Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance)

ANNEX VIII

METHODS OF ANALYSIS TO CONTROL ILLEGAL PRESENCE OF NO LONGER AUTHORISED ADDITIVES IN FEED

Important notes:

More sensitive methods of analysis than the methods of analysis mentioned in this Annex can be used to detect the illegal presence of no longer authorised additives in feed.

The methods of analysis mentioned in this Annex shall be used for confirmatory purposes.

A. DETERMINATION OF METHYL BENZOQUATE

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

1. Purpose and scope

This method makes it possible to determine the level of methyl benzoquate in feed. The limit of quantification 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, equivalent to HPLC grade

3.3. HPLC mobile phase

Mixture of methanol (3.2) and water (equivalent to 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, c = 2 %

Dilute 20,0 ml methanesulfonic acid to 1 000 ml with methanol (3.2).

3.5. Hydrochloric acid solution, c = 10 %

Dilute 100 ml hydrochloric acid (ρ_{20}1,18 g/ml) to 1 000 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

4.6. Membrane filters, 0,45 µm

5. Procedure

5.1. General

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

5.1.2. A recovery test shall 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 shall be similar in type to that of the sample and on analysis methyl benzoquate must 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 ^{o}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 dryness on the rotary evaporator (4.2) operating under reduced pressure at 40 ^{o}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 chromatographic 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 constant 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 = weight 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 must be similar to the estimated amount of methyl benzoquate in the sample extract.

Only the height of the methyl-benzoquate peak shall 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 no 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 shall be at least 90 %.

8. Results of a collaborative study

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

	Blank	**Meal 1**	**Pellet 1**	**Meal 2**	**Pellet2**
Mean [mg/kg]	ND	4,5	4,5	8,9	8,7
s_r [mg/kg]	—	0,3	0,2	0,6	0,5
CV_r [%]	—	6,7	4,4	6,7	5,7
s_R [mg/kg]	—	0,4	0,5	0,9	1,0
CV_R [%]	—	8,9	11,1	10,1	11,5
Recovery [%]	—	92,0	93,0	92,0	89,0

ND = 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, %.

B. DETERMINATION OF OLAQUINDOX

2-[N-2'-(hydroxyethyl)carbamoyl]-3-methylquinoxaline-N^1,N^4-dioxide

1. Purpose and scope

This method makes it possible to determine the level olaquindox in feed. The limit of quantification is 5 mg/kg.

2. Principle

The sample is extracted by a water-methanol mixture. The content of olaquindox is determined by reversed-phase high-performance liquid chromatography (HPLC) using an UV detector.

3. Reagents

3.1. Methanol.

3.2. Methanol, equivalent to HPLC grade.

3.3. Water, equivalent to HPLC grade.

3.4. Mobile phase for HPLC.

Water (3.3)-methanol (3.2) mixture, 900 +100 (V + V).

3.5. Standard substance: pure olaquindox 2-[N-2'-(hydroxyethyl)carbamoyl]-3-methylquinoxaline-N^1,N^4-dioxide, E 851.

3.5.1. *Olaquindox stock standard solution, 250 µg/ml*

Weigh to the nearest 0,1 mg 50 mg of olaquindox (3.5) in a 200 ml graduated flask and add ca. 190 ml water. Then place the flask for 20 min. into an ultrasonic bath (4.1). After ultrasonic treatment bring the solution to room temperature, make up to the mark with water and mix. Wrap the flask with aluminium foil and store in a refrigerator. This solution must be prepared fresh each month.

3.5.2. *Olaquindox intermediate standard solution, 25 µg/ml*

Transfer 10,0 ml of the stock standard solution (3.5.1) into a 100 ml graduated flask, make up to the mark with the mobile phase (3.4) and mix. Wrap the flask with aluminium foil and store in a refrigerator. This solution must be prepared fresh each day.

3.5.3. *Calibration solutions*

Into a series of 50 ml graduated flasks transfer 1,0, 2,0, 5,0, 10,0, 15,0 and 20,0 ml of the intermediate standard solution (3.5.2). Make up to the mark with the mobile phase (3.4) and mix. Wrap the flasks with aluminium foil. These solutions correspond to 0,5, 1,0, 2,5, 5,0, 7,5 and 10,0 µg of olaquindox per ml respectively.

