European Commission

JRC TECHNICAL REPORTS

Re-validation of a method for the determination of diclazuril in poultry feed by collaborative study

In the frame of the Revision of Commission Regulation (EC) No 152/2009

Ursula Vincent, Zigmas Ezerskis and Christoph von Holst

2016

EUR 27954

Re-validation of a method for the determination of diclazuril in poultry feed by collaborative study

JRC Science Hub
https://ec.europa.eu/jrc

JRC101940

EUR 27954

ISBN 978-92-79-58903-4 (PDF)

ISSN 1831-9424 (online)

doi:10.2787/973816 (online)

How to cite: Authors; title; EUR; doi

Table of contents

Acknowledgements 2

Executive summary 3

1. Introduction and purpose of the study 4
2. Design of the study 5
3. Statistics 5
4. Test item 6
   - 4.1. Preparation 6
   - 4.2. Homogeneity 7
   - 4.3. Distribution 7
5. Reference values and their uncertainties 7
6. Evaluation of results 8
   - 6.1. Additional information extracted from the questionnaire 13
7. Discussion and recommendations 13

References 16

List of abbreviations and definitions 17

List of figures 18

List of tables 19

Annexes 20

Acknowledgements

All laboratories participating in this exercise and listed below are kindly acknowledged.

Institute name	Country
Federal Laboratory for the Safety of the Food Chain (FLVVT)	Belgium
Huvepharma AD / Biovet AD	Bulgaria
Estonian Veterinary and Food Laboratory, Tallinn VFL, Chromatography Department	Estonia
Service Commun des Laboratoires des ministères du budget, des comptes publics, de la fonction publique et de la réforme de l'état (MBCPFPRE), de l'économie, des finances et de l'industrie (MEFI), Laboratoire de RENNES; SCL-L35, section Additifs de l'alimentation animale	France
Federal Institute for Risk Assessment (BfR), National Reference Laboratory for Feed Additives, Unit Contaminants Department Safety in the Food Chain	Germany
Landeslabor Berlin Brandenburg FB II-4: Futtermittel, Düngemittel, Pflanzenschutz	Germany
Thüringer Landesanstalt für Landwirtschaft (TLL)	Germany
Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Futtermittelinstitut Stade	Germany
LUFA Speyer, Department feeding stuffs	Germany
National Food Chain Safety Office, Food and Feed Safety Directorate, Feed Investigation National reference Laboratory	Hungary
Department of Pharmacology and Toxicology, National Veterinary Research Institute	Poland
University in Ljubljana, Veterinary Faculty , National Veterinary Institute - Unit for Pathology of Animal Nutrition and Environmental Hygiene	Slovenia
Laboratorio de Diagnóstico General (LDG)	Spain
Station de recherche, Agroscope Liebefeld-Posieux ALP	Switzerland
National Reference Laboratory of the Central Institute for Supervising and Testing in Agriculture, Ústřední kontrolní a zkušební ústav zemědělský, Praha - ÚKZÚZ	The Czech Republic

The EURL-FA Control is also thankful to Mr. Jean Charoud-Got (JRC-IRMM, Standards for Innovation and Sustainable Development Unit) for the homogenisation of the bulk test feed materials and to Mr. Mostafa Chedin (EURL-FA Control) for his support in the LC-MS/MS measurements for the homogeneity study.

Executive summary

The European Union Reference Laboratory for Feed Additives (EURL-FA), hosted by the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC), a Directorate General of the European Commission, is, among other tasks, mandated to provide scientific support to National Reference Laboratories (NRLs) in the field of the control of authorised feed additives [1]. In this frame the EURL-FA has been contacted in June 2013 by an official control laboratory and its related National Reference Laboratory regarding severe technical problems when implementing an official method established by Commission Regulation (EC) No 152/2009 [2] for the determination of diclazuril, a coccidiostat authorised as feed additive by EU legislation [3]. The EURL-FA first conducted a survey among all NRLs for feed additives control & authorisation to gather their experiences with the Community method for the determination of diclazuril at authorised additive level in feed. The information provided by the NRL's hinted at a major flaw in a specific part of the method description of the Commission Regulation. Following the survey and reporting the issue to the Directorate General for Health and Food Safety (DG SANTE), the method described in Commission Regulation (EC) No 152/2009 [2] was evaluated by the EURL-FA Control in order to identify the technical reason for this problem. The study performed at the EURL-FA Control confirmed the lack of fitness for purpose reported by the laboratories due to a significant error in the method description as given in the Commission Regulation [2]. The method was therefore modified at the EURL-FA Control and the results from the single-laboratory validation of this method showed satisfactory performance characteristics. Moreover, a modification of Commission Regulation (EC) No 152/2009 was considered necessary, to remove the error in the corresponding method description. The EURL-FA organised a collaborative study among the NRLs in order to evaluate the fitness for purpose of the optimised method, which corrects the error found in the method description.

The aim of the collaborative study was to assess the method performance characteristics of the corrected method based on high performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV or HPLC-DAD) for the determination of diclazuril at additive level in feed. The required target limits of detection and quantification were respectively 0.1 mg kg^{-1} and 0.5 mg kg^{-1}. According to the standard operational procedure (SOP) of the corrected method, the test portion, after addition of an internal standard, was extracted with acidified methanol and an aliquot of the extract was purified on a C_{18} solid phase extraction (SPE) cartridge, containing 5000 mg of the SPE sorbent. Diclazuril was eluted from the cartridge with a mixture of acidified methanol and water. After evaporation, the residue was dissolved in a mixture of N,N-dimethylformamide (DMFA) / water. The content of diclazuril was determined by ternary gradient reversed-phase HPLC using a spectrophotometry detector measuring at 280 nm. In total 5 samples corresponding to 4 blind duplicates and 1 blank feed were sent to 15 laboratories and 14 laboratories reported results. Statistical evaluation revealed that the relative standard deviation for repeatability (RSD_r) ranged from 4.5 % to 11.2 % and the relative standard deviation for reproducibility (RSD_R) varied between 14.3 % and 18.1 %. The method showed acceptable within-laboratory and between-laboratory precision, since the calculated HORRAT values were in all cases below the critical value of 1.5. In addition, no false positive result was found.
Based on the data of the collaborative study and on the discussions at the EURL-FA workshop in 2015 the EURL-FA will submit to DG Health and Food Safety (SANTE) a recommendation for the revision of the current Community method. The recommendation will include a proposal for the final text of the method for its inclusion in a revised version of Regulation (EC) No 152/2009.

