The difference between the results of two parallel determinations carried out on the same sample must not exceed 0,4 in absolute value for a starch content lower than 40 % and 1,1 % relative for starch contents equal or higher than 40 %

▼ M5**7. Observations**

- 7.1. If the sample contains more than 6 % of carbonates, calculated in terms of calcium carbonate, they must be destroyed by treatment with an exactly appropriate quantity of dilute sulphuric acid before determination of the total optical rotation.
- 7.2. In the case of products with a high lactose content, such as powdered milk serum or skimmed milk powder, proceed as follows after adding 80 ml of ethanol (3.5). Fit a reflux condenser to the flask and immerse the latter in a water bath at 50 °C for 30 minutes. Leave to cool and continue the analysis as indicated in 5.3.
- 7.3. Following feed materials, in case they are present in significant amount in feedingstuffs, are known to give rise to interferences when determining the starch content by polarimetric method and whereby incorrect results could be yielded:
 - (sugar) beet products such as (sugar) beet pulp, (sugar) beet molasses, (sugar) beet pulp-molassed, (sugar) beet vinasse, (beet) sugar,
 - citrus pulp,
 - linseed; linseed expeller; linseed extracted,
 - rape seed; rape seed expeller; rape seed extracted; rape seed hulls,
 - sunflower seed; sunflower seed extracted; sunflower seed, partially decorticated, extracted,
 - copra expeller; copra extracted,
 - potato pulp,
 - dehydrated yeast,
 - products rich in inulin (e.g. chips and meal of Jerusalem artichokes);
 - greaves.

▼ M3**2. DETERMINATION OF CRUDE PROTEIN****1. Purpose and scope**

This method makes it possible to determine the crude protein content of feedingstuffs on the basis of the nitrogen content, determined according to the Kjeldahl method.

2. Principle

The sample is digested by sulfuric acid in the presence of a catalyst. The acid solution is made alkaline with sodium hydroxide solution. The ammonia is distilled and collected in a measured quantity of sulfuric acid, the excess of which is titrated with a standard solution of sodium hydroxide.

3. Reagents

- 3.1 Potassium sulfate.
- 3.2 Catalyst: copper (II) oxide CuO or copper (II) sulfate pentahydrate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
- 3.3 Granulated zinc.
- 3.4 Sulfuric acid, $\rho_{20} = 1,84 \text{ g/ml}$.
- 3.5 Sulfuric acid $c(\frac{1}{2}\text{H}_2\text{SO}_4) = 0,5 \text{ mol/l}$.
- 3.6 Sulfuric acid $c(\frac{1}{2}\text{H}_2\text{SO}_4) = 0,1 \text{ mol/l}$.
- 3.7 Methyl red indicator; dissolve 300 mg of methyl red in 100 ml of ethanol, $\sigma = 95-96 \%$ (v/v)
- 3.8 Sodium hydroxide solution (Technical grade may be used) $\beta = 40 \text{ g/100 ml}$ (m/v: 40 %).
- 3.9 Sodium hydroxide solution $c = 0,25 \text{ ml/l}$.
- 3.10 Sodium hydroxide solution $c = 0,1 \text{ mol/l}$.
- 3.11 Granulated pumice stone, washed in hydrochloric acid and ignited.
- 3.12 Acetanilide (m.p. = 114 °C, N = 10,36 %)
- 3.13 Sucrose (nitrogen free).

▼ **M3****4. Apparatus**

Apparatus suitable for performing digestion, distillation and titration according to the Kjeldahl procedure.

5. Procedure*5.1 Digestion*

Weigh 1 g of the sample to the nearest 0,001 g and transfer the sample to the flask of the digestion apparatus. Add 15 g of potassium sulfate (3.1.), an appropriate quantity of catalyst (3.2) (0,3 to 0,4 g of copper (II) oxide or 0,9 to 1,2 g of copper (II) sulfate pentahydrate), 25 ml of sulfuric acid (3.4) and a few granules of pumice stone (3.11) and mix. Heat the flask moderately at first, swirling from time to time if necessary until the mass has carbonized and the foam has disappeared; then heat more intensively until the liquid is boiling steadily. Heating is adequate if the boiling acid condenses on the wall of the flask. Prevent the sides from becoming overheated and organic particles from sticking to them. When the solution becomes clear and light green continue to boil for another two hours, then leave to cool.

