ISSN 0378-6978

Official Journal

of the European Communities

L 257

Volume 24

10 September 1981

English edition

Legislation

Contents

Acts whose titles are printed in light type are those relating to day-to-day management of agricultural matters, and are generally valid for a limited period.

The titles of all other Acts are printed in bold type and preceded by an asterisk.

II

(Acts whose publication is not obligatory)

COMMISSION

FIRST COMMISSION DIRECTIVE

of 28 July 1981

laying down Community methods of analysis for verifying that certain additives used in foodstuffs satisfy criteria of purity

(81/712/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to the Council Directive of 23 October 1962 on the approximation of the laws of the Member States concerning the colouring matters authorized for use in foodstuffs intended for human consumption (1), as last amended by Directive 78/144/EEC (2), and in particular Article 11 (2) thereof,

Having regard to Council Directive 64/54/EEC of 5 November 1963 on the approximation of the laws of the Member States concerning the preservatives authorized for use in foodstuffs intended for human consumption (3), as last amended by Directive 79/40/EEC (4), and in particular Article 8 (2) thereof.

Having regard to Council Directive 70/357/EEC of 13 July 1970 on the approximation of the laws of the Member States concerning the antioxidants authorized for use in foodstuffs intended for human consumption (5), as last amended by Directive 78/143/EEC (6), and in particular Article 5 (2) thereof.

Whereas these provisions lay down that Community methods of analysis shall be established for verifying that these additives satisfy general and specific criteria of purity;

Whereas a first series of methods for which the studies have been completed should now be adopted;

Whereas the measures provided for in this Directive are in accordance with the opinion of the Standing Committee on Foodstuffs,

HAS ADOPTED THIS DIRECTIVE:

Article 1

The Member States shall prescribe that the analyses necessary for verifying that certain additives used in foodstuffs satisfy the general and specific criteria of

(1) OJ No 115, 11. 11. 1962, p. 2645/62.
(2) OJ No L 44, 15. 2. 1978, p. 20.
(3) OJ No 12, 27. 1. 1964, p. 161/64.
(4) OJ No L 13, 19. 1. 1979, p. 50.
(5) OJ No L 157, 18. 7. 1970, p. 31.
(6) OJ No L 44, 15. 2. 1978, p. 18.

purity shall be carried out according to the methods described in Annex II, the scope of which is laid down in Annex I.

Article 2

The Member States shall bring into force the laws, regulations or administrative provisions necessary to comply with this Directive not later than 20 February 1983. They shall forthwith inform the Commission thereof.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 28 July 1981.

For the Commission
Karl-Heinz NARJES
Member of the Commission

ANNEX I

SCOPE OF THE COMMUNITY METHODS OF ANALYSIS FOR VERIFYING THAT CERTAIN ADDITIVES USED IN FOODSTUFFS MEET PURITY CRITERIA

I. INTRODUCTION

. . . . . .

II. COLOURING MATTERS

II.1. Determination of substances extractable with diethyl ether from water-soluble sulphonated organic colouring matters used in foodstuffs using Annex II, method 1.

III. PRESERVATIVES

III.1. Determination of formic acid, formates and of other oxidizable impurities in acetic acid (E 260), potassium acetate (E 261), sodium diacetate (E 262) and calcium acetate (E 263) using Annex II, method 2.

III.2. Determination of non-volatile substances in propionic acid (E 280) using Annex II, method 3.

III.3. Determination of the loss of mass on drying of sodium nitrite (E 250) using Annex II, method 4.

III.4. Limit test for salicylic acid in ethyl *p*-hydroxybenzoate (E 214), ethyl *p*-hydroxybenzoate, sodium salt (E 215), *n*-propyl *p*-hydroxybenzoate (E 216), *n*-propyl *p*-hydroxybenzoate, sodium salt (E 217), methyl *p*-hydroxybenzoate (E 218) and methyl *p*-hydroxybenzoate, sodium salt (E 219) using Annex II, method 5.

III.5. Determination of free acetic acid in sodium diacetate (E 262) using Annex II, method 6.

III.6. Determination of sodium acetate in sodium diacetate (E 262) using Annex II, method 7.

III.7. Limit test for determination of aldehydes in sorbic acid (E 200) in sodium, potassium and calcium sorbates (E 201, E 202, E 203) and in propionic acid (E 280) using Annex II, method 8.

IV. ANTIOXIDANTS

IV.1. Determination of the number of peroxide groups of lecithins (E 322) using Annex II, method 9.

IV.2. Determination of toluene-insoluble substances in lecithins (E 322) using Annex II, method 10.

IV.3. Limit test for reducing substances in sodium, potassium and calcium lactates (E 325, E 326 and E 327) using Annex II, method 11.

IV.4. Determination of volatile acids in orthophosphoric acid (E 338) using Annex II, method 12.

IV.5. Limit test for nitrates in orthophosphoric acid (E 338) using Annex II, method 13.

IV.6. Determination of water-insoluble substances in mono-, di- and tri-sodium orthophosphate and mono-, di- and tri-potassium orthophosphates (E 339(i), E 339(ii), E 339(iii), E 340(i), E 340(ii), E 340(iii)) using Annex II, method 14.

V. GENERAL

V.1. Determination of pH in food additives using Annex II, method 15.

ANNEX II

METHODS OF ANALYSIS RELATING TO THE CRITERIA OF PURITY OF FOOD ADDITIVES

INTRODUCTION

1. Preparation of the analysis sample

1.1. *General*

The mass of the laboratory sample intended for analysis must normally be 50 g unless a larger quantity is required for a specific determination.

1.2. *Sample preparation*

The sample shall be made homogeneous prior to analysis.

1.3. *Preservation*

The prepared sample shall always be kept in an air-tight and moisture-tight container and stored so that deterioration is prevented.

2. Reagents

2.1. *Water*

2.1.1. Wherever mention is made of water for solution, dilution or washing purposes, distilled water, or demineralized water of at least equivalent purity, is intended.

2.1.2. Wherever reference is made to 'solution' or 'dilution' without further indication of a reagent, an aqueous solution is intended.

2.2. *Chemicals*

All chemicals shall be of analytical reagent quality except where otherwise specified.

3. Equipment

3.1. *List of equipment*

The list of equipment contains only those items with a specialized use and items with a particular specification.

3.2. *Analytical balance*

Analytical balance means a balance with a sensitivity of 0·1 mg or greater.

4. Expression of results

4.1. *Results*

The result stated in the official analysis report shall be the mean value of at least two determinations the repeatability of which is satisfactory.

4.2. *Calculation of percentage*

Unless otherwise stated the results shall be expressed as a percentage by mass of the original sample as received at the laboratory.

4.3. *Number of significant figures*

The number of significant figures in the result so expressed shall be governed by the precision of the method.

METHOD 1

DETERMINATION OF SUBSTANCES EXTRACTABLE WITH DIETHYL ETHER FROM WATER-SOLUBLE SULPHONATED ORGANIC COLOURING MATTERS INTENDED FOR FOODSTUFFS

1. **Scope and field of application**

The method determines substances extractable with diethyl ether in water soluble sulphonated organic colouring matters which have not been mixed with any support.

2. **Definition**

Substances extractable with diethyl ether: the content of material as determined by the method specified.

3. **Principle**

Extract the colouring matter with diethyl ether and weigh the extracted residue after evaporation of the ether.

4. **Reagents**

4.1. Diethyl ether, dry, peroxide-free (dried with the aid of freshly calcined calcium chloride).

5. **Apparatus**

5.1. Soxhlet apparatus with flask.

5.2. Desiccator, containing freshly activated silica gel or equivalent desiccant with a water content indicator.

5.3. Analytical balance.

5.4. Oven, thermostatically controlled at 85 ± 2 °C.

6. **Procedure**

Accurately weigh, to the nearest 10 mg, about 10 g of the sample of the colouring matter on a piece of filter paper. Fold the paper, put it into a paper thimble and close the latter with some fat-free cotton wool. Extract for six hours with diethyl ether (4.1) in a Soxhlet extraction apparatus (5.1). Evaporate the ether at as low a temperature as possible. Place

the Soxhlet flask, which has been previously weighed, with the residue in the oven (5.4) at 85 ± 2 °C for 20 minutes to dry. Transfer the flask to a desiccator (5.2), cover with a loose-fitting lid and allow to cool. Weigh the flask and residue.

Repeat the drying and weighing until two successive weighings differ by less than 0·5 mg. Should an increase in mass occur, the lowest recorded reading will be used in the calculation.

7. **Expression of results**

7.1. *Formula and method of calculation*

The content of substances extractable with ether, as a percentage of the sample, is given by:

$$\frac{m_1 \times 100}{m_0}$$

where:

m_1 = mass in grams of the residue after evaporation,

m_0 = initial mass in grams of the sample taken.

7.2. *Repeatability*

The difference between the results of two determinations when carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 20 mg per 100 g of sample.

METHOD 2

DETERMINATION OF FORMIC ACID, FORMATES AND OTHER OXIDIZABLE IMPURITIES IN ACETIC ACID (E 260), POTASSIUM ACETATE (E 261), SODIUM DIACETATE (E 262) AND CALCIUM ACETATE (E 263)

1. **Scope and field of application**

The method determines formic acid, formates and other oxidizable impurities, expressed as formic acid in:

— acetic acid (E 260),

— potassium acetate (E 261),

— sodium diacetate (E 262),

— calcium acetate (E 263).

