FIFTH COMMISSION DIRECTIVE

of 25 March 1974

establishing Community methods of analysis for the official control of feedingstuffs

(74/203/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, as last amended by the Act [2] annexed to the Treaty [3] concerning the Accession of new Member States to the European Economic Community and the European Atomic Energy Community, 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 Directives No 71/250/EEC of 15 June 1971 [4], No 71/393/EEC of 18 November 1971 [5], No 72/199/EEC of 27 April 1972 [6] and No 73/46/EEC of 5 December 1972 [7] have already established a number of Community methods of analysis; whereas the progress of work since then makes it advisable to adopt a fifth 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

The Member States shall require that analyses for official controls of feedingstuffs as regards the content of starch and of starch degradation products of high molecular weight in feedingstuffs containing beet cossettes, beet bulp, dried beet tops or leaves, potato pulp, dried yeasts, products rich in inulin or greaves be carried out according to the method 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 shall be applicable to the method described in Annex I to this Directive.

Article 2

The Member States shall require that analyses for official controls of feedingstuffs as regard their contents of amprolium, ethopabate, dinitolmide (DOT), nicarbazin and menadione (vitamin K_3) be carried out according to 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 the part dealing with preparation of the sample to be analysed, shall be applicable to the methods described in Annex II to this Directive.

Article 3

The Member States shall, not later than 1 November 1974, bring into force the laws, regulations or administrative provisions necessary to comply with this Directive. They shall forthwith notify the Commission thereof.

Article 4

This Directive is addressed to the Member States.

Done at Brussels, 25 March 1974.

For the Commission

The President

François-Xavier ORTOLI

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[1] OJ No L 170, 3. 8. 1970, p. 2.
[2] OJ No L 73, 27. 3. 1972, p. 14.
[3] OJ No L 73, 27. 3. 1972, p. 5.
[4] OJ No L 155, 12. 7. 1971, p. 13.
[5] OJ No L 279, 20. 12. 1971, p. 7.
[6] OJ No L 123, 29. 5. 1972, p. 6.
[7] OJ No L 83, 30. 3. 1973, p. 21.

ANNEX I

DETERMINATION OF STARCH

— pancreatic method —

1. **Purpose and field of application**

The method makes it possible to determine the content of starch and of starch degradation products of high molecular weight in feedingstuffs containing beet cossettes, beet pulp, dried beet tops or leaves, potato pulp, dried yeasts, products rich in inulin (e.g. cossettes and meal of Jerusalem artichoke) and products containing greaves. The determination should only be carried out when microscopic examination shows that significant quantities of starch are present in the sample.

2. **Principle**

Sugars present in the sample are extracted with ethanol. The starch in the extracted residue is reduced to sugar with pancreatin. The sugars formed are hydrolysed with hydrochloric acid and the glucose formed is determined by the Luff-Schoorl method. The quantity of glucose thus obtained, multiplied by a constant factor, gives the starch content of the sample.

3. **Reagents**

3.1. 90 % (v/v) ethanol, neutral to phenolphthalein.

3.2. Pentan-1-01 (amyl alcohol) a.p.

3.3. Toluene

3.4. Butter solution : dissolve in water 9·078 g potassium dihydrogen phosphate KH_2PO_4 and 11·876 g disodium hydrogen phosphate $Na_2HPO_4\ 2H_2O$. Make up to 1 litre with water.

3.5. Sodium chloride solution 0·2 N.

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

3.7. Carrez II solution : dissolve in water 10·6 g potassium ferrocyanide $K_4\ /Fe\ (CN_6)/\ 3H_2O$. Make up to 100 ml with water.

3.8. Hydrochloric acid N.

3.9. Hydrochloric acid a.p., approximately 8N, d : 1·125.

3.10. Sodium hydroxide solution a.p., approximately 10 N, d : 1·33.

3.11. Indicator : 0·1 % (w/v) methylorange solution.

3.12. Pancreatin in powder form, as per the directions in point 8. Keep in stoppered flasks, protected from light and moisture.

3.13. Luff-Schoorl reagent : Pour the citric acid solution (3.13.2) into the sodium carbonate solution (3.13.3) stirring carefully during the addition. Then add the copper sulphate solution (3.13.1) and make up to 1 litre with water. Leave to stand overnight and filter. Check the normality of the reagent thus obtained (Cu 0·1 N ; $Na_2\ CO_3$ 2N). The pH of the solution must be approximately 9·4.

3.13.1. Copper sulphate solution : dissolve 25 g copper sulphate a.p. $Cu\ SO_4.5H_2O$ in 100 ml water.

3.13.2. Citric acid solution : dissolve 50 g citric acid a.p. $C_6H_8O_7.H_2O$ in 50 ml water.

3.13.3. Sodium carbonate solution : dissolve 143·8 g anhydrous sodium carbonate a.p. in approximately 300 ml hot water. Leave to cool.

3.14. Granules of pumice stone purified by boiling in hydrochloric acid, washing in water and drying.

3.15. 30 % (w/v) solution of potassium iodide a.p.

3.16. Sulphuric acid, approximately 6 N, d : 1·18.

3.17. Sodium thiosulphate solution 0·1 N.

3.18. Starch solution : add a mixture of 5 g soluble starch in 30 ml water to 1 litre boiling water. Boil for 3 minutes, leave to cool. This solution should be freshly prepared.

4. Apparatus

4.1. Extractor (see diagram p. 12), consisting of :

4.1.1. A wide-necked 500 ml conical flask ;

4.1.2. A reflux condenser fitted with a bung to the conical flask ;

4.1.3. A sliding spindle in the centre of the reflux condenser, fitted with a hook at its lower end. A peg to hold the spindle firm ;

4.1.4. A metal container for suspending on the spindle hook (4.1.3) and holding the filtration crucible (4.1.5) ;

4.1.5. A filtration crucible for rapid filtration ; max. size of pores : 90 to 150 microns (e.g. porosity one, approximately 30 ml in capacity ;

4.1.6. Filter papers, suitable in shape and size for the filtration crucible.

4.2. Incubator, set at 38 °C.

4.3. 200 ml graduated flasks with a standard ground glass joint and reflux condenser.

4.4. 100 ml graduated flasks with a standard ground glass joint and reflux condenser.

5. Procedure

5.1. *Preparation of the sample*

Crush the sample so that the whole of it will pass through a 0·5 mm sieve. (ISO sieve of R 565 conforms).