5.2 Distillation

Add carefully enough water to ensure complete dissolution of the sulfates. Allow to cool and then add a few granules of zinc (3.3).

Place in the collecting flask of the distillation apparatus an exactly measured quantity of 25 ml of sulfuric acid (3.5) or (3.6) depending on the presumed nitrogen content. Add a few drops of methyl red indicator (3.7).

Connect the digestion flask to the condenser of the distillation apparatus and immerse the end of the condenser in the liquid contained in the collecting flask to a depth of at least 1 cm (see observation 8.3). Slowly pour 100 ml of sodium hydroxide solution (3.8) into the digestion flask without loss of ammonia (see observation 8.1).

Heat the flask until the ammonia has distilled over.

5.3 Titration

Titrate the excess sulfuric acid in the collecting flask with sodium hydroxide solution (3.9) or (3.10) depending on the concentration of the sulfuric acid used, until the end point is reached.

5.4 Blank test

To confirm that the reagents are free from nitrogen, carry out a blank test (digestion, distillation and titration) using 1 g of sucrose (3.13) in place of the sample.

6. Calculation of results

The content of crude protein is calculated according to the following formula:

$$\frac{(V_0 - V_1) \times c \times 0,014 \times 100 \times 6,25}{m}$$

Where,

V_0 = Volume (ml) of NaOH (3.9 or 3.10) used in the blank test.

V_1 = Volume (ml) of NaOH (3.9 or 3.10) used in the sample titration.

c = Concentration (mol/l) of sodium hydroxide (3.9 or 3.10).

m = Mass (g) of sample.

7. Verification of the method*7.1 Repeatability*

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

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- 0,2 % in absolute value, for crude protein contents of less than 20 %;
- 1,0 % relative to the higher value, for crude protein contents from 20 % to 40 %;
- 0,4 % in absolute value, for crude protein contents of more than 40 %.

7.2 *Accuracy*

Carry out the analysis (digestion, distillation and titration) on 1,5 to 2,0 g of acetanilide (3.12) in the presence of 1 g of sucrose (3.13); 1 g acetanilide consumes 14,80 ml of sulfuric acid (3.5). Recovery must be at least 99 %.

8. Observations

- 8.1 Apparatus may be of the manual, semi-automatic or automatic type. If the apparatus requires transference between the digestion and distillation steps, this transfer must be carried out without loss. If the flask of the distillation apparatus is not fitted with a dropping funnel, add the sodium hydroxide immediately before connecting the flask to the condenser, pouring the liquid slowly down the side.
- 8.2 If the digest solidifies, recommence the determination using a larger amount of sulfuric acid (3.4) than that specified above.
- 8.3 For products with a low nitrogen content, the volume of sulfuric acid (3.6) to be placed in the collecting flask may be reduced, if necessary, to 10 or 15 ml and made up to 25 ml with water.

▼B**3. DETERMINATION OF CRUDE PROTEIN DISSOLVED BY PEPSIN AND HYDROCHLORIC ACID****1. Purpose and scope**

This method makes it possible to determine the fraction of crude protein dissolved by pepsin and hydrochloric acid under defined conditions. It is applicable to all feedingstuffs.

2. Principle

The sample is heated for 48 hours at 40 °C in a solution of pepsin hydrochloride. The suspension is filtered and the nitrogen content of the filtrate determined according to the method for the determination of crude protein.

3. Reagents

- 3.1 Hydrochloric acid, d: 1.125.
- 3.2 Hydrochloric acid 0.075 N.
- 3.3 2.0 U/mg pepsin; pepsin activity is defined in the method described in Part 4 of this Annex and must be established according to that method.
- 3.4 About 0.2 % (w/v) freshly prepared solution of pepsin in hydrochloric acid (3.2): activity: 400 U/l.
- 3.5 Anti-foaming emulsion (eg silicone).
- 3.6 All the reagents listed under 3 in the method for the determination of crude protein.