1. Introduction and purpose of the study

Within the European Union feed additives are authorised according to Regulation (EC) No 1831/2003 [3] requiring various criteria to be fulfilled including the need of providing suitable methods of analysis for official control in feedingstuffs. Coccidiosis is a major disease in poultry as well as in many other hosts. Coccidiostats are the only anti-protozoal substances still authorised as feed additives and constitute the main choice to fight against coccidiosis. The conditions of use are given in the respective Commission Regulations authorising the feed additive, specifying individually for each additive important aspects such as the target animal, the inclusion level of the active substance in the feed and – in the case of cocciodiostats – the duration of the period before slaughter (withdrawal period) when the use of these substances is prohibited. Additionally, as all anti-microbial substances, coccidiostats may be a risk for human health because the presence of their residues in foodstuffs could cause toxic effects, directly in sensitive individuals and also indirectly because their widespread usage could be responsible for the promotion of resistant strains of microorganisms. Analytical methods for the accurate control of coccidiostats are therefore crucial to ensure the safety of the products, entering the food chain as additives to animal feed. Diclazuril is authorised in the EU according to various regulations as displayed in Annex 1. For the determination of diclazuril at additive level, an official method exists [2]. Based on the evaluation reports of the EURL-FA Authorisation, this method was included in these regulations and became therefore mandatory to be used for official control. However, concerns were recently raised by laboratories responsible to carry out the official controls in the Member States, as regards the non-acceptable performance characteristics of this method for the determination of diclazuril at additive level in feed. Indeed, when applying the method as described in Commission Regulation (EC) No 152/2009, a German official control laboratory reported to its NRL for Feed Additives Control and to the EURL-FA Control that diclazuril was not detected during the analysis. The Belgian NRL having experience with the method informed the EURL-FA that they identified an error in the method description as regards the amount of sorbent to be used for the solid-phase extraction (SPE) cartridge, which was afterwards confirmed by the German NRL who implemented the method both as described and as performed by the Belgian NRL.

The objectives of the work performed by the EURL-FA Control were therefore (i) to verify whether or not the lack of suitability of the method was only due to a typing error in the method description (the amount of sorbent in the SPE cartridge should be 5000 mg instead of the described 100 mg); (ii) to verify the fitness for purpose of the modified method (with 5000 mg as sorbent amount); (iii) to organise a collaborative study to assess the method performance characteristics of the modified method; and (iv) to provide DG SANTE with a consolidated opinion of the EURL-FA and the associated networks of NRLs on the need for revision of Commission Regulation (EC) No 152/2009 [2] as regards the method for the determination of diclazuril in feed.

The method was verified in compound poultry feed containing diclazuril at concentrations close to the authorised level as specified in the regulations given in Annex 1.

This report summarises the outcome of the inter-comparison exercise.

2. Design of the study

The inter-laboratory study was conducted by the EURL-FA Control for Feed Additives, hosted at the European's Commission Joint Research Centre (JRC) Institute for Reference Materials and Measurements (IRMM).

Sixteen laboratories, consisting of six NRLs for feed additives authorisation, four NRLs for feed additives control, two laboratories from feed additives' providers, a Swiss laboratory expert in the determination of coccidiostats and four official control laboratories volunteered to take part in this collaborative study. One laboratory withdrew from the study before the dispatch of the samples due to internal workload and lack of resources. Finally, fourteen laboratories representing ten countries reported results. The participating laboratories were reminded that all samples should be analysed using the method description provided by the EURL-FA. The participating laboratories were also informed that major deviation as regards the non-respect of the protocol, the deadline and/or the specified units for reporting would lead to the exclusion of their results from the statistical evaluation.

The results were used to obtain the final evaluation of the method in detecting and quantifying diclazuril in poultry feed for its official control.

The validation period had a duration of 46 days, from the dispatch of the materials (sent to the laboratories through express courier on 14 April 2015) to the deadline to report the results (29 May 2015). The results were reported both electronically by e-mail and on paper by fax or normal mail (chromatograms).

The collaborative study participants received a detailed method protocol (Annex 2), the 5 samples to be analysed and ad-hoc electronic forms to report the results of the analyses (an example is reported in Annex 3). This was crucial in order to avoid the manual transfer of data and thereby reducing possible errors in this step. The laboratories were asked to report on the content of diclazuril as mean value obtained for the measurement of 2 aliquots of the same sample bag. In this study the emphasis was placed on quantitative analyses and the result was expressed in mg kg^{-1}. The laboratories also received a questionnaire form (Annex 4) to report any comments on the method and/or minor deviations they may have performed.

After the deadline, the EURL-FA Control collected all results and performed the data evaluation and the statistical treatment. The results were presented at the EURL-FA Control & Authorisation annual workshops in Geel (Belgium) in November 2015.

3. Statistics

The design of the study and the target performance characteristics for the evaluation of the modified LC-UV method to detect diclazuril in feed samples were selected according to internationally accepted guidelines for method validation [5]. The quantitative results submitted by the laboratories at first were used to estimate average and standard deviations under repeatability and reproducibility conditions by applying the analysis of variance approach as recommended in ISO 5725:1994 [6].

Extreme values were not included in the statistical evaluation for different reasons. An initial screening excluded obvious experimental deviation from the protocol. Furthermore, as indicated by the IUPAC protocol [5] the initial reported valid data must be purged of all outliers flagged by the harmonised outlier removal procedure. This procedure basically consists of the sequential application of the Cochran and Grubbs tests (at 2.5% probability level, 1 tail for Cochran and 2 tails for Grubbs) until no further outliers are flagged or until a drop of 22.2% (= 2/9) in the original number of laboratories providing valid data would occur.

The precision data obtained from the collaborative study were then compared with predicted acceptable levels of precision. These levels, as estimated by the Horwitz equation [7], provide an indication as to whether the method is sufficiently precise for the level of analyte being measured. This indication is expressed by the HorRat value [8]. For the between-laboratories reproducibility, the HorRat value is a ratio of the measured reproducibility precision with the precision calculated by the Horwitz equation (Eq. 1) for a method measuring at that particular level of analyte:

$$\text{HorRat value} = \frac{\text{RSD}_\text{R}}{\text{RSD}_{\text{R}_\text{Horwitz}}} \qquad (1)$$

where RSD_R is the relative standard deviation of reproducibility obtained by ANOVA, and $\text{RSD}_{\text{RHorwitz}}$ is the calculated standard deviation of reproducibility using the Horwitz equation (Eq. 2).

$$\text{RSD}_{\text{R}_\text{Horwitz}} = 2^{(1-0.5 \log \text{C})} \qquad (2)$$

where C is the mass concentration expressed in power of 10 (i.e. 1 mg/g = 10^{-3}).

