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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)

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.

The general provisions set out in Part 1 (Introduction) of the Annex to the First Commission Directive No 71/250/EEC of 15 June 1971 establishing Community methods of analysis for the official control of feedingstuffs shall be applicable to the methods described in Annex I to this Directive.

Article 2

The Member States shall require that analyses for official controls of feedingstuffs, for the purpose of detecting and identifying antibiotics of the tetracycline group and also for determining the levels of chlortetracycline, oxytetracycline, tetracycline, oleandomycin, tylosin, and virginiamycin in feedingstuffs, be carried out using the methods described in Annex II to this Directive.

The general provisions set out in Part 1 (Introduction) of the Annex to the First Commission Directive No 71/250/EEC of 15 June 1971, with the exception of those concerning the preparation of the sample for analysis, shall be applicable to the methods described in Annex II to this Directive.

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.

Article 4

This Directive is addressed to the Member States.

Done at Brussels, 27 April 1972.

For the Commission

The President

S. L. MANSHOLT

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[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.

ANNEX I

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, with the exception of those feedingstuffs which contain beet chips, beet pulp, dried beet tops or leaves, potato pulp, dehydrated yeast, products rice in inulin (e.g. chips and meal of Jerusalem artichokes) or greaves.

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.

3.2 1·128 % (w/v) hydrochloric acid.

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

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 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, homogenize 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.

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), homogenize 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, homogenize, filter and measure the optical rotation as indicated in the 2nd and 3rd paragraphs of 5.2.

6. Calculation of results

The starch content as a percentage of the sample is calculated as follows:

6.1 *Measurement by polarimetry*

$$\text{Percentage of starch} = \frac{2000\,(P - P')}{[\alpha]^{20°}_{D}}$$

where:

P = total optical rotation in degrees;

P' = optical rotation in degrees of substances soluble in 40 % ethanol;

$[\alpha]^{20°}_{D}$ = specific optical rotation of the pure starch. The conventionally accepted values for this factor are the following:

+ 185·9°: rice starch,

+ 195·4°: 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 saccharimetry*

$$\text{Percentage of starch} = \frac{2000}{[\alpha]^{20°}_{D}} \cdot \frac{(2\,N \cdot 0{\cdot}665)\,(S - S')}{} = \frac{26{\cdot}6\,N\,(S - S')}{[\alpha]^{20°}_{D}}$$

where:

S = total optical rotation in saccharimetric degrees;

S' = optical rotation in saccharimetric degrees of substances soluble in 40 % ethanol;

N = weight in g of sucrose in 100 ml of water giving an optical rotation of 100 saccharimetric degrees in a 200 mm tube. The weight varies as follows according to the type of saccharimeter used:
16·29 g for French saccharimeters,
26·00 g for German saccharimeters,
20·00 g for other saccharimeters;

$[\alpha]^{20°}_{D}$ = specific optical rotation of the 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 starch contents of less than 40 %, and 1 %, in relative value, for starch contents of 40 % or more.

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 ab 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.

2. DETERMINATION OF CRUDE PROTEIN

1. Purpose and scope

This method makes it possible to determine conventionally 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 wet combustion. The acid solution is alkalized with a sodium hydroxide solution. The ammonia released is removed by distillation and collected in a measured quantity of sulphuric acid, the excess of which is titrated with a solution of sodium hydroxide.

3. Reagents

3.1 Potassium sulphate A.R.

3.2 Catalyst: cupric oxide CuO A.R. or crystallized cupric sulphate $CuSO_4 \cdot 5H_2O$ A.R. or mercury or mercuric oxide HgO A.R.

3.3 Granulated zinc A.R.

3.4 Sulphuric acid A.R., d: 1·84.

3.5 Sulphuric acid 0·1 N.

3.6 Sulphuric acid 0·5 N.

3.7 Methyl red indicator: dissolve 300 mg of methyl red in 100 ml of 95 to 96 % (v/v) ethanol.

3.8 40 % solution (w/v) of sodium hydroxide.

3.9 Sodium hydroxide solution 0·1 N.

3.10 Sodium hydroxide solution 0.25 N.

3.11 Saturated solution of sodium sulphide A.R.

3.12 8 % solution (w/v) of sodium thiosulphate, $Na_2S_2O_3 \cdot 5H_2O$ A.R.

3.13 Granulated pumice stone, washed in hydrochloric acid and ashed.

4. Apparatus

Apparatus for digestion by combustion and for distillation by the Kjeldahl method (see observation 7.1).