These solutions must be prepared fresh each day.

4. Apparatus

4.1. Ultrasonic bath

4.2. Mechanical shaker

4.3. HPLC equipment with variable wavelength ultraviolet detector or diode array detector

4.3.1. Liquid chromatographic column, 250 mm × 4 mm, C_{18}, 10 µm packing, or equivalent

4.4. Membrane filters, 0,45 µm

5. Procedure

Note: Olaquindox is light sensitive. Carry out all procedures under subdued light or use amber glassware.

5.1. General

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

5.1.2. A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of olaquindox, similar to that present in the sample. To fortify at a level of 50 mg/kg, transfer 10,0 ml of the stock standard solution (3.5.1) to a 250 ml conical flask and evaporate the solution to ca. 0,5 ml. Add 50 g of the blank feed, mix thoroughly and leave for 10 min. mixing again several times before proceeding with the extraction step (5.2).

Note: For the purpose of this method the blank feed shall be similar in type to that of the sample and olaquindox must not be detected.

5.2. Extraction

Weigh to the nearest 0,01 g, approximately 50 g of the sample. Transfer to a 1 000 ml conical flask, add 100 ml of methanol (3.1) and place the flask for 5 min. in the ultrasonic bath (4.1). Add 410 ml water and leave in the ultrasonic bath for further 15 min. Remove the flask from the ultrasonic bath, shake it for 30 min. on the shaker (4.2) and filter through a folded filter. Transfer 10,0 ml of the filtrate into a 20 ml graduated flask, make up to the mark with water and mix. An aliquot is filtered through a membrane filter (4.4). (see 9. Observation) Proceed to the HPLC determination (5.3).

5.3. HPLC determination

5.3.1. *Parameters:*

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

Analytical column (4.3.1)	
Mobile Phase (3.4):	water (3.3)-methanol (3.2) mixture, 900 + 100 (V + V)
Flow rate:	1,5-2 ml/min.
Detection wavelength:	380 nm
Injection volume:	20 µl –100 µl

Check the stability of the chromatographic system, injecting several times the calibration solution (3.5.3) containing 2,5 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. *Calibration graph*

Inject each calibration solution (3.5.3) several times and determine the mean peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights (areas) of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

5.3.3. *Sample solution*

Inject the sample extract (5.2) several times using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the olaquindox peaks.

6. Calculation of the results

From the mean height (area) of the olaquindox peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.3.2).

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

$$w = \frac{c \times 1000}{m}$$

in which:

c = olaquindox concentration of the sample extract (5.2) in µg/ml
m = weight of the test portion in g (5.2).

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 (5.2) and the calibration solution (3.5.3) containing 5,0 µg/ml are compared.

7.1.1. *Co-chromatography*

A sample extract (5.2) is fortified by addition of an appropriate amount of calibration solution (3.5.3). The amount of added olaquindox must be similar to the amount of olaquindox found in the sample extract.

Only the height of the olaquindox peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its height, must be within ± 10 % of the original width of the olaquindox peak of the unfortified sample extract.

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 ± 2 nm.

(b) Between 220 and 400 nm, the sample and standard spectra recorded at the peak apex of the chromatogram, must not be different for those parts of the spectrum within the range 10 %-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 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 %-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 peak 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 15 % relative to the higher result for olaquindox contents between 10 and 200 mg/kg.

7.3. Recovery

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

8. Results of a collaborative study

An EC collaborative study was arranged in which four piglet feed samples including one blank feed were analysed by up to 13 laboratories. The results are given below:

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**
L	13	10	11	11
n	40	40	44	44
mean [mg/kg]	—	14,6	48,0	95,4
S_r [mg/kg]	—	0,82	2,05	6,36
S_R [mg/kg]	—	1,62	4,28	8,42
CV_r [%]	—	5,6	4,3	6,7
CV_R [%]	—	11,1	8,9	8,8
Nominal content				
[mg/kg]	—	15	50	100
recovery %	—	97,3	96,0	95,4

L = number of laboratories
n = number of single values
S_r = standard deviation of repeatability
S_R = standard deviation of reproducibility
CV_r = coefficient of variation of repeatability
CV_R = coefficient of variation of reproducibility.