5.2. *Extraction*

Weigh to the nearest mg 2 g of the sample and place in the filtration crucible (4.1.5), the bottom of which has first been covered with a filter paper (4.1.6) moistened with ethanol (3.1). Place in the conical flask (4.1.1) 55 ml ethanol (3.1) and a few granules of pumice stone (3.14). Place the filtration crucible in the metal container (4.1.4) and suspend the latter on the spindle hook (4.1.3). Place the reflux condenser over the conical flask and lower the spindle so that the bottom of the crucible just touches the surface of the ethanol. Peg the spindle firmly at this level. Bring the ethanol to boiling point and keep boiling for 3 hours. Then leave to cool and raise the spindle (4.1.3) to bring the crucible as high up as possible in the conical flask. Carefully unstopper the conical flask and allow 45 ml water to flow down along the sides of the flask. Replace the reflux condenser over the Erlenmeyer flask and keep the filtration crucible 10 cm above the surface of the liquid. Bring the liquid to boiling point and keep boiling for 3 hours. Then leave to cool, unstopper the conical flask and withdraw the crucible from the container.

5.3. *Saccharification and hydrolysis*

Place the crucible on a vacuum flask and dry under suction. Transfer the extraction residue to a mortar and grind finely. Using approximately 60 ml water, transfer the powder quantitatively to a 200 ml graduated flask with a standard ground glass joint and add a few drops of amyl alcohol (3.2). Connect a reflux condenser to the flask. Heat to boiling point and keep boiling for 1 hour. Then leave to cool and disconnect the reflux condenser. Add 25 ml buffer solution (3.4), 250 mg pancreatin (3.12), 2·5 ml sodium chloride solution (3.5) and 10 drops toluene (3.3). Shake for 2 minutes, place the flask in the incubator (4.2) and keep there for 21 hours, shaking occasionally. Then leave to cool to room temperature.

Add 5 ml Carrez I solution (3.6) and shake for one minute. Then add 5 ml Carrez II solution (3.7) and shake once again for one minute. Make up to volume with water, mix and filter. Using a pipette, take 50 ml filtrate and place in a 100 ml graduated flask (one may also work on 100 ml filtrate in a 200 ml graduated flask). Add a few drops of indicator (3.11) and acidify with hydrocholoric acid 8 N (3.9) until the indicator turns red. Then add an extra 6·25 ml hydrochloric acid 8 N (3.9) (12·50 ml if working on 100 ml filtrate). Connect the reflux condenser to the flask, bring the solution to boiling point and keep boiling for 1 hour. Leave to cool, neutralize with the sodium hydroxide solution 10 N (3.10) until the indicator turns yellow. Then acidify slightly by adding a little hydrochloric acid N ('3.8), make up to volume with water and mix. Determine the glucose content according to the **Luff-Schoorl method as shown in 5.4.**

5.4 *Titration according to Luff-Schoorl*

Using a pipette, take 25 ml Luff-Schoorl reagent (3.13) and place in a 300 ml conical flask ; add 25 ml, accurately measured, of the solution obtained in 5·3, this should not contain more than 60 mg of glucose. Add two granules of pumice stone (3.14), heat, while shaking manually, over a medium flame and bring the liquid to boiling point in approximately 2 minutes. Immediately place the conical flask on a wire gauze fitted with an asbestos screen which has a hole approximately 6 cm in diameter. Under the wire gauze a flame has first been lit, and this is regulated in such a way that only the bottom of the conical flask is heated. Then connect a reflux condenser to the conical flask. As soon as this is done, boil for 10 minutes exactly. Cool immediately in cold water and after approximately 5 minutes, titrate as follows :

Add 10 ml potassium iodide solution (3.15) and, immediately afterwards, but with care, (because of the risk of extensive foaming) 25 ml sulphuric acid 6 N (3.16). Then titrate with the sodium thiosulphate solution 0·1 N (3.17) until a dull yellow colouring appears, add a few drops of starch indicator (3.18) and complete the titration.

Carry out the same titration on an accurately measured mixture of 25 ml Luff-Schoorl reagent (3.13) and 25 ml water, after adding 10 ml potassium iodide solution (3.15) and 25 ml sulphuric acid 6 N (3.16), without bringing to the boil.

5.5. *Blank test*

Carry out a blank test, applying the procedure described in 5.3 and 5.4, but without a sample.

6. Calculation of results

Using the table in the Annex, determine the quantity of glucose in mg corresponding to the difference between the results of the two titrations (expressed in ml of sodium thiosulphate 0·1 N) and relating both to the sample analysis and the blank test.

The content of starch as a percentage of sample is given by the formula :

$$0{\cdot}72\ (a - b)$$

where :

a = mg of glucose relating to the sample ;
b = mg of glucose relating to the blank test (see observation 7.2).

7. Observations

7.1 Where partially or totally dextrinated starch and lactose are simultaneously present in the sample, the result may be high by 0·5 to 3·0 %. In such cases, the actual starch content is obtained as follows :

(a) Determine the content of reducing sugars in the ethanolic extract obtained in 5.2, and express the result as a percentage of glucose ;

(b) Determine the content of water-soluble reducing sugars in the sample, and express the result as a percentage of glucose ;

(c) Deduct the result obtained in (a) from that obtained in (b) and multiply the difference by 0·9 ;

(d) Deduct the value obtained in (c) from the starch content obtained by applying the method and calculating as shown in 6.

7.2 The quantity of glucose in relation to the blank test is normally 0·25 mg. It may not be greater than 0·50 mg.

8. Directions relating to pancreatin

Physical aspect : yellowish-white, amorphous powder.

Glucose content : the quantity of glucose of the blank test (see 5.5) is normally 0·25 mg. A result greater than 0·50 mg indicates that pancreatin can no longer be used.