4. Apparatus

- 4.1 Water bath or incubator, set at 40 °C ± 1 °C.
- 4.2 Kjeldahl digestion and distillation apparatus.

5. Procedure5.1 *Preparation of solution* (see observation 7.2)

Weigh 2 g of the sample to the nearest mg and place in a 500 ml graduated flask. Add 450 ml of pepsin hydrochloride solution (3.4) previously heated to 40 °C and shake to prevent the formation of agglomerates. Check that the pH of the suspension is less than 1.7. Place the flask in the water bath or incubator (4.1) and leave there for 48 hours. Shake after 8, 24 and 32 hours. After 48 hours, add 15 ml of

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hydrochloric acid (3.1), cool to 20 °C, make up to volume with water and filter.

5.2 Digestion

Take 250 ml of the filtrate and place in the flask of the distillation apparatus (4.2). Add the reagents necessary for digestion indicated in the second sentence of 5.1 of the method for the determination of crude protein. Homogenize and bring to the boil. If any foam should form, add a few drops of anti-foaming emulsion (3.5). Continue to boil vigorously until the water has been almost completely evaporated. Reduce the heat and carefully eliminate the last traces of water.

When the solution becomes clear and colourless (or light green if a copper-based catalyst is used), continue to boil for another hour. Leave to cool.

5.3 Distillation and titration

Proceed as indicated in 5.2 and 5.3 of the method for the determination of crude protein.

5.4 Blank test

Carry out a blank test applying the same procedure but omitting the sample to be analyzed.

6. Calculation of results

Subtract the volume of sulphuric acid consumed in the blank test from that consumed by the test sample. 1 ml of sulphuric acid 0.1 N corresponds to 1.4 mg of nitrogen.

Multiply the quantity of nitrogen by the factor 6.25. Express the result as a percentage of the sample.

Repeatability

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

- 0.4, in absolute value, for contents of less than 20 %;
- 2.0 %, in relative value, for contents of not less than 20 % and not more than 40 %;
- 0.8, in absolute value, for contents of more than 40 %.

7. Observations

- 7.1 The values obtained by this method have no direct connection with digestibility *in vivo*.
- 7.2 Products with an oil or fat content exceeding 10 % must first be defatted by extraction with petroleum ether (B.P. 40 to 60 °C).

4. ESTIMATION OF PEPSIN ACTIVITY**1. Purpose and scope**

This method makes it possible to establish the activity of the pepsin used in the determination of crude protein dissolved by pepsin and hydrochloric acid.

2. Principle

Haemoglobin is treated with pepsin in a hydrochloric acid medium under defined conditions. The non-hydrolyzed fraction of the protein is precipitated in trichloroacetic acid. Sodium hydroxide and Folin-Ciocalteu reagent are added to the filtrate. The optical density of this solution is measured at 750 nm and the corresponding quantity of tyrosine is read from a calibration curve.

Definition: The unit of pepsin is defined as being the quantity of that enzyme which, under the conditions of the method, liberates per minute, a quantity of hydroxyaryl groups which, when stained with the Folin-Ciocalteu reagent, has an optical density corresponding to that of one μ mole of tyrosine stained in the same manner.

