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 IV

METHODS OF ANALYSIS TO CONTROL THE LEVEL OF AUTHORISED ADDITIVES IN FEED

F.DETERMINATION OF DICLAZURIL

(+)-4-chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl)phenyl] acetonitrile

1. Purpose and scope

The method makes it possible to determine the level of diclazuril in feed and premixtures. The limit of detection is 0,1 mg/kg, the limit of quantification is 0,5 mg/kg.

2. Principle

After addition of an internal standard, the sample is extracted with acidified methanol. For feed, an aliquot of the extract is purified on a C_{18} solid phase extraction cartridge. Diclazuril is eluted from the cartridge with a mixture of acidified methanol and water. After evaporation, the residue is dissolved in DMF/water. For premixtures, the extract is evaporated and the residue is dissolved in DMF/water. The content of diclazuril is determined by ternary gradient reversed-phase high-performance liquid chromatography (HPLC) using a UV detector.

3. Reagents

3.1. Water, equivalent to HPLC-grade

3.2. Ammonium acetate

3.3. Tetrabutylammonium hydrogen sulphate (TBHS)

3.4. Acetonitrile, equivalent to HPLC grade

3.5. Methanol, equivalent to HPLC grade

3.6. N, N-dimethylformamide (DMF)

3.7. Hydrochloric acid, ρ_{20} = 1,19 g/ml

3.8. Standard substance: diclazuril II-24: (+)-4-chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl) phenyl] acetonitrile with guaranteed purity, E771

3.8.1. *Diclazuril stock standard solution, 500 µg/ml*

Weigh to the nearest 0,1 mg, 25 mg of diclazuril standard substance (3.8) in a 50 ml graduated flask. Dissolve in DMF (3.6), make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.8.2. *Diclazuril standard solution, 50 µg/ml*

Transfer 5,0 ml of the stock standard solution (3.8.1) into a 50 ml graduated flask, make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9. Internal standard substance: 2,6 dichloro-α-(4-chlorophenyl)-4-(4,5 dihydro-3,5-dioxo-1,2,4-triazine-2 (3H) — yl) α-methylbenzene-acetonitrile

3.9.1. *Internal stock standard solution, 500 µg/ml*

Weigh to the nearest 0,1 mg 25 mg of internal standard substance (3.9) in a 50 ml graduated flask. Dissolve in DMF (3.6), make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9.2. *Internal standard solution, 50 µg/ml*

Transfer 5,0 ml of the internal stock standard solution (3.9.1) into a 50 ml graduated flask, make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9.3. *Internal standard solution for premixtures, p/1 000 mg/ml*

(p = nominal content of diclazuril in the premixture in mg/kg)

Weigh to the nearest 0,1 mg p/10 mg of the internal standard substance in a 100 ml graduated flask, dissolve in DMF (3.6) in a ultrasonic bath (4.6), make up to the mark with DMF and mix. Wrap the flask with aluminium foil or use amber flask and store in a refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.10. Calibration solution, 2 µg/ml.

Pipet 2,0 ml diclazuril standard solution (3.8.2) and 2,0 ml internal standard solution (3.9.2) into a 50 ml graduated flask. Add 16 ml DMF (3.6), make up to the mark with water and mix. This solution must be prepared freshly before use.

3.11. C_{18} solid phase extraction cartridge, e.g. Bond Elut, size: 1 cc, sorbent weight: 100 mg.

3.12. Extraction solvent: acidified methanol.

Pipet 5,0 ml hydrochloric acid (3.7) into 1 000 ml of methanol (3.5), and mix.

3.13. Mobile phase for HPLC

3.13.1. Eluent A: ammonium acetate — tetrabutylammonium hydrogen sulphate solution.

Dissolve 5 g ammonium acetate (3.2) and 3,4 g TBHS (3.3) in 1 000 ml water (3.1) and mix.