2. **Definition**

Formic acid, formates and other oxidizable impurities content: the content of formic acid, formates and other oxidizable impurities as determined by the method specified.

3. **Principle**

The solution of the sample is treated with excess of standard potassium permanganate in alkaline conditions to form manganese dioxide. The manganese dioxide and excess potassium permanganate are determined iodometrically in acid conditions and the concentration of oxidizable impurities calculated and expressed as formic acid.

4. **Reagents**

4.1. Potassium iodide.

4.2. Potassium permanganate, 0·02 mol/l.

4.3. Sodium carbonate (anhydrous).

4.4. Sodium thiosulphate, 0·1 mol/l.

4.5. Starch solution (approximately 1 % m/v).

4.6. Dilute sulphuric acid: add 90 ml of sulphuric acid (ρ_{20} = 1·84 g/ml) to water and dilute to 1 l.

5. **Apparatus**

5.1. Water bath, boiling.

5.2. Analytical balance.

6. **Procedure**

If the test sample is the free acid, accurately weigh, to the nearest 10 mg, about 10 g of the sample and dilute with 70 ml of water and add a solution containing 10 g of anhydrous sodium carbonate (4.3) in 30 ml of water. If the sample is a salt, accurately weigh, to the nearest 10 mg, about 10 g of the sample and dissolve in 100 ml of water. Add 1 g anhydrous sodium carbonate (4.3) and shake to dissolve. Add 20 ml of 0·02 mol/l potassium permanganate (4.2) and heat on a boiling water bath for 15 minutes. Cool the mixture. Add 50 ml of dilute sulphuric acid (4.6) and 0·5 g of potassium iodide (4.1). Swirl the mixture until all precipitated manganese dioxide has redissolved. Titrate with 0·1 mol/l sodium thiosulphate (4.4) until the solution becomes pale yellow in colour. Add a few drops of starch solution (4.5) and continue the titration until the solution becomes colourless.

7. **Expression of results**

7.1. *Formula and method of calculation*

The percentage of formic acid, formates and of other oxidizable impurities, expressed as formic acid, is given by:

$$\frac{2{\cdot}3b}{m_0} \times \left(\frac{100a}{b} - V\right)$$

where:

a = molarity of potassium permanganate,

b = molarity of sodium thiosulphate,

m_0 = initial mass in grams of the sample taken,

V = volume in millilitres of 0·1 mol/l sodium thiosulphate used in the titration.

7.2. *Repeatability*

The difference between the results of two determinations when carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 5 mg per 100 g of sample.

8. **Notes**

8.1. A volume of 11·3 ml of 0·1 mol/l sodium thiosulphate is equivalent to 0·2 % formic acid in a 10 g sample.

8.2. If there is no formate present, the volume required will be 20 ml, but if there is more than 0·27 % (m/m) of formic acid present, there will be insufficient excess of potassium permanganate and a fixed minimum volume of 8 ml will be obtained. In this case repeat the determination using a smaller sample weight.

METHOD 3

DETERMINATION OF NON-VOLATILE SUBSTANCES IN PROPIONIC ACID (E 280)

1. **Scope and field of application**

The method determines non-volatile substances in propionic acid (E 280).

2. **Definition**

The content of non-volatile material in propionic acid: the content of non-volatile material as determined by the method specified.

3. **Principle**

The sample is evaporated and then dried at 103 ± 2 °C and the residue determined gravimetrically.

4. **Apparatus**

4.1. Evaporation vessel, silica or platinum and of sufficient size to contain 100 g of sample.

4.2. Oven, electrically heated, thermostatically controlled at 103 ± 2 °C.

4.3. Analytical balance.

4.4. Water bath, boiling.

4.5. Desiccator, containing freshly activated silica gel or equivalent desiccant with water content indicator.

5. **Procedure**

Weigh, to the nearest 0·1 g, 100 g of the sample of propionic acid into a previously dried and weighed vessel (4.1). Evaporate over a boiling water bath in a fume cupboard (4.4). When all the propionic acid has evaporated, place in an oven (4.2) at 103 ± 2 °C for one hour. Place in a desiccator and allow to cool and then weigh. Repeat the heating, cooling and weighing operations until the difference between two successive weighings is less than 0·5 mg. Should an increase in mass occur the lowest recorded reading will be used in the calculation.

6. **Expression of results**

6.1. *Formula and method of calculation*

The non-volatile matter content, calculated as a percentage of the sample, is given by:

$$\frac{100 \times m_1}{m_0}$$

where:

m_1 = mass in grams of the residue after evaporation,

m_0 = mass in grams of the sample taken.

6.2. *Repeatability*

The difference between the results of two determinations, carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 5 mg per 100 g of sample.

METHOD 4

DETERMINATION OF THE LOSS OF MASS ON DRYING OF SODIUM NITRITE (E 250)

1. **Scope and field of application**

The method determines the loss of mass on drying of sodium nitrite (E 250).

2. **Definition**

The moisture content of sodium nitrite; the loss of mass on drying as determined by the method specified.

3. **Principle**

The loss of mass on drying is obtained by heating in an oven at 103 ± 2 °C, weighing and calculation of the loss in mass.

4. **Apparatus**

4.1. Oven, electrically heated, thermostatically controlled at 103 ± 2 °C.

4.2. Weighing dish, flat-bottomed, glass, of diameter 60 to 80 mm and depth at least 25 mm, with loose-fitting lid.

4.3. Desiccator, containing freshly activated silica gel or equivalent desiccant with water content indicator.

4.4. Analytical balance.

5. **Procedure**

Remove the lid from the weighing dish (4.2) and heat dish and lid in the oven (4.1) at 103 ± 2 °C for one hour. Replace the lid and place the dish (4.2) with its lid in the desiccator (4.3) and allow to cool to room temperature. Weigh the covered dish (4.2) to the nearest

10 mg. Accurately weigh, to the nearest 10 mg, approximately 10 g of sample into the covered dish. Remove the lid and place both dish and lid in the oven (4.1) for one hour at 103 ± 2 °C. Replace the lid and allow the covered dish to cool to room temperature in the desiccator (4.3). Weigh it to the nearest 10 mg. Repeat the heating, cooling and weighing until the difference between two successive weights is less than 10 mg. Should an increase in mass occur, the lowest recorded reading will be used in the calculation.

6. **Expression of results**

6.1. *Formula and method of calculation*

The loss of mass on drying, calculated as a percentage by mass of the sample, is given by:

$$\frac{100 \times (m_2 - m_3)}{(m_2 - m_1)}$$

where:

m_1 = mass in grams of the dish,

m_2 = mass in grams of the dish and sample before drying,

m_3 = mass in grams of the dish and sample after drying.

6.2. *Repeatability*

The difference between the results of two determinations, carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 100 mg per 100 g of sample.

METHOD 5

LIMIT TEST FOR SALICYLIC ACID IN ETHYL *p*-HYDROXYBENZOATE (E 214), ETHYL-*p*-HYDROXYBENZOATE, SODIUM SALT (E 215), *n*-PROPYL *p*-HYDROXYBENZOATE (E 216), *n*-PROPYL *p*-HYDROXYBENZOATE, SODIUM SALT (E 217), METHYL *p*-HYDROXYBENZOATE (E 218), METHYL *p*-HYDROXYBENZOATE, SODIUM SALT (E 219)

1. **Scope and field of application**

The method detects salicylic acid in ethyl *p*-hydroxybenzoate (E 214), *n*-propyl *p*-hydroxybenzoate (E 216), and methyl *p*-hydroxybenzoate (E 218) and in their sodium salts (E 215, E 217 and E 219).

2. **Definition**

The detection of the limit test concentration of salicylic acid: the limit test result as determined by the method specified.

3. **Principle**

A violet colouration is produced from the reaction of ammonium iron (III) sulphate with a solution of the sample. Its intensity is compared with that produced by a reference solution.

4. **Reagents**

4.1. Ammonium iron (III) sulphate solution, 0·2 % m/v. Prepare by dissolving 0·2 g of ammonium iron (III) sulphate dodecahydrate in 50 ml of water, add 10 ml of nitric acid, 10 % v/v, and dilute to 100 ml with water.

4.2. Ethanol, 95 % v/v.

4.3. Salicylic acid solution, 0·1 g/l.

4.4. Sulphuric acid, 1 mol/l.

5. **Apparatus**

5.1. Nessler cylinders, graduated at 50 ml. Total volume approximately 60 ml.

6. **Procedure**

6.1. *Ethyl, n-propyl and methyl p-hydroxybenzoate samples*

6.1.1. Weigh, to the nearest 1 mg, 0·1 g of the sample and dissolve in 10 ml of 95 % v/v ethanol (4.2). Transfer the solution to a graduated Nessler cylinder (5.1) and dilute to 50 ml with water. Stir and add 1 ml of ammonium iron (III) sulphate solution (4.1) while stirring. Allow to stand for one minute.

6.1.2. Prepare a comparison solution at the same time by repeating 6.1.1, but replacing the 0·1 g of sample by 1 ml of salicylic acid solution (4.3).

