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.

7.1.1. Co-chromatography

A sample extract is fortified by addition of an appropriate amount of calibration solution (3.9.3). The amount of added robenidine should be similar to the estimated amount of robenidine found in the sample extract.

Only the height of the robenidine peak should be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within approximately 10 % of the original width.

7.1.2. Diode-array detection

The results are evaluated according to the following criteria :

(a) the wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within approximately 2 nm ;

(b) between 250 and 400 nm, the sample and standard spectra recorded at the peak apex on the chromatogram, must not be different for those parts of the spectrum within the range 10 to 100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte ;

(c) between 250 and 400 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 to 100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. *Repeatability*

The difference between the results of two parallel determinations carried out on the same sample must not exceed 10 % of the higher result for robenidine content higher than 15 mg/kg.

7.3. *Recovery*

For a fortified blank sample the recovery should be at least 85 %.

8. **Results of a collaborative study**

A collaborative study was arranged by the Community in which four samples of poultry and rabbit feed, in meal or pelleted form were analysed by 12 laboratories. Duplicate analyses were performed on each sample. The results are given in the table below :

	Poultry		Rabbit	
	Meal	Pellet	Meal	Pellet
Mean mg/kg	27,00	27,99	43,6	40,1
S_r (mg/kg)	1,46	1,26	1,44	1,66
CV_r (%)	5,4	4,5	3,3	4,1
S_r (mg/kg)	4,36	3,36	4,61	3,91
CV_R (%)	16,1	12,0	10,6	9,7
Recovery (%)	90,0	93,3	87,2	80,2

S_r = standard deviation of repeatability.
CV_r = coefficient of variation of repeatability
S_R = standard deviation of reproducibility
CV_R = coefficient of variation of reproducibility.

2. DETERMINATION OF METHYL BENZOQUATE

7-benzyloxy-6-butyl-3-methoxycarbonyl-4-quinolone

1. **Purpose and scope**

This method is for the determination of methyl benzoquate in feedingstuffs. The lower limit of determination is 1 mg/kg.

2. **Principle**

Methyl benzoquate is extracted from the sample with methanolic methanesulfonic acid solution. The extract is purified with dichloromethane, by ion-exchange chromatography and then again with dichloromethane. The methyl benzoquate content is determined by reversed-phase high-performance liquid chromatography (HPLC) with an UV detector.

3. **Reagents**

3.1. *Dichloromethane*

3.2. Methanol, HPLC grade

3.3. *HPLC mobile phase:*

mixture of methanol (3.2) and water (HPLC grade) 75 + 25 (v + v).

Filter through a 0,22 µm filter (4.5) and degas the solution (e.g. by ultrasonification for 10 minutes).

3.4. *Methanesulfonic acid solution,* $\sigma = 2\ \%$

Dilute 20,0 ml methanesulfonic acid to 1000 ml with methanol (3.2).

3.5. *Hydrochloric acid solution,* $\sigma = 10\ \%$

Dilute 100 ml hydrochloric acid (P_{20} c. 1,18 g/ml) to 1000 ml with water.

3.6. *Cation-exchange resin Amberlite CG-120 (Na), 100 to 200 mesh*

The resin is pretreated before use : slurry 100 g resin with 500 ml hydrochloric acid solution (3.5) and heat on a hot plate to boiling, stirring continuously. Allow to cool and decant off the acid. Filter through a filter paper under vacuum. Wash the resin twice with 500 ml portions of water and then with 250 ml of methanol (3.2). Rinse the resin with a further 250 ml portion of methanol and dry by passing air through the filter cake. Store the dried resin in a stoppered bottle.

3.7. *Standard substance : pure methyl benzoquate (7-benzyloxy-6-butyl-3-methoxycarbonyl-4-quinolone)*

3.7.1. Methyl benzoquate stock standard solution, 500 µg/ml

Weigh to the nearest 0,1 mg, 50 mg of standard substance (3.7), dissolve in methanesulfonic acid solution (3.4) in a 100 ml graduated flask, make up to the mark and mix.