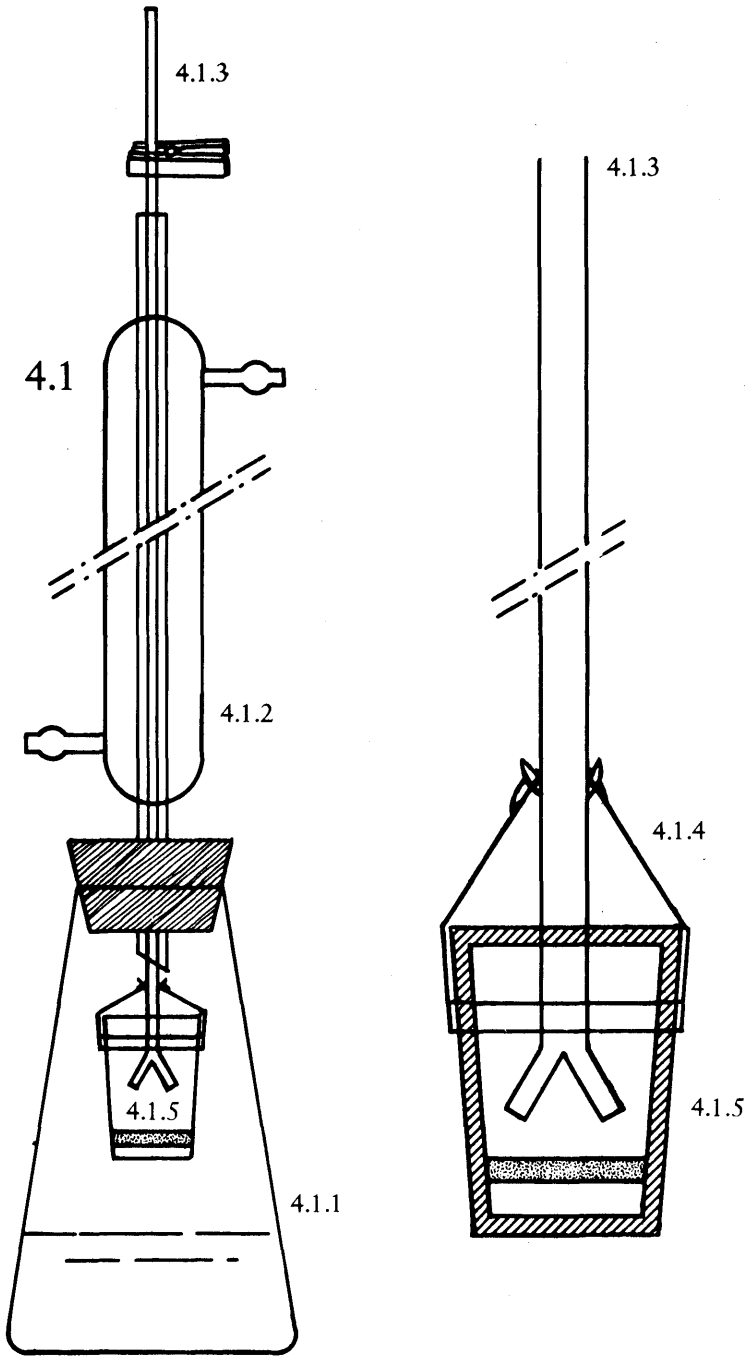
Check for iodine consumption : suspend 62·5 mg pancreatin in approximately 50 ml water heated to 25 to 30 °C. Add 1 ml iodine solution 0·1 N. Stir for 2 minutes. Titrate with a solution of sodium thiosulphate 0·1 N in the presence of starch indicator. Consumption of iodine solution by pancreatin must not exceed 0·5 ml.

Check for amylolytic activity : mix 100 ml starch solution (3.18), 5 ml buffer solution (3.4), 0·5 ml sodium chloride solution (3.5) and 62·5 mg pancreatin. Heat the mixture to 25 to 30 °C, stir for 2 minutes. Add 1 ml iodine solution 0·1 N. The blue colouration must have disappeared within 15 minutes exactly of the addition of the iodine solution.

Table of values for 25 ml Luff-Schoorl reagent

ml of $Na_2S_2O_3$ 0·1 N ; 2 minutes heating ; 10 minutes boiling

$Na_2S_2O_3$ 0·1 N	Glucose, fructose, invert sugars $C_6H_{12}O_6$		Lactose $C_{12}H_{22}O_{11}$		Maltose $C_{12}H_{22}O_{11}$		$Na_2S_2O_3$ 0·1 N
ml	mg	difference	mg	difference	mg	difference	ml
1	2·4		3·6		3·9		1
		2·4		3·7		3·9	
2	4·8		7·3		7·8		2
		2·4		3·7		3·9	
3	7·2		11·0		11·7		3
		2·5		3·7		3·9	
4	9·7		14·7		15·6		4
		2·5		3·7		4·0	
5	12·2		18·4		19·6		5
		2·5		3·7		3·9	
6	14·7		22·1		23·5		6
		2·5		3·7		4·0	
7	17·2		25·8		27·5		7
		2·6		3·7		4·0	
8	19·8		29·5		31·5		8
		2·6		3·7		4·0	
9	22·4		33·2		35·5		9
		2·6		3·8		4·0	
10	25·0		37·0		39·5		10
		2·6		3·8		4·0	
11	27·6		40·8		43·5		11
		2·7		3·8		4·0	
12	30·3		44·6		47·5		12
		2·7		3·8		4·1	
13	33·0		48·4		51·6		13
		2·7		3·8		4·1	
14	35·7		52·2		55·7		14
		2·8		3·8		4·1	
15	38·5		56·0		59·8		15
		2·8		3·9		4·1	
16	41·3		59·9		63·9		16
		2·9		3·9		4·1	
17	44·2		63·8		68·0		17
		2·9		3·9		4·2	
18	47·1		67·7		72·2		18
		2·9		4·0		4·3	
19	50·0		71·7		76·5		19
		3·0		4·0		4·4	
20	53·0		75·7		80·9		20
		3·0		4·1		4·5	
21	56·0		79·8		85·4		21
		3·1		4·1		4·6	
22	59·1		83·9		90·0		22
		3·1		4·1		4·6	
23	62·2		88·0		94·6		23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ANNEX II

1. DETERMINATION OF AMPROLIUM

/Chloride hydrochloride of 1-(4-amino-2-propyl-5-pyrimidylmethyl-2-picolinium/

1. Purpose and scope

The method makes it possible to determine the quantity of amprolium in feedingstuffs, concentrates and premixes. The lower limit of the determination is 40 ppm.

2. Principle

The sample is extracted with diluted methanol. The extract is purified on a column of aluminium oxide and treated with a methanol solution of 2,7-dihydroxydnapthalene, potassium ferricyanide, potassium cyanide and sodium hydroxide, forming a purple colour complex. Amprolium is determined by spectrophotometry at 530 nm.

