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

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 $\leq 4\ ^{o}C$ 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 $\leq 4\ ^{o}C$ 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.

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