5. Procedure

5.1 *Digestion*

Weight 1 g of the sample to the nearest mg and place in the flask of the digestion apparatus. Add 10 g of potassium sulphate (3.1), an appropriate quantity of catalyst (3.2) (0·3 to 0·4 g of cupric oxide or 0·9 to 1·2 g of cupric sulphate or a drop of mercury or 0·6 to 0·7 g of mercuric oxide), 25 ml of sulphuric acid (3.4) and a few granules of pumice stone (3.13). Homogenize. Heat the flask moderately at first, shaking from time to time, until the mass has carbonized and the foam has disappeared; then heat more intensively until the liquid is boiling steadily. Prevent the sides from becoming overheated and organic particles from sticking to them. When the solution becomes clear and colourless (or light green if a copper-based catalyst is used), continue to boil for another hour, then leave to cool.

5.2 *Distillation*

Carefully add 250 to 350 ml of water, stirring all the while to dissolve the sulphates completely; leave to cool. 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 sulphuric acid 0·1 N (3.5) or 0·5 N (3.6) depending on the presumed nitrogen content (see observation 7.2), and add a few drops of methyl red indicator (3.7).

Connect the 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 7.3). Slowly pour 100 ml of 40 % sodium hydroxide solution (3.8) into the flask through the dropping funnel. If a mercury-based catalyst has been used, also add either 10 ml of sodium sulphide solution (3.11), or 25 ml of sodium thiosulphate solution (3.12).

Heat the flask in such a way that approximately 150 ml of liquid is distilled in 30 minutes. At the end of this time, check the pH of the resulting distillate with litmus paper. If the reaction is alkaline, continue distillation. Discontinue when the distillate becomes neutral to litmus paper. During distillation keep the colouration under observation and shake the contents of the collecting flask from time to time. If the liquid turns yellow, immediately add an exactly measured volume of sulphuric acid 0·1 N (3.5) or 0·5 N (3.6).

5.3 *Titration*

In the collecting flask titrate the excess sulphuric acid with sodium hydroxide solution 0·1 N (3.9) or 0·25 N (3.10), depending on the normality of the sulphuric acid used, until the colour turns pale yellow.

5.4 *Verification of the method*

To establish whether the reagents are free from nitrogen, carry out a blank test (distillation and titration) omitting the sample to be analyzed. To check the accuracy of the method, carry out the analysis (digestion, distillation and titration) on 1·5 to 2·0 g of acetanilide A.R. (m.p. 114 °C; % N: 10·36) in the presence of 1 g of nitrogen-free sucrose; 1 g of acetanilide consumes 14·80 ml of sulphuric acid 0·5 N.

6. Calculation of results

Determine the volume of sulphuric acid consumed. 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:

— 0·2, in absolute value, for crude protein contents of less than 20 %;

— 1·0 %, in relative value, for contents of not less than 20 % and not more than 40 %;

— 0·4, in absolute value, for contents of more than 40 %.

7. Observations

7.1 Certain apparatus requiring transference between digestion and distillation may be used. If such apparatus is used, the transfer must be carried out without loss.

7.2 For products with a low nitrogen content, the volume of sulphuric acid 0·1 N 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.

7.3 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 sides of the condenser so that is does not mix with the acid solution.

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. Procedure

5.1 *Preparation of solution* (see observation 7.2)

Weigh 2 g of the sample to the mearest 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 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 established 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 μmole of tyrosine stained in the same manner.

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 ($Na_2WO_4 . 2H_2O$), 25 g of sodium molybdate ($Na_2MoO_4 . 2H_2O$) 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 ($Li_2SO_4 . 2H_2O$), 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

[1] Determine the nitrogen content by the semi-micro Kjeldahl method (theoretical content: 17·7 % of nitrogen).

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 °C ± 0·1 °C by ultrathermostat.

4.2 Spectrophotometer.

4.3 Chronometer, accuracy: 1 second.

4.4 pH-meter.

5. Procedure

5.1 *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 °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 °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 °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 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.

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 necessaty, 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.

Substract 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.

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:

free gossypol: $E^{1\%}_{1\,cm} = 625$

total gossypol: $E^{1\%}_{1\,cm} = 600$

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

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

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).

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.

ANNEX II

1. DETECTION AND IDENTIFICATION OF ANTIBIOTICS OF THE TETRACYCLINE GROUP

1. Purpose and scope

This method makes it possible to detect and identify antibiotics of the tetracycline group in feedingstuffs containing at least 0.1 ppm of antibiotics, in concentrates and in premixes.

2. Principle

The sample is extracted with a mixture of methanol and hydrochloric acid. The extract and reference solutions for comparison are subjected to ascending paper chromatography. The antibiotics are detected and identified by comparing their Rf values with those of the standard substances, either by fluorescence in UV light (high antibiotic contents) or by bioautography on an agar medium inoculated with *B. cereus*.