6.1.3. Compare the colouring in the sample solution with that appearing in the comparison solution.

6.2. *Sodium salts of ethyl, n-propyl and methyl p-hydroxybenzoate samples*

6.2.1. Repeat 6.1.1 acidifying to pH 5 using 1 mol/l sulphuric acid (4.4) before dilution to 50 ml.

6.2.2. Repeat 6.1.2.

6.2.3. Repeat 6.1.3.

7. **Expression of results**

7.1. *Limit test interpretation*

If the reddish-violet colour appearing in the sample solution tube is more intense than that appearing in the comparison solution tube, the test is positive and the sample contains more than 0·1 % salicylic acid.

7.2. *Sensitivity*

The limit of detection of the test is 30 mg of salicylic acid per 100 g of sample.

7.3. *Observations*

The results of two limit tests carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall be identical.

METHOD 6

DETERMINATION OF FREE ACETIC ACID IN SODIUM DIACETATE (E 262)

1. **Scope and field of application**

The method determines acetic acid in sodium diacetate (E 262).

2. **Definition**

The acetic acid content: the content of acetic acid as determined by the method specified.

3. **Principle**

Direct titration of the acetic acid in the sample using standard sodium hydroxide solution and phenolphthalein indicator.

4. **Reagents**

4.1. Phenolphthalein solution 1% (m/v) in ethanol.

4.2. Sodium hydroxide, 1 mol/l.

5. **Apparatus**

5.1. Analytical balance.

6. **Procedure**

Weigh, to the nearest 1 mg, about 3 g of the test sample and dissolve in about 50 ml of water. Add two or three drops of phenolphthalein indicator solution (4.1) and titrate with 1 mol/l sodium hydroxide (4.2) until a red tint persists for five seconds.

7. **Expression of results**

7.1. *Formula and method of calculation*

The acetic acid content, as a percentage of the sample mass, is given by:

$$\frac{6{\cdot}005 \times V \times c}{m_0}$$

where:

V = volume in millilitres of sodium hydroxide (4.2) required,

c = concentration of the sodium hydroxide solution in mol/l,

m_0 = initial mass in grams of the sample taken.

7.2. *Repeatability*

The difference between the results of two determinations when carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 500 mg per 100 g of sample.

8. **Comment**

A volume of 20 ml is obtained when 3 g of a sample containing 40 % acetic acid is titrated with 1 mol/l sodium hydroxide.

METHOD 7

DETERMINATION OF SODIUM ACETATE IN SODIUM DIACETATE (E 262)

1. **Scope and field of application**

The method determines sodium acetate and water, expressed as sodium acetate, in sodium diacetate (E 262).

2. **Definition**

Sodium acetate content: the content of sodium acetate and water expressed as sodium acetate as determined by the method specified.

3. **Principle**

The sample is dissolved in glacial acetate acid, before titration, with standard perchloric acid, using crystal violet as indicator.

4. **Reagents**

4.1. Glacial acetic acid $\rho_{20\,°C} = 1{\cdot}049$ g/ml) (for non-aqueous titrations).

4.2. Crystal violet, CI No 42555 indicator solution, 0·2 % (m/v) in glacial acetic acid.

4.3. Potassium hydrogen phtalate, $C_8H_5KO_4$.

4.4. Acetic anhydride $(CH_3CO)_2O$.

4.5. Perchloric acid, 0·1 mol/l in glacial acetic acid. This must be prepared and standardized as follows:

Weigh P g of perchloric acid solution into a 1 000 ml volumetric flask fitted with a ground-glass stopper. The quantity P is calculated from the formula:

$$P = \frac{1\,004.6}{m}$$

where m is the concentration (per cent m/m) of perchloric acid determined by alkalimetric titration (70 to 72 % m/m acid is the most suitable). Add about 100 ml of glacial acetic acid and then a quantity, Q g, of acetic anhydride in successive small portion, stirring and cooling the mixture continuously during the additions. The quantity Q may be calculated from the formula:

$$Q = \frac{(567 \times P) - 5\,695}{a}$$

where P is the weighed amount of perchloric acid and a is the concentration (per cent m/m) of the acetic anhydride. Stopper the flask and allow to stand for 24 hours in a dark place, then add sufficient glacial acetic acid to produce 1 000 ml of solution. The solution prepared in this way is practically anhydrous. Standardize the solution against potassium hydrogen phthalate as follows:

Weigh accurately, to the nearest 0·1 mg, about 0·2 g of potassium hydrogen phthalate, previously dried at 110 °C for two hours, and dissolve in 25 ml of glacial acetic acid in a titration flask, warming gently. Cool, add two drops of a 0·2 % (m/m) crystal violet solution (4.2) in glacial acetic acid and titrate with the perchloric acid solution until the colour of the indicator changes to pale green. Carry out a blank titration using the same volume of solvent and deduct the value of the blank from the value found in the actual determination. Each 20·42 mg of potassium hydrogen phthalate is equivalent to 1 ml of 0·1 mol/l perchloric acid.

5. **Apparatus**

5.1. Analytical balance.

6. **Procedure**

Weigh, to the nearest 0·5 mg, about 0·2 g of the sample and dissolve in 50 ml of glacial acetic acid (4.1). Add a few drops of crystal violet indicator solution (4.2) and titrate to a pale green end-point, using standard 0·1 mol/l perchloric acid (4.5).

7. **Expression of results**

7.1. *Formula and method of calculation*

The sodium acetate content, as defined in section 2 (definition), expressed as a percentage by weight of the sample, is given by the following formula:

$$\frac{8{\cdot}023 \times V \times c}{m_0}$$

where:

V = volume in millilitres of the standard perchloric acid (4.5) used,

c = molarity of the perchloric acid solution (4.5),

m_0 = initial mass in grams of the sample taken.

7.2. *Repeatability*

The difference between the results of two determinations when carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 1·5 g per 100 g of sample.

8. **Observations**

The reagents used in this method are toxic and explosive and need careful handling.

METHOD 8

LIMIT TEST FOR ALDEHYDES IN SORBIC ACID (E 200), SODIUM, POTASSIUM AND CALCIUM SORBATES (E 201, E 202, E 203) AND PROPIONIC ACID (E 280)

1. **Scope and field of application**

The method detects aldehydes, expressed as formaldehyde, in:

— sorbic aid (E 200),

— sodium, potassium and calcium sorbates (E 201, E 202, E 203),

— propionic acid (E 280).

2. **Definition**

The detection of the limit test concentration of aldehydes: the limit test result as determined by the method specified.

3. **Principle**

The aldehydes in the test solution, and the formaldehyde in a comparison solution, react with Schiff's reagent to produce red coloured complexes, the intensities of which are compared.

4. **Reagents**

4.1. Formaldehyde standard solution (0·01 mg/ml): prepared by dilution of concentrated formaldehyde solution (400 mg/ml).

4.2. Schiff's reagent.

5. **Procedure**

5.1. Weigh, to the nearest 1 mg, about 1 g of the sample, add to 100 ml of water and shake. Filter the solution if necessary and treat 1 ml of filtrate or sample solution with 1 ml of Schiff's reagent (4.2). At the same time, treat 1 ml of formaldehyde comparison solution (4.1) with 1 ml of Schiff's reagent (4.2).

5.2. Compare the colour in the sample solution with that appearing in the comparison solution.

6. **Expression of results**

6.1. *Limit test interpretation*

If the red colour appearing in the sample solution tube is more intense than that appearing in the comparison solution tube, the test is positive and the sample contains more than 0·1 % aldehydes, expressed as formaldehyde.

6.2. *Sensitivity*

The limit of detection of this test is 30 mg of formaldehyde per 100 g of sample.

6.3. *Observations*

The result of two limit tests when carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall be identical.

METHOD 9

DETERMINATION OF THE PEROXIDE NUMBER IN LECITHINS (E 322)

1. **Scope and field of application**

The method determines the peroxide number in lecithins (E 322).

2. **Definition**

Peroxide number of lecithins: the result obtained as determined by the method specified.

3. **Principle**

Oxidation of potassium iodide by the peroxides of lecithin and titration of the iodine liberated using standard sodium thiosulphate solution.

4. **Reagents**

4.1. Acetic acid glacial.

4.2. Chloroform.

4.3. Potassium iodide.

4.4. Sodium thiosulphate, 0·1 mol/l or 0·01 mol/l.

4.5. Starch solution (approximately 1 % m/v).

5. **Apparatus**

5.1. Analytical balance.

5.2. Apparatus, as shown in the figure, consisting of:

5.2.1. round-bottomed flask, 100 ml;

5.2.2. reflux condenser;

5.2.3. glass tube, 250 mm long and 22 mm internal diameter, fitted with ground glass joints;

5.2.4. micro beaker (external dimension of 20 mm diameter and 35 to 50 mm height).

6. **Procedures**

6.1. Place 10 ml of glacial acetic acid (4.1) and 10 ml of chloroform (4.2) in the 100 ml flask (5.2.1). Fit the glass tube (5.2.3) and reflux condenser (5.2.2) and gently boil the mixture for two minutes to expel all dissolved air. Dissolve 1 g of potassium iodide (4.3) in 1·3 ml of water and add this solution to the mixture in the flask (5.2.1) taking care that the boiling is not interrupted.

If a yellow colour appears at this stage the determination must be rejected and repeated using fresh reagents.

