COMMISSION

SIXTH COMMISSION DIRECTIVE

of 20 December 1974

establishing Community methods of analysis for the official control of feedingstuffs

(75/84/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 to 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 Commission 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), No 73/46/EEC of 5 December 1972 (7) and No 74/203/EEC of 25 March 1974 (8) have already established a number of Community methods of analysis; whereas the progress of work since then makes it advisable to adopt a sixth 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 their content of buquinolate, sulphaquinoxaline and furazolidone be carried out in accordance with the methods described in the Annex 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 the Annex to this Directive.

Article 2

The Member States shall not later than 1 November 1975 bring into force the laws, regulations or administrative provisions necessary to comply with this Directive. They shall forthwith notify the Commission thereof.

Article 3

This Directive is addressed to the Member States.

Done at Brussels, 20 December 1974.

For the Commission

The President

François-Xavier ORTOLI

(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.
(8) OJ No L 108, 22. 4. 1974, p. 7.

ANNEX

1. DETERMINATION OF BUQUINOLATE

(ethyl-4-hydroxy-6,7-diisobutoxy-3-quinoline carboxylate)

1. Purpose and scope

The method makes it possible to determine the quantity of buquinolate in feedingstuffs, concentrates and premixes. The lower limit of determination is 10 ppm. Decoquinate interferes in the determination.

2. Principle

The sample is extracted with chloroform: The extract is evaporated to dryness, the residue is dissolved in chloroform and the solution is then subjected to thin-layer chromatography. The buquinolate is eluted with ethanol and determined spectrophotofluoremetrically by comparison with standard solutions.

3. Reagents

3.1. Chloroform a.p.

3.2. 96 % (V/V) ethanol a.p.

3.3. Mixture of chloroform and ethanol: mix 10 volumes of chloroform (3.1) with one volume of ethanol (3.2).

3.4. 80 % (V/V) ethanol a.p.

3.5. Silica gel G for thin-layer chromatography.

3.6. Standard substance: pure buquinolate.

3.7. Standard solutions:

3.7.1. Standard solution of 0·2 mg of buquinolate per ml: Weigh out 50 mg, to within 0·1 mg, of standard substance (3.6). Dissolve in chloroform (3.1) in a 250 ml volumetric flask by warming in a water-bath at 50° C. Leave to cool to room temperature, make up the volume with chloroform (3.1) and mix.

3.7.2. Working standard solutions: Transfer 5, 10, 15, 20 and 25 ml aliquots of the solution (3.7.1) into 25 ml volumetric flasks. Make up the volume with chloroform (3.1) and mix. Prepare immediately before use. These solutions contain respectively 0·04, 0·08, 0·12, 0·16 and 0·20 mg of buquinolate per ml.

4. Apparatus

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

4.2. Shaker.

4.3. Centrifuge, with 15 ml tubes with ground-glass stoppers.

4.4. Water bath at 50° C.

4.5. Equipment for thin-layer chromatography.

4.6. Glass-plates for thin-layer chromatography, 200 × 200 mm, treated as follows. Spread on the plates a uniform layer 0·5 mm thick of silica gel G (3.5) and leave to dry in the air for 15 minutes. Keep the plates in the drying oven (4.11) for two hours and transfer into a dessicator containing dehydrating silica gel. Ready-made plates are suitable if they give results similar to those for the plates treated as indicated above.

4.7. 0·50 ml micropipettes.

4.8. Zone collector for thin-layer chromatography.

4.9. Short-wavelength ultraviolet lamp.

4.10. Spectrophotofluorimeter fitted with a xenon lamp, and two monochromators.

4.11. Drying oven equipped with a fan and regulated to 100° C.

4.12. Rotary vacuum evaporator, with 250 ml flask.

5. Procedure

5.1. *Preparation of the sample*

Grind the sample so that the whole of it will pass through a sieve with a 1 mm mesh (in accordance with recommendation ISO R 565).