The acceptance limit for the HorRat value is considered to be 1.5 to 2.0.

4. Test item

4.1. Preparation

Three test items were used in this exercise and were produced by the EURL-FA Control by spiking milled blank poultry feed with diclazuril standard solutions. The main ingredients of the feed were corn (40%), wheat (22%), sunflower by-product (11%), soya (10%) and limestone (7%). The feed contained 40% starch, 16% proteins, 4% fat and its moisture was 11%. The test items were first tested at the EURL-FA Control laboratories using High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS) to ensure that no contamination by any authorised coccidiostat was present. The feed was ground and sieved to obtain particle sizes ranging from 100 µm to 800 µm.

In total 5 samples corresponding to two spiked materials sent as blind duplicates and 1 blank feed were sent to 15 laboratories and 14 laboratories reported results. The first test item consisted of a blank poultry feed spiked with diclazuril at 0.9 mg kg^{-1} (MAT 1). The second test item was a blank poultry feed spiked with diclazuril at 1.5 mg kg^{-1} (MAT 2) and the third test item was the poultry feed left as a blank (MAT 3). Homogeneous impregnation was ensured by thorough agitation of the feed after adding the spiking solution. The solvents in excess were subsequently evaporated until the feed appeared as a powder with no aggregates. The bulk materials of MAT 1 and MAT 2 were subsequently homogenised using a tubular-like mixer and divided into 120 g sub-samples using a balance. All sub-samples were filled into aluminium bags, sealed under vacuum, labelled and stored at -20 °C until further dispatch and/or analysis. The blank material MAT 3 was also distributed using the same type of bags sealed under vacuum and stored at -20 °C. All bags were labelled ensuring a random number encoding. Each participant received two bags of MAT 1 and of MAT 2 (blind replicates) and one bag of MAT 3.

The assigned values (x_a) for the concentration of diclazuril in the samples were calculated from the formulation, according to the IUPAC harmonized protocol [9]. The uncertainties for the assigned values (u_a) were calculated according to the ISO Guide for the Expression of Uncertainty in Measurement (GUM) [10].

4.2. Homogeneity

To assess the homogeneity of the test items produced, 10 bags of each test item were randomly selected. Two aliquots from each bag were extracted and further analysed in duplicate by LC-MS/MS according to the multi-analyte method implemented at the EURL-FA Control. The mean concentration values of the sub-sample duplicates were subjected to analysis of variance and the results evaluated according to the ISO 13528:2005 [11] and to the IUPAC protocol [9]. The results obtained for each of the test items showed that the homogeneity of the test items was sufficient (Annex 5) to proceed with the inter-comparison exercise.

4.3. Distribution

All samples were dispatched to participants by IRMM on 14 April 2015. Each participant received:

a) Two bags containing approximately 120 g of test item MAT 1 (blind replicates),

b) Two bags containing approximately 120 g of test item MAT 2 (blind replicates),

c) One bag containing approximately 120 g of test item MAT 3,

d) An accompanying letter with instructions for sample handling and reporting (including the individual lab code) (Annex 6) and

e) A "Confirmation of receipt" form to be sent back to IRMM after receipt of the test items (Annex 6).

f) The reporting sheet to be used to report the results was sent in an electronic message on 07 May 2015 to each participant (Annex 3).

5. Reference values and their uncertainties

The assigned value for the total content in diclazuril in poultry feed was set as the nominal value calculated from the formulation, following the IUPAC protocol [9] and corrected for purity according to Equation (3).

$$x_a = \frac{m_{dicl}}{M} \times \frac{V_2 \times V_4}{V_1 \times V_3} \times p \times 10 \qquad \text{Eq. (3)}$$

where

x_a is the assigned concentration of diclazuril, in mg kg^{-1} of feed

m_{dicl} is the weighed mass of the diclazuril pure reference standard, in mg

V_1 is the volume of solvent added for the stock standard solution, in ml

V_2 is the volume of stock standard solution of diclazuril used to produce the spiking solution, in ml

V_3 is the total volume of the spiking solution, in ml

V_4 is the volume of the spiking solution used for spiking the blank feed with diclazuril, in ml

M is the mass of blank feed to be spiked, in g

p is the purity of the diclazuril standard substance as declared on the certificate of analysis provided by the supplier, in %.

The associated standard uncertainty to the assigned values is given by Equation (4) according to the ISO Guide for the Expression of Uncertainty in Measurement (GUM) [10].

$$RSu_a = \sqrt{\sum RSu_i^2} \qquad \text{Eq. (4)}$$

where

RSu_a is the relative standard uncertainty associated to the assigned concentration, and

RSu_i is the relative standard uncertainty associated to each component of Equation (3).

Note 1: The type B uncertainties on the volume, the mass and the purity are assumed to follow a rectangular distribution. Following the Eurachem guide, the calculations of the standard uncertainty u_i and the relative standard uncertainty $RSDu_i$ were done according to Eq. (3) and Eq. (4) respectively.

$$u_i = \frac{reported\_half\_range}{\sqrt{3}} \qquad \text{Eq. (5)}$$

and the relative standard uncertainty is

$$RSDu_i = \frac{u_i}{i} \qquad \text{Eq. (6)}$$

Table 1 displays the assigned values (x_a) with the associated uncertainties (u_a) for diclazuril in the two spiked test items. Overall mean concentrations obtained during the homogeneity study are included for comparison.

Table 1: Assigned concentrations (x_a) in mg kg^{-1} of feed, expressed as mean value ± expanded uncertainty (k=2) and target standard deviations in %. The mean concentrations ($C_{homogeneity}$) obtained during the homogeneity study are given for information.