▼ **B****3. Reagents**

- 3.1 Hydrochloric acid 0.2 N.
- 3.2 Hydrochloric acid 0.06 N.
- 3.3 Hydrochloric acid 0.025 N.
- 3.4 5 % solution (w/v) of trichloroacetic acid.
- 3.5 Sodium hydroxide solution 0.5 N.
- 3.6 Folin-Ciocalteu reagent. Place 100 g of sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$), 25 g of sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) and 700 ml of water in a 2 litre round-bottomed flask fitted with a standard ground-glass joint. Add 50 ml of phosphoric acid (d: 1.71) and 100 ml of concentrated hydrochloric acid (d: 1.19), connect a reflux condenser to the flask, bring to the boil and keep the solution gently boiling for 10 hours. Leave to cool, detach the reflux condenser, add 175 g of lithium sulphate ($\text{Li}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$), 50 ml of water and 1 ml of bromine. Boil for 15 minutes to eliminate excess bromine.
- Leave to cool, transfer the solution to a 1 litre graduated flask, make up to volume with water, homogenize and filter. No greenish coloration must remain. Before use, dilute 1 volume of the reagent with 2 volumes of water.
- 3.7 Haemoglobin solution: Weigh a quantity of haemoglobin (approx. 2 g of protein substratum determined according to Anson) corresponding to 354 mg of nitrogen ⁽¹⁾ and place in a 200 ml flask fitted with a standard ground-glass joint. Add a few ml of hydrochloric acid (3.2), connect the flask to the vacuum pump and shake until the haemoglobin has completely dissolved. Release the vacuum and, while shaking, add hydrochloric acid (3.2) to make up to 100 ml. *Prepare immediately before use.*
- 3.8 Standard tyrosine solution: Dissolve 181.2 mg of tyrosine in the hydrochloric acid (3.1) and make up to 1 litre with the same acid (stick solution). Take 20.0 ml and dilute to 100 ml with hydrochloric acid (3.1). 1 ml of this solution contain 0.2 μmole of tyrosine.

4. Apparatus

- 4.1 Water bath set at $25\text{ }^\circ\text{C} \pm 0.1\text{ }^\circ\text{C}$ by ultrathermostat.
- 4.2 Spectrophotometer.
- 4.3 Chronometer, accuracy: 1 second.
- 4.4 pH-meter.

5. Procedure5.1 *Preparation of the solution (see observation 7.1)*

Dissolve 150 mg of pepsin in 100 ml of hydrochloric acid (3.2). Pipette 2 ml of the solution into a 50 ml graduated flask and make up to volume with hydrochloric acid (3.3). The pH, checked with the pH-meter, must be 1.6 ± 0.1 . Immerse the flask in the water bath (4.1).

5.2 *Hydrolysis*

Pipette 5.0 ml of haemoglobin solution (3.7) into a test tube, heat to $25\text{ }^\circ\text{C}$ in the water bath (4.1), add 1.0 ml of the pepsin solution obtained in 5.1 and mix with a glass rod thickened at one end, with about 10 back-and-forth movements. Leave the test tube in the water bath at $25\text{ }^\circ\text{C}$ for exactly 10 minutes, timed from the addition of the pepsin solution (duration and temperature must be strictly observed). Then add 10.0 ml of trichloroacetic acid solution (3.4) previously heated to $25\text{ }^\circ\text{C}$, homogenize and filter through a dry filter.

5.3 *Development of coloration and measurement of optical density*

Pipette 5.0 ml of the filtrate into a 50 ml Erlenmeyer flask, add 10.0 ml of sodium hydroxide solution (3.5) and, shaking constantly, 3.0 ml of dilute Folin-Ciocalteu reagent (3.6). After 5 to 10 minutes, determine

⁽¹⁾ Determine the nitrogen content by the semi-micro Kjeldahl method (theoretical content: 17.7 % of nitrogen).

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the optical density of the solution with the spectrophotometer at 750 nm in cells 1 cm thick against water.

5.4 Blank test

For each determination, carry out a blank test as follows:

Pipette 5.0 ml of haemoglobin solution (3.7) into a test tube, heat to 25 °C in the water bath (4.1), add 10.0 ml of trichloroacetic acid solution (3.4) previously heated to 25 °C, homogenize, then add 1.0 ml of the pepsin solution obtained in 5.1. Mix with a glass rod and leave the test tube in the water bath (4.1) at 25 °C for exactly 10 minutes. Homogenize and filter through a dry filter. Follow the procedure indicated in 5.3.

5.5 Calibration curve

Place 1.0, 2.0, 3.0, 4.0 and 5.0 ml aliquots of standard tyrosine solution (3.8), corresponding to 0.2, 0.4, 0.6, 0.8 and 1.0 µmoles of tyrosine respectively in 50 ml Erlenmeyer flasks. Complete the series with a reference solution free from tyrosine. Make up the volumes to 5.0 ml with hydrochloric acid (3.1). Add 10.0 ml of sodium hydroxide solution (3.5) and, shaking constantly, 3.0 ml of dilute Folin-Ciocalteu reagent (3.6). Measure the optical density as indicated in the last sentence of 5.3. Trace the calibration curve by plotting the optical densities against the quantities of tyrosine.