5.2. *Extraction*

Weigh out, to within 1 mg, a quantity of the finely divided and homogeneous sample containing about 1·25 mg of buquinolate. Place the test portion in a 250 ml conical flask (4.1) and add 100 ml of chloroform (3.1). Mix, stopper the flask, and shake for one hour using the shaker (4.2). Decant, filter and discard the first millilitres of the filtrate.

Transfer 80 ml of the clear filtrate into a 150 ml beaker, or into a 250 ml flask fitted to the rotary evaporator (4.12). Evaporate nearly to dryness on a water-bath (4.4), dissolve the oily residue, using repeatedly a few millilitres of chloroform (3.1) and transfer quantitatively the liquids into a 10 ml volumetric flask, using a funnel with a thin stem. Make up the volume with chloroform (3.1) and mix. If the solution is not clear, centrifuge for three minutes at 3 000 rpm using a stoppered tube.

5.3. *Thin-layer chromatography*

Using a micropipette (4.7), deposit *in spots* on a plate for thin layer chromatography (4.6), at intervals of 2 cm, volumes of 0·25 ml of the extract obtained in 5.2 and of the five working standard solutions (3.7.2).

Develop the chromatogram with chloroform (3.1) until the solvent front has practically reached the upper edge of the plate, then dry with the aid of a current of air. Develop with the chloroform-ethanol mixture (3.3) until the solvent front has travelled about 12 cm. Let the solvents evaporate. Expose the chromatogram to ultraviolet light (4.9) and using a needle, mark the boundary of the stain of buquinolate spot (Rf-value 0·4 to 0·6).

5.4. *Elution*

Collect the silica gel from each marked zone, using a zone collector (4.8), and place in centrifuge tubes. Add to each tube 10 ml of ethanol (3.4), shake for 20 minutes, then centrifuge for five minutes at 3 000 rpm. Decant the clear solutions into 50 ml conical flasks (4.1).

5.5. *Measurement of fluorescence*

Set the scale of the spectrophotofluorimeter (4.10) at 100 with the aid of the eluate from the most concentrated standard solution, using the excitation wavelength between 200 and 280 nm that gives the most intense fluorescence and an emission wavelength of 375 nm.

Under these conditions, measure the fluorescence of the other eluates (5.4). From the values obtained, determine the quantity (A) of buquinolate in mg in the 10 ml of eluate from the sample.

6. Calculation of results

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

$$\frac{A}{P} \cdot 50\,000$$

in which:

A = quantity in mg of buquinolate determined by spectrofluorometric measurement.

P = weight of test portion in grammes.

7. **Repeatability**

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

50 % relative to the higher result for buquinolate contents between 10 and 20 ppm;

10 ppm in absolute value for contents between 20 and 100 ppm;

10 % relative to the higher result for contents between 100 and 5 000 ppm;

500 ppm in absolute value for contents between 5 000 and 10 000 ppm;

5 % relative to the higher result for contents above 10 000 ppm.

2. DETERMINATION OF SULPHAQUINOXALINE

[(2-4-aminobenzenesulphonamido) quinoxaline]

1. **Purpose and scope**

The method makes it possible to determine the quantity of sulphaquinoxaline in feeding-stuffs, concentrates and premixes. The lower limit of determination is 20 ppm. Other sulphonamides and arsanilic acid interfere with the determination.

2. **Principle**

The sample is extracted with dimethylformamide and chloroform. The sulphaquinoxaline is hydrolyzed in alkaline medium. After neutralization, the amino derivative formed is diazotized and coupled with N-2-aminoethyl-1-naphthylamine. The optical density of the solution is measured at 545 nm.

3. **Reagents**

3.1. N,N-dimethylformamide a.p.

3.2. Chloroform a.p.

3.3. Ethanol absolute.

3.4. Alkaline brine: Dissolve 10 g sodium hydroxide a.p. and 25 g sodium chloride a.p. in water. Make up to 500 ml with water and mix.