3. Reagents

3.1. Methanol a.p.

3.2. Diluted methanol : mix two volumes of methanol (3.1) with one volume of water.

3.3. 0·2 % solution (w/v) of potassium ferricyanide $K_3Fe(CN)_6$ a.p. This solution is stable for two weeks.

3.4. 1 % solution (w/v) of potassium cyanide a.p. This solution is stable for two weeks.

3.5. 1·125 % solution (w/v) of sodium hydroxide a.p.

3.6. Methanolic sodium hydroxide solution : dilute 15 ml of the solution (3.5) to 200 ml with methanol (3.1).

3.7. 0·0025 % solution (w/v) of 2,7-dihydroxynapthalene : dissolve 25 mg of 2,7-dihydroxynaphthalene a.p. in methanol (3.1) and make it up to 1 000 ml with methanol (3.1).

3.8. Colour reagent : Transfer 90 ml of 2,7-dihydroxynaphthalene solution (3.7) to a 250 ml conical flask (4.1), add 5 ml of potassium ferricyanide solution (3.3) and mix well. Then add 5 ml of potassium cyanide solution (3.4), stopper the flask and mix well. Leave to stand for 30 to 35 minutes, add 100 ml of methanolic sodium hydroxide solution (3.6), mix and filter through a filter crucible (4.3). Use this reagent in the 75 minutes following filtration.

3.9. Aluminium oxide for column chromatography. Before use, stir 100 g of aluminium oxide with 500 ml of water for 30 minutes, filter the slurry, wash the aluminium oxide on the filter 3 times with 50 ml of methanol (3.1) drying each time by suction, leave to stand overnight and then dry for 2 hours at 100 °C in a vacuum drier. Put in a desiccator to cool. Check strength by subjecting a specified quantity of standard solution (3.11) to analysis, starting from point 5.2. The recovery rate of the amprolium must be 100 % ± 4 %.

3.10. Standard substance : pure amprolium complying with the characteristics below.

Melting point (decomposition) : 248 °C.
Molecular extinction coefficient at both 265 and 235 nm in distilled water : $11{\cdot}0 \times 10^3$.

3.11. Standard solution : Weigh out to within 0·1 mg, 50 mg of standard substance (3.10). Dissolve in diluted methanol (3.2) in a 500 ml volumetric flask, make up the volume with the same solvent and homogenize. Dilute 10·0 ml to 50 ml with diluted methanol (3.2) in a volumetric flask and mix well. 1 ml of this solution contains 20 µg of amprolium.

4. Apparatus

4.1. 50, 250 and 500 ml conical flasks with ground-glass stoppers.

4.2. Stirrer.

4.3. Filtering crucible, porosity G3, diameter : 60 mm.

4.4. Glass tube for chromatography (interior diameter : 9 mm, length : 400 to 500 mm).

4.5. Centrifuge with 25 ml tubes with ground-glass stoppers.

4.6. Spectrophotometer, with 10 mm cells.

5. Procedure

5.1. *Extraction and purification*

5.1.1. *Feedingstuffs and premixes*

Weigh out to within 1 mg, 10 g of the finely divided and homogenized sample. For premixes weigh out 3 to 6 grams, within 1 mg. Place the test portion in a 250 ml conical flask (4.1) and add exactly 100 ml of diluted methanol (3.2). Shake for 60 minutes and filter. Dilute with diluted methanol (3.2) if necessary to obtain a solution containing 5 to 15 µg of amprolium per ml.

Insert a cotton wool plug into the lower end of a chromatographic tube (4.4), and tamp in 5 g of aluminium oxide (3.9) and then run in 25·0 ml of the extract. Let the liquid run through the column, discard the first 5 ml and collect the next 12 ml in a graduated test-tube.

5.1.2. *Concentrates*

Weigh out to within 1 mg 0·5 g of the finely divided and homogenized sample, place it in a 500 ml conical flask (4.1), add 250 ml of diluted methanol (3.2) shake or stir for 60 minutes and filter. Dilute 5·0 ml of the filtrate to 200 ml with diluted methanol (3.2) in a volumetric flask.

5.2. *Development of colour and measurement of the optical density*

Transfer 5·0 ml of the solution obtained in 5.1.1 or 5.1.2 into centrifuge tube A (4.5). Place 5·0 ml of diluted methanol (3.2) in centrifuge tube B (4.5). Add to each tube 10·0 ml of colouring reagent (3.8), stopper the tubes, mix and leave to stand for 18 minutes. Then centrifuge for 3 minutes and decant solutions A and B in 50 ml conical flasks (4.1).

Immediately measure the optical density of solution A at 530 nm in the spectrophotometer, using solution B as a control. Determine the quantity of amprolium by referring to the calibration curve (5.3).

5.3. *Calibration curve*

Pipette into centrifuge tubes (4.5) volumes of 1·0, 2·0, 3·0, 4·0 and 5·0 ml respectively of the standard solution (3.11). Make the volumes of the first four tubes up to 5·0 ml with diluted methanol (3.2). Add to all five tubes 10·0 ml of colouring reagent (3.8), stopper the tubes, mix and leave to stand for 18 minutes. Then centrifuge for 3 minutes and decant the solutions into 50 ml conical flasks (4.1).

Immediately measure the optical density of the solutions at 530 nm in the spectrophotometer, using a mixture of 5 ml ciluted methanol (3.2) and 10 ml of colour reagent (3.8) as a control. Plot the calibration curve, using the optical density values as the ordinates and the corresponding quantities of amprolium in mg as the abcissa.

6. Calculation of results

6.1. *Feedingstuffs and premixes*

The amprolium content in mg per kg of sample is given by the formula

$$\frac{A}{W} \cdot F \cdot 20\,000$$

in which :

A = quantity of amprolium in mg as determined by photometric measurement.
W = weight of the test portion in g.
F = coefficient of dilution (possibly worked out in 5.1.1).

6.2. *Concentrates*

The amprolium content per cent of sample is given by the formula

$$\frac{A}{W} \cdot 200$$

in which :

A = quantity of amprolium in mg as determined by phototmetric measurement.
W = weight of the test portion in g.

7. **Repeatability**

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

10 ppm, in absolute value, for amprolium contents below 100 ppm ;
10 %, in relative value, for contents between 100 and 5 000 ppm ;
500 ppm, in absolute value, for contents between 5 000 and 10 000 ppm ;
5 %, in relative value, for contents above 10 000 ppm.