3. Reagents and culture medium

3.1 *Buffer solution, pH 3.5*

Citric acid monohydrate A.R.	10·256 g
*di*Sodium hydrogen phosphate $Na_2HPO_4 \cdot 2H_2O$ A.R.	7·45 g
Acetone A.R.	300 ml
Distilled water to	1000 ml

3.2 *Phosphate buffer solution, pH 5.5*

Potassium dihydrogen phosphate KH_2PO_4 A.R.	130·86 g
*di*Sodium hydrogen phosphate $Na_2HPO_4 \cdot 2H_2O$ A.R.	6·947 g
Distilled water to	1000 ml

3.3 Eluent I: Mixture of pure nitromethane/pure chloroform/1,3-dichloropropan-2-ol: 20/10/1·5 by volume. prepare immediately before use.

3.4 Eluent II: Mixture of pure nitromethane/pure chloroform/2-picoline: 20/10/3 by volume. Prepare immediately before use.

3.5 Mixture of pure methanol/hydrochloric acid (d: 1·19): 98/2 by volume.

3.6 Hydrochloric acid 0·1 N.

3.7 Ammonia, d: 0·91.

3.8 Standard substances: chlortetracycline, oxytetracycline, tetracycline, the activity of which is expressed in terms of hydrochloride.

3.9 *Micro-organism: B. cereus ATCC No 11.778*

Maintenance of the parent strain, preparation of the spore suspension and inoculation of the culture medium: follow the directions given in 3.1 and 3.2 of the method for the determination of chlortetracycline, oxytetracycline and tetracycline contents by diffusion on agar which is described in Part 2 of this Annex.

3.10 *Culture medium*[1]

Glucose	1 g
Tryptic peptone	10 g
Meat extract	1·5 g
Yeast extract	3 g
Agar	20 g
Distilled water to	1000 ml

Adjust the pH to 5·8 immediately before use.

3.11 0·1 % (w/v) 2,3,5-triphenyltetrazolium chloride solution and 5 % (w/v) glucose solution.

[1] Any commercial culture medium of similar composition and giving the same results may be used.

4. Apparatus

4.1 Apparatus for ascending paper chromatography (height of paper: 25 cm). Schleicher and Schüll paper (2040b or 2043b) or equivalent.

4.2 Centrifuge.

4.3 Incubator set at 30 °C.

4.4 U.V. lamp for the detection of fluorescence.

4.5 Glass plates approximately 20 × 30 cm for bioautography.

5. Standard solutions

5.1 *Stock solutions*

Using hydrochloric acid (3.6), prepare from the standard substances (3.8) solutions with concentrations corresponding to 500 μg per ml of chlortetracycline-HCl, of oxytetracycline-HCl, and of tetracycline-HCl.

5.2 *Reference solutions for detections by UV light*

Dilute the solutions (5.1) with the phosphate buffer solution (3.2) to obtain solutions with concentrations corresponding to 100 μg per ml of chlortetracycline-HCl, of oxytetracycline-HCl and of tetracycline-HCl.

5.3 *Reference solutions for detection by bioautography*

Dilute the solutions (5.1) with the phosphate buffer solution (3.2) to obtain solutions with concentrations corresponding to 5 μg per ml of chlortetracycline-HCl, of oxytetracycline-HCl and of tetracycline-HCl.

6. Extraction

When the presumed antibiotic content is less than 10 ppm, either the homogenized sample or the finest fraction separated by sieving may be used, since the antibiotics are to be found mainly in this fraction.

Suspend the sample in the mixture (3.5) and centrifuge. Collect the supernatant liquid and use it directly or dilute, if necessary, with the mixture (3.5) to obtain antibiotic concentrations of approximately 100 μg per ml (6.1) and 5 μg per ml (6.2).

7. Detection and identification

7.1 *Chromatography*

Immerse the paper in the buffer solution, pH 3·5 (3.1). Remove the excess liquid by pressing the paper between sheets of dry filter paper. Then place volumes of 0·01 ml of the reference solutions (5.2 and 5.3) and of the extract (6.1 and 6.2) on the paper. To give a good separation, the paper must have the correct moisture content; if necessary, leave to dry a little.

Develop by ascending chromatography. Use eluent I (3.3) for detection by bioautography and eluent II (3.4) for detection by UV light. When the solvent front has climbed 15 to 20 cm (approx. 1 hour 30 minutes), stop chromatography and dry the paper.

7.2 *Detection by UV light*

If the antibiotic level is greater than 1 μg per cm^2, after the chromatogram has been treated with ammonia vapours (3.7) golden yellow fluorescent spots will be seen on irradiation under the UV lamp (4.4).

7.3 *Detection by bioautography*

Pour the culture medium (3.10), previously inoculated with *B, cereus* (3.9), into glass plates (4.5) and place the paper on the culture medium. After 5 minutes' contact, detach the paper and place it on another spot in the culture medium, where it will remain during the incubation period. The incubate overnight at 30 °C. If an antibiotic of the tetracycline group is present, light inhibition zones will appear in the cloudy culture medium.

To fix the chromatogram, the solution (3.11) is vaporized on the paper, after incubation.

