TWELFTH COMMISSION DIRECTIVE 93/117/EC

of 17 December 1993

establishing Community analysis methods for official control of feedingstuffs

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European 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 Regulation (EEC) No 3768/85 [2], and in particular Article 2 thereof,

Whereas Directive 70/373/EEC requires that official controls on feedingstuffs for the purpose of checking compliance with requirements arising under quality and composition provisions laid down by law, regulation or administrative action shall be carried out using Community sampling and analysis methods;

Whereas Community analysis methods for the additives robenidine and methyl benzoquate should be established for use in checking compliance with the conditions for its use in animal feedingstuffs;

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 conducted in the course of official checks on feedingstuffs to identify their robenidine and methyl benzoquate content be made using the methods described in the Annex hereto.

Article 2

Member States shall bring into force the laws, regulations and administrative provisions required for compliance with this Directive by 30 November 1994 at the latest and shall forthwith inform the Commission thereof.

When Member States adopt these provisions, these shall contain a reference to this Directive or shall be accompanied by such reference at the time of their official publication. The procedure for such reference shall be adopted by Member States.

Article 3

This Directive shall enter into force on the third day following that of its publication in the *Official Journal of the European Communities.*

Done at Brussels, 17 December 1993.

For the Commission

René STEICHEN

Member of the Commission

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[1] OJ No L 170, 3. 8. 1970, p. 2.
[2] OJ No L 362, 31. 12. 1985, p. 8.

ANNEX

1. DETERMINATON OF ROBENIDINE

1,3-bis[(4-chlorobenzylidene)amino] guanidine-hydrochloride

1. **Purpose and scope**

This method is for the determination of robenidine in feedingstuffs. the lower limit of determination is 5 mg/kg.

2. **Principle**

The sample is extracted with acidified methanol. The extract is dried and an aliquot portion subjected to a clean-up on an aluminium oxide column. Robenidine is eluted from the column with methanol, concentrated, and made up to a suitable volume with mobile phase. The content of robenidine is determined by reversed-phase high-performance liquid chromatography (HPLC) using an UV detector.

3. **Reagents**

3.1. *Methanol*

3.2. *Acidified methanol*

Transfer 4,0 ml hydrochloric acid ($P_{20}c$, 1,18 g/ml) into a 500 ml graduated flask, make up to the mark with methanol (3.1) and mix. This solution should be freshly prepared before use.

3.3. *Acetonitrile, HPLC grade*

3.4. *Molecular sieve*
Type 3A, 8 to 12 mesh beads (1,6-2,5 mm beads, crystalline alumino-silicate, diameter of pores 0,3 mm)

3.5. *Aluminium oxide: acidic activity grade I for column chromatography*

Transfer 100 g aluminium oxide into a suitable container and add 2,0 ml of water. Stopper and shake for approximately 20 minutes. Store in a well stoppered container.

3.6. *Potassium dihydrogen phosphate solution, c = 0,025 mol/l*

Dissolve 3,40 g of potassium dihydrogen phosphate in water (HPLC grade) in a 1000 ml graduated flask, make up to the mark and mix.

3.7. *Di-sodium hydrogen phosphate solution, c = 0,025 mol/l*

Dissolve 3,55 g of anhydrous (or 4,45 g of dihydrate or 8,95 g of dodecahydrate) di-sodium hydrogen phosphate in water (HPLC grade) in a 1,000 ml graduated flask, make up to the mark and mix.

3.8. *HPLC mobile phase*

Mix together the following reagents :

650 ml acetonitrile (3.3),

250 ml water (HPLC-grade),

50 ml potassium di-hydrogen phosphate solution (3.6),

50 ml di-sodium hydrogen phosphate solution (3.7).

Filter through a 0,22 µm filter (4.6) and degas the solution, (e.g. by ultrasonification for 10 minutes).

3.9. *Standard substance*

Pure robenidine : 1.3-bis[4-chlorobenzylidene)amino] guanidine hydrochloride, E 750.

3.9.1. Robenidine stock standard solution : 300 µg/ml :

Weigh to the nearest 0,1 mg, 30 mg of robenidine standard substance (3.9). Dissolve in acidified methanol (3.2) in a 100 ml graduated flask, make up to the mark with the same solvent and mix. Wrap the flask with aluminium foil and store in a dark place.

3.9.2. Robenidine intermediate standard solution : 12 µg/ml

Transfer 10,0 ml of the stock standard solution (3.9.1) into a 250 ml graduated flask, make up to the mark with the mobile phase (3.8) and mix. Wrap the flask with aluminium foil and store in a dark place.