2. DETERMINATION OF ETHOPABATE

(methyl-4-acetamido-2-ethoxybenzoate)

1. Purpose and scope

The method makes it possible to determine the quantity of ethopabate in feedingstuffs, concentrates and premixes. The lower limit of the determination is 2 ppm.

2. Principle

The sample is extracted with diluted methanol. The solution is acidified and extracted with chloroform. The chloroform extract is washed first with an alkaline solution and then with water. The purified extract is concentrated, the ethopabate is hydrolysed with diluted hydrochloric acid. The amino derivative thus formed is diazotized and coupled with 2-aminoethyl-1-naphthylamine. The coloured complex is extracted with butanol and the optical density of the solution is measured at 555 nm.

3. Reagents

3.1. Methanol a.p.

3.2. 50 % (v/v) methanol : mix equal volumes of methanol (3.1) and water.

3.3. Hydrochloric acid a.p., d : 1·19.

3.4. 1/10th dilute hydrochloric acid : Dilute 10·0 ml of hydrochloric acid (3.3), to 100 ml with water.

3.5. Approximately 0·3 N hydrochloric acid : Dilute 25 ml of hydrochloric acid (3.3), to 1 000 ml with water.

3.6. Chloroform a.p.

3.7. 4 % (w/v) sodium carbonate solution : Dissolve 40·0 g of anhydrous sodium carbonate a.p. in water and make up to 1 000 ml with water.

3.8. 0·2 % (w/v) sodium nitrite solution : Dissolve 100 mg of sodium nitrite a.p. in water and make up to 50 ml with water in a volumetric flask. Prepare immediately before use.

3.9. 1·0 % (w/v) ammonium sulphamate solution : Dissolve 500 mg of ammonium sulphamate a.p. in water and make up to 50 ml water in a volumetric flask. Prepare immediately before use.

3.10. 0·2 % (w/v) 2-aminoethyl-1-naphthylamine solution : Dissolve 100 mg of 2-aminoethyl-1-naphthylamine a.p. in water and make up to 50 ml with water in a volumetric flask. Prepare immediately before use.

3.11. Anhydrous sodium chloride a.p.

3.12. n-butanol a.p.

3.13. Standard substance : pure ethopabate.

3.14. Standard solutions :

3.14.1 Solution of 0·040 mg of ethopabate per ml : Weigh out 40 mg to within 0·1 mg of standard substance (3.13). Dissolve in methanol (3.2) in a 100 ml volumetric flask ; make up the volume with the same solvent and mix. Dilute 10·0 ml to 100 ml with methanol (3.2) in a volumetric flask and mix. This solution is stable for a month.

3.14.2. Solution of 0·016 mg of ethopabate per 20 ml : Dilute 5·0 ml of the solution (3.14.1) to 250 ml with methanol (3.2) in a volumetric flask and mix well. Prepare before use.

4. Apparatus

4.1. 250 ml conical flasks, with ground-glass stoppers.

4.2. 100 ml separating funnels, with ground-glass stoppers.

4.3. Shaker.

4.4. Rotary vacuum evaporator, with 250 ml flasks.

4.5. Water bath.

4.6. Centrifuge, with 50 ml and 15 ml tubes, with ground-glass stoppers.

4.7. Air condenser, with ground-glass joint.

4.8. Spectrophotometer, with 10 mm cells.

5. **Procedure**

5.1. *Extraction*

Weigh out to within 1 mg, a quantity of the finely divided and homogenized sample, containing about 80 µg of ethopabate. Place the test portion in a 250 ml conical flask (4.1) and add 100·0 ml of diluted methanol (3.2). Mix, stopper the flask and shake for 1 hour with the aid of a shaker (4.3). Decant, filter and discard the first mls of the filtrate.

5.2. *Purification*

N.B. All operations under this point must be carried out rapidly.
Transfer 20·0 ml of the clear extract into a 100 ml separating funnel (4.2), add 5·0 ml of 1/10th diluted hydrochloric acid (3.4) and 20·0 ml of chloroform (3.6). Shake, first carefully and then vigorously for 3 minutes. Leave to stand until the zones separate and collect the chloroform phase in a second 100 ml separating funnel (4.2).

Extract the acid phase twice more with 20·0 ml of chloroform (3.6). Collect the chloroform extracts in the second separating funnel and discard the acid phase. Add to the combined chloroform solution 10 ml of sodium carbonate solution (3.7), shake for 3 minutes and leave to stand until the phases separate. Collect the chloroform phase in a third 100 ml separating, funnel (4.2) and discard the aqueous phase. Add to the chloroform solution 10 ml of sodium carbonate solution (3.7), shake for 3 minutes and leave to stand until the phases separate.

Collect the chloroform phase in a fourth 100 ml separating funnel (4.2), wash twice consecutively with 25·0 ml of water each time, separate the aqueous phases and quantitatively collect the chloroform extract in a 250 ml balloon flask (4.4). Combine the aqueous phases together, in one of the separating funnels ; rinse each empty funnel with a few ml chloroform ; shake the aqueous phase with the same ml chloroform, allow phases to separate, and transfer the chloroform phase to the chloroform extract collected in the flask.

5.3. *Hydrolysis*

Evaporate the chloroform extract down to about 2 ml on a 50 °C water bath with the aid of the rotary vacuum evaporator (4.4). Dissolve the residue in 2 to 3 ml of methanol (3.1), and transfer quantitatively the solution in a 50 ml centrifuge tube (4.6) with the aid of two 10 ml portions and one 5 ml portion of 0·3 N hydrochloric acid (3.5). Fit the air condenser (4.7), add a few glass beads, shake well, plunge the tube in a bath of boiling water and keep it there for 45 minutes. Then cool under a stream of cold running water.

5.4. *Development of colour, and measurement of the optical density*

Add 1·0 ml of sodium nitrite solution (3.8), stir and leave to stand for 2 minutes. Add 1·0 ml of ammonium sulphamate solution (3.9), shake and leave to stand for 2 minutes. Add 1·0 ml of 2-aminoethyl-1-naphthylamine solution (3.10), stir and leave to stand for 10 minutes. Add 5·0 g of sodium chloride (3.11) and 10·0 ml of n-butanol (3.12), shake vigorously until the sodium chloride has completely dissolved.