6. Calculation of results

From the calibration curve read the quantity of tyrosine, in µmoles, corresponding to the optical density of the coloured solution, corrected on the basis of the blank value.

The pepsin activity, in µmoles, of tyrosine at 25 °C, per mg and per minute, is calculated by using the formula:

$$\text{Units per mg (U/mg)} = \frac{0 \cdot 32 a}{P}$$

where:

a = quantity of tyrosine, in µmoles, read from the calibration curve;

p = weight in mg of the quantity of pepsin added in 5.2.

7. Observations

7.1 The quantity of pepsin to be dissolved must be such that, on final photometric measurement, an optical density of 0.35 ± 0.035 is obtained.

7.2 Two units per mg obtained by this method correspond to:

3.64 Anson milliunits /mg (µmoles of tyrosine/mg · min at 35.5 °C) or

36 400 commercial units/g (µmoles of tyrosine/g in 10 min at 35.5 °C).

5. DETERMINATION OF FREE AND GOSSYPOL**1. Purpose and scope**

This method makes it possible to determine the levels of free gossypol, total gossypol and chemically related substances in cottonseed, cottonseed meal and cottonseed cake and in compound feedingstuffs containing these substances where more than 20 ppm are present.

2. Principle

The gossypol is extracted in the presence of 3-aminopropan-1-ol, either with a mixture of propan-2-ol and hexane, for the determination of free gossypol, or with dimethylformamide, for the determination of total gossypol. The gossypol is converted by aniline into gossypol-dianiline, the optical density of which is measured at 440 nm.

3. Reagents

3.1 Propan-2-ol-hexane mixture: mix 60 parts by volume of propan-2-ol A.R. with 40 parts by volume of *n*-hexane.

3.2 Solvent A: Place in a 1 litre graduated flask approximately 500 ml of propan-2-ol-hexane mixture (3.1), 2 ml of 3-aminopropan-1-ol, 8 ml of glacial acetic acid and 50 ml of water. Make up to volume with the propan-2-ol-hexane mixture (3.1). This reagent is stable for one week.

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- 3.3 Solvent B: Pipette 2 ml of 3-aminopropan-1-ol and 10 ml of glacial acetic acid into a 100 ml graduated flask. Cool to room temperature and make up to volume with N, N-dimethylformamide. This reagent is stable for one week.
- 3.4 Aniline A.R.: *If the optical density in the blank test exceeds 0.022*, distil the aniline over zinc dust, discarding the first and last 10 % fractions of the distillate. Refrigerated and stored in a brown, stoppered glass flask, this reagent will keep for several months.
- 3.5 Standard gossypol solution A: Place 27.9 mg of gossypol acetate in a 250 ml graduated flask. Dissolve and make up to volume with solvent A (3.2). Pipette 50 ml of this solution into a 250 ml graduated flask and make up to volume with solvent A. The gossypol concentration of this solution is 0.02 mg per ml. Leave to stand for one hour at room temperature before use.
- 3.6 Standard gossypol solution B: Place 27.9 mg of gossypol acetate in a 50 ml graduated flask, Dissolve and make up to volume with solvent B (3.3). The gossypol concentration of this solution is 0.5 mg per ml.

Standard gossypol solutions A and B will remain stable for 24 hours if protected from the light.

4. Apparatus

- 4.1 Mixer (tumbler): approximately 35 rpm.
- 4.2 Spectrophotometer.

5. Procedure**5.1 Test sample**

The amount of test sample used depends on the presumed gossypol content of the sample. It is preferable to work with a small test sample and a relatively large aliquot part of the filtrate, so as to obtain sufficient gossypol for precise photometric measurement to be possible. *For the determination of free gossypol* in cottonseed, cottonseed meal and cottonseed cake, the test sample should not exceed 1 g; for compound feedingstuffs, it may be as much as 5 g. A 10 ml aliquot part of filtrate is suitable in most cases; it should contain 50 to 100 µg of gossypol. *For the determination of total gossypol*, the test sample should be between 0.5 and 5 g, that a 2 ml aliquot part of filtrate will contain 40 to 200 µg of gossypol.