6.2. Accurately weigh, to the nearest 1 mg, about 1 g of the sample and, after a further two minutes of boiling, add the weighed sample to the contents of the flask (5.2.1) again taking care that the boiling remains continuous. For this purpose the sample should be contained in a micro-beaker (5.2.4) which may be lowered through the glass tube (5.2.3) with a glass rod, the bottom of which has been suitably shaped as shown in the diagram. The condenser (5.2.2) may be removed for a short time. Continue boiling for three to four minutes. Stop heating and immediately disconnect the condenser (5.2.2). Quickly add 50 ml of water through the glass tube (5.2.3). Remove the glass tube (5.2.3) and cool the flask (5.2.1) to room temperature under the water tap. Titrate with sodium thiosulphate (0·1 mol/l or 0·01 mol/l) (4.4) until the aqueous layer becomes pale yellow. Add 1 ml of starch solution (4.5) and continue the titration until the blue colour is discharged. Shake the flask (5.2.1) well during the titration to ensure the complete extraction of iodine from the non-aqueous layer.

6.3. Obtain a blank titration value by repeating the complete procedure 6.1 and 6.2, but without adding the sample.

7. **Expression of results**

7.1. *Formula and method of calculation*

The peroxide number in the sample, in milliequivalents per kilogram, is given by:

$$\frac{1\,000 \times a \times (V_1 - V_2)}{m_0}$$

where:

V_1 = volume in millilitres of thiosulphate solution required for the titration of the sample (6.2),

V_2 = volume in millilitres of thiosulphate solution required for the titration of the blank (6.3),

a = concentration of sodium thiosulphate solution in mol/l,

m_0 = initial mass in grams of the sample taken.

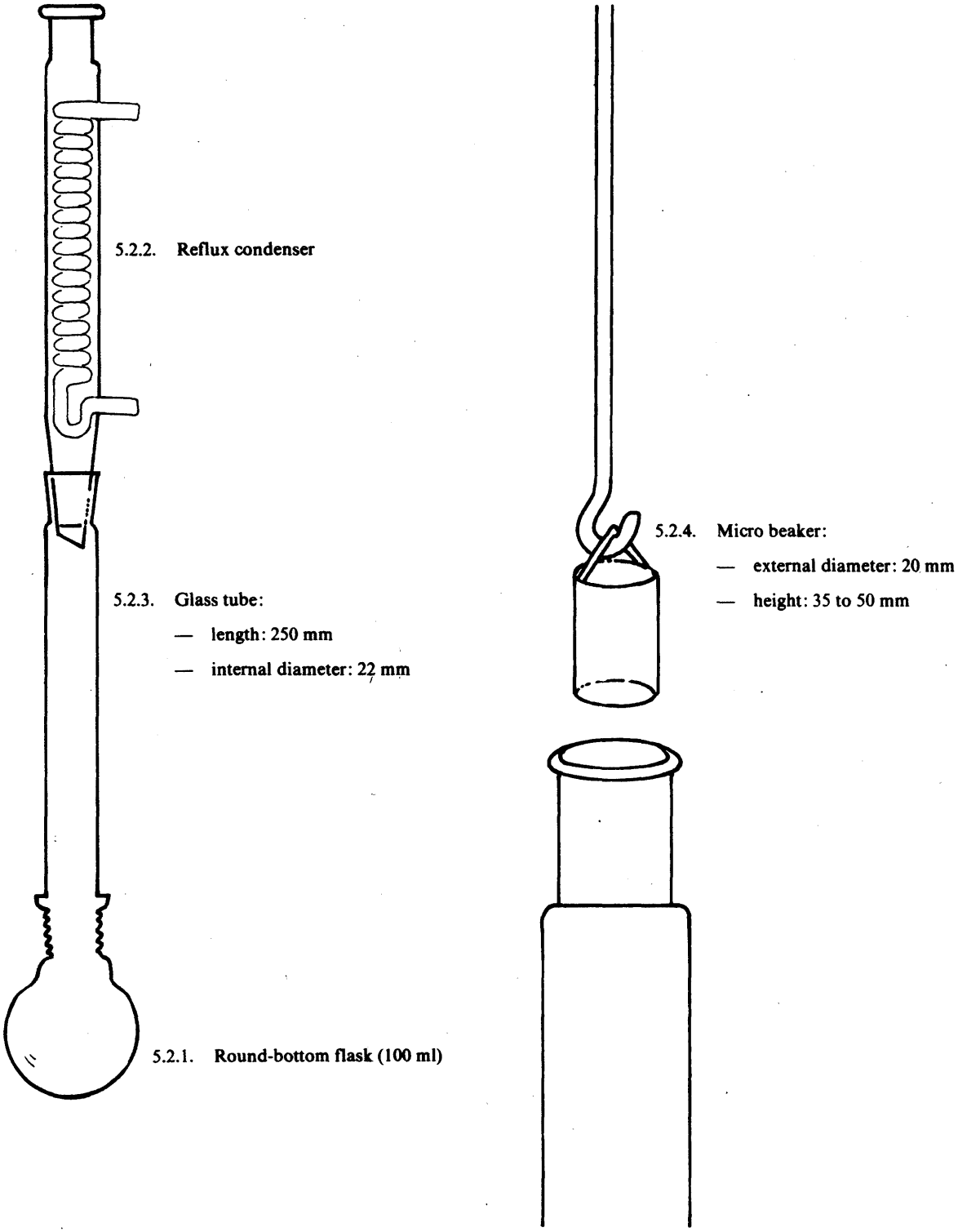
7.2. *Repeatability*

The difference between the results of two determinations carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 0·5 (expressed as a peroxide number in milliequivalents per kilogram of sample).

8. **Notes**

8.1. The choice of the concentration of the sodium thiosulphate used depends on the anticipated titration value. If less than 0·5 ml of 0·1 mol/l sodium thiosulphate is required, repeat the determination using 0·01 mol/l sodium thiosulphate.

8.2. The determination should not be carried out in strong light.



Apparatus for the determination of peroxide number in lecithins

METHOD 10

DETERMINATION OF TOLUENE-INSOLUBLE MATTER IN LECITHINS (E 322)

1. **Scope and field of application**

The method determines the toluene-insoluble matter in lecithins (E 322).

2. **Definition**

The toluene-insoluble matter content: the content of toluene-insoluble matter as determined by the method specified.

3. **Principle**

The sample is dissolved in toluene, filtered, and the residue dried and weighed.

4. **Reagents**

4.1. Toluene.

5. **Apparatus**

5.1. Sintered glass crucible, 30 ml capacity, G 3 or equivalent porosity.

5.2. Drying oven, electrically heated and thermostatically controlled at 103 ± 2 °C.

5.3. Water bath, operating at a temperature not exceeding 60 °C.

5.4. Desiccator, containing freshly activated silica gel or equivalent desiccant with a water content indicator.

5.5. Conical flask of 500 ml.

5.6. Vacuum pump.

5.7. Analytical balance.

6. **Procedure**

6.1. Dry a 30 ml sintered glass crucible (5.1) in an oven at 103 ± 2 °C (5.2). Transfer the crucible to desiccator (5.4), allow to cool and then weigh.

6.2. Thoroughly mix the sample of lecithins, if necessary after warming in a water bath (5.3). Weigh, to the nearest 1 mg, about 10 g of the sample into a conical flask (5.5). Add 100 ml of toluene (4.1) and swirl the mixture until all the lecithin has apparently dissolved. Filter the solution through the sintered glass crucible (5.1). Wash the conical flask (5.5) with 25 ml of toluene (4.1) and pass the washings through the crucible (5.1). Repeat this process with another 25 ml of toluene (4.1). Remove excess toluene from the crucible (5.1) by suction.

6.3. Dry the crucible (5.1) in the drying oven (5.2) at 103 ± 2°C for two hours. Place in desiccator (5.4) and allow to cool. Weigh the crucible and residue when cool.

6.4. Repeat 6.3 until the difference in weight between two successive weighings is less than 0·5 mg.

Should an increase in mass occur, the lowest recorded reading will be used in the calculation.

7. **Expression of results**

7.1. *Formula and method of calculation*

The content of toluene insoluble substances is given by:

$$\frac{100\,(m_2 - m_1)}{m_0}$$

where:

m_1 = mass in grams of the empty crucible (6.1),

m_2 = mass in grams of the crucible and residues (6.4),

m_0 = initial mass in grams of the sample taken.

7.2. *Repeatability*

The difference between the results of two determinations carried out in simultaneous or rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 30 mg per 100 g of sample.

METHOD 11

LIMIT TEST FOR REDUCING SUBSTANCES IN SODIUM, POTASSIUM AND CALCIUM LACTATES (E 325, E 326, E 327)

1. **Scope and field of application**

The test detects qualitatively reducing substances in:

— sodium lactate (E 325),

— potassium lactate (E 326),

— calcium lactate (E 327).

2. **Definition**

The detection of the limit test concentration of reducing substances: the limit test result as determined by the method specified.

3. **Principle**

Fehling's solution is reduced by substances capable of exhibiting reducing action. Such substances will normally be reducing sugars.

4. **Reagents**

4.1. Fehling's solution A: 6·93 g of copper sulphate pentahydrate is dissolved in water and made up 100 ml with water.

4.2. Fehling's solution B: 34·6 g of potassium sodium tartrate and 10 g of sodium hydroxide are dissolved in water and made up to 100 ml with water.

5. **Procedures**

Weigh, to the nearest 1 mg, about 1 g of the sample and dissolve in 10 ml of warm water. Add 2 ml of Fehling's solution A (4.1) and 2 ml of Fehling's solution B (4.2) and then boil the mixture for one minute and observe whether a colour change occurs. The precipitation of calcium sulphate, which sometimes occurs, does not interfere.