Draw off the supernatant butanolic solution with the aid of a pipette, and transfer it to a 15 ml centrifuge tube (4.6) and centrifuge. Then measure the optical density E_A with a spectrophotometer at 555 nm using n-butanol (3.12) as blank.

5.5. *Control test*

Carry out a control test, using the same procedure, starting from point 5·2, on 20·0 ml of diluted methanol (3.2). Measure the optical density F_B at 555 nm using n-butanol (3.12) as blank.

5.6. *Standard test*

Carry out a standard test, using the same procedure, starting from point 5·2, on 20·0 ml of standard solution (3.14.2). Measure the optical density E_C at 555 nm using n-butanol (3.12) as blank.

6. **Calculation of results**

The ethopabate content in mg per kg of sample is given by the formula

$$\frac{(E_A - E_B)}{(E_C - E_B} \cdot \frac{80}{W}$$

in which :

E_A = optical density of the solution from the sample.
E_B = optical density of the solution resulting from the control test.
E_C = optical density of the solution resulting from the standard test.
W = weight of test portion in g.

7. **Repeatability**

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

20 %, in relative value, for contents of ethopabate below 7·5 ppm ;
1·5 ppm, in absolute value, for contents between 7·5 and 10 ppm ;
15 %, in relative value, for contents above 10 ppm.

3. DETERMINATION OF DINITOLMIDE (DOT)

(3·5-dinitro-o-toluamide)

1. Purpose and scope

The method makes it possible to determine the quantity of dinitolmide (DOT) in feedingstuffs, concentrates and premixes. Nitrofurane derivatives may interfere. The lower limit of the determination is 40 ppm.

2. Principle

The sample is extracted with acetronitrile. The extract is purified on aluminium oxide and filtered. An aliquot of the filtrate is evaporated to dryness. The residue is dissolved in dimethylformamide and treated with ethylenediamine forming a purple complex. Dinitolmide is determined by spectrophotometry at 560 nm.

3. Reagents

3.1. 85 % (v/v) acetonitrile : mix 850 ml of pure acetonitrile and 150 ml of water. Distil the mixture and collect the fraction which boils between 75 and 77 °C before use.

3.2. Aluminium oxide for column chromatography. Calcinate at 750 °C for at least 2 hours, cool in desiccator and keep in a brown glass bottle with a ground-glass stopper. Before use humidify as follows : place in a brown glass bottle 10 g of aluminium oxide and 0·7 ml of water, stopper, heat for 5 minutes in a bath of boiling water, shaking virgorously, let it cool, still shaking. Check strength by subjecting to analysis, starting from point 5.1, a determined quantity of standard solution (3.6). The recovery rate of the dinitolmide must be 100 % ± 2 %.

3.3. 95 % (v/v) N,N-dimethylformamide : mix 95·0 ml of N,N-dimethylformamide a.p. and 5·0 ml of water.

3.4. Diaminoethane a.p. maximum water content : 2·0 %.

3.5. Standard substance : pure 3·5-dinitro-o-toluamide complying with the characteristics below :

melting point : 177 °C ;
molecular extinction coefficient at 248 nm in acetonitrile : $13{\cdot}1 \times 10^3$;
molecular extinction coefficient at 266 nm in N,N-dimethylformamide : $10{\cdot}1 \times 10^3$.

3.6. Standard solution : weigh out, to within 0·1 mg, 40 mg of standard substance (3.5), dissolve with acetonitrile (3.1) in a 200 ml volumetric flask, make up the volume with the same solvent and mix. Dilute 20·0 ml to 100 ml with acetonitrile (3.1) in a volumetric flask and mix. 1 ml of this solution contains 40 µg of dinitolmide.

4. Apparatus

4.1. 250 ml conical flask with ground-glass stopper.

4.2. Reflux condenser with ground-glass joint.

4.3. Filtering crucible, porosity G 3, diameter 60 mm.

4.4. Vacuum filter (such as Witt apparatus)

4.5. Water bath, set at 50 °C.

4.6. Spectrophotometer, with 10 mm cells.

5. Procedure

5.1. *Extraction and purification*

Weigh out to within 1 mg, 10 g of the finely divided and homogenized sample. For concentrates and premixes, weigh out 1g, to within 1 mg. Place the test portion in a 250 ml conical flask (4.1) and add 65 ml of acetonitrile (3.1). Mix, fit reflux condenser (4.2) to the flask and heat in the water bath (4.5) for 30 minutes, shaking continuously. Cool under stream of cold water. Add 20 g of aluminium oxide (3.2), shake for 3 minutes, leave to settle.

Place a 100 ml-volumetric flask in the vacuum filter (4.4), fit filtering crucible (4.3) and filter the solution, using suction. Then transfer the remaining solids into the crucible with the aid of a few ml of acetonitrile (3.1) and suck the residue dry. Release the vacuum, suspend the cake again in a few ml of acetonitrile (3.1) and evacuate again. Repeat these last operations until the volume of the filtrate reaches about 95 ml. Make up to 100 ml with acetonitrile (3.1) and mix. If necessary, dilute an aliquot with acetonitrile (3.1) to obtain a solution containing 5 to 15 µg of dinitolmide per ml.

5.2. *Development of colour and measurement of the optical density*

Pipette into three 50 ml beakers A, B and C respectively, 4·0 ml of the solution obtained in 5.1. Also add *to beaker C only* 1·0 ml of standard solution (3.6). Place the three beakers on the water bath (4.5), placed under a well ventilated hood, and evaporate until dry in a current of dry air. Cool the three beakers to room temparature.

Add 10·0 ml of N,N-dimethylformamide (3.3) in beaker A and 2·0 ml in beakers B and C respectively, leave in contact for a few minutes, stirring a little, until the residue completely dissolves. Then add 8·0 ml of diaminoethane (3.4) in beakers B and C and mix. Exactly 5 minutes after adding the diaminoethane measure the optical density of the three solutions in the spectrophotometer (4.6) at 560 nm using the N,N-dimethylformamide (3.3) as a blank.

6. Calculation of results

The dinitolmide content in mg per kg of sample is given by the formula :

$$\frac{(E_B - E_A)}{(E_C - E_B)} \cdot \frac{F}{W} \cdot 1\,000$$

in which :

E_A = optical density of solution A (blank).
E_B = optical density of solution B (sample).
E_C = optical density of solution C (internal standard).
W = weight of test portion in grams.
F = coefficient of dilution (possibly worked out in 5.1).