9. Observation

Although the method has not been validated for feeds containing more than 100 mg/kg of olaquindox, it may be possible to obtain satisfactory results by taking a smaller sample weight and/or diluting the extract (5.2) to reach a concentration within the range of the calibration graph (5.3.2).

C. DETERMINATION OF AMPROLIUM

1-[(4-amino-2-propylpyrimidin-5-yl)methyl]-2-methyl-pyridinium chloride hydrochloride

1. Purpose and Scope

This method makes it possible to determine the level of amprolium in feed and premixtures. The detection limit is 1 mg/kg, the limit of quantification is 5 mg/kg.

2. Principle

The sample is extracted with a methanol-water mixture. After dilution with the mobile phase and membrane filtration the content of amprolium is determined by cation exchange high performance liquid chromatography (HPLC) using a UV detector.

3. Reagents

3.1. Methanol.

3.2. Acetonitrile, equivalent to HPLC grade.

3.3. Water, equivalent to HPLC grade.

3.4. Sodium dihydrogen phosphate solution, c = 0,1 mol/l.

Dissolve 13,8 g of sodium dihydrogen phosphate monohydrate in water (3.3) in a 1 000 ml graduated flask, make up to the mark with water (3.3) and mix.

3.5. Sodium perchlorate solution, c = 1,6 mol/l.

Dissolve 224,74 g of sodium perchlorate monohydrate in water (3.3) in a 1 000 ml graduated flask, make up to the mark with water (3.3) and mix.

3.6. Mobile phase for HPLC (see observation 9.1).

Mixture of acetonitrile (3.2), sodium dihydrogen phosphate solution (3.4) and sodium perchlorate solution (3.5), 450+450+100 (v+v+v). Prior to use filter through a 0,22 µm membrane filter (4.3) and degas the solution (e.g. in the ultrasonic bath (4.4) for at least 15 minutes).

3.7. Standard substance: pure amprolium, 1-[(4-amino-2-propylpyrimidin-5-yl)methyl]-2-methyl-pyridinium chloride hydrochloride, E 750 (see 9.2).

3.7.1. *Amprolium stock standard solution, 500 µg/ml*

Weigh to the nearest 0,1 mg, 50 mg of amprolium (3.7) in a 100 ml graduated flask, dissolve in 80 ml methanol (3.1) and place the flask for 10 min. in an ultrasonic bath (4.4). After ultrasonic treatment bring the solution to room temperature, make up to the mark with water and mix. At a temperature of ≤ 4 ^{o}C the solution is stable for 1 month.

3.7.2. *Amprolium intermediate standard solution, 50 µg/ml*

Pipette 5,0 ml of the stock standard solution (3.7.1) into a 50 ml graduated flask, make up to the mark with the extraction solvent (3.8) and mix. At a temperature of ≤ 4 ^{o}C the solution is stable for 1 month.

3.7.3. *Calibration solutions*

Transfer 0,5, 1,0 and 2,0 ml of the intermediate standard solution (3.7.2) into a series of 50 ml graduated flasks. Make up to the mark with the mobile phase (3.6) and mix. These solutions correspond to 0,5, 1,0 and 2,0 µg of amprolium per ml respectively. These solutions must be prepared freshly before use.

3.8. Extraction solvent.

Methanol (3.1)-water mixture 2+1 (v+v).

4. Apparatus

4.1. HPLC equipment with injection system, suitable for injection volumes of 100 µl.

4.1.1. Liquid chromatographic column 125 mm × 4 mm, cation exchange Nucleosil 10 SA, 5 or 10 μm packing, or equivalent.

4.1.2. UV detector with variable wavelength adjustment or diode array detector.

4.2. Membrane filter, PTFE material, 0,45 μm.

4.3. Membrane filter, 0,22 μm.

4.4. Ultrasonic bath.

4.5. Mechanical shaker or magnetic stirrer.

5. Procedure

5.1. General

5.1.1. *Blank feed*

For the performance of the recovery test (5.1.2) a blank feed shall be analysed to check that neither amprolium nor interfering substances are present. The blank feed shall be similar in type to that of the sample and amprolium or interfering substances must not be detected.