6. **Expression of results**

6.1. *Limit test interpretation*

If there is a colour change after boiling (5), the test is positive and the presence of reducing substances is indicated.

6.2. *Sensitivity*

The limit of detection for reducing substances reacting is 100 mg glucose per 100 g of sample.

6.3. *Observations*

6.3.1. The results of two limit tests carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall be identical.

6.3.2. All of the Fehling solutions react if 2 % glucose is present in the sample.

METHOD 12

DETERMINATION OF VOLATILE ACIDS IN ORTHOPHOSPHORIC ACIDS (E 338)

1. **Scope and field of application**

The method determines volatile acids, expressed as acetic acid, in orthophosphoric acid (E 338).

2. **Definition**

Volatile acid content: the content of volatile acids, expressed as acetic acid, as determined by the method specified.

3. **Principle**

Water is added to the sample and the solution is distilled. The distillate is titrated against standard sodium hydroxide solution and the acidity calculated and expressed as acetic acid.

4. **Reagents**

4.1. Pheolphthalein solution, 1 % (m/v) in ethanol.

4.2. Sodium hydroxide, 0·01 mol/l.

5. **Apparatus**

5.1. Distillation apparatus including a spray trap.

6. **Procedure**

Weigh, to the nearest 50 mg, about 60 g of the sample and place the weighed sample and 75 ml of freshly boiled cooled water in the distillation flask fitted with the spray trap (5.1). Mix and then distil about 50 ml.

Titrate the distillate with standard 0·01 mol/l sodium hydroxide (4.2) using phenolphthalein (4.1) as indicator. Continue the titration until the first red tint in the solution persists for 10 seconds.

7. **Expression of results**

7.1. *Formula and method of calculation*

The content of volatile acids, expressed as milligrams per kilogram of acetic acid, is given by:

$$\frac{600 \times V}{m_0}$$

where:

V = volume in millilitres of 0·01 mol/l sodium hydroxide solution used for neutralization,

m_0 = mass in grams of the orthophosphoric acid sample.

7.2. *Repeatability*

The difference between the results of two determinations carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 1 mg per 100 g of sample.

METHOD 13

LIMIT TEST FOR NITRATE IN ORTHOPHOSPHORIC ACID (E 338)

1. **Scope and field of application**

This method detects nitrates in orthophosphoric acid (E 338).

2. **Definition**

The detection of the limit test concentration of nitrate, expressed as sodium nitrate; the limit test result as determined by the method specified.

3. **Principle**

The sample is added to indigo carmine solution in a concentrated sulphuric acid medium. The blue colouration present is discharged by oxidizing agents including nitrates.

4. **Reagents**

4.1. Indigo carmine solution, 0·18 % (m/v): dissolve 0·18 g of sodium indigotin disulphonate in water and make up to 100 ml with water.

4.2. Sodium chloride solution, 0·05 % (m/v).

4.3. Sulphuric acid concentrated (ρ_{20} = 1·84 g/ml).

5. **Procedure**

Measure 2 ml of the sample and dilute to 10 ml with the sodium chloride solution (4.2). Add 0·1 ml of carmine indigo solution (4.1) and then slowly add 10 ml of concentrated sulphuric acid (4.3), cooling during the addition. Note whether the blue colouration of the solution persists for five minutes.

6. **Expression of results**

6.1. *Limit test interpretation*

If the blue colouration is discharged within five minutes the test is positive and the content of oxidizing agents, expressed as sodium nitrate, is greater than 5 mg/kg.

6.2. *Observations*

6.2.1. Carry out a blank test.

6.2.2. The results of two limit tests when carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall be identical.

6.2.3. Indigo carmine solution should not be used if it has been prepared for more than 60 days.

6.2.4. If a positive result is obtained the sample may contain nitrates and other oxidizing agents and the test must be repeated using ISO Method 3709 (1976) 'Phosphoric acid for industrial use (including foodstuffs) — determination of oxides of nitrogen content — 3,4-xylenol spectrophometric method'.

METHOD 14

DETERMINATION OF WATER-INSOLUBLE SUBSTANCES PRESENT IN MONO-, DI- AND TRI-SODIUM ORTHOPHOSPHATES AND MONO-, DI- AND TRI-POTASSIUM ORTHOPHOSPHATES (E 339(i), E 339(ii), E 339(iii), E 340(i), E 340(ii) AND E 340(iii))

1. **Scope and field of application**

The method determines water-insoluble matter in:

— mono-sodium orthophosphate (E 339(i)),

— di-sodium orthophosphate (E 339(ii)),

— tri-sodium orthophosphate (E 339(iii)),

— mono-potassium orthophosphate (E 340(i)),

— di-potassium orthophosphate (E 340(ii)),

— tri-potassium orthophosphate (E 340(iii)).

2. **Definition**

Water insoluble matter: the content of water-insoluble matter as determined by the method specified.

3. **Principle**

The sample is dissolved in water and filtered through a suitable porcelain crucible. After washing and drying the residue is weighed and calculated as water-insoluble matter.

4. **Apparatus**

4.1. Sintered porcelain crucible, porosity G 3 or equivalent.

4.2. Desiccator, containing freshly activated silica gel with a water content indicator, or equivalent desiccant.

4.3. Oven, thermostatically controlled at 103 ± 2 °C.

4.4. Polypropylene beaker, 400 ml.

4.5. Water bath, boiling.

5. **Procedure**

Weigh, to the nearest 10 mg, about 10 g of the sample of phosphate and dissolve in 100 ml of hot water by bringing to the boil in a polypropylene beaker (4.4) and maintaining on a hot water bath (4.5) for 15 minutes. Filter the solution through a previously cleaned, dried and weighed crucible (4.1). Wash the insoluble residue with hot water. Place the crucible with residue in the oven (4.3) and dry at 103 ± 2 °C for two hours.

Place the crucible in the desiccator and allow to cool and weigh the crucible.

Repeat the drying, cooling and weighing until the difference in weight of two successive weighings is less than 0·5 mg. Should an increase in mass occur the lowest recorded reading will be used in the calculation.

6. **Expression of results**

6.1. *Formula and method of calculation*

The content of water-insoluble matter in the sample is given by:

$$\frac{m_1}{m_0} \times 100$$

where:

m_1 = mass in grams of the residue after drying,

m_0 = mass in grams of the sample taken.

6.2. *Repeatability*

The difference between the results of two determinations carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 10 mg per 100 g of sample.

METHOD 15

DETERMINATION OF THE pH OF FOOD ADDITIVES

1. **Scope and field of application**

The method outlines general instructions on how to determine the pH of food additives.

2. **Definition**

The pH of a food additive: the pH value as determined by the method specified.

3. **Principle**

The pH value of an aqueous solution of the dissolved or slurried sample is conventionally determined using a glass electrode, reference electrode and pH meter.

4. **Reagents**

4.1. Calibrate the instrument using the following buffer solutions:

4.1.1. Buffer solution pH 6·88 at 20 °C, consisting of equal volume 0·05 mol/l potassium dihydrogen phosphate (KH_2PO_4) and 0·05 mol/l disodium hydrogen ortho phosphate dyhidrate ($Na_2HPO_4 \cdot 2H_2O$).

4.1.2. Buffer solution pH 4 at 20 °C, consisting of 0·05 mol/l potassium hydrogen phthalate ($C_8H_5KO_4$).

4.1.3. Buffer solution pH 9·22 at 20 °C, consisting of 0·05 mol/l sodium borate ($Na_2B_4O_7 \cdot 10H_2O$).

4.2. Saturated or 3 mol/l potassium chloride solution, or other suitable solution prescribed by the electrode manufacturer, to fill the reference electrode.

4.3. Distilled water, carbon dioxide-free, having a pH between 5 and 6.

5. **Apparatus**

5.1. pH meter, with an accuracy within 0·01 pH units.

5.2. Electrodes, either a combined glass electrode or single glass and reference electrodes together with suitable clamps to hold the electrodes.

5.3. Magnetic stirrer, with heater element.

5.4. Thermometer, calibrated over the range 0 to 100 °C.

6. **Procedure**

6.1. *Standardization of the pH meter*

The glass electrodes must be set using the instructions given by the manufacturer. The pH reading from the electrodes must be regularly checked by comparison with buffer solutions of known pH.

Electrodes should be washed with water and then gently wiped with a soft tissue or should be rinsed with water and then twice with the next sample/standard solution before being placed in the next sample/standard solution to be used.

If the sample to be considered has an acid pH, the buffer solutions used to check the pH reading should be those of pH 4 (4.1.2) and pH 6·88 (4.1.1). If the sample to be analyzed has an alkaline pH, the buffer solutions to be used to check the pH reading should be those of pH 9·22 (4.1.3) and pH 6·88 (4.1.1).

6.2. *Measurement of the sample solution*

The concentration of sample to be used or the sample preparation procedure to be adopted is as prescribed in the appropriate Community food additive Directive.

Prepare the sample solution as directed using distilled water (4.3) and then adjust to 20 °C while stirring. Stop the stirring, place the glass electrodes in the solution and after two minutes note the pH on the pH meter (5.1).

7. **Expression of results**

7.1. *Repeatability*

The difference between the results of two determinations when carried out simultaneously or in rapid succession on the same sample, by the same analyst, under the same conditions, shall not exceed 0·05 pH unit.