7. Repeatability

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

10 ppm, in absolute value, for contents of dinitolmide below 100 ppm ;
10 %, in relative value, for contents between 100 and 5 000 ppm ;
500 ppm, in absolute value, for contents between 5 000 and 10 000 ppm ;
5 %, in relative value, for contents above 10 000 ppm.

4. DETERMINATION OF NICARBAZIN

(equimolecular mixture of 4,4'-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine)

1. Purpose and scope

The method makes it possible to determine the quantity of nicarbazin in feedingstuffs, concentrates and premixes containing not more than 5 % grassmeal. Nitrofuran derivatives, acetylenheptine and carbadox may interfere. The lower limit of the determination is 20 ppm.

2. Principle

The sample is extracted with N,N-dimethylformamide. The extract is purified by chromatography on a column of aluminium oxide ; the nicarbazin is eluted with ethanol. The eluate is treated with ethanolic sodium hydroxide ; forming a yellow colour. Nicarbazin is determined by spectrophotometry at 430 nm.

3. Reagents

3.1. N,N-dimethylformamide a.p.

3.2. Aluminium oxide for column chromatography. Calcinate at 750 °C for at least 2 hours, cool in desiccator and keep in a brown glass bottle with a ground-glass stopper. Before use, check strength by subjecting to analysis, starting from point 5.2, a determined quantity of standard solution (3.8.3). The recovery rate of the nicarbazin must be 100 % ± 2 %.

3.3. 95 % (v/v) ethanol.

3.4. 80 % (v/v) ethanol.

3.5. 50 % (w/v) sdium hydroxide a.p. solution.

3.6. 1 % (w/v) ethanolic sodium hydroxide : put 1 ml of sodium hydroxide solution (3.5) in a 50 ml volumetric flask ; make up the volume with 80 % ethanol (3.4). Prepare at the time of use.

3.7. Standard substance : pure nicarbazin, molecular extinction coefficient at 362 nm in N,N-dimethylformamide : $37{\cdot}8 \times 10^3$.

3.8. Standard solutions :

3.8.1. Solution of 1·25 mg of nicarbazin per ml : weigh out, to within 0·1 mg, 125 mg of standard substance (3·7). Dissolve in 75 ml of N,N-dimethylformamide (3.1) in a 100 ml volumetric flask, heating slightly, Allow to cool, make up the volume with the same solvent and mix. Keep away from light.

3.8.2. Solution of 0·125 mg of nicarbazin per ml : Dilute 10·0 ml of the solution (3.8.1), to 100 ml with N,N-dimethylformamide (3.1) in a volumetric flask and mix.

3.8.3. Solution of 0·025 mg of nicarbazin per ml : Dilute 20·0 ml of the solution (3.8.2), to 100 ml with N,N-dimethylformamide (3.1) in a volumetric flask and mix.

4. Apparatus

4.1. 250 ml conical flask, with ground-glass stopper.

4.2. Reflux condenser with ground-glass joint.

4.3. Boiling water bath.

4.4. Centrifuge, with 120 ml tubes.

4.5. Glass tube for chromatography (interior diameter : 25 mm, length : 300 mm).

4.6. Spectrophotometer, with 10 mm cells.

4.7. Burette marked in 1/10th mls.

5. Procedure

5.1. *Extraction*

Weigh out, to within 1 mg, 10 g of the finely divided and homogenized sample. For concentrates and premixes, weigh out 1 g, to within 1 mg. Place the test portion in a 250 ml conical flask (4.1) and add exactly 100 ml of N,N-dimethylformamide (3.1). Mix, fit reflux

condenser (4.2) on the flask and heat on the water-bath (4.3) for 15 minutes, shaking from time to time. Cool under a stream of cold water. Then pour the supernatant layer into a centrifuge tube (4.4) and centrifuge for about 3 minutes. If necessary, dilute 25·0 ml of the supernatant layer with N,N-dimethylformamide (3.1), to obtain a solution containing 2·0 to 10 µg of nicarbazin per ml.

5.2 *Chromatography*

Run into a tube for chromatography (4.5) a slurry of 30 g of aluminium oxide (3.2) in N,N-dimethylformamide (3.1). Let the liquid level fall to 1 cm above the column of aluminium oxide and then put into the column 25·0 ml of the extract obtained in 5.1. Allow the liquid to flow through, not letting the column get dry, and wash the column with three 10 ml portions of N,N-dimethylformamide (3.1). Then elute with 70 ml of 95 % ethanol (3.3). Eliminate the first 10 ml of the eluate and collect the rest, dividing it up as follows :

one 5 ml portion (a) ;
one 50 ml portion (b) in a volumetric flask ;
one 5 ml portion (c).

Check that portions (a) and (c) do not turn yellow when ethanolic sodium hydroxide (3.6) is added. Continue the operations on portion (b) as shown in 5.3.

5.3 *Development of colour and measurement of the optical density*

Pipette 20·0 ml of portion (b) of the eluate in two separate 25 ml volumetric flasks A and B. Add to flask A 50 ml of ethanolic sodium hydroxide (3.6) and to flask B 5.0 ml of 95 % methanol (3.3).
Mix well.

Within the next five minutes measure the optical density of both solutions at 430 nm, using a mixture of 20·0 ml of 95 % methanol (3.3) and 5.0 ml of ethanol solution of sodium hydroxide (3.6) as a blank.

Subtract the value of the optical density of solution B from that of solution A. From this value determine the quantity of nicarbazin referring to the calibration curve (5.4).

5.4 *Calibration*

Subject 25·0 ml of the standard solution (3.8.3) to chromatography as shown in 5.2. Pour portion (b) of the eluate into the graduated burette (4.7) and distribute it in 25 ml volumetric flasks in respective volumes of 2·0, 4·0, 6·0, 8·0 and 10·0 ml (corresponding to 0·025, 0·050, 0·075, 0·100 and 0·125 mg of nicarbazin respectively). To each flask add 5.0 ml of ethanolic sodium hydroxide (3.6), make the volume up with 95 % ethanol (3.3) and mix well.

Within the next five minutes measure the optical density of the solutions at 430 nm, using a mixture of 20·0 ml of 95 % ethanol (3.3) and 5.0 ml of ethanolic sodium hydroxide (3.6) as a control.

Trace the calibration curve, using the optical density values as the ordinates and the corresponding quantities of nicarbazin in mg as the abcissa.