5.1.2. *Recovery test*

A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of amprolium, similar to that present in the sample. To fortify at a level of 100 mg/kg, transfer 10,0 ml of the stock standard solution (3.7.1) to a 250 ml conical flask and evaporate the solution to approximately 0,5 ml. Add 50 g of the blank feed, mix thoroughly and leave for 10 min. mixing again several times before proceeding with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample to be analysed is fortified with a quantity of amprolium similar to that already present in the sample. This sample is analysed together with the unfortified sample and the recovery can be calculated by subtraction.

5.2. Extraction

5.2.1. *Premixtures (content < 1 % amprolium) and feed*

Weigh to the nearest 0,01 g, 5-40 g of the sample depending on the amprolium content into a 500 ml conical flask and add 200 ml extraction solvent (3.8). Place the flask in the ultrasonic bath (4.4) and leave for 15 minutes. Remove the flask from the ultrasonic bath and shake it for 1 h on the shaker or stir on the magnetic stirrer (4.5). Dilute an aliquot of the extract with the mobile phase (3.6) to an amprolium content of 0,5-2 μg/ml and mix (see observation 9.3). Filter 5-10 ml of this diluted solution on a membrane filter (4.2). Proceed to the HPLC determination (5.3).

5.2.2. *Premixtures (content ≥ 1 % amprolium)*

Weigh to the nearest 0,001 g, 1-4 g of the premixture depending on the amprolium content into a 500 ml conical flask and add 200 ml extraction solvent (3.8). Place the flask in the ultrasonic bath (4.4) and leave for 15 minutes. Remove the flask from the ultrasonic bath and shake it for 1 h on the shaker or stir on the magnetic stirrer (4.5). Dilute an aliquot of the extract with the mobile phase (3.6) to an amprolium content of 0,5-2 μg/ml and mix. Filter 5-10 ml of this diluted solution on a membrane filter (4.2). Proceed to the HPLC determination (5.3).

5.3. HPLC determination

5.3.1. *Parameters:*

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

Liquid chromatographic	
column (4.1.1):	125 mm × 4 mm, cation exchange Nucleosil 10 SA, 5 or 10 µm packing, or equivalent
Mobile phase (3.6):	Mixture of acetonitrile (3.2), sodium dihydrogen phosphate solution (3.4) and sodium perchlorate solution (3.5), 450+450+100 (v+v+v).
Flow rate:	0,7-1 ml/min
Detection wavelength:	264 nm
Injection volume:	100 µl

Check the stability of the chromatographic system, injecting several times the calibration solution (3.7.3) containing 1,0 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. *Calibration graph*

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

5.3.3. *Sample solution*

Inject the sample extract (5.2) several times using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the amprolium peaks.

6. Calculation of the results

From the mean height (area) of the amprolium peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.3.2).

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

$$w = \frac{V \times c \times f}{m}$$

[mg/kg]

in which:

V = volume of the extraction solvent (3.8) in ml according to 5.2 (i.e. 200 ml)
c = amprolium concentration of the sample extract (5.2) in µg/ml
f = dilution factor according to 5.2
m = weight of the test portion in g.

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 (5.2) and the calibration solution (3.7.3) containing 2,0 µg/ml are compared.

7.1.1. *Co-chromatography*

A sample extract (5.2) is fortified by addition of an appropriate amount of calibration solution (3.7.3). The amount of added amprolium must be similar to the amount of amprolium found in the sample extract.

Only the height of the amprolium peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its height, must be within ± 10 % of the original width of the amprolium peak of the unfortified sample extract.

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 ± 2 nm.

(b) Between 210 and 320 nm, the sample and standard spectra recorded at the peak apex of the chromatogram, must not be different for those parts of the spectrum within the range 10 %-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 210 and 320 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 %-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 peak 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:

— 15 % relative to the higher value for amprolium contents from 25 mg/kg to 500 mg/kg,
— 75 mg/kg for amprolium contents between 500 mg/kg and 1 000 mg/kg,
— 7,5 % relative to the higher value for amprolium contents of more than 1 000 mg/kg.

7.3. Recovery

For a fortified (blank) sample the recovery shall be at least 90 %.