The analysis should be carried out at a room temperature of about 20 °C.

5.2 Determination of free gossypol

Place the test sample in a ground-necked 250 ml flask, the bottom of the flask having been covered with crushed glass. Using a pipette, add 50 ml of solvent A (3.2), stopper the flask and mix for one hour in the mixer. Filter through a dry filter and collect the filtrate in a small ground-necked flask. During filtration, cover the funnel with a watch glass. Pipette identical aliquot parts of filtrate containing 50 to 100 µg of gossypol into each of two 25 ml graduated flasks (A and B). If necessary, make up the volume to 10 ml with solvent A (3.2). Then make the contents of flask (A) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the sample solution.

Pipette 10 ml of solvent A (3.2) into each of two other 25 ml graduated flasks (C and D). Make the contents of flask (C) up to volume with the propan-2-ol-hexane mixture (3.1). This solution will be used as a reference solution against which to measure the blank test solution.

Add 2 ml of aniline (3.4) to each of flasks (D) and (B). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour.

Determine the optical density of the blank test solution (D) by comparison with the reference solution (C), and the optical density of the sample solution (B) by comparison with the reference solution (A), in the spectrophotometer at 440 nm using 1 cm glass cells.

Subtract the optical density of the blank test solution from that of the sample solution (= corrected optical density). From this value calculate the free gossypol content as indicated in 6.

▼B**5.3 Determination of total gossypol**

Place a test sample containing 1 to 5 mg of gossypol in a 50 ml graduated flask and add 10 ml of solvent B (3.3). At the same time, prepare a blank test, placing 10 ml of solvent B (3.3) in another 50 ml graduated flask. Heat the two flasks for 30 minutes over a boiling water bath. Cool to room temperature and make the contents of each flask up to volume with the propan-2-ol-hexane mixture (3.1). Homogenize and leave to settle for 10 to 15 minutes, then filter and collect the filtrates in ground-necked flasks.

Pipette 2 ml of the sample filtrate into each of two 25 ml graduated flasks, and 2 ml of the blank test filtrate into each of two other 25 ml flasks. Make the contents of one flask from each series up to 25 ml with the propan-2-ol-hexane mixture (3.1). These solutions will be used as reference solutions.

Add 2 ml of aniline (3.4) to each of the other two flasks. Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to 25 ml with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour.

Determine the optical density as indicated in 5.2 for free gossypol. From this value calculate the total gossypol content as indicated in 6.

6. Calculation of results

Results may be calculated either from the specific optical density (6.1), or by reference to a calibration curve (6.2).

6.1 From the specific optical density

The specific optical densities, under the conditions described, will be the following:

$$\text{free gossypol: } E \frac{1\%}{1 \text{ cm}} = 625$$

$$\text{total gossypol: } E \frac{1\%}{1 \text{ cm}} = 600$$

The free or total gossypol content of the sample is calculated by using the following formula:

$$\% \text{ gossypol} = \frac{E \cdot 1250}{E_{1 \text{ cm}}^1 \cdot p \cdot a}$$

where:

E = corrected optical density, determined as indicated in 5.2;

p = test sample in g;

a = aliquot part of the filtrate in ml.

6.2 From a calibration curve**6.2.1 Free gossypol**

Prepare 2 series of five 25 ml graduated flasks. Pipette aliquots of 2.0, 4.0, 6.0, 8.0 and 10.0 ml of standard gossypol solution A (3.5) into each series of flasks. Make up the volumes to 10 ml with solvent A (3.2). Complete each series with a 25 ml graduated flask containing only 10 ml of solvent A (3.2) (blank test).

Make the volume of the flasks in the first series (including the flask for the blank test) up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

Add 2 ml of aniline (3.4) to each flask in the second series (including the flask for the blank test). Heat for 30 minutes over a boiling water bath to develop the colour. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in µg).