Test item	Measurand	$x_a \pm U_a$ (k=2) mg kg^{-1}	$C_{homogeneity} \pm u_{homogeneity}$ (k=2) (mg kg^{-1})
		Assigned	**Observed (n=20)**
MAT 1	Diclazuril	**0.909 ± 0.066**	**0.879 ± 0.015**
MAT 2	Diclazuril	**1.514 ± 0.080**	**1.445 ± 0.028**
		Formulation	**Homogeneity**

6. Evaluation of results

In total 14 laboratories delivered results for the 2 duplicate materials and the blank material. However, after checking the rigorous application of the protocol in the

collaborative study, only 10 were considered valid. Indeed, four laboratories made significant modifications to the protocol such as:

- a change of the composition of the mobile phase, of the elution gradient and of the flow rate,
- a change of the test portion size (5 g instead of 50 g described in the method protocol), of the sample preparation (extraction and clean-up) conditions including a change in the amount of prescribed sorbent (500 mg were used instead of 5000 mg), in the composition of the mobile phase and of the elution gradient,
- a change of the amount of sorbent used (e.g. 360 mg were used instead of 5000 mg),
- a change of the test portion size (25 g instead of 50 g), of the mobile phase composition, and of the elution mode (isocratic instead of gradient)

and it was therefore decided not to take their results into account.

Other laboratories modified the injection volume, the column type and/or length, the column temperature, an adaptation of the flow rate, the filtration/centrifugation before SPE clean-up, no filtration of the final extract, or used different (than prescribed by the protocol) concentrations of the diclazuril stock solution. It was, however, decided that these modifications were minor and their reported results were taken into account in the statistical evaluation. By analysing 2 duplicate spiked materials the participants provided 40 valid results for the diclazuril analysis. Other results were further excluded as outliers after Cochran and Grubb's tests as highlighted in Table 2.

As indicated by the IUPAC protocol [5] the initial reported valid data must be purged of all outliers flagged by the harmonised outlier removal procedure This procedure basically consists of the sequential application of the Cochran and Grubbs tests (at 2.5% probability level, 1 tail for Cochran and 2 tails for Grubbs) until no further outliers are flagged or until a drop of 22.2% (= 2/9) in the original number of laboratories providing valid data would occur. This limit was always respected as the maximum number of outliers was 1/10 laboratories.

Table 2 shows that no false-negatives and no false-positives were found for diclazuril in the tested poultry feed.

Table 2: Diclazuril results

LAB	MAT 1	MAT 1	MAT 2	MAT 2	MAT 3
Target value	**0.909**	**0.909**	**1.514**	**1.514**	**0.000**
L01	1.035	1.090	1.640	1.560	<LOQ
L02	1.000	0.999	1.519	1.554	<LOD
L03	0.987	0.909	1.351	1.466	<LOD
L04	0.994	1.028	1.507	1.519	<0.25
L05	1.085	1.115	1.674	1.630	< LOD
L08	9.750	9.859	14.075	14.951	<0.5
L09	0.676	0.913	1.258	1.273	<LOD
L10	0.853	1.247	1.169	1.924	< LOD
L11	1.094	1.140	1.647	1.702	< LOQ
L12	1.078	0.921	1.597	1.399	<LOD
L13	1.073	1.061	1.596	1.548	<LOQ
L14	1.431	1.365	1.953	1.891	< LOD
L15	0.881	0.807	1.224	1.192	<LOD
L16	0.926	0.912	1.488	1.368	<LOD

	Cochran exclusion
	Grubb's exclusion
	Non-compliant laboratory exclusion

The resulting precision data indicating the within-lab repeatability (RDS_r) and the between-lab reproducibility (RDS_R) are outlined in Table 3.

Table 3: Precision data for diclazuril in poultry compound feed

	No of laboratories	Targ. (mg kg^{-1})	Av. (mg kg^{-1})	s_r (mg kg^{-1})	RSDr (%)	s_R (mg kg-1)	RSDR (%)	HorR
MAT 1	14 (4)	0.9	1.0	0.11	11.2	0.18	18.1	1.14
MAT 2	14 (5)	1.5	1.5	0.07	4.5	0.21	14.3	0.95

Targ.: Target concentration; Av: average concentration; s_r: within-laboratory standard deviation (repeatability); RSDr: relative within-laboratory standard deviation (repeatability); s_R: between-laboratory standard deviation (reproducibility); RSDR: relative between-laboratory standard deviation; HoR : HorRat value for reproducibility. The number between brackets indicates the number of laboratories identified as outliers due to deviation from the protocol or by statistical tests

The repeatability relative standard deviation represented in Figure 1 was evaluated per test material and was 4.5% for MAT2 and 11.2% for MAT1.

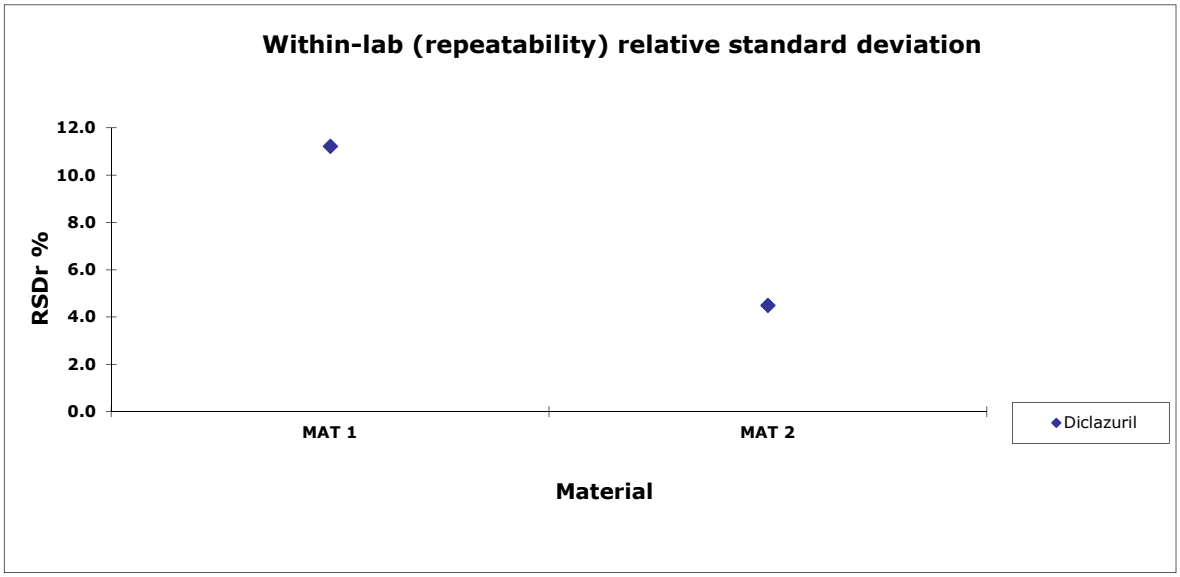


Figure 1: Repeatability relative standard deviation

Figure 2 displays the reproducibility relative standard deviation which was 14.3% for MAT2 and 18.2% for MAT1.