8. Results of a collaborative study

A collaborative study was arranged in which three poultry feeds (sample 1-3), one mineral feed (sample 4) and one premix (sample 5) were analysed. The results are given in the following table:

	Sample 1 (blank feed)	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
L	14	14	14	14	15
n	56	56	56	56	60
mean [mg/kg]	—	45,5	188	5 129	25 140
s_r [mg/kg]	—	2,26	3,57	178	550
CVr [%]	—	4,95	1,9	3,46	2,2
s_R [mg/kg]	—	2,95	11,8	266	760
CV_R [%]	—	6,47	6,27	5,19	3,0
nominal content [mg/kg]	—	50	200	5 000	25 000

L = number of laboratories
n = number of single values
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.

9. Observations

9.1. If the sample contains thiamine, the thiamine peak in the chromatogram appears shortly before the amprolium peak. Following this method amprolium and thiamine must be separated. If the amprolium and thiamine are not separated by the column (4.1.1) used in this method, replace up to 50 % of the acetonitrile portion of the mobile phase (3.6) by methanol.

9.2. According to the British Pharmacopoeia, the spectrum of an amprolium solution (c = 0,02 mol/l) in hydrochloric acid (c = 0,1 mol/l) shows maxima at 246 nm and 262 nm. The absorbance shall amount to 0,84 at 246 nm and 0,8 at 262 nm.

9.3. The extract must always be diluted with the mobile phase, because otherwise the retention time of the amprolium peak may shift significantly, due to changes in the ionic strength.

D. DETERMINATION OF CARBADOX

Methyl 3-(2-quinoxalinylmethylene)carbazate N^1,N^4-dioxide

1. Purpose and scope

This method makes it possible to determine the level of carbadox in feed, premixtures and preparations. The detection limit is 1 mg/kg. The limit of quantification is 5 mg/kg.

2. Principle

The sample is equilibrated with water and extracted with methanol-acetonitrile. For feed, an aliquot portion of the filtered extract is subjected to clean-up on an aluminium oxide column. For premixtures and preparations an aliquot portion of the filtered extract is diluted to an appropriate

concentration with water, methanol and acetonitrile. The content of carbadox is determined by reversed-phase high-performance liquid chromatography (HPLC) using a UV detector.

3. Reagents

3.1. Methanol.

3.2. Acetonitrile, equivalent to HPLC grade.

3.3. Acetic acid, w = 100 %.

3.4. Aluminium oxide: neutral, activity grade I.

3.5. Methanol-acetonitrile 1 + 1 (v + v).

Mix 500 ml of methanol (3.1) with 500 ml of acetonitrile (3.2).

3.6. Acetic acid, σ = 10 %.

Dilute 10 ml acetic acid (3.3) to 100 ml with water.

3.7. Sodium acetate.

3.8. Water, equivalent to HPLC grade.

3.9. Acetate buffer solution, c = 0,01 mol/l, pH = 6,0.

Dissolve 0,82 g of sodium acetate (3.7) in 700 ml of water (3.8) and adjust the pH to 6,0 with acetic acid (3.6). Transfer to a 1 000 ml graduated flask, make up to the mark with water (3.8) and mix.

3.10. Mobile phase for HPLC.

Mix 825 ml of acetate buffer solution (3.9) with 175 ml of acetonitrile (3.2).

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

3.11. Standard substance.

Pure carbadox: Methyl 3-(2-quinoxalinylmethylene)carbazate N^1,N^4-dioxide, E 850.

3.11.1. *Carbadox stock standard solution, 100 μg/ml (see Note 5. Procedure):*

Weigh to the nearest 0,1 mg, 25 mg of carbadox standard substance (3.11) into a 250 ml graduated flask. Dissolve in methanol-acetonitrile (3.5) by ultrasonification (4.7). After ultrasonic treatment bring the solution to room temperature, make up to the mark with methanol-acetonitrile (3.5) and mix. Wrap the flask with aluminium foil or use amber glassware and store in a refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.11.2. *Calibration solutions*

Transfer 2,0, 5,0, 10,0, and 20,0 ml of the stock standard solution (3.11.1) into a series of 100 ml calibrated flasks. Add 30 ml of water, make up to the mark with methanol-acetonitrile (3.5) and mix. Wrap the flasks with aluminium foil. These solutions correspond to 2,0, 5,0, 10,0 and 20,0 μg/ml of carbadox respectively.

Calibration solutions must be freshly prepared before use.

Note: For the determination of carbadox in feed containing less than 10 mg/kg, calibration solutions with a concentration below 2,0 µg/ml must be prepared.