8. **Note**

This method is only applicable to those pH requirements in Community food additives Directives where the food additive is dissolved or slurried in water.

COMMISSION DECISION

of 28 July 1981

on the list of establishments in the Federative Republic of Brazil approved for the purpose of importing fresh beef and veal and meat of domestic solipeds into the Community

(81/713/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 72/462/EEC of 12 December 1972 on health and veterinary inspection problems upon importation of bovine animals and swine and fresh meat from third countries [1], and in particular Articles 4 (1) and 18 (1) (a) and (b) thereof,

Whereas establishments in non-member countries cannot be authorized to export fresh meat to the Community unless they satisfy the general and special conditions laid down in Directive 72/462/EEC;

Whereas the Federative Republic of Brazil has forwarded, in accordance with Article 4 (3) of Directive 72/462/EEC, a list of the establishments authorized to export to the Community;

Whereas Community on-the-spot inspections have shown that the hygiene standards of many of these establishments are sufficient and they may therefore be entered on a first list, established according to Article 4 (1) of the said Directive, of establishments from which importation of fresh meat may be authorized;

Whereas the case of the other establishments proposed by Brazil must be re-examined on the basis of additional information regarding their hygiene standards and their ability to adapt quickly to the Community legislation;

Whereas, in the meantime and so as to avoid any abrupt interruption of existing trade flows, these establishments may be authorized temporarily to continue their exports of fresh meat to those Member States prepared to accept them;

Whereas it will therefore be necessary to re-examine and, if necessary, amend this Decision in the light of measures taken to this end and improvements made;

Whereas it should be recalled that imports of fresh meat are also subject to other Community veterinary legislation, particularly as regards health protection requirements, including the special provisions for Denmark, Ireland and the United Kingdom;

Whereas the conditions of importation of fresh meat from establishments appearing on the list annexed to this Decision remain subject to provisions laid down elsewhere and to the general provisions of the Treaty; whereas, in particular, the importation from non-member countries and the re-exportation to other Member States of certain categories of meat, such as meat weighing less than three kilograms or meat containing residues of certain substances which are not yet covered by harmonized Community rules, remain subject to the health legislation of the importing Member State;

Whereas the measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,

HAS ADOPTED THIS DECISION:

Article 1

1. The establishments in the Federative Republic of Brazil listed in the Annex are hereby approved for the purposes of the importation of fresh beef and veal and meat of domestic solipeds into the Community.

2. Imports from those establishments shall remain subject to the Community veterinary provisions laid down elsewhere, and in particular those concerning health protection requirements.

Article 2

1. Member States shall prohibit imports of the categories of fresh meat referred to in Article 1 (1)

[1] OJ No L 302, 31. 12. 1972, p. 28.

coming from establishments other than those listed in the Annex.

2. This prohibition, however, shall not apply until 1 May 1982 to establishments which are not listed in the Annex but which have been officially approved and proposed by the Brazilian authorities as of 1 July 1981 pursuant to Article 4 (3) of Directive 72/462/EEC, unless a decision is taken to the contrary, in accordance with Article 4 (1) of the above-mentioned Directive, before 1 May 1982.

The Commission shall forward the list of these establishments to the Member States.

Article 3

This Decision shall enter into force on 1 October 1981.

Article 4

This Decision shall be reviewed and, if necessary, amended before 1 March 1982.

Article 5

This Decision is addressed to the Member States.

Done at Brussels, 28 July 1981.

For the Commission

The President

Gaston THORN

ANNEX

LIST OF ESTABLISHMENTS

I. BOVINE MEAT

A. **Slaughterhouses and cutting premises**

Establishment No	Address
0005	Cooperativa Rural Serrana Ltda, Tupanciretã, Rio Grande do Sul
0226	Frigorífico Bordon SA, Bagé, Rio Grande do Sul
0385	Frigorífico Mouran SA, Andradina, São Paulo
0458	Frigorífico União SA, Presidente Epitácio, São Paulo
0834	Frigorífico Kaiowa SA, Presidente Venceslau, São Paulo
0906	Frigorífico T. Maia SA, Governador Valadares, Minas Gerais
1602	Bon Beef Indústria e Comércio de Carnes SA, Vinhedo, São Paulo
1651	Frigorífico Extremo Sul SA, Pelotas, Rio Grande do Sul
1926	Frigorífico Anselmi SA, Indústria de Carnes, Derivados e Conservas, Pelotas, Rio Grande do Sul

B. **Slaughterhouses**

Establishment No	Address
0076	SA Frigorífico Anglo-Barretos, São Paulo

C. **Cutting premises**

Establishment No	Address
0001	Cia de Alimentos do Brasil SA (COMABRA), Osasco, São Paulo

II. HORSEMEAT

Slaughterhouses and cutting premises

Establishment No	Address
0003	Frigorífico Yukijirushi do Paraná SA, Curitiba, Paraná
0924	Matadouro e Frigorífico Industrial SA (MAFISA), Belo Jardim, Pernambuco

III. COLD STORES

Establishment No	Address
0072	Cefri Centrais de Estocagem Frigorificada Ltda, Mairinque, São Paulo
0078	Interfrio SA Comercial e Industrial, Pelotas, Rio Grande do Sul
0535	Matadouro e Frigorífico Industrial SA (MAFISA), Recife, Pernambuco
0933	Companhia Brasileira de Armazenamento (CIBRAZEM), Rio de Janeiro
0966	C. Sola, Comércio e Exportação SA, Três Rios, Rio de Janeiro
1075	Frigorífico de Cotia SA, Santos, São Paulo
1127	Companhia Brasileira de Armazenamento (CIBRAZEM), Curitiba, Paraná
1599	Martini Meat SA, Comércio, Importação e Exportação de Carnes, Paranaguá, Paraná
1945	Departamento Estadual de Portos Riós e Canais, Rio Grande, Rio Grande do Sul
1958	Avante SA Produtos Alimentícios, Santos, São Paulo
2176	Frimorite Frigorífico Ltda, São Gonçalo, Rio de Janeiro

COMMISSION DECISION

of 28 July 1981

amending the lists of establishments in the Argentine Republic and in the Republic of Uruguay approved for the purpose of importing fresh beef and veal, sheepmeat and meat of domestic solipeds into the Community

(81/714/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 72/462/EEC of 12 December 1972 on health and veterinary inspection problems upon importation of bovine animals and swine and fresh meat from third countries (1), and in particular Articles 4 (1) and 18 (1) (a) and (b) thereof,

Whereas lists of establishments in Argentina and Uruguay, approved for the purposes of the importation of fresh beef and veal, sheepmeat and meat of domestic solipeds into the Community, were drawn up initially by the Commission Decision of 25 November 1980, as amended by Decisions 81/91/EEC (2) and 81/92/EEC (3), and as last amended by the Decision of 15 July 1981;

Whereas further on-the-spot inspections have shown that the hygiene standards of other establishments proposed by Argentina and Uruguay have been improved and may now be considered to be satisfactory; whereas these establishments may therefore be entered on the lists drawn up in accordance with Article 4 (1) of Directive 72/462/EEC;

Whereas it is therefore necessary to supplement the lists of establishments;

Whereas the measures provided for in this Decision are in accordance with the opinion of the Standing Veterinary Committee,

HAS ADOPTED THIS DECISION:

Article 1

The Annex to Decision 81/91/EEC is hereby replaced by Annex A to this Decision.

Article 2

The Annex to Decision 81/92/EEC is hereby replaced by Annex B to this Decision.

Article 3

This Decision is addressed to the Member States.

Done at Brussels, 28 July 1981.

For the Commission

The President

Gaston THORN

(1) OJ No L 302, 31. 12. 1972, p. 28.
(2) OJ No L 58, 5. 3. 1981, p. 39.
(3) OJ No L 58, 5. 3. 1981, p. 43.

ANNEX A

LIST OF ESTABLISHMENTS

I. BOVINE MEAT

A. Slaughterhouses and cutting premises

Establishment No	Address
8	Corporación argentina de productores de carnes (CAP) cuatreros, Daniel Cerri, Buenos Aires
9	Corporación argentina de productores de carnes (CAP) yuqueri, Concordia, Entre Ríos
13	Cia Swift de la Plata SA, Rosarie, Santa Fé
15	Frigorífico Colón SA, Colon, Entre Ríos
16	Frigorífico regional Santa Elena SA, Santa Elena, Entre Ríos
20	SA Frigorífico Monte Grande Ltda, Monte Grande, Buenos Aires
89	Frigorífico Carcarana SACI, Carcarana, Santa Fé
249	Industrias frigoríficas Nelson SACIA, Nelson, Santa Fé
1113	La Morocha SAAICF, Villa Mercedes, San Luis
1333	Frigorífico argentino San Antonio (FASA), Parana, Entre Ríos
1344	Vizental y Cia SACIA, Ramirez, Entre Ríos
1352	Frigorífico Meatex Ciafiiesa, Alejandro Korn, Buenos Aires
1373	Frigorífico el Gentenario SA, Venado Tuerto, Santa Fé
1383	Barreca Hnos, Vivorata, Buenos Aires
1399	Frigorífico regional industria argentina SAIC (FRIA), Casilda, Santa Fé
1404	Pedro Hnos SAICIFA, Monte Chingolo, Buenos Aires
1408	Subpga SACIEI, Berazategui, Buenos Aires
1905	Frigorífico Yaguune SACIFA, Gonzalez Catan, Buenos Aires
1918	Cocarsa Cia de carneceros SAICAI, San Fernando, Buenos Aires
1920	Frigorífico rioplatense SAICIF, General Pacheco, Buenos Aires
1921	San Telmo SACIAFIF, Mar de Plata, Buenos Aires
1930	Vizental y Cia SACIA, San José, Entre Ríos
1970	Frigorífico regional industrias alimenticias reconquista SA, Reconquista, Santa Fé
1984	Matadero y Frigorífico regional de Azul SAGIC, Azul, Buenos Aires
1989	Corporación argentina de productores de carnes (CAP), Rosario, Santa Fé
2012	Frigorífico el Duranzillo IFCA SAIFCA, Río Segundo, Córdoba
2019	Abastecedora delfino SACI, Tres Lomas, Buenos Aires
2052	Matadero y Frigorífico Antartico SAIC, Gonzalez Catan, Buenos Aires
2064	Frigorífico Siracusa SAACIIF, Bahia Blanca, Buenos Aires
2065	Frigorífico mediterraneos SAICIFA, Pajas Blancas
2067	Cia elaboradora de productos animales SAI CAGT, Pontevedra, Buenos Aires
2072	Frigorífico ganadero SACIA FIGMS, Curuzu Cuatia, Corrientes
2080	Caucan SA, Ezeiza, Buenos Aires