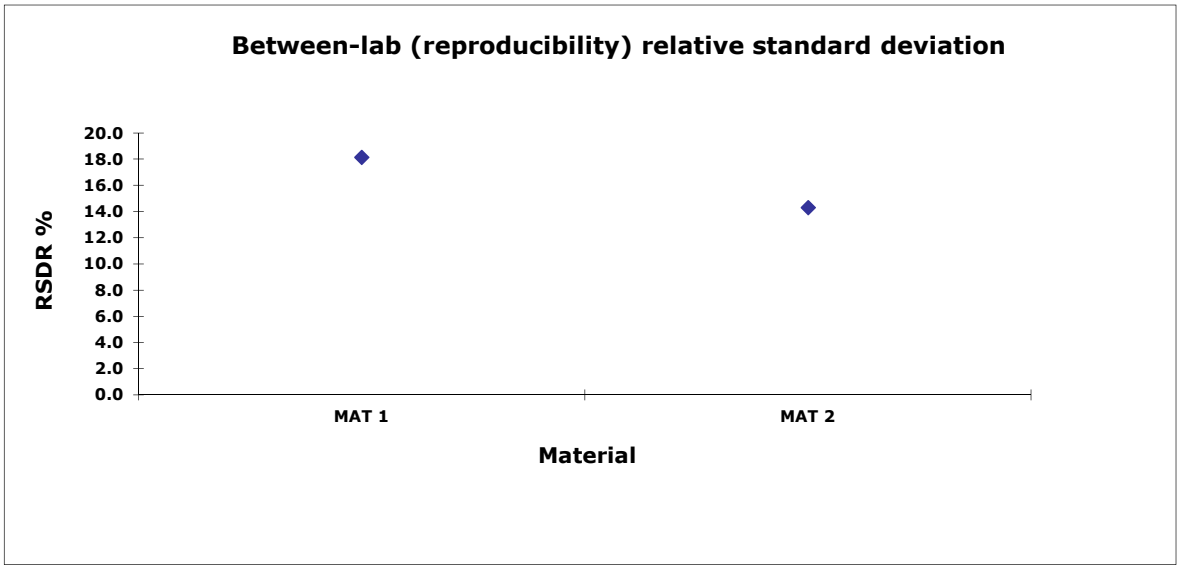


Figure 2: Reproducibility relative standard deviation

The reproducibility of the method was then compared with the precision estimated by the Horwitz equation and was expressed as the HorRat value (Figure 3).

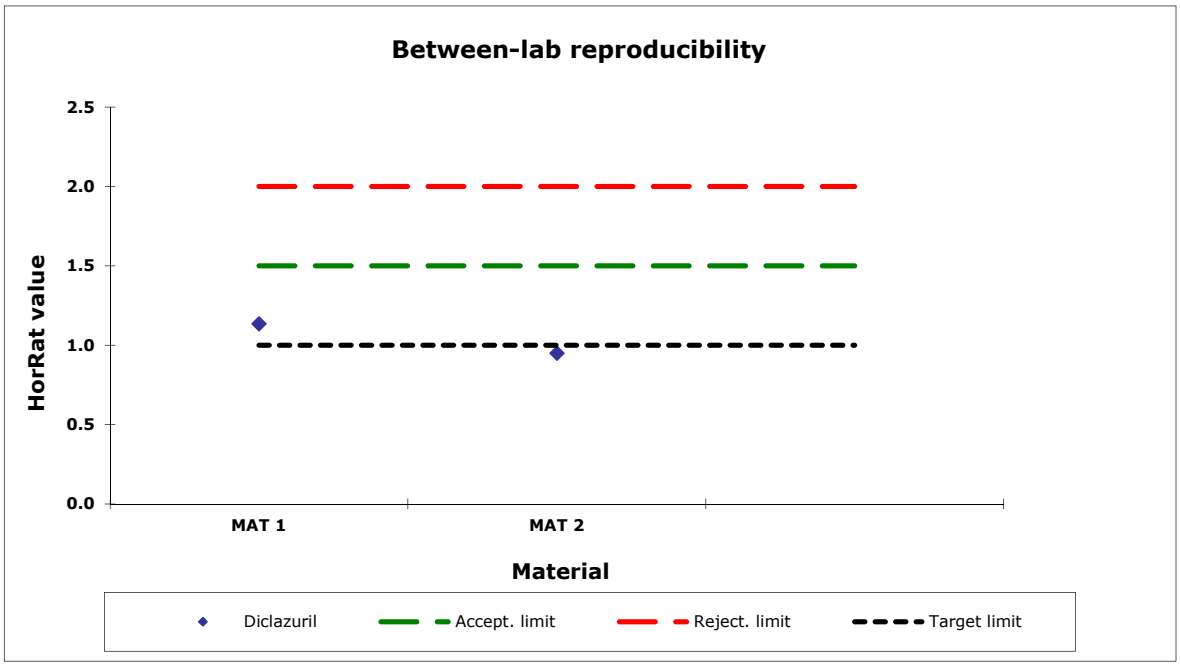


Figure 3: Reproducibility, HorRat value

As highlighted from the graph in Figure 3, the HorRat value was always below 1.5. From these results it is evident that the method is fit for determining diclazuril in poultry compound feed at authorised concentrations levels.

In addition to the initial aim, an evaluation of the trueness of the method was also performed. The trueness, represented by the analytical recovery and indicating the closeness of agreement between the reported result and the target value is represented in Figure 4. For all materials the trueness within the acceptance limits of 80% and 120%. This would allow a reliable quantification of diclazuril and an appropriate check of the labelling.