3.5. Concentrated hydrochloric acid a.p., d = 1·18.

3.6. 0·1 % (m/V) sodium nitrite solution: Dissolve 100 mg sodium nitrite a.p. in water, make up to 100 ml with water and mix. Prepare immediately before use.

3.7. 0·5 % (m/V) ammonium sulphamate solution: Dissolve 500 mg ammonium sulphamate a.p. in water, make up to 100 ml with water and mix. Prepare immediately before use.

3.8. 0·1 % (m/V) N-2-aminoethyl-1-naphthylamine dihydrochloride solution: Dissolve 100 mg of N-2-aminoethyl-1-naphthylamine dihydrochloride a.p. in 0·1 % (V/V) hydrochloric acid a.p.; make up to 100 ml with the same acid and mix. Prepare immediately before use.

3.9. Standard substance: pure sulphaquinoxaline.

3.10. Standard solution: Weigh out 250 mg, to within 0·1 mg, of standard substance (3.9). Dissolve in 50 ml sodium hydroxide solution (25 ml 0·1 N sodium hydroxide a.p. solution + 25 ml water), make up to 500 ml with water and mix. Dilute 5 ml to 100 ml with water. 1 ml of this solution contains 25 μg of sulphaquinoxaline.

4. **Apparatus**

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

4.2. Shaker.

4.3. Sintered glass funnel, porosity 3, 80 mm diameter, with filter flask.

4.4. 250 ml separating funnels.

4.5. 50, 100, 250 and 500 ml volumetric flasks.

4.6. Test tubes, 150 mm × 25 mm.

4.7. Steam bath.

4.8. Spectrophotometer, with *20 mm* cells.

5. **Procedure**

5.1. *Preparation of the sample*

Grind the sample so that the whole of it will pass through a sieve with 1 mm mesh (in accordance with recommendation ISO R 565).

5.2. *Extraction*

Weigh out, to within 1 mg, a quantity of the finely divided and homogeneous sample containing between 0·25 and 1·25 mg of sulphaquinoxaline. Place the test portion in a 250 ml conical flask (4.1) and add 20 ml N,N-dimethylformamide (3.1). Mix and heat the flask on the steam bath (4.7) for 20 minutes. Leave to cool under a stream of cold water. Add 60 ml of chloroform (3.2), stopper the flask and shake for 30 minutes with the aid of a shaker (4.2).

Filter the liquid through a sintered funnel (4.3) under mild suction. Rinse the filter flask with four 5 ml portions of chloroform (3.2) and pass the rinsings through the funnel. Transfer the filtrate to a separating funnel (4.4), rinse the filter flask with about 15 ml chloroform (3.2) and transfer the rinsings to the separating funnel.

5.3. *Hydrolysis*

Add to the separating funnel 50 ml of alkaline brine (3.4) and 5 ml ethanol (3.3). Thoroughly mix the layers avoiding emulsion formation, either by slow inversion of the funnel about 20 times or by rotating it about the horizontal axis of the stem and the stopper. Allow the layers to separate (separation is usually complete in about 15 minutes).

Transfer the upper layer (aqueous layer) to a 250 ml volumetric flask (4.5). Repeat the extraction of the chloroform layer with three further 50 ml portions of alkaline brine (3.4), adding each aqueous extract to the contents of the volumetric flask. Make up the volume with water and mix.

Transfer 25 ml of the solution to a 50 ml volumetric flask (4.5), add 5 ml hydrochloric acid (3.5), make up the volume with water and mix. Filter if necessary, discarding the first 15 ml of filtrate. Transfer 10 ml aliquots of the solution to two test tubes (4.6), A and B.

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

To each tube add 2 ml of sodium nitrite solution (3.6), mix and leave to stand for three minutes. Add 2 ml of ammonium sulphamate solution (3.7), mix and leave to stand for two minutes. Add 1 ml of N-2-aminoethyl-1-naphthylamine dihydrochloride solution (3.8) to tube A and 1 ml water to tube B. Mix thoroughly the contents of each tube. By means of a water pump apply a slight vacuum to the tubes through rubber connections in order to remove dissolved nitrogen.