3.12. Water-[methanol-acetonitrile] (3.5) mixture, 300 + 700 (v + v).

Mix 300 ml of water with 700 ml of the mixture of methanol-acetonitrile (3.5).

4. Apparatus

4.1. Laboratory shaker or magnetic stirrer.

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

4.3. Glass column (length 300 to 400 mm, internal diameter approximately 10 mm) with sintered glass frit and draw-off valve.

Note: a glass column fitted with a stopcock or a glass column with a tapered end may also be used; in this case, a small glass-wool plug is inserted into the lower end and it is tamped down using a glass rod.

4.4. HPLC equipment with injection system, suitable for injection volumes of 20 µl.

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

4.4.2. UV detector with variable wavelength adjustment or diode array detector operating in the range of 225 to 400 nm.

4.5. Membrane filter, 0,22 µm.

4.6. Membrane filter, 0,45 µm.

4.7. Ultrasonic bath.

5. Procedure

Note: Carbadox is light-sensitive. Carry out all procedures under subdued light or use amber glassware or glassware wrapped in aluminium foil.

5.1. General

5.1.1. *Blank feed*

For the performance of the recovery test (5.1.2) a blank feed shall be analysed to check that neither carbadox nor interfering substances are present. The blank feed shall be similar in type to that of the sample and on analysis carbadox or interfering substances must not be detected.

5.1.2. *Recovery test*

A recovery test shall be carried out by analysing the blank feed (5.1.1) which has been fortified by the addition of a quantity of carbadox, similar to that present in the sample. To fortify at a level of 50 mg/kg, transfer 5,0 ml of the stock standard solution (3.11.1) to a 200 ml conical flask. Evaporate the solution to approximately 0,5 ml in a stream of nitrogen. Add 10 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample is fortified with a quantity of carbadox, similar to that already present in the sample. This sample is analysed, together with the unfortified sample and the recovery can be calculated by subtraction.

5.2. Extraction

5.2.1. *Feed*

Weigh to the nearest 0,01 g, 10 g of the sample and transfer to a 200 ml conical flask. Add 15,0 ml of water, mix, and equilibrate for 5 min. Add 35,0 ml of methanol-acetonitrile (3.5), stopper and shake for 30 min. on the shaker or stir on the magnetic stirrer (4.1). Filter the solution through a glass fibre filter paper (4.2). Retain this solution for the purification step (5.3).

5.2.2. *Premixtures (0,1 %-2,0 %)*

Weigh to the nearest 0,001 g, 1 g of the unground sample and transfer to a 200 ml conical flask. Add 15,0 ml of water, mix, and equilibrate for 5 min. Add 35,0 ml of methanol-acetonitrile (3.5), stopper and shake for 30 min. on the shaker or stir on the magnetic stirrer (4.1). Filter the solution through a glass fibre filter paper (4.2).

Pipet an aliquot of filtrate into a 50 ml calibrated flask. Add 15,0 ml of water, make up to the mark with methanol-acetonitrile (3.5) and mix. The carbadox concentration of the final solution shall be approximately 10 μg/ml. An aliquot is filtered through a 0,45 μm filter (4.6).

Proceed to the HPLC determination (5.4).

5.2.3. *Preparations (> 2 %)*

Weigh to the nearest 0,001 g, 0,2 g of the unground sample and transfer to a 250 ml conical flask. Add 45,0 ml of water, mix, and equilibrate for 5 min. Add 105,0 ml of methanol-acetonitrile (3.5), stopper and homogenise. Sonicate (4.7) the sample for 15 min. followed by shaking or stirring for 15 min. (4.1). Filter the solution through a glass fibre filter paper (4.2).

Dilute an aliquot of filtrate with the mixture of water-methanol-acetonitrile (3.12) to a final carbadox concentration of 10-15 μg/ml (for a 10 % preparation, the dilution factor is 10). An aliquot is filtered through a 0,45 μm filter (4.6).

Proceed to the HPLC determination (5.4).

5.3. Purification

5.3.1. *Preparation of the aluminium oxide column*

Weigh 4 g of aluminium oxide (3.4) and transfer it to the glass column (4.3).

5.3.2. *Sample purification*

Apply 15 ml of the filtered extract (5.2.1) to the aluminium oxide column and discard the first 2 ml of eluate. Collect the next 5 ml and filter an aliquot through a 0,45 μm filter (4.6).