7.4 *Identification*

The relative Rf values of antibiotics of the tetracycline group are given below. These values may vary slightly according to the quality of the paper and its moisture content:

Chlortetracycline (CTC)	0·60
Tetracycline (TC)	0·40
Oxytetracycline (OTC)	0·20
4-epi-CTC	0·15

4-epi-TC	0·13
4-epi-OTC	0·10

The antibiotic activity of the 'epi' compounds is less than that of the normal compounds.

2. DETERMINATION OF CHLORTETRACYCLINE, OXYTETRACYCLINE AND TETRACYCLINE

A. BY DIFFUSION ON AGAR

1. Purpose and scope

This method makes it possible to determine the levels of chlortetracycline (CTC), oxytetracycline (OTC) and tetracycline (TC) in feedingstuffs, concentrates and premixes where more than 5 ppm are present. Contents of less than 5 ppm may be estimated by graphic interpolation.

2. Principle

For contents of 50 ppm or less, the sample is extracted with dilute formamide. For contents greater than 50 ppm, it is extracted with a mixture of acetone, water and hydrochloric acid, for the determination of CTC, and with a mixture of methanol and hydrochloric acid for the determination of OTC and TC.

The extracts are then diluted and their antibiotic activity determined by measuring the diffusion of the CTC, OTC or TC on an agar medium seeded with *B. cereus*. 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: B. cereus, ATCC No 11.778

3.1 *Maintenance of the parent strain*

Inoculate with *B. cereus* a tube of sloped agar taken from culture medium (4.1) *free from methylene blue and boric acid.* Incubate overnight at approximately 30°C. Keep the culture in a refrigerator and re-inoculate sloped agar with it every 14 days.

3.2 *Preparation of the spore suspension*

Collect the bacteria from a tube of sloped agar (3.1) using 2 to 3 ml of physiological saline (4.5). With this suspension, seed a Roux flask containing 300 ml of culture medium (4.1), *free from methylene blue and boric acid,* with 3 to 4 % agar concentration. Incubate for 3 to 5 days at 28 to 30 °C, then collect the spores in 15 ml of ethanol (4.6) after checking sporulation under a microscope, and homogenize. This suspension will keep in a refrigerator for 5 months or more.

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 antibiotic used, will give the largest possible inhibition zones that are still clear. This quantity is usually between 0·2 and 0·3 ml per 1000 ml. The culture medium is inoculated at between 50 and 60 °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
'Tween 80'	1 ml
Phosphate buffer solution, pH 5·5 (4.2)	10 ml
5 % (w/v) boric acid solution	15 ml
0·5 % solution of methylene blue ethanol	4 ml
Distilled water to	1000 ml

Adjust to pH 5·8 before use.

[1] Any commercial culture medium of similar composition and giving the same results may be used.

4.2 *Phosphate buffer solution, pH 5·5*

Potassium dihydrogen phosphate KH_2PO_4 A.R.	130·86 g
*di*Sodium hydrogen phosphate $Na_2HPO_4 \cdot 2H_2O$ A.R.	6·947 g
Distilled water to	1000 ml

4.3 Phosphate buffer solution, pH 5·5, diluted to 1/10.

4.4 *Phosphate buffer solution, pH 8*

Potassium dihydrogen phosphate KH_2PO_4 A.R.	1·407 g
*di*Sodium hydrogen phosphate $Na_2HPO_4 \cdot 2H_2O$ A.R.	57·539 g
Distilled water to	1000 ml

4.5 Sterile physiological saline.

4.6 20 % (v/v) ethanol.

4.7 Hydrochloric acid 0·1 N.

4.8 70 % (v/v) formamide: prepare fresh before use and adjust the pH to 4·5 using sulphuric acid approximately 2 N.

4.9 Mixture of pure acetone/water/hydrochloric acid (d: 1·19): 65/33/2 by volume.

4.10 Mixture of pure methanol/hydrochloric acid (d: 1·19): 98/2 by volume.

4.11 Standard substances: CTC, OTC, TC, the activity of which is expressed in terms of hydrochloride.

5. Standard solutions

5.1 *Chlortetracycline*

Using hydrochloric acid (4.7), prepare from the standard solution (4.11) a stock solution with a concentration corresponding to 500 μg per ml of chlortetracycline-HCl. This solution will keep for one week in a refrigerator.
From this stock solution, prepare a standard working solution S_8 with a concentration corresponding to 0·2 μg per ml of chlortetracycline-HCl. Dilution is carried out using the phosphate buffer solution, pH 5·5, diluted to 1/10 (4.3), to which 0.01 % of amido black has been added[1].