3.7.2. Methyl benzoquate intermediate standard solution, 50 µg/ml

Transfer 5,0 ml of methyl benzoquate stock standard solution (3.7.1) into a 50 ml graduated flask, make up to the mark with methanol (3.2) and mix.

3.7.3. Calibration solutions

Transfer 1,0, 2,0, 3,0, 4,0 and 5,0 ml of methyl benzoquate intermediate standard solution (3.7.2) into a series of 25 ml graduated flasks. Make up to the mark with the mobile phase (3.3) and mix. These solutions have concentrations of 2,0, 4,0, 6,0, 8,0 and 10,0 µg/ml methyl benzoquate respectively. These solutions must be freshly prepared before use.

4. **Apparatus**

4.1. *Laboratory shaker*

4.2. *Rotary film evaporator*

4.3. *Glass column (250 mm × 15 mm) fitted with a stopcock and reservoir of approximately 200 ml capacity*

4.4. *HPLC equipment with variable wavelength ultraviolet detector or diode-array detector*

4.4.1. Liquid chromatographic column : 300 mm × 4 mm, C-18, 10 µm packing or equivalent

4.5. *Membrane filters, 0,22 µm*

4.5. *Membrane filters, 0,45 µm*

5. **Procedure**

5.1. *General*

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

5.1.2. A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of methyl benzoquate, similar to that present in the sample. To fortify at a level of 15 mg/kg, add 600 µl of the stock standard solution (3.7.1) to 20 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 methyl benzoquate should not be detected.

5.2. *Extraction*

Weigh to the nearest 0,01 g, approximately 20 g of the prepared sample and transfer to a 250 ml conical flask. Add 100,0 ml of methanesulfonic acid solution (3.4) and shake mechanically (4.1) for 30 minutes. Filter the solution through a filter paper and retain the filtrate for the liquid-liquid partition step (5.3).

5.3. *Liquid-liquid partition*

Transfer into a 500 ml separating funnel containing 100 ml of hydrochloric acid solution (3.5), 25,0 ml of the filtrate obtained in (5.2). Add 100 ml dichloromethane (3.1) to the funnel and shake for one minute. Allow the layers to separate and run off the lower (dichloromethane) layer into a 500 ml round-bottomed flask. Repeat the extraction of the aqueous phase with two further 40-ml portions of dichloromethane and combine these with the first extract in the round-bottomed flask. Evaporate the dichloromethane extract down to dryness on the rotary evaporator (4.2) operating under reduced pressure at 40° C. Dissolve the residue in 20 to 25 ml methanol (3.2), stopper the flask and retain the whole of the extract for ion-exchange chromatography (5.4).

5.4. *Ion-exchange chromatography*

5.4.1. Preparation of the cation-exchange column

Insert a plug of glass wool into the lower end of a glass column (4.3). Prepare a slurry of 5,0 g of the treated cation-exchange resin (3.6) with 50 ml of hydrochloric acid (3.5), pour into the glass column and allow to settle. Run out the excess acid to just above the resin surface and wash the column with water until the effluent is neutral to litmus. Transfer 50 ml methanol (3.2) onto the column and allow to drain down to the resin surface.

5.4.2. Column chromatography

By means of a pipette, carefully transfer the extract obtained in (5.3) onto the column. Rinse the round-bottomed flask with two portions of 5 to 10 ml methanol (3.2) and transfer these washings to the column. Run the extract down to the resin surface and wash the column with 50 ml methanol, ensuring that the flow rate does not exceed 5 ml per minute. Discard the effluent. Elute the methyl benzoquate from the column using 150 ml of methanesulfonic acid solution (3,4) and collect the column eluate in a 250 ml conical flask.