B. **Cutting premises**

Establishment No	Address
18	Quickfood, Buenos Aires
273	Frigorífico guardia nacional SA, Guardia Nacional 1166, Cap. Federal
1122	Frigorífico Lafayette SAICAG, Lafayette 1740, Cap. Federal
1311	Frymat SAICFA, Buenos Aires 3680, Santa Fé
1920 a.	Frigorífico rioplatense SAICIF, General Pacheco, Buenos Aires

II. SHEEP MEAT

Slaughterhouses and cutting premises

Establishment No	Address
8	Corporación argentina de productores de carnes (CAP) cuatreros, Daniel Cerri, Buenos Aires
9	Corporación argentina de productores de carnes (CAP) yuqueri, Concordia, Entre Ríos
14	Corporación argentina de productores de carnes (CAP) Rio Grande, Tierra del fuego
97	Corporación argentina de productores de carnes (CAP), Pto. deseado, Santa Cruz
286	Frigorífico San Jorge SAIC, Bo Industrial, Comodoro Rivadavia
1352	Frigorífico Meatex Ciafiiesa, Alejandro Korn, Buenos Aires
1408	Subpga SACIEI, Berazategui, Buenos Aires
2006	Vizental y Cia SACIA, General Pico, La Pampa
2044	Frigorífico Siracusa SAACIIF, Comodoro Rivadavia, Chubut
2062	Finexcor, Pernal, Buenos Aires
2072	Frigorífico ganadero SACIA FIGMS, Curuzu Cuatia, Corrientes

III. HORSE MEAT

Slaughterhouses and cutting premises

Establishment No	Address
351	SA Indio Pampa ICAG, Trenque Lauquen, Buenos Aires
1369	Frigorífico Felmar SA, San Francisco, Buenos Aires
1400	Frigorífico Juchco SCA, Gialeguay, Entre Ríos
1451	Lamar SRL, Mercedes, Buenos Aires
2028	Lamar SRL, Resistencia, Chaco

IV. COLD STORES

Establishment No	Address
152	Comalfri, Pilar, Buenos Aires
267	Frymat SACIFA, Santa Fé
308	Frigorífico americano de morris Neremberg Ltda SA, Boulogne sur mer 260/2, Cap. Federal
391	Frigorífico Siracusa SAACIIF, Avellaneda, Buenos Aires
1326	Establecimiento azul SRL, Azul, Buenos Aires
1838	Guaicos SAIIF, Osvaldo Cruz 3047, Cap. Federal

ANNEX B

I. BOVINE MEAT

Slaughterhouses and cutting premises

Establishment No	Address
1	Codadesa, Ruta 39, km 143, departamento de Maldonado
2	Colonia, Tarariras, departamento de Colonia
3	Carrasco, Camino Carrasco 5, departamento de Canelones
7	Infrinsa, Ruta Brigadier-General Juan A. Lavalleja, km 391, Ciudad de Melo, departamento de Cerro Largo
8	Canelones, Ciudad de Canelones, departamento de Canelones
12	Tacuarembo, Rutas 5 y 26, departamento de Tacuarembo
14	Efcsa (durazno), Santa Bernardina, departamento de Durazno
20	Comargen, Ruta 67 y Elias Regules, las Piedras, departamento de Canelones
106	Improgan, Camino Santos Lugares, km 4, la Pas, departamento de Canelones
344	San Jacinto, Ruta 7, km 59,5, San Jacinto, departamento de Canelones
394	Cybaran, La Caballada, departamento de Salto

II. SHEEP MEAT

Establishment No	Address
1	Codadesa, Ruta 39, km 143, departamento de Maldonado
2	Colonia, Tarariras, departamento de Colonia
3	Carrasco, Camino Carrasco 5, departamento de Canelones
7	Infrinsa, Ruta Brigadier-General Juan A. Lavalleja, km 391, Ciudad de Melo, departamento de Cerro Largo
8	Canelones, Ciudad de Canelones, departamento de Canelones
12	Tacuarembo, Rutas 5 y 25, departamento de Tacuarembo
14	Efcsa (durazno), Santa Bernardina, departamento de Durazno
20	Comargen, Ruta 67 y Elias Regules, las Piedras, departamento de Canelones
106	Improgan, Camino Santos Lugares, km 4, la Pas, departamento de Canelones
344	San Jacinto, Ruta 7, km 59,5, San Jacinto, departamento de Canelones
394	Cybaran, La Caballada, departamento de Salto

III. HORSE MEAT

Establishment No	Address
303	Clay, Ruta 7, km 40, departamento de Canelones

IV. COLD STORES

Establishment No	Address
10	Frigorífico Modelo SA (Planta Propios), Br. Batten Ordones 3029, departamento de Montevideo
87	Santos Arbiza, Colombia 1257, departamento de Montevideo
175	Corfrisa, Las Piedras, departamento de Canelones
903	Acer, Rambla Baltasar Brum 3653, departamento de Montevideo

NINTH COMMISSION DIRECTIVE

of 31 July 1981

establishing Community methods of analysis for the official control of feedingstuffs

(81/715/EEC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Directive 70/373/EEC of 20 July 1970 on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs (1), as last amended by the Act of Accession of Greece, and in particular Article 2 thereof,

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

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

Whereas the measures provided for in this Directive are in accordance with the opinion of the Standing Committee for Feedingstuffs,

HAS ADOPTED THIS DIRECTIVE:

Article 1

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

Article 2

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

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 31 July 1981.

For the Commission
The President
Gaston THORN

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

ANNEX

1. DETERMINATION OF AVOPARCIN BY DIFFUSION IN AN AGAR MEDIUM

1. PURPOSE AND SCOPE

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

2. PRINCIPLE

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

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

3.1. **Maintenance of stock culture**

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

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

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

4. CULTURE MEDIA AND REAGENTS

4.1. **Culture medium** [2]

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

pH 6·5 (after sterilization).

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

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

---

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

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

4.4. Sodium hydroxide, 2 M solution.

4.5. Phosphate buffer, 0·1 M:

Potassium dihydrogen phosphate, KH_2PO_4:13·6 g.

Water to 1 000 ml.

Adjust pH to 4·5.

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

4.7. Standard substance: avoparcin sulphate of known activity.

5. STANDARD SOLUTIONS

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

5.1. **For premixes**

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

S_8 4·0 µg/ml

S_4 2·0 µg/ml

S_2 1·0 µg/ml

S_1 0·5 µg/ml

5.2: **For feedingstuffs**

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

S_8 2·0 µg/ml

S_4 1·0 µg/ml

S_2 0·5 µg/ml

S_1 0·25 µg/ml

6. PREPARATION OF THE EXTRACT AND ASSAY SOLUTIONS

6.1. **Premixes**

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

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

6.2. **Feedingstuffs**

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

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

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

7. ASSAY PROCEDURE

7.1. **Inoculation of the assay medium**

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

7.2. **Preparation of the plates**

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

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

7.3. **Incubation**

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

8. EVALUATION

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

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

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

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

$$\text{(b) SH} = \frac{7\,S_8 + 4\,S_4 + S_2 - 2\,S_1}{10}$$

Similarly, calculate the 'best fit' points for the extract lowest level (UL) and the extract highest level (UH) by substituting U_1, U_2, U_4 and U_8 for S_1, S_2, S_4 and S_8 in the above formulae.

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

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

If the lines are found to be non-parallel, either U_1 and S_1 or U_8 and S_8 may be discarded and SL, SH, UL and UH calculated, using the alternative formulae, to give alternative 'best fit' lines:

$$\text{(a')}\ \text{SL} = \frac{5\,S_1 + 2\,S_2 - S_4}{6} \text{ or } \frac{5\,S_2 + 2\,S_4 - S_8}{6}$$

$$\text{(b')}\ \text{SH} = \frac{5\,S_4 + 2\,S_2 - S_1}{6} \text{ or } \frac{5\,S_8 + 2\,S_4 - S_2}{6}$$

and similarly for UL and UH. The alternative 'best fit' lines should be checked for parallelism as before. 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:

For four levels

$$\text{(c) log. A} = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \times 0{\cdot}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

For three levels

$$\text{(d) log. A} = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4) \times 0{\cdot}401}{U_4 + S_4 - U_1 - S_1}$$

or

$$\text{(d') log. A} = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8) \times 0{\cdot}401}{U_8 + S_8 - U_2 - S_2}$$

Real activity = supposed activity × relative activity.