Then prepare by successive dilutions (1 + 1), using the buffer solution (4.3), the following concentrations:

S_4	0·1 μg/ml
S_2	0·05 μg/ml
S_1	0·025 μg/ml

5.2 *Oxytetracycline*

Proceeding as indicated in 5.1, prepare, from a stock solution with a concentration corresponding to 400 μg per ml of oxytetracycline-HCl, a standard working solution S_8 containing 1·6 μg per ml of oxytetracycline-HCl, and the following concentrations:

S_4	0·8 μg/ml
S_2	0·4 μg/ml
S_1	0·2 μg/ml

5.3 *Tetracycline*

Proceeding as indicated in 5.1, prepare, from a stock solution with a concentration corresponding to 500 μg per ml of tetracycline-HCl, a standard working solution S_8 containing 1·0 μg per ml of tetracycline-HCl and the following concentrations:

S_4	0·5 μg/ml
S_2	0·25 μg/ml
S_1	0·125 μg/ml

6. Extraction

6.1 *Contents of 50 ppm or less*

To test sample add formamide (4.8) in the quantities indicated in the table below. Shake for 30 minutes on a shaking platform. Then dilute immediately with the phosphate buffer solution (4.3) according to the indications given in the table below to obtain the concentration U_8. The formamide concentration of this solution must not exceed 40 %.

[1] Amido black is used to make evident the inhibition zones of the standard solutions (blue rings).

Centrifuge or decant to obtain a clear solution. Then prepare the concentrations U_4, U_2 and U_1 by successive dilutions (1 + 1) using the phosphate buffer solution (4.3).

Antibiotic	CTC		OTC		TC	
Presumed content in ppm	10	50	10	50	10	50
Test sample in g	10	10	24	9.6	20	10
ml of formamide (4.8)	100	100	80	100	80	100
ml of phosphate buffer solution (4.3)	dil. 1 : 5 (a)	dil. 1 : 25 (b)	70	200	120	dil. 1 : 5 (a)
U_8 concentration in μg/ml	0·2	0·2	1·6	1·6	1.0	1·0

(a) Take 20 ml of extract and make up to 100 ml in a graduated flask with the buffer solution.
(b) Take 4 ml of extract and make up to 100 ml in a graduated flask with the buffer solution.

6.2 *Contents greater than 50 ppm*

6.2.1 *Chlortetracycline*

To a test sample of 2 to 10 g, depending on the presumed antibiotic content of the sample or its manufacturer's guarantee, add 20 times its volume of mixture (4.9). Shake for 30 minutes on a shaking platform. The pH must remain below 3 during extraction; if necessary, readjust to pH 3 (using 10 % acetic acid for mineral compounds). Take an aliquot part of the extract and adjust the pH to 5·5 using the phosphate buffer solution, pH 8 (4.4) in the presence of bromocresol green (turning from yellow to blue). Dilute, using the phosphate buffer solution, pH 5·5, diluted to 1/10 (4.3), to obtain the concentration U_8 (see 6.1).

Then prepare the concentrations U_4, U_2 and U_1 by successive dilutions (1 + 1) using the phosphate buffer solution (4.3).

6.2.2 *Oxytetracycline and tetracycline*

Proceed as indicated in 6.2.1, using the mixture (4.10) instead of the mixture (4.9).

7. Determination method

7.1 *Inoculation of the culture medium*

Inoculate at 50 to 60 °C the basic medium for the determination (4.1) with the spore 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 extract and of standard solution 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 trays consisting of flat glass plates fitted with a perfectly level aluminium or plastic ring, 200 mm in diameter and 20 mm high.

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 for approximately 18 hours at 28 to 30 °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:

$$\frac{(U_1 + U_2 + U_4 \times U_8 - S_1 - S_2 - S_4 - S_8) \cdot 0{\cdot}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

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.

B. BY TURBIDIMETRY

1. Purpose and scope

This method makes it possible to determine the levels of chlortetracycline (CTC), oxytetracycline (OTC) and tetracycline (TC) where concentrations are greater than 1 g per kg, provided there is no interference from other substances clouding the extracts. This method is quicker than diffusion on agar.

2. Principle

For the determination of CTC, the sample is extracted with a mixture of acetone, water and hydrochloric acid, and with a mixture of methanol and hydrochloric acid for the determination of OTC and TC.

The extracts are then diluted and their antibiotic effect determined by measuring the light transmission of a culture medium which has been seeded with *Staphylococcus aureus* and to which the antibiotic has been added. The light transmission depends on the antibiotic concentration.

3. Micro-organism: Staphylococcus aureus K 141[1]

3.1 *Maintenance of the parent strain*

Inoculate with *S. aureus* a tube of sloped agar taken from the culture medium (4.1), *to which 1·5 to 3 % of agar has been added* (depending on the quality). Incubate overnight at 37 °C. Keep the culture in a refrigerator and re-inoculate sloped agar with it every 4 weeks. At the same time prepare sub-cultures for laboratory use.