After 10 minutes measure the optical densities E_A and E_B of the solutions with the spectrophotometer (4.8) at 545 nm using water as blank. From the value $E_A - E_B$ determine the amount (A) of sulphaquinoxaline present in the sample solution by reference to a previously prepared calibration curve (5.5).

5.5. *Calibration curve*

Transfer into a series of 100 ml volumetric flasks (4.5) volumes of 2, 4, 6, 8 and 10 ml of the standard solution (3.10) corresponding to 50, 100, 150, 200 and 250 micrograms of sulphaquinoxaline. Add 8 ml hydrochloric acid (3.5) to each flask, make up the volume with water and mix.

Pipette 10 ml of each solution (equivalent to 5, 10, 15, 20 and 25 microgrammes sulphaquinoxaline) into test tubes (4.6). Develop the colour reaction as indicated under point 5.4, first paragraph. Measure the optical densities at 545 nm using water as blank. Trace the calibration curve, using the optical density values as ordinates and the corresponding quantities of sulphaquinoxaline in microgrammes as abcissae.

6. **Calculation of results**

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

$$\frac{A}{P} \cdot 50$$

in which:

A = quantity of sulphaquinoxaline in microgrammes as determined by photometric measurement.

P = weight of test portion in grammes.

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 sulphaquinoxaline contents between 20 and 100 ppm;

10 % relative to the higher result for contents between 100 and 5 000 ppm;

500 ppm in absolute value for contents between 5 000 and 10 000 ppm;

5 % relative to the higher result for contents above 10 000 ppm.

3. DETERMINATION OF FURAZOLIDONE

[(3-(5-nitrofurfurylideneamino)-oxazolidin-2-one)*]*

1. **Purpose and scope**

The method makes it possible to determine the quantity of furazolidone in feedingstuffs, concentrates and premixes. The lower limit of determination is 10 ppm.

2. **Principle**

The furazolidone is extracted with acetone, after a preliminary extraction of the sample with light petroleum to remove fat. The extract is purified by chromatography on a column of aluminium oxide and the furazolidone is eluted with acetone. The acetone eluate is evaporated to dryness and the residue dissolved in pentanol. Furazolidone is then extracted from the pentanol with aqueous urea solution and the optical density of the extract is measured at 375 nm.

3. **Reagents**

3.1. Acetone a.p.

3.2. Aluminium oxide for chromatography, neutral, 100 to 240 mesh, prepared as follows: stir 500 g of the aluminium oxide with one litre of hot distilled water and decant the supernatant liquid. Repeat this procedure twice, and finally filter using a Buchner funnel. Dry the aluminium oxide at 105° C to constant weight.

3.3. Pentyl acetate a.p.

3.4. Pentanol a.p. (material containing mixed isomers is acceptable).

3.5. Light petroleum, boiling range 40 to 60° C.

3.6. Urea solution. Mix 90 g of urea a.p. with 100 ml of water, warm gently to ensure complete solution.

3.7. Standard substance: pure furazolidone.

3.8. Standard solution: Weigh out to within 0·1 mg, 25 mg of standard substance (3.7), dissolve in acetone (3.1) in a 250 ml volumetric flask (4.1), make up to the volume with acetone (3.1) and mix. 1 ml of this solution contains 100 μg of furazolidone.

4. **Apparatus**

4.1. Amberglass 100 and 250 ml volumetric flasks.

4.2. Amberglass 100 ml separating funnels.

4.3. Suitable extraction apparatus, e.g. Soxhlet or Twisselmann.

4.4. Extraction thimbles, 25 × 80 mm or 28 × 100 mm.

4.5. Glass tubes for chromatography, internal diameter: 10 mm, length 300 mm.

4.6. Steam bath.

4.7. Spectrophotometer with 10 mm cells.

5. Procedure

N.B. All procedures should be carried out in subdued light.