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.

9. REPEATABILITY

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

— 2 mg/kg, in absolute value, for contents of avoparcin from 2 and 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.

2. DETERMINATION OF MONENSIN SODIUM BY DIFFUSION IN AN AGAR MEDIUM

1. PURPOSE AND SCOPE

The method is for the determination of monensin sodium in feedingstuffs and premixes. The lower limit of determination is 10 mg/kg (10 ppm) (1).

2. PRINCIPLE

The sample is extracted with 90 % methanol. The extract is submitted to appropriate procedures according to the monensin sodium content of the sample. The antibiotic activity is determined by measuring the diffusion of monensin sodium in an agar medium inoculated with Bacillus subtilis. Diffusion is shown by the formation of zones of inhibition of the micro-organism. The diameter of these zones is taken to be in direct proportion to the logarithm of the antibiotic concentration over the range of antibiotic concentrations employed. The sensitivity of this assay system is reduced in the presence of sodium ions.

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

3.1. **Maintenance of stock culture**

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

3.2. **Preparation of the spore suspension** (2)

Harvest the growth from a recently prepared agar slope (3.1) by means of 2 to 3 ml of sterile water. Use this suspension to inoculate 300 ml of culture medium (4.1) contained in a Roux flask and incubate for three to five days at 30 °C. Harvest the growth in 15 ml of 20 % ethanol (4.3), after having checked sporulation under the microscope and mix well. This suspension may be kept for at least five months at about 4 °C.

4. CULTURE MEDIA AND REAGENTS

4.1. **Culture medium** (3)

Tryptone	10·0 g
Yeast extract	3·0 g
Meat extract	1·5 g
Glucose	1·0 g
Agar (according to quality)	10·0 to 20·0 g
Water	1 000 ml

pH 6·5 (after sterilization).

4.2. **Assay medium**

Glucose	10·0 g
Yeast extract	2·5 g
Dipotassium hydrogen phosphate, K_2HPO_4	0·69 g
Potassium dihydrogen phosphate, KH_2PO_4	0·45 g
Agar (according to quality)	10·0 to 20·0 g
Water	1 000 ml

pH 6 (after sterilization).

(1) 1 mg monensin sodium is equivalent to 1 000 UK units.
(2) Other methods may be used provided that it has been established that they give similar spore suspensions.
(3) Any commercial culture medium of similar composition and giving the same results may be used.

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

4.4. Methanol, anhydrous.

4.5. Methanol 90 % (v/v): dilute 900 ml of methanol (4.4) with 100 ml of water.

4.6. Methanol 50 % (v/v): dilute 500 ml of methanol (4.4) with 500 ml of water.

4.7. Aluminium oxide, granulated (alcoa F, 20 mesh; activated alumina UG1: F. Lancaster and Co., or equivalent).

4.8. Standard substances: monensin sodium of known activity (e.g. from the International Laboratory for Biological Standards, Central Veterinary Laboratory, Weybridge, UK-Surrey KT15 3NB).

5. APPARATUS

5.1. Rotary vacuum evaporator, with a 250 ml round-bottom flask.

5.2. Glass tube for chromatography, internal diameter: 25 mm, length: 400 mm, with an open end of 2 mm diameter.

5.3. Glass tube for chromatography, internal diameter: 11 mm, length: approximately 300 mm, with an open end of 2 mm diameter.

6. STANDARD SOLUTIONS

Dissolve an accurately weighed quantity of the standard substance (4.8) in methanol (4.4) and dilute to give a stock solution containing 800 µg monensin sodium per ml. Stored in stoppered flasks at 4 °C, this solution is stable for up to two weeks.

From this stock solution prepare by successive dilution with 50 % methanol (4.6) the following solutions:

S_8 8·0 µg/ml

S_4 4·0 µg/ml

S_2 2·0 µg/ml

S_1 1·0 µg/ml

7. PREPARATION OF THE EXTRACT

7.1. **Extraction**

7.1.1. *Premixes*

Weigh out a quantity of sample of 2 g, add 100 ml of 90 % methanol (4.5), homogenize and centrifuge for a few minutes. Dilute the supernatant solution with 50 % methanol (4.6) to obtain an expected monensin sodium content of 8 µg/ml ($= U_8$).

7.1.2. *Feedingstuffs with a level of monensin sodium not lower than 50 ppm*

Weigh out a quantity of sample of 10 to 20 g, add 100 ml of 90 % methanol (4.5), homogenize for 15 minutes and leave to settle.

Insert a cotton-wool plug at the narrow end of a glass tube (5.2) and add aluminium oxide (4.7) with gentle tapping until the column reaches 75 to 80 mm high.

Decant the extract on to the aluminium oxide column and collect the filtrate. Dilute 30 ml of the filtrate to 50 ml with water. Make subsequent dilutions with 50 % methanol (4.6) to obtain an expected monensin sodium content of 8 µg/ml ($= U_8$).

7.1.3. *Feedingstuffs with a level of monensin sodium lower than 50 ppm (up to the limit of 10 ppm)*

Weigh out a quantity of sample of 10 to 20 g, add 100 ml of 90 % methanol (4.5) and homogenize for 15 minutes. Centrifuge till clear.

For a sample containing 20 ppm of monensin sodium, take 40 ml of the supernatant liquid. For a sample containing 10 ppm, take 80 ml and evaporate to dryness under vacuum on a rotary evaporator (5.1) at not more than 40 °C. Dissolve the residue in 10 ml of 90 % methanol (4.5).

Insert a cotton-wool plug at the narrow end of a glass tube (5.3) and add aluminium oxide (4.7) with gentle tapping until the column reaches 75 to 80 mm high.

Decant the methanolic solution of the residue on to the aluminium oxide column and collect the filtrate. Wash the column with 10 ml of 90 % methanol (4.5) and combine the washings with the filtrate.

Evaporate the solution to dryness under vacuum on a rotary evaporator (5.1) at less than 40 °C. Dissolve the residue in 10 ml of anhydrous methanol (4.4) and make up to 20 ml with water. Centrifuge the solution at at least 4 000 r/min for at least five minutes. Make subsequent dilutions with 50 % methanol (4.6) to obtain an expected monensin sodium content of 8 μg/ml (= U_8).

7.2. **Assay solutions**

From solution U_8 prepare solutions U_4 (expected content: 4 μg/ml), U_2 (expected content: 2 μg/ml) and U_1 (expected content: 1 μg/ml) by means of successive dilution (1 + 1) with 50 % methanol (4.6).

8. ASSAY PROCEDURE

8.1. **Inoculation of the assay medium**

Inoculate the assay medium (4.2) with the spore suspension (3.2) at 50 to 60 °C. By preliminary trials on plates with assay medium (4.2) determine the quantity of spore suspension required to give the largest and clearest zones of inhibition with the various concentrations of monensin sodium.

8.2. **Preparation of the plates**

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

Pour into the plates a quantity of the medium (4.2), inoculated as in 8.1, 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.

8.3. **Incubation**

Incubate the plates for approximately 18 hours at 35 to 37 °C.

9. EVALUATION

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

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

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

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

$$\text{(b) } SH = \frac{7\,S_8 + 4\,S_4 + S_2 - 2\,S_1}{10}$$

Similarly, calculate the 'best fit' points for the extract lowest level (UL) and the extract highest level (UH) by substituting U_1, U_2, U_4 and U_8 for S_1, S_2, S_4 and S_8 in the above formulae.

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

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

If the lines are found to be non-parallel, either U_1 and S_1 or U_8 and S_8 may be discarded and SL, SH, UL and UH calculated, using the alternative formulae, to give alternative 'best fit' lines:

$$\text{(a') } SL = \frac{5\,S_1 + 2\,S_2 - S_4}{6} \text{ or } \frac{5\,S_2 + 2\,S_4 - S_8}{6}$$

$$\text{(b') } SH = \frac{5\,S_4 + 2\,S_2 - S_1}{6} \text{ or } \frac{5\,S_8 + 2\,S_4 - S_2}{6}$$

and similarly for UL and UH. The alternative 'best fit' lines should be checked for parallelism as before. 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:

For four levels

$$\text{(c) } \log. A = \frac{(U_1 + U_2 + U_4 + U_8 - S_1 - S_2 - S_4 - S_8) \times 0{\cdot}602}{U_4 + U_8 + S_4 + S_8 - U_1 - U_2 - S_1 - S_2}$$

For three levels

$$\text{(d) } \log. A = \frac{(U_1 + U_2 + U_4 - S_1 - S_2 - S_4) \times 0{\cdot}401}{U_4 + S_4 - U_1 - S_1}$$

or

$$\text{(d') } \log. A = \frac{(U_2 + U_4 + U_8 - S_2 - S_4 - S_8) \times 0{\cdot}401}{U_8 + S_8 - U_2 - S_2}$$

Real activity = supposed activity × relative activity.

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.

10. REPEATABILITY

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

— 20 % related to the highest value for contents of monensin sodium from 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.