3.2 *Preparation of the inoculum*

24 hours before use, re-inoculate sloped agar with a sub-culture and incubate overnight at 37 °C. Suspend all the culture contained in a tube of agar in approximately 2 ml of the basic medium (4.1), then transfer the suspension under sterile conditions into approximately 100 ml of the same basic medium (4.1). Incubate in a water bath at 37 °C until the growth of the strain enters its logarithmic phase (1 hour 30 minutes to 2 hours).

4. Culture media and reagents

4.1 *Basic medium for the determination*[2]

Peptone	5 g
Yeast extract	1·5 g
Meat extract	1·5 g
Sodium chloride	3·5 g
Glucose	1·0 g
Potassium dihydrogen phosphate KH_2PO_4 A.R.	1·32 g
di Potassium hydrogen phosphate K_2HPO_4 A.R.	3·68 g
Distilled water to	1 000 ml

pH after sterilization: 6·8 to 7·0.

4.2 *Phosphate buffer solution, pH 4·5*

Potassium dihydrogen phosphate KH_2PO_4 A.R.	13·6 g
Distilled water to	1 000 ml

[1] This strain, isolated by the LUFA at Kiel, grows more rapidly than *S. aureus ATCC 6538 P*.
[2] Any commercial culture medium of similar composition and giving the same results may be used.

4.3 Hydrochloric acid 0·1 N.

4.4 Mixture of pure acetone/water/hydrochloric acid (d: 1·19): 65/33/2 by volume.

4.5 Mixture of pure methanol/hydrochloric acid (d: 1·19): 98/2 by volume.

4.6 Approximately 10 % (w/v) formaldehyde solution.

4.7 Standard substances: CTC, OTC, TC, the activity of which is expressed in terms of hydrochloride.

5. Standard solution

Using hydrochloric acid (4.3), prepare from the standard substance (4.7) a stock solution with a concentration corresponding to 400 to 500 μg per ml of CTC-HCl, OTC-HCl or TC-HCl. This solution will keep for one week in a refrigerator.

6. Extraction

6.1 *Chlortetracycline*

Place a 1 to 2 g test sample in a 200 or 250 ml graduated flask. Add approximately 100 ml of the mixture (4.4) and shake for 30 minutes on a shaking platform. Make up to volume with the phosphate buffer solution, pH 4·5 (4.2). Homogenize and leave to settle.

6.2 *Oxytetracycline and tetracycline*

Place a 1 to 2 g test sample in a 200 or 250 ml graduated flask. Add approximately 100 ml of the mixture (4.5) and shake for 30 minutes on a shaking platform. Make up to volume with the phosphate buffer solution, pH 4·5 (4.2). Homogenize and leave to settle.

7. Determination method

7.1 *Preparation of the standard series and of the extract*

Dilute the standard solution (5) and the extract (6) with the phosphate buffer solution, pH 4·5 (4.2), to obtain a series of concentrations. For each determination, a calibration curve is drawn from the respective concentration, permitting the interpolation of at least two values relating to the extract. The dilutions should be chosen according to the conditions under which the strain is grown, which may vary from one laboratory to another. The procedure is generally the following:

7.1.1 *Chlortetracycline*

Dilute the standard solution (5) with the phosphate buffer solution (4.2) to obtain a standard working solution with a concentration corresponding to 0·2 μg per ml of CTC-HCl. Then, using the phosphate buffer solution (4.2), prepare in test tubes, as indicated below, 6 dilutions, each dilution in duplicate.

ml of standard working solution	ml of phosphate buffer solution (4.2)	Concentration of CTC-HCl (μg/ml)
0·7	0·3	0·14
0·6	0·4	0·12
0·55	0·45	0·11
0·45	0·55	0·09
0·4	0·6	0·08
0·3	0·7	0·06

Dilute the extract (6.1) with the phosphate buffer solution (4.2) to obtain a presumed CTC-HCl concentration of 0·12 μg per ml. Place 1 ml of this solution in each of 2 tubes, and 0·75 ml (— 0·09 μg) in each of 2 other tubes. Make the volume of the latter two tubes up to 1 ml with the phosphate buffer solution (4.2).

7.1.2 *Oxytetracycline and tetracycline*

Dilute the standard solution (5) with the phosphate buffer solution (4.2) to obtain a standard working solution with a concentration corresponding to 0·6 μg per ml of OTC-HCl or of TC-HCl. Then, using the phosphate buffer solution (4.2), prepare in test tubes, as indicated below, 7 dilutions, each dilution in duplicate.

ml of standard working solution	ml of phosphate buffer solution (4.2)	Concentration of OTC-HCl or TC-HCl (μg/ml)
0·9	0·1	0·54
0·8	0·2	0·48
0·7	0·3	0·42
0·6	0·4	0·36
0·4	0·6	0·24
0·3	0·7	0·18
0·2	0·8	0·12

Dilute the extract (6.2) with the phosphate buffer solution (4.2) to obtain a presumed OTC-HCl or TC-HCl concentration of 0·48 μg per ml. Place 1 ml of this solution in each of 2 tubes, and 0·5 ml (= 0·24 μg) in each of 2 other tubes. Makes the volume of the latter two tubes up to 1 ml with the phosphate buffer solution (4.2).