6. Calculation of results

The nicarbazin content in mg per kg of sample is given by the formula

$$\frac{A}{W} \cdot F \cdot 10\,000$$

in which :

A = quantity of nicarbazin in mg as determined by photometric measurement ;
W = weight of test portion in grams ;
F = coefficient of dilution (possibly worked out in 5.1).

7. Repeatability

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

10 ppm, in absolute value, for contents of nicarbazin below 100 ppm ;
10 %, in relative value, for contents between 100 and 5 000 ppm ;
500 ppm, in absolute value, for contents between 5 000 and 10 000 ppm ;
5 %, in relative value, for contents above 10 000 ppm.

5. DETERMINATION OF MENADIONE (VITAMIN K_3)

1. Purpose and scope

The method makes it possible to determine the quantity of menadione (vitamin K_3) in feedingstuffs, concentrates and premixes. The lower limit of the determination is 1 ppm.

2. Principle

The sample is extracted with diluted ethanol. The mixture is clarified with tannin solution and centrifuged. The extract is treated with a solution of sodium carbonate ; the liberated menadione is extracted with 1,2-dichloroethane. The dichloroethane extract is treated, according to its menadione content, either directly or after evaporation, with 2,4-dinitrophenylhydrazine in solution in ethanol acidified with hydrochloric acid. The obtained hydrazone treated with ammonia in excess gives rise to a blue-green coloured complex the optical density of which is measured at 635 nm.

3. Reagents

3.1. 96 % (v/v) ethanol.

3.2. Ethanol (3.1) diluted to 40 % with water.

3.3. 10 % (w/v) tannin solution, prepared from purified powdered tannin.

3.4. 1,2-dichloroethane a.p.

3.5. 10 % (w/v) solution of anhydrous sodium carbonate p.a.

3.6. 37 % (w/v) hydrochloric acid, d = 1·19.

3.7. Absolute ethanol a.p.

3.8. 2,4-dinitrophenylhydrazine reagent : Dissolve 40 mg 2,4-dinitrophenylhydrazine a.p. in about 40 ml boiling absolute ethanol (3.7), allow to cool and transfer into a 50 ml volumetric flask. Add 1 ml hydrochloric acid (3.6) and make up to volume with absolute ethanol (3.7). Prepare immediately before use.

3.9. 25 % (w/v) ammonia, d = 0·91.

3.10. Ammoniacal ethanol : mix one volume of ethanol (3.7) with one volume of ammonia (3.9).

3.11. Standard solutions of menadione : Dissolve 20 mg menadione (vitamin K_3) in 1,2-dichloroethane (3.4) and make up to 200 ml. Dilute aliquots of this stock solution with 1,2-dichloroethane (3.4) to obtain a series of solutions with menadione concentrations between 2 and 10 µg per ml. These solutions must be freshly prepared.

4. Apparatus

4.1. Mechanical shaker.

4.2. Centrifuge (3 000 to 5 000 r.p.m.).

4.3. 100 and 250 ml separators, with ground glass stoppers.

4.4. Rotary vacuum evaporator, with 250 ml flasks.

4.5. Water bath.

4.6. Spectrophotometer, with 10 mm cells.

5. Procedure

N.B. All operations must be carried out away from direct light, using apparatus of amber glass where necessary.

5.1. *Test Sample*

From the finely divided sample, take a test sample according to the presumed menadione content, e.g. :

0·1 to 5·0 g for concentrates and premixes ;
20 to 30 g for feedingstuffs.

Transfer the test sample to a 250 ml flask with ground glass stopper without delay.

5.2 *Extraction*

Add to the test sample exactly 96 ml dilute ethanol (3.2) and shake mechanically for 15 minutes at room temperature. Then add 4·0 ml tannin solution (3.3), mix, transfer the extract into a centrifuge tube, centrifuge at 3 000 to 5 000 r.p.m. and decant.

Place 20 to 40 ml, accurately measured, of the extract in a 250 ml separator, add by pipette 50 ml 1,2-dichloroethane (3.4), mix and add by pipette 20 ml sodium carbonate solution (3.5). Shake vigorously for 30 seconds and then collect the dichloroethane phase in a 100 ml separator. Add 20 ml water, shake again for 15 seconds, collect the dichloroethane phase and remove traces of water with strips of filter paper.

For concentrates and premixes, take an aliquot part of the extract and dilute with 1,2-dichloroethane (3.4) to obtain a menadione concentration of 2 to 10 µg per ml. For feedingstuffs, evaporate to dryness an aliquot part of the extract under reduced pressure in an atmosphere of nitrogen on a water bath at 40 °C. Rapidly treat the residue with 1,2-dichloroethane (3.4) to obtain a solution containing 2 to 10 µg menadione per ml.

5.3. *Hydrazone formation*

Transfer 2·0 ml of the dichloroethane extract obtained in 5·2 to a 10 ml volumetric flask and add 3·0 ml 2,4-dinitrophenylhydrazine reagent (3.8), securely stopper the flask with a cork or teflon stopper so as to prevent evaporation and heat for two hours at 70 °C on a water bath. Allow to cool, add 3·0 ml ammoniacal ethanol (3.10), mix, make up to volume with absolute ethanol (3.7) and mix again.

5.4. *Measurement of the optical density*

Measure the optical density of the blue-green coloured complex by the spectrophotometer at 635 nm by comparison with a reagent blank obtained by treating 2·0 ml 1,2-dichloroethane (3.4) as indicated in 5.3.

Determine the quantity of menadione by reference to a calibration curve established for each series of analyses.

5.5. *calibration curve*

Treat 2·0 ml of the menadione standard solutions (3.11) as described in 5.3. Measure the optical density as indicated in 5.4. Plot the calibration curve with the optical density values as ordinates and the corresponding quantities of menadione in µg as abscissae.

6. **Calculation of results**

Calculate the menadione content of the sample by taking account of the weight of the test sample and of the dilations carried out in the course of analysis.

Express the result in mg menadione per kg.

7. **Repeatability**

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

— 20 %, in relative value, for menadione contents less than 10 ppm ;
— 2 ppm, in absolute value, for contents between 10 and 14 ppm ;
— 15 %, in relative value, for contents between 14 and 100 ppm ;
— 15 ppm, in absolute value, for contents between 100 and 150 ppm ;
— 10 %, in relative value, for contents greater than 150 ppm.