▼B**6.2.2 Total gossypol**

Prepare six 50 ml graduated flasks. In the first flask place 10 ml of solvent B (3.3), and in the others 2.0, 4.0, 6.0, 8.0 and 10.0 ml of standard gossypol solution B (3.6) respectively. Make the contents of each flask up to 10 ml with solvent B (3.3). Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1) and homogenize.

Place 2.0 ml of these solutions in each of two series of six 25 ml graduated flasks. Make the contents of the flasks in the first series up to 25 ml with the propan-2-ol-hexane mixture (3.1) (reference series).

Add 2 ml of aniline (3.4) to each flask in the second series. Heat for 30 minutes over a boiling water bath. Cool to room temperature, make up to volume with the propan-2-ol-hexane mixture (3.1), homogenize and leave to stand for one hour (standard series).

Determine as indicated in 5.2 the optical density of the solutions in the standard series by comparison with the corresponding solutions in the reference series. Trace the calibration curve by plotting the optical densities against the quantities of gossypol (in µg).

6.3 Repeatability

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

- 15 %, in relative value, for gossypol contents of less than 500 ppm;
- 75 ppm, in absolute value, for contents of not less than 500 ppm and not more than 750 ppm;
- 10 %, in relative value, for contents of more than 750 ppm.

▼B

ANNEX II

▼M4▼B

4. DETERMINATION OF TYLOSIN

— by diffusion on agar —

1. Purpose and scope

This method makes it possible to determine the tylosin content of feeding-stuffs, concentrates and premixes where more than 2 ppm are present.

2. Principle

The sample is treated with a pH 8 phosphate buffer solution, previously heated to 80 °C, and then extracted with methanol. After centrifuging, the extract is diluted and its antibiotic activity determined by measuring the diffusion of the tylosin on an agar medium seeded with *Sarcina lutea*. The diffusion is made evident by the formation of inhibition zones in the presence of the micro-organism. The diameter of these zones is directly proportional to the logarithm of the antibiotic concentration.

3. Micro-organism: *Sarcina lutea* ATCC No 9341

3.1 Maintenance of the parent strain

Inoculate with *Sarcina lutea* a tube of sloped agar taken from the culture medium (4.1), adjust to pH 7.0. Incubate overnight at approximately 35 °C. Keep the culture in a refrigerator and re-inoculate sloped agar with it every month.

3.2 Preparation of the bacteria suspension

Collect the bacteria from a recently prepared tube of sloped agar (3.1) using 2 to 3 ml of physiological saline (4.4). With this suspension seed a Roux flask containing 250 ml of the culture medium (4.1), adjusted to pH 7.0. Incubate for 24 hours at 35 °C, then collect the bacteria in 25 ml of physiological saline (4.4). Homogenize, and dilute this suspension to obtain approximately 75 % light transmission at 650 nm.

If kept in a refrigerator this suspension may be used for one week.

By preliminary tests on plates with the basic medium for the determination (4.1), establish the quantity of inoculum which, for the different concentrations of tylosin used, will give the largest possible inhibition zones that are still clear. The culture medium is inoculated at 48 to 50 °C.

4. Culture media and reagents

4.1 Basic medium for the determination ⁽¹⁾

Glucose	1 g
Tryptic peptone	10 g
Meat extract	1.5 g
Yeast extract	3 g
Agar, according to quality	10 to 20 g
Distilled water to	1000 ml

Adjust immediately before use to pH 7.0 for maintenance of the parent strain and preparation of the bacteria suspension, and to pH 8.0 for the determination.

4.2 Phosphate buffer solution, pH 8

Potassium dihydrogen phosphate KH_2PO_4 A.R.	0.523 g
diPotassium hydrogen phosphate K_2HPO_4 A.R.	16.730 g

⁽¹⁾ Any commercial culture medium of similar composition and giving the same results may be used

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Distilled water to	1000 ml
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4.3 Phosphate buffer solution, pH 7

Potassium dihydrogen phosphate KH_2PO_4 A.R.	5.5 g
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<i>di</i> Potassium hydrogen phosphate K_2HPO_4 A.R.	13.6 g
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Distilled water to	1000 ml
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4.4 Sterile physiological saline.**4.5 Pure methanol.****4.6 40 % (v/v) methanol.****4.7 Mixture of phosphate buffer solution (4.2)/ pure methanol: 60/40 by volume.****4.8 Standard substance: tylosin of known activity.****5. Standard solutions**

Dry the standard substance (4.8) for 3 hours at 60 °C in a vacuum oven (5 mm of mercury). Weigh 10 to 50 mg into a graduated flask, dissolve in 5 ml of methanol (4.5) and dilute the solution with the phosphate buffer solution, pH 7 (4.3), to obtain a tylosin-base concentration of 1000 µg per ml.