7.2 *Inoculation of the culture medium*

Inoculate the basic medium for the determination (4.1) with the inoculum (3.2) to obtain with the photometer at 590 nm 85 % light transmission in a 5 cm cell or 92 % transmission in a 2 cm cell, the apparatus being set at 100 % transmission on the non-inoculated basic medium (4.1).

7.3 *Seeding*

Place 9 ml of the inoculated culture medium (7.2) in each tube (7.1.1 or 7.1.2). The tubes must be filled under clean but not necessarily sterile conditions.

7.4 *Incubation*

Incubation must be carried out in a water bath whose temperature is kept uniform at 37 °C ± 0·1 °C by stirring. The incubation period chosen (generally 2 hours 30 minutes to 3 hours) must be such that it will be possible to trace transmission curves with gradients suitable for accurate measurement. Then block further growth by rapidly injecting 1 ml of formaldehyde solution (4.6) into each tube.

7.5 *Measurement of growth*

Measure the transmissions with the photometer at 590 nm, setting the apparatus at 100 % transmission on the clearest standard solution (corresponding to the highest antibiotic content). Since the different tubes will show slight differences of turbidity, at least 2 cm, and preferably 5 cm, cells should be used.

8. Calculation of results

Trace the calibration curve on millimetre graph paper by plotting the photometric transmissions against the antibiotic concentrations. Interpolate on the curve the transmission values of the extract. Calculate the antibiotic content of the sample.

9. Repeatability

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

3. DETERMINATION OF OLEANDOMYCIN

— by diffusion on agar —

1. Purpose and scope

This method makes it possible, even in the presence of tetracyclines, to determine the oleandomycin content of feedingstuffs, concentrates and premixes, where more than 0.5 ppm are present.

2. Principle

The sample is extracted with a dilute methanol solution of tri(hydroxymethylamino)methane. After centrifuging, the extract is diluted and its antibiotic activity determined by measuring the diffusion of the oleandomycin on an agar medium seeded with *B. cereus*. 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: B. cereus K 250 TR[1] (resistant to tetracyclines)

3.1 *Maintenance of the parent strain*

Inoculate with *B. cereus* a tube of sloped agar taken from the culture medium (4.1) to which 100 μg per 5 ml of oxytetracycline has been added. Incubate overnight at approximately 30 °C. Keep the culture in a refrigerator and re-inoculate sloped agar with it every 4 weeks.

3.2 *Preparation of the spore suspension*

Collect the bacteria from a tube of sloped agar (3.1) using approximately 3 ml of physiological saline (4.3). With this suspension, seed a Roux flask containing 300 ml of culture medium (4.1) *which has a 3 to 4 % agar concentration.* Incubate for 3 to 5 days at 28 to 30 °C, then collect the spores in 15 ml of ethanol (4.4) after checking sporulation under a microscope, and homogenize. This suspension will keep in a refrigerator for 5 months or more.

By preliminary tests on plates with the basic medium for the determination (4.2), establish the quantity of inoculum which, for the different concentrations of oleandomycin used, will give the largest possible inhibition zones that are still clear. This quantity is usually between 0·1 and 0·2 ml per 1000 ml. The culture medium is inoculated at 60 °C.

4. Culture media and reagents

4.1 *Medium for maintenance of the parent strain*[2]

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 the pH to 6·5 immediately before use.

4.2 *Basic medium for the determination*[2]

Medium (4.1) adjusted to pH 8·8.

4.3 Sterile physiological saline.

4.4 20 % (v/v) ethanol.

4.5 Pure methanol.

4.6 0·5 % (w/v) solution of tri(hydroxymethylamino)methane A.R.

4.7 *Extraction solution*

Pure methanol	50 ml
Distilled water	50 ml
Tri(hydroxymethylamino)methane A.R.	0·5 g

4.8 Standard substance: oleandomycin of known activity.

5. Standard solution

Dissolve some of the standard substance (4.8) in 5 ml of methanol (4.5) and dilute with the solution (4.6) to obtain an oleandomycin concentration of 100 μg per ml.

From this stock solution, prepare a standard working solution S_8 containing 0.1 μg per ml of oleandomycin by diluting with the solution (4.6). Then prepare by successive dilutions (1 + 1), using the solution (4.6), the following concentrations:

S_4	0·05 μg/ml
S_2	0·025 μg/ml
S_1	0·0125 μg/ml

6. Extraction

Take a test sample of 2 to 10 g, depending on the presumed oleandomycin content of the sample, add 100 ml of the solution (4.7) and shake for 30 minutes on a shaking platform.