5.5. *Liquid-liquid partition*

Transfer the eluate obtained in (5.4.2) into a 1 litre separating funnel. Rinse the conical flask with 5 to 10 ml methanol (3.2) and combine the washings with the contents of the separating funnel. Add 300 ml of hydrochloric acid solution (3.5) and 130 ml of dichloromethane (3.1). Shake for 1 minute and allow the phases to separate. Run off the lower (dichloromethane) layer into a 500 ml round-bottomed flask. Repeat the extraction of the aqueous phase with two further 70 ml portions of dichloromethane and combine these extracts with the first in the round-bottomed flask.

Evaporate the dichloromethane extract down to dyrness on the rotary evaporator (4.2) operating under reduced pressure at 40° C. Dissolve the residue in the flask with approximately 5 ml of methanol (3.2) and transfer this solution quantitatively to a 10 ml graduated flask. Rinse the round-bottomed flask with a further two portions of 1 to 2 ml of methanol and transfer these to the graduated flask. Make up to the mark with methanol and mix. An aliquot portion is filtered through a membrane filter (4.6). Reserve this solution for HPLC-determination (5.6).

5.6. *HPLC determination*

5.6.1. Parameters

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

— liquid chromatograhic column (4.4.1),

— HPLC mobile phase : methanol-water mixture (3.3),

— flow rate : 1 to 1,5 ml/minute,

— detection wavelength : 265 nm,

— Injection volume : 20 to 50 µl.

Check the stability of the chromatographic system, injecting the calibration solution (3.7.3) containing 4 µg/ml several times, until contant peak heights or areas and retention times are achieved.

5.6.2. Calibration graph

Inject each calibration solution (3.7.3) several times and measure the peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights or areas of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

5.6.3. Sample solution

Inject the sample extract (5.5) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the methyl benzoquate peaks.

6. Calculation of results

Determine the concentration of the sample solution in µg/ml from the mean height (area) of the methyl benzoquate peaks of the sample solution by reference to the calibration graph (5.6.2).

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

$$w = \frac{c \times 40}{m}$$

in which :

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

m = mass of the test portion 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 (3.7.3) containing 10 µg/ml are compared.

7.1.1. Co-chromatography

A sample extract is fortified by addition of an appropriate amount of the intermediate standard solution (3.7.2). The amount of added methyl benzoquate should be similar to the estimated amount of methyl benzoquate in the sample extract.

Only the height of the methyl-benzoquate peak should be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within approximately 10 % of the original width,

7.1.2. Diode-array detection

The results are evaluated according to the following criteria :

(a) the wavelength of maximum absorption of the sample and of the standard spectra recorded at the peak apex on the chromatogram must be the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within approximately 2 nm ;

(b) between 220 and 350 nm, the sample and standard spectra recorded at the peak apex on the chromatogram must not be different for those parts of the spectrum within the range 10 to 100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte ;

(c) between 220 and 350 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10 to 100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

7.2. *Repeatability*

The difference between the results of two parallel determinations carried out on the same sample must not exceed : 10 % relative to the higher result for methyl benzoquate contents between 4 and 20 mg/kg.

7.3. *Recovery*

For a fortified blank sample the recovery should be at least 90 %.

8. **Results of a collaborative study**

Five samples were analysed by 10 laboratories. Duplicate analyses were performed on each sample.

Results

	Blank	Meal 1	Pellet 1	Meal 2	Pellet 2
Mean (mg/kg)	n.d.	4,50	4,50	8,90	8,70
S_r (mg/kg)	—	0,30	0,20	0,60	0,50
CV_r (%)	—	6,70	4,40	6,70	5,70
S_r (mg/kg)	—	0,40	0,50	0,90	1,00
CV_R (%)	—	8,90	11,10	10,10	11,50
Recovery (%)	—	92,00	93,00	92,00	89,00

n.d. = not detected.

S_r = standard deviation of repeatability.

CV_r = coefficient of variation of repeatability.

S_R = standard deviation of reproducibility.

CV_R = coefficient of variation of reproducibility.