Prepare a standard working solution S_8 containing 2 µg per ml of tylosin base from this stock solution by diluting with the mixture (4.7).

Then prepare by successive dilutions (1 + 1), using the mixture (4.7), the following concentrations:

S_4	1 µg/ml
S_2	0.5 µg/ml
S_1	0.25 µg/ml

6. Extraction

For concentrates, take a 10 g test sample; for premixes and feedingstuffs, a 20 g test sample. Add 60 ml of phosphate buffer solution, pH 8 (4.2), previously heated to 80 °C, and homogenize for 2 minutes (domestic mixer, Ultra-turrax, etc.).

Leave to stand for 10 minutes, add 40 ml of methanol (4.5) and homogenize for 5 minutes. Centrifuge the extract and dilute an aliquot part with the mixture (4.7) to obtain a presumed tylosin concentration of 2 µg per ml (= U_8). Then prepare the concentrations U_4 , U_2 and U_1 by successive dilutions (1 + 1) using the mixture (4.7).

For contents of less than 10 ppm, evaporate the extract until dry in a rotary evaporator at 35 °C and dissolve the residue in 40 % methanol (4.6).

7. Determination method**7.1 Inoculation of the culture medium**

Inoculate at 48 to 50 °C the basic medium for the determination (4.1), adjust to pH 8.0, with the bacteria suspension (3.2).

7.2 Preparation of the trays

Diffusion on agar is carried out in trays using 4 concentrations of the standard solution (S_8 , S_4 , S_2 , S_1) and 4 concentrations of the extract (U_8 , U_4 , U_2 , U_1). The 4 concentrations of standard solution and of extract must be placed in each tray.

Choose trays, therefore, which are large enough to allow at least 8 holes 10 to 13 mm in diameter to be made in the agar medium. Calculate the quantity of inoculated culture medium (7.1) needed to provide a uniform covering approximately 2 mm thick. The test should preferably be carried out on flat trays consisting of glass plates filled with a perfectly level aluminium or plastic ring, 200 mm in diameter and 20 mm high.

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Pipette into the holes accurately measured quantities of between 0.10 and 0.15 ml of antibiotic solution, depending on the diameter of the holes.

For each sample repeat the diffusion at least 4 times with each concentration so that each determination comprises an evaluation of 32 inhibition zones.

7.3 Incubation

Incubate the trays overnight at 35 to 37 °C.

8. Evaluation

Measure the diameter of the inhibition zones, preferably by projection. Record the measurements on semi-logarithmic paper, plotting the logarithm of the concentrations against the diameter of the inhibition zones. Trace the lines of the standard solution and of the extract. Provided there is no interference the two lines will be parallel.

The logarithm of the relative activity is calculated by using the following formula:

$$\frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \cdot 0.602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

Real activity = presumed activity × relative activity.

9. Repeatability

The difference between the results of two parallel determinations carried out on the same sample must not exceed 10% in relative value.

▼M2**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 ⁽²⁾

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

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

⁽²⁾ Other methods may be used provided that it has been established that they give similar bacterial suspensions.

▼ **M2**

chloride solution (4.3). This suspension may be kept for one week at about 4 °C.

4. Culture media and reagents4.1. *Culture and assay medium* ^(*)

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.

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 solutions6.1. *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).

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

▼ **M2**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:

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

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

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

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

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.

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.

▼ M2

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:

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

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

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.

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

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

For three levels

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

or

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

Activity of sample extract = activity of relevant standard $\times A$

$$(u_8 = s_8 \times A)$$

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 virginiamycin per kilogram of feeding-stuff.

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 virginiamycin 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.