[1] Strain isolated by the LUFA at Kiel.
[2] Any commercial culture medium of similar composition and giving the same results may be used.

Centrifuge, take an aliquot part of the extract and dilute with the solution (4.6) to obtain a presumed oleandomycin concentration of 0·1 μg per ml (= U_8). Then prepare the concentrations U_4, U_2 and U_1 by successive dilutions (1 + 1) using the solution (4.6).

7. Determination method

7.1 *Inoculation of the culture medium*

Inoculate at 60 °C the basic medium for the determination (4.2) with the spore 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 four 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 trays consisting of flat glass plates fitted with a perfectly level aluminium or plastic ring 200 mm in diameter and 20 mm high.

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 for approximately 18 hours at 28 to 30 °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:

$$\frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \cdot 0{\cdot}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

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.

4. DETERMINATION OF TYLOSIN

— by diffusion on agar —

1. Purpose and scope

This method makes it possible to determine the tylosin content of feedingstuffs, 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
*di*Potassium hydrogen phosphate K_2HPO_4 A.R.	16·730 g
Distilled water to	1000 ml

4.3 *Phosphate buffer solution, pH 7*

Potassium dihydrogen phosphate KH_2PO_4 A.R.	5·5 g
di Potassium hydrogen phosphate K_2HPO_4 A.R.	13·6 g
Distilled water to	1000 ml

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 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.).

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

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 fitted with a perfectly level aluminium or plastic ring, 200 mm in diameter and 20 mm high.

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:

$$\frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \cdot 0{\cdot}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

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.

5. DETERMINATION OF VIRGINIAMYCIN

— by diffusion on agar —

1. Purpose and scope

This method makes it possible to determine the virginiamycin content of feedingstuffs, concentrates and premixes where more than 2 ppm are present.

2. Principle

The sample is extracted with a 'Tween 80' methanol solution. After centrifuging or filtering, the extract is diluted and its antibiotic activity determined by measuring the diffusion of the virginiamycin 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 *S. Lutea* a tube of sloped agar taken from the culture medium (4.1). Incubate overnight at approximately 35 °C. Keep the culture in a refrigerator and re-inoculate sloped agar with it every 14 days.

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 (4.3). With this suspension, seed a Roux flask containing 250 ml of the culture medium (4.1). Incubate for 24 hours at 35 °C, then collect the bacteria in 25 ml of physiological saline (4.3). 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 virginiamycin 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 reagent

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 the pH to 6.5 before use.

4.2 *Phosphate buffer solution, pH 6*

Potassium dihydrogen phosphate KH_2PO_4 A.R.	8·0 g
*di*Potassium hydrogen phosphate K_2HPO_4 A.R.	2·0 g
Distilled water to	1000 ml

4.3 Sterile physiological saline.

4.4 Pure methanol.

4.5 Mixture of phosphate buffer solution (4.2)/pure methanol: 80/20 by volume.

4.6 0·5 % (w/v) 'Tween 80' methanol solution.

4.7 Standard substance: virginiamycin of known activity.

5. Standard solutions

Prepare a methanol solution of the standard substance (4.7) containing 800 μg per ml of virginiamycin. From this stock solution prepare a standard working solution S_8 containing 1 μg per ml of virginiamycin by diluting with the mixture (4.5). Then prepare by successive dilutions (1 + 1), using the mixture (4.5), the following concentrations:

S_4	0·5 μg/ml
S_2	0·25 μg/ml
S_1	0·125 μg/ml

6. Extraction

6.1 *Products with a virginiamycin content of 50 ppm or less*

Take a test sample of 10 to 20 g, add 100 ml of the solution (4.6) and shake for 30 minutes on a shaking platform. Centrifuge or filter, take 20 ml of the clear solution and evaporate until dry in a rotary evaporator. Dissolve the residue in 20 ml or more of the mixture (4.5) to obtain a presumed virginiamycin concentration of 1 μg per ml (= U_8). Then prepare the concentrations U_4, U_2 and U_1 by successive dilutions (1 + 1) using the mixture (4.5).

[1] Any commercial culture medium of similar composition and giving the same results may be used.

6.2 *Products with a virginiamycin content greater than 50 ppm*

Take a test sample of 1 to 10 g, add 100 ml of solution (4.6) and shake for 30 minutes on a shaking platform. Centrifuge or filter, then dilute with the mixture (4.5) to obtain a presumed virginiamycin concentration of 1 μg per ml (= U_8). Then prepare the concentrations U_4, U_2 and U_1 as indicated in 6.1.

7. Determination method

7.1 *Inoculation of the culture medium*

Inoculate the basic medium for the determination (4.1) at 48 to 50 °C with the suspension of bacteria (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 fitted with a perfectly level aluminium or plastic ring, 200 mm in diameter and 20 mm high.

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 for approximately 18 hours at 28 to 30 °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:

$$\frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \cdot 0{\cdot}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

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.