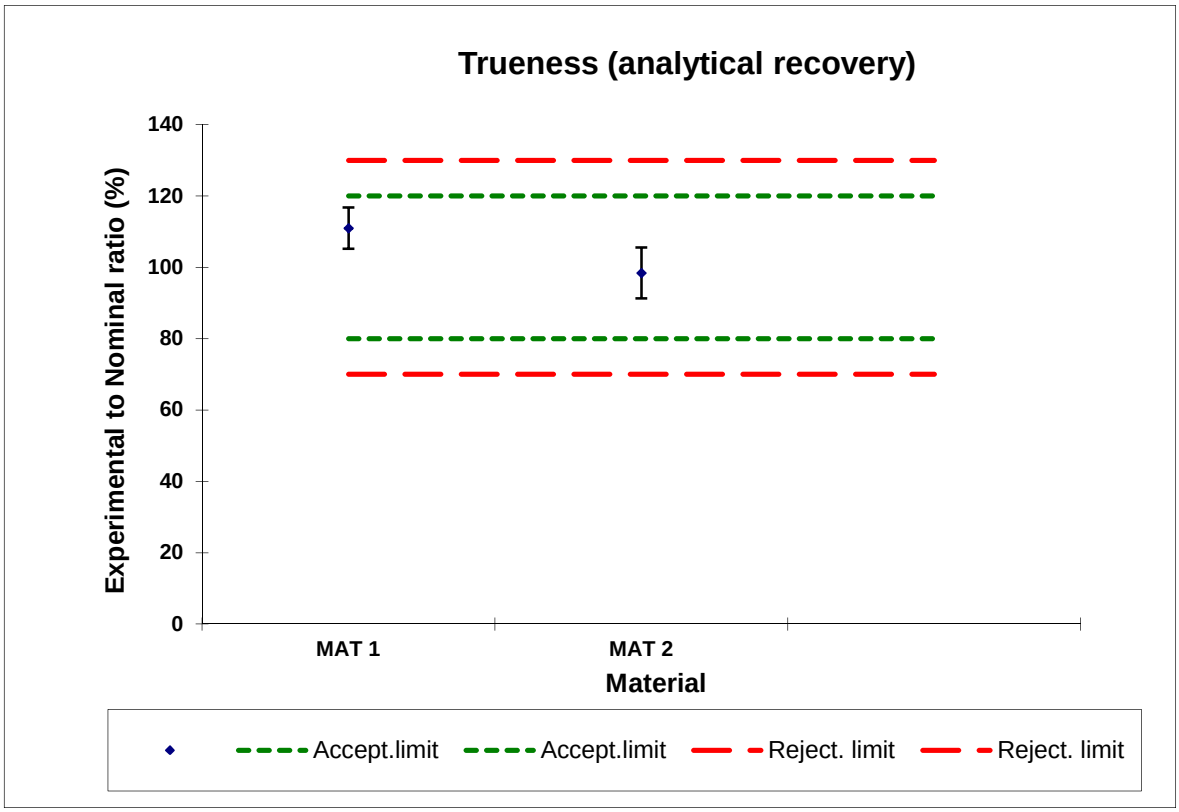


Figure 4: Trueness per material

The error bars represent 2 x standard error (between-laboratory standard deviation/square root of the number of valid measurements); acceptance range: 80%-120%, rejection limits: 70% and 130%.

6.1. Additional information extracted from the questionnaire

Additional information was gathered from the questionnaire filled in by the participants. A compilation of the method parameters used by the participants is given in Annex 7.

7. Discussion and recommendations

Following an alert from official control laboratories, the method for the determination of diclazuril in feedingsuffs described in Commission Regulation (EC) No 152/2009 [2] was re-evaluated by the EURL-FA. The initial method was modified as regards the amount of sorbent to be used for the solid phase extraction of diclazuril and in-house validated before being proposed for validation through a collaborative study involving National Reference Laboratories, Official Control Laboratories and feed additives providers' laboratories. The main aim was to assess the method performance characteristics of the modified method based on HPLC-UV or HPLC-DAD for the determination of diclazuril at additive level in poultry. The required target limits of detection and quantification were respectively 0.1 mg kg^{-1} and 0.5 mg kg^{-1}.

Since the collaborative study demonstrated the fitness for purpose of the method for the determination of diclazuril in poultry compound feed, it can therefore be recommended that the current Commission Regulation (EC) No 152/2009 [2] should be revised as regards the method description for the determination of diclazuril.

However, the NRLs and Official Control Laboratories often report that e.g.:

- the method is tedious and time consuming,

- the quantification is not reliable when matrix interferences at the retention time of diclazuril are present in the samples,
- a difference in the recovery of the analyte in relation to the internal standard which can potentially lead to a to a biased result.

On the whole, several NRLs recommend switching to a LC-MS/MS based method with a modified clean-up protocol that proved to be fit for the purpose in single-laboratory validation.

The following options were discussed:

- Option 1: Removing the method from the Regulation No 152/2009. This would allow flexibility for utilizing :
    - single-laboratory validated LC-MS methods that demonstrated to perform satisfactorily in previous proficiency tests organised by the EURL-FA Control,
    - the modified HPLC-UV method validated during the current exercise, and eventually
    - the upcoming EN standard for multi-coccidiostats and antibiotics

- Option 2: Substituting the current HPLC-UV method in Regulation No 152/2009 by the modified method validated during the current exercise. It would then be compulsory to use this modified method when performing official controls of diclazuril in animal feed.

During the discussions at the annual workshops of the EURL-FA Authorisation and Control respectively, the majority of the laboratories advocated for a revision of the Community method but allowing more freedom in applying flexible chromatographic conditions (e.g. isocratic flow as alternative to the gradient and different columns). The laboratories were reluctant having LC-MS based methods for official control stating that analytical results produced by these methods may have expanded uncertainties of ca. 40% and furthermore, that the flexibility of choosing methods might last only for 2-3 years until the EN standard becomes available and therefore compulsory for official control, according to the cascade approach of Regulation (EC) 882/2004. However, it was suggested by the Czech NRL to retain LC-MS based methods for the analysis of diclazuril in *fatty* feed (only). As an additional option it was suggested that the corrected Community method could be retained in the updated regulation, including in the description of the method the possibility of using other *equivalent* methods (e.g. LC-MS based).

Finally, the laboratories were asked to express their opinion by anonymous voting. The results for 16 voting laboratories from the EURL-FA Authorisation network were as follows:

- Withdrawal of the Community method: 19% (3 laboratories);
- Substituting the current Community method by the corrected one: 75% (12 laboratories);
- No opinion: 6% (1 laboratory).