5.1. *Preparation of the sample*

Grind the sample so that the whole of it will pass through a sieve with a 1 mm mesh (in accordance with recommendation ISO R 565).

5.2. *Extraction*

Weigh out to within 1 mg, 5 to 20 g of the finely divided and homogeneous sample (containing not more than 1 mg of furazolidone) into an extraction thimble (4.4) and transfer it to the extraction apparatus (4.3). Extract with light petroleum (3.5), ensuring, in the case of a Soxhlet apparatus, 13 to 17 cycles of solvent; if other extractors are used, allow not less than 30 minutes for this stage. Remove the thimble from the apparatus, drain off the residual solvent and dry the thimble and the extracted feed in a current of warm air.

Place the dried thimble and contents in a clean extraction apparatus and extract with acetone (3.1), allowing at least 25 cycles of solvent when using a Soxhlet apparatus. The exact conditions for achieving complete extraction with any particular apparatus should be predetermined. Evaporate the acetone extract to a volume of 5 to 10 ml on the steam bath (4.6), and cool to room temperature.

5.3. *Chromatography*

Insert a plug of glass wool into the lower end of a chromatography tube (4.5) and poke it down with a suitable rod to a thickness of 2 to 3 mm. Prepare a slurry of aluminium oxide (3.2) with acetone (3.1), pour into the tube and allow to settle. The prepared column should be about 200 mm in height. Allow the acetone layer to drain down to the top of the column.

Transfer the acetone extract obtained in 5.2 from the flask to the column, rinse the flask several times with acetone (3.1) and transfer the liquid onto the column. Place a suitable flask under the column and elute the furazolidone with acetone (3.1); the total volume of acetone used, including that used for rinsing, should be about 150 ml.

5.4. *Extraction and measurement of the optical density*

Evaporate the acetone eluate (5.3) just to dryness on a steam bath (4.6). (On occasions a small quantity of diacetone alcohol, produced by condensation of acetone on the aluminium oxide may be left but this will not interfere with the subsequent extractions.) Dissolve the residue in 10 ml pentanol (3.4) and transfer the solution to a separating funnel (4.2). Repeat the process using 10 ml pentyl acetate (3.3) as a rinse liquid. Finally rinse the vessel which contained the extract residue with 10 ml of urea solution (3.6), add this to the separating funnel and shake fairly vigorously for two minutes.

Allow the phases to separate for a period of three to four minutes before transferring the aqueous extract to a 100 ml volumetric flask (4.1). Repeat the rinsing and extraction stages with four further 10 ml aliquots of urea solution (3.6) and transfer the aqueous extracts to the volumetric flask. Dilute the contents of the volumetric flask to 100 ml with urea solution (3.6) and mix. Measure the optical density of the solution in the spectrophotometer (4.7) at 375 nm against urea solution (3.6) in the reference cell. Determine the quantity of furazolidone by referring to the calibration curve (5.5).

5.5. *Calibration curve*

Prepare four chromatographic columns as described in 5.3, first paragraph. Pipette into separate columns volumes of 2·5, 5, 7·5 and 10 ml respectively of the standard solution (3.8). Wash each of the four columns with 150 ml acetone (3.1) and

continue as in paragraph 5.4. Plot the calibration curve, using the optical density values as ordinates and the corresponding quantities of furazolidone in μg as abscissae.

6. **Calculation of results**

The furazolidone content in mg per kg is given by the formula

$$\frac{A}{P}$$

in which:

A = quantity of furazolidone in microgrammes as determined by photometric measurement.

P = weight of test portion in grammes.

7. **Repeatability**

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

50 % relative to the higher result for furazolidone contents between 10 and 20 ppm;

10 ppm in absolute value for contents between 20 and 100 ppm;

10 % relative to the higher result for contents between 100 and 5 000 ppm;

500 ppm in absolute value for contents between 5 000 and 10 000 ppm;

5 % relative to the higher result for contents above 10 000 ppm.