Proceed to the 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):	300 mm × 4 mm, C_{18}, 10 µmpacking or equivalent
Mobile phase (3.10):	Mixture of acetate buffer solution (3.9) and acetonitrile (3.2), 825 + 175 (v+v)
Flow rate:	1,5-2 ml/min.
Detection wavelength:	365 nm
Injection volume:	20 µl

Check the stability of the chromatographic system, injecting the calibration solution (3.11.2) containing 5,0 µg/ml several times, until constant peak heights (areas) and retention times are achieved.

5.4.2. *Calibration graph*

Inject each calibration solution (3.11.2) 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/ml as the abscissae.

5.4.3. *Sample solution*

Inject the sample extract [(5.3.2) for feed, (5.2.2) for premixtures and (5.2.3) for preparations] several times and determine the mean peak height (area) of the carbadox peaks.

6. Calculation of the results

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

6.1. Feed

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

$$w = \frac{c \times V_1}{m}$$

[mg/kg]

in which:

c = carbadox concentration of the sample extract (5.3.2) in µg/ml
V_1 = extraction volume in ml (i.e. 50)
m = weight of the test portion in g.

6.2. Premixtures and preparations

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

$$w = \frac{c \times V_2 \times f}{m}$$

[mg/kg]

in which:

c = carbadox concentration of the sample extract (5.2.2 or 5.2.3) in µg/ml
V_2 = extraction volume in ml (i.è. 50 for premixtures; 150 for preparations)
f = dilution factor according to 5.2.2 (premixtures) or 5.2.3 (preparations)
m = weight of the test portion in g.

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 (3.11.2) containing 10,0 µg/ml are compared.

7.1.1. *Co-chromatography*

A sample extract is fortified by addition of an appropriate amount of calibration solution (3.11.2). The amount of added carbadox must be similar to the estimated amount of carbadox found in the sample extract.

Only the height of the carbadox peak shall 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 + 2 nm;

(b) between 225 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 225 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

For contents of 10 mg/kg and higher, the difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result.

7.3. Recovery

For a fortified (blank) sample the recovery shall be at least 90 %.

8. Results of a collaborative study

A collaborative study was arranged in which 6 feed, 4 premixtures and 3 preparations were analysed by 8 laboratories. Duplicate analyses were performed on each sample. (More detailed information on this collaborative study can be found in the *Journal of the AOAC, Volume 71, 1988, p. 484-490*). The results (excluding outliers) are shown below:

TABLE 1

Results of the collaborative study for feed

	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**
L	8	8	8	8	8	8
n	15	14	15	15	15	15
Mean (mg/kg)	50,0	47,6	48,2	49,7	46,9	49,7
Sr (mg/kg)	2,9	2,69	1,38	1,55	1,52	2,12
CVr (%)	5,8	5,6	2,9	3,1	3,2	4,3
SR (mg/kg)	3,92	4,13	2,23	2,58	2,26	2,44
CVR (%)	7,8	8,7	4,6	5,2	4,8	4,9
Nominal content (mg/kg)	50,0	50,0	50,0	50,0	50,0	50,0

TABLE 2

Results of the collaborative study for premixtures and preparations

	Premixtures				**Preparations**		
	A	**B**	**C**	**D**	**A**	**B**	**C**
L	7	7	7	7	8	8	8
n	14	14	14	14	16	16	16
Mean (g/kg)	8,89	9,29	9,21	8,76	94,6	98,1	104
Sr (g/kg)	0,37	0,28	0,28	0,44	4,1	5,1	7,7
CVr (%)	4,2	3,0	3,0	5,0	4,3	5,2	7,4
SR (g/kg)	0,37	0,28	0,4	0,55	5,4	6,4	7,7
CVR (%)	4,2	3,0	4,3	6,3	5,7	6,5	7,4
Nominal content (g/kg)	10,0	10,0	10,0	10,0	100	100	100

L = number of laboratories
n = number of single values
Sr = standard deviation of repeatability
CVr = coefficient of variation of repeatability
SR = standard deviation of reproducibility
CVR = coefficient of variation of reproducibility.

Status:
Point in time view as at 31/12/2020.

Changes to legislation:
There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, ANNEX VIII.