The same voting exercise was conducted during the EURL-FA Control workshop; 17 laboratories took part in the voting and the results were as follows:

- Withdrawal of the Community method: 47% (8 laboratories);
- Substituting the current Community method by the corrected one: 53% (9 laboratories).

It was therefore agreed that the EURL-FA will submit to the European Commission a recommendation for the revision of the current Community method, described in Commission Regulation (EC) 152/2009. The recommendation will include a proposal for the final text of the method, drafted by the EURL-FA and consolidated after review by the NRLs (Authorisation and Control networks), for its inclusion in the revised Regulation. The consolidated text of the method is given in Annex 8.

References

[1] Commission Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, *Official Journal of the European Union*, L 165 (2004) 1 – 141.

[2] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, *Official Journal of the European Union*, L 54 (2009) 85 – 89.

[3] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, *Official Journal of the European Union*, L 268 (2003) 29 – 43.

[4] Regulation (EC) No 767/2009 of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC, *Official Journal of the European Union*, L 229 (2009) 1 – 28

[5] Horwitz W., "Protocol for the design, conduct and interpretation of method-performance studies", Pure and Applied Chemistry, (1995), 67, 331.

[6] ISO 5725:1994, "Accuracy (trueness and precision) of measurement methods and results, parts 1 to 6." In "Statistical methods for quality control." Geneva: ISO Standards Handbook, International Standardization Organisation. (ISBN 92-67-10321-0).

[7] W Horwitz, L R Kamps, K W Boyer, "Quality assurance in the analysis of foods and trace constituents" J Assoc Off Anal Chem, (1980), 63, 1344

[8] Peeler JT, Horwitz W, Albert R, "Precision parameters of standard methods of analysis for dairy products", J Assoc Off Anal Chem. (1989) Sep-Oct;72(5):784

[9] Thompson M., Ellison S., Wood R., The international harmonized protocol for the proficiency testing of analytical chemistry laboratories. Pure Appl. Chem., Vol. 78, No. 1, pp. 145–196, 2006

[10] "Evaluation of measurement data - Guide to the Expression of Uncertainty in Measurement" (GUM 1995 with minor corrections) (2008). Joint Committee for Guides in Metrology (JCGM/WG 1 - BIPM, IEC, IFCC, ILAC, ISO, IUPAC, IUPAP and OIML).

[11] ISO 13528 - *"Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparisons"*, issued by ISO-Geneva (CH), International Organization for Standardization.

[12] W. Horwitz, R. Albert, J. AOAC Int., 89 (2006) 1095 – 1109.

List of abbreviations and definitions

EURL-FA: European Reference Laboratory for Feed Additives

EURL-FA Control: European Reference Laboratory for Feed Additives Control

NRL: National Reference Laboratory

DG: Directorate General

HPLC or LC: High Performance Liquid chromatography

LC-MS: High Performance Liquid Chromatography coupled to mass spectrometry

LC-MS/MS: High Performance Liquid Chromatography tandem mass spectrometry

LC-UV or LC-DAD: High Performance Liquid chromatography coupled to spectrophotometric detection – UV: ultra-violet; DAD: (Photo)Diode Array Detection

C_{18}: Carbon 18

SPE: solid-phase extraction

RSD_r: relative standard deviation for repeatability; within-lab repeatability

RSD_R: relative standard deviation for reproducibility; between-lab reproducibility

ANOVA: ANalysis Of VAriances

List of figures

Figure 1: Repeatability relative standard deviation

Figure 2: Reproducibility relative standard deviation

Figure 3: Reproducibility, HorRat value

Figure 4: Trueness per material

List of tables

Table 1: Assigned concentrations (xa) in mg kg^{-1} of feed, expressed as mean value ± expanded uncertainty (k=2) and target standard deviations in %.

Table 2: Diclazuril results

Table 3: Precision data for diclazuril in poultry compound feed

Annexes

Annex 1 : EU Regulations authorising diclazuril as a feed additive

Regulation	Target animal	Minimum content	Maximum content	Amendment		Holder of authorisation	Name	Legislation
COMMISSION REGULATION (EU) No 1118/2010	chicken for fattening	1	1	COMMISSION IMPLEMENTING REGULATION (EU) No 160/2013	Change holder	From Janssen Pharmaceutica to Eli Lilly	Clinacox	Regulation (EC) No 131/2003
COMMISSION REGULATION (EU) No 169/2011	Guinea fowls	1	1	COMMISSION IMPLEMENTING REGULATION (EU) No 160/2013	Change holder	From Janssen Pharmaceutica to Eli Lilly	Clinacox	Regulation (EC) No 131/2003
COMMISSION IMPLEMENTING REGULATION (EU) No 888/2011	Turkeys for fattening	1	1	COMMISSION IMPLEMENTING REGULATION (EU) No 160/2013	Change holder	From Janssen Pharmaceutica to Eli Lilly	Clinacox	Regulation (EC) No 131/2003
COMMISSION REGULATION (EC) No 971/2008	Rabbits	1	1	COMMISSION IMPLEMENTING REGULATION (EU) No 160/2013	Change holder	From Janssen Pharmaceutica to Eli Lilly	Clinacox	Directive 70/524/EEC
COMMISSION IMPLEMENTING REGULATION (EU) No 667/2013	Chicken reared for laying	1	1			Janssen Pharmaceutica	Clinacox	Regulation (EC) No 131/2003
COMMISSION IMPLEMENTING REGULATION (EU) 2015/46	Chickens for fattening Turkeys for fattening Guinea fowl for fattening and for breeding	0.8	1.2			Huvepharma	Coxiril	Regulation (EC) No 131/2003
COMMISSION IMPLEMENTING REGULATION (EU) 2015/1417	Rabbits	1	1			Huvepharma	Coxiril	Regulation (EC) No 131/2003

Annex 2 : Method protocol

Work Instruction

EURL-FA Control

Determination of Diclazuril in poultry compound feed at feed additive levels with High Performance Liquid Chromatography coupled to UV detection (HPLC-UV)