3.13.2. Eluent B: acetonitrile (3.4).

3.13.3. Eluent C: methanol (3.5).

4. Apparatus

4.1. Mechanical shaker

4.2. Equipment for ternary gradient HPLC

4.2.1. Liquid chromatographic column, Hypersil ODS, 3 µm packing, 100 mm x 4,6 mm, or equivalent

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

4.3. Rotary film evaporator

4.4. Membrane filter, 0,45 µm

4.5. Vacuum manifold

4.6. Ultrasonic bath

5. Procedure

5.1. General

5.1.1. *Blank feed*

A blank feed shall be analysed to check that neither diclazuril nor interfering substances are present. The blank feed shall be similar in type to that of the sample and on analysis diclazuril or interfering substances shall 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 diclazuril similar to that present in the sample. To fortify at a level of 1 mg/kg add 0,1 ml of the stock standard solution (3.8.1) to 50 g of a blank feed, mix thoroughly and leave for 10 min. mixing again several times before proceeding (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 diclazuril, 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 approximately 50 g of the sample. Transfer to a 500 ml conical flask, add 1,0 ml internal standard solution (3.9.2), 200 ml extraction solvent (3.12) and stopper the flask. Shake the mixture on the shaker (4.1) overnight. Allow to settle for 10 minutes. Transfer a 20 ml aliquot of the supernatant to a suitable glass container and dilute with 20 ml water. Transfer this solution on an extraction cartridge (3.11), and pass through by applying vacuum (4.5). Wash the cartridge with 25 ml of a mixture of extraction solvent (3.12) and water, 65 + 35 (V + V). Discard the collected fractions and elute the compounds with 25 ml of a mixture of extraction solvent (3.12) and water, 80 + 20 (V + V). Evaporate this fraction until it had just reached dryness by means of the rotary evaporator (4.3) at 60 ^{o}C. Dissolve the residue in 1,0 ml DMF (3.6), add 1,5 ml of water (3.1) and mix. Filter through a membrane filter (4.4). Proceed to the HPLC determination (5.3).

5.2.2. *Premixtures*

Weigh to the nearest 0,001 g approximately 1 g of the sample. Transfer to a 500 ml conical flask, add 1,0 ml internal standard solution (3.9.3), 200 ml extraction solvent (3.12) and stopper the flask. Shake the mixture overnight on the shaker (4.1). Allow to settle for 10 minutes. Transfer an aliquot of 10 000/p ml (p = nominal content of diclazuril in the premix in mg/kg) of the supernatant to a round bottomed flask of suitable size. Evaporate until it had just reached dryness, under reduced pressure at 60 ^{o}C by means of the rotary evaporator (4.3). Redissolve the residue in 10,0ml DMF (3.6), add 15,0 ml water (3.1) and mix. 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 (4.2.1)	100 mm × 4,6 mm, Hypersil ODS, 3 µm packing, or equivalent	
Mobile phase:	Eluent A (3.13.1):	Aqueous solution of ammonium acetate and tetrabutyl-ammonium hydrogen sulphate
	Eluent B (3.13.2):	acetonitrile
	Eluent C (3.13.3):	methanol
Elution mode:	— linear gradient — initial conditions: A + B + C = 60 + 20 + 20 (V + V + V) — after 10 min. gradient elution during 30 min. to: A + B + C = 45 + 20 + 35 (V + V + V) Flush with B during 10 min.	
Flow rate:	1,5-2 ml/min.	
Injection volume:	20 µl	
Detector wavelength:	280 nm.	

Check the stability of the chromatographic system, injecting several times the calibration solution (3.10), containing 2 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. *Calibration solution*

Inject 20 µl of the calibration solution (3.10) several times and determine the mean peak height (area) of the diclazuril and internal standard peaks.

5.3.3. *Sample solution*

Inject 20 µl of the sample solution (5.2.1 or 5.2.2) several times and determine the mean peak height (area) of the diclazuril and internal standard peaks.