3.9.3. Calibration solutions

Into a series of 50 ml calibrated flasks, transfer 5,0, 10,0, 15,0, 20,0 and 25,0 ml of the intermediate standard solution (3.9.2). Make up to the mark with mobile phase (3.8) and mix. These solutions correspond to 1,2, 2,4, 3,6, 4,8 and 6,0 µg/ml of robenidine respectively. These solutions must be freshly prepared before use.

4. Apparatus

4.1. *Glass column*

Constructed of amber glass fitted with a stopcock and a reservoir of approximately 150 ml capacity, internal diameter 10 to 15 mm, length 250 mm.

4.2. *Laboratory wrist-action shaker*

4.3. *Rotary film evaporator*

4.4. *HPLC equipment with variable wavelength ultraviolet detector or diode array detector operating in the range of 250 to 400 mm*

4.4.1. Liquid chromatographic column : 300 mm × 4 mm, C_{18}, 10 µm packing or equivalent

4.5. *Glass fibre filter paper* (Whatman GF/A or equivalent)

4.6. *Membrane filters, 0,22 µm*

4.7. *Membrane filters, 0,45 µm*

5. Procedure

Note : Robenidine is light-sensitive. Amber glassware should be used in all operations.

5.1. *General*

5.1.1. A blank feed should be analysed to check that neither robenidine nor interfering substances are present.

5.1.2. A recovery test should be carried out by analysing the blank feed (5.1.1) which has been fortified by addition of a quantity of robenidine, similar to that present in the sample. To fortify at a level of 60 mg/kg, transfer 3,0 ml of the stock standard solution (3.9.1) to a 250 ml conical flask. Evaporate the solution to c. 0,5 ml in a stream of nitrogen. Add 15 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Note : for the purpose of this method, the blank feed should be similar in type to that of the sample and on analysis robenidine should not be detected.

5.2. *Extraction*

Weigh to the nearest 0,01 g, approximately 15 g of the prepared sample. Transfer to a 250 ml conical flask and add 100,0 ml of acidified methanol (3.2), stopper and shake for one hour on the shaker (4.2). Filter the solution through a glass fibre filter paper (4.5) and collect the whole filtrate in a 150 ml conical flask. Add 7,5 g molecular sieve (3.4), stopper and shake for five minutes. Filter immediately through a glass-fibre filter paper. Retain this solution for the purification step (5.3).

5.3. *Purification*

5.3.1. Preparation of the aluminium-oxide column

Insert a small glass-wool plug into the lower end of a glass column (4.1) and tamp it down using a glass rod. Weigh out 11,0 g of the prepared aluminium oxide (3.5) and transfer to the column. Care should be taken to minimize the exposure to the atmosphere during this stage. Gently tap the loaded column at its lower end to settle the aluminium oxide.

5.3.2. Sample purification

Transfer onto the column by pipette 5,0 ml of the sample extract prepared in (5.2). Rest the pipette tip close to the column wall and allow the solution to be absorbed onto the aluminium oxide. Elute the robenidine from the column using 100 ml methanol (3.1), at a flow rate of 2 to 3 ml/minute and collect the eluate in a 250 ml round bottomed flask. Evaporate the methanol solution to dryness under reduced pressure at 40 C by means of a rotary film evaporator (4.3). Re-dissolve the residue in 3 to 4 ml of mobile phase (3.8) and transfer quantitatively to a 10 ml graduated flask. Rinse the flask with several 1 to 2 ml portions of mobile phase and transfer these rinsings to the graduated flask. Make up to the mark with the same solvent and mix. An aliquot is filtered through a 0,45 µm (4.7). Reserve this solution for HPLC determination (5.4).

5.4. *HPLC determination*

5.4.1. Parameters

The following conditions are offered for guidance, other conditions may be used provided they yield equivalent results :

Liquid chromatographic column (4.4.1),

HPLC mobile phase (3.8),

Flow rate : 1,5 to 2 ml/minute,

Detector wavelength : 317 nm,

Injection volume : 20 to 50 µl.

Check the stability of the chromatographic system, injecting the calibration solution (3.9.3) containing 3,6 µg/ml several times, until constant peak heights and retention times are achieved.

5.4.2. Calibration graph

Inject each calibration solution (3.9.3) several times and measure the peak heights (areas) for each concentration. Plot a calibration curve using the mean peak heights or areas of the calibration solutions as the ordinates and corresponding concentrations in µg per ml as abscissae.

5.4.3. Sample solution

Inject the sample extract (5.3.2) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the robenidine peaks.

6. Calculation of results

From the mean height (area) of the robenidine peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.4.2).

The content of robenidine w (mg/kg) in the sample is given by the following formula :

$$w = \frac{c \times 200}{m}$$

in which :

c = robenidine concentration of the sample solution in µg/ml,

m = mass of the test position in grams.

7. Validation of the results

7.1. *Identity*

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract and the calibration solution (3.9.3) containing 6 µg/ml are compared.