6. Calculation of the results

6.1. Feeds

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

$$w = \frac{h_{d,s} \times h_{i,c}}{h_{i,s} \times h_{d,c}} \times \frac{c_{d,c} \times 10\ V}{m}$$

[mg/kg]

where:

$h_{d,s}$ = peak height (area) of diclazuril in the sample solution (5.2.1)
$h_{i,s}$ = peak height (area) of the internal standard in the sample solution (5.2.1)
$h_{d,c}$ = peak height (area) of diclazuril in the calibration solution (3.10)
$h_{i,c}$ = peak height (area) of the internal standard in the calibration solution (3.10)

$c_{d,c}$ = diclazuril concentration in the calibration solution in µg/ml (3.10)
m = weight of the test portion in g
V = volume of the sample extract according to 5.2.1 (i.e. 2,5 ml)

6.2. Premixtures

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

$$w = \frac{h_{d,s} \times h_{i,c}}{h_{i,s} \times h_{d,c}} \times \frac{c_{d,c} \times 0{,}02V \times p}{m}$$

[mg/kg]

where:

$h_{d,c}$ = peak height (area) of diclazuril in the calibration solution (3.10)
$h_{i,c}$ = peak height (area) of the internal standard in the calibration solution (3.10)
$h_{d,s}$ = peak height (area) of diclazuril in the sample solution (5.2.2)
$h_{i,s}$ = peak height (area) of the internal standard in the sample solution (5.2.2)
$c_{d,c}$ = diclazuril concentration in the calibration solution in µg/ml (3.10)
m = weight of the test portion in g
V = volume of the sample extract according to 5.2.2 (i.e. 25 ml)
p = nominal content of diclazuril in mg/kg in the premixture

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 (5.2.1 or 5.2.2) and the calibration solution (3.10) are compared.

7.1.1. *Co-chromatography*

A sample extract (5.2.1 or 5.2.2) is fortified by addition of an appropriate amount of calibration solution (3.10). The amount of added diclazuril must be similar to the amount of diclazuril found in the sample extract.

Only the height of the diclazuril peak and the internal standard 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 diclazuril peak or the internal standard 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 230 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 230 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:

— 30 % relative, to the higher value for diclazuril contents from 0,5 mg/kg to 2,5 mg/kg,
— 0,75 mg/kg for diclazuril contents between 2,5 mg/kg and 5 mg/kg,
— 15 % relative to the higher value for diclazuril contents of more than 5 mg/kg.

7.3. Recovery

For a fortified (blank) sample the recovery shall be at least 80 %.

8. Results of a collaborative study

A collaborative study was arranged in which 5 samples were analysed by 11 laboratories. These samples consisted of two premixtures; one was mixed with an organic matrix (O 100) and the other with an inorganic matrix (A 100). The theoretical content is 100 mg diclazuril per kg. The three mixed feeds for poultry were made by 3 different producers (NL) (L1/Z1/K1). The theoretical content is 1 mg diclazuril per kg. The laboratories were instructed to analyse each of the samples once or in duplicate. (More detailed information on this collaborative study can be found in the *Journal of AOAC International, Volume 77, No 6, 1994, p. 1359-1361)*. The results are given in the following table.

	Sample 1A 100	**Sample 2O 100**	**Sample 3L1**	**Sample 4Z1**	**Sample 5K1**
L	11	11	11	11	6
n	19	18	19	19	12
Mean	100,8	103,5	0,89	1,15	0,89
S_r (mg/kg)	5,88	7,64	0,15	0,02	0,03
CV_r (%)	5,83	7,38	17,32	1,92	3,34
S_R (mg/kg)	7,59	7,64	0,17	0,11	0,12
CV_R (%)	7,53	7,38	18,61	9,67	13,65
Nominal content (mg/kg)	100	100	1	1	1

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

The diclazuril response must have been previously demonstrated to be linear over the range of concentrations being measured.

Status:
Point in time view as at 27/01/2009.

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