March 2015

Contents

1. Aim 25
2. Scope 25
3. Definition 25
4. Description 25
4.1 Principle 25
4.2 Reagents and materials 25
4.2.1 Water, HPLC grade or equivalent (e.g. milli-Q purified water) 26
4.2.2 Acetonitrile, HPLC gradient grade or hypergrade LC-MS, minimum 99.9% purity 26
4.2.3 Methanol, HPLC grade or hypergrade LC-MS 26
4.2.4 N,N-Dimethylformamide (DMFA), minimum 99% purity 26
4.2.5 Ammonium acetate, minimum 99% purity 26
4.2.6 Tetrabutylammonium hydrogen sulphate (TBHS), minimum 99% purity 26
4.2.7 Hydrochloric acid, ρ_{20} = 1.19 g ml^{-1}, minimum 37% purity 26
4.2.8 Extraction solvent – acidified methanol 26
4.2.9 Mixture of extraction solvent and water (65%:35%, v/v) 26
4.2.10 Mixture of extraction solvent and water (80%:20%, v/v) 26
4.2.11 Mobile phase for HPLC: 26
4.3 Reference standards 26
4.3.1 Diclazuril, minimum 98% purity 26
4.3.2 Bis-Diclazuril, minimum 97% purity, to be used as internal standard (I.S.) for Diclazuril (4.3.1) 26
4.4 Standard solutions 26
4.4.1 Stock standard solutions, ca. 0.5 mg ml^{-1} 26
4.4.1.1 Diclazuril 26
4.4.1.2 Bis-Diclazuril (I.S.) 27
4.4.2 Intermediate standard solutions, ca. 0.05 mg ml^{-1} 27
4.4.2.1 Diclazuril 27
4.4.2.2 Bis-Diclazuril (I.S.) 28
4.4.3 Calibration solution, ca. 0.001 mg ml^{-1} (Diclazuril) and ca. 0.002 mg ml^{-1} (I.S.) 28
4.4.4 Calibration solution, ca. 0.002 mg ml^{-1} (Diclazuril) and ca. 0.002 mg ml^{-1} (I.S.) 28
4.4.5 Calibration solution, ca. 0.003 mg ml^{-1} (Diclazuril) and ca. 0.002 mg ml^{-1} (I.S.) 28
4.4.6 Calibration solution, ca. 0.004 mg ml^{-1} (Diclazuril) and ca. 0.002 mg ml^{-1} (I.S.) 28
4.4.7 Calibration solution, ca. 0.005 mg ml^{-1} (Diclazuril) and ca. 0.002 mg ml^{-1} (I.S.) 28
4.5 Apparatus 29
4.5.1 HPLC system consisting of the following: 29
4.5.2 HPLC column: Hypersil ODS, 3 µm packing, 100 mm x 4.6 mm ID or equivalent 29
4.5.3 HPLC column guard: Hypersil ODS, 3 µm packing, 10 mm x 4.0/4.6 mm ID or equivalent 29
4.5.4 Mechanical shaker 29
4.5.5 Rotary evaporator 29

4.5.6 Ultrasonic bath 29
4.5.7 Balances, one analytical of 10 g capacity or greater with 0.1 mg readability 29
4.5.8 Vacuum pump, for solid phase extraction (SPE) applications 29
4.5.9 Vacuum manifold, for SPE applications 29
4.5.10 Membrane filter, 0.45 µm 29
4.5.11 Disposable syringe, 5 ml 29
4.5.12 SPE cartridge, C18 phase, size - 20 cc, sorbent weight - 5000 mg 29
4.5.13 Variable-volume air or positive displacement piston pipettes, suitable for pipetting volumes ranged from 10 to 5000 µl 29
4.5.14 Graduated flask, of 50 ml 29
4.5.15 Graduated flask, of 1000 ml 29
4.5.16 Glass graduated cylinder, of 25 ml 29
4.5.17 Glass graduated cylinder, of 50 ml 29
4.5.18 Glass graduated cylinder, of 100 ml 29
4.5.19 Conical flask, of 100 ml, with stopper 29
4.5.20 Conical flask, of 250 ml, with stopper 29
4.5.21 Conical flask, of 500 ml, with stopper 29
4.5.22 Graduated glass pipette, of 20 ml 29
4.5.23 Round bottom flask, of 50 ml 29
4.5.24 Storage vial, of 5 ml with cap 29
4.5.25 Glass vial, of 1.5 ml for HPLC with cap 29
4.6 Procedure 29
4.6.1 General 29
4.6.2 Extraction 30
4.6.3 HPLC determination 30
4.7 Calculation of the results 31
4.8 Normative references 31

1. Aim

This working instruction describes a method based on reversed-phase high-performance liquid chromatography (HPLC) coupled to UV detection for the determination of Diclazuril content in animal compound feed at feed additive levels.

2. Scope

The method makes it possible to determine the level of Diclazuril in feed. The limit of detection is 0.1 mg/kg, the limit of quantification is 0.5 mg/kg.

3. Definition

None

4. Description

IMPORTANT NOTE: a check of the calibration status of all equipment having impact on the quality (e.g. balances, pipettes,…) by the method operator is compulsory before performing any measurement. All used equipment shall be calibrated and/or validated at the time of the measurement; if this status is not achieved, no further experiment shall be performed and appropriate measures shall be taken according to the established procedures.

4.1 Principle

After addition of an internal standard, the sample is extracted with acidified methanol. An aliquot of the extract is purified on a C18 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 the mixture of N,N-dimethylformamide (DMFA) / water. The content of Diclazuril is determined by ternary gradient reversed-phase high-performance liquid chromatography (HPLC) using a UV detector.

4.2 Reagents and materials

WARNING – Avoid inhalation of and exposure to the toxic standard materials and solutions thereof. Work in a fume-hood when handling the solvents and solutions. Wear safety glasses, protective clothing and avoid skin contact.

Use only reagents recognised as analytical grade at least unless otherwise stated.

4.2.1 Water, HPLC grade or equivalent (e.g. milli-Q purified water)

4.2.2 Acetonitrile, HPLC gradient grade or hypergrade LC-MS, minimum 99.9% purity

4.2.3 Methanol, HPLC grade or hypergrade LC-MS

4.2.4 N,N-Dimethylformamide (DMFA), minimum 99% purity

4.2.5 Ammonium acetate, minimum 99% purity

4.2.6 Tetrabutylammonium hydrogen sulphate (TBHS), minimum 99% purity

4.2.7 Hydrochloric acid, ρ_{20} = 1.19 g ml^{-1}, minimum 37% purity

4.2.8 Extraction solvent – acidified methanol

Pipet 5.0 ml of hydrochloric acid (4.2.7) into 1000 ml graduated flask (4.5.15), make up to the mark with methanol (4.2.3) and mix.

4.2.9 Mixture of extraction solvent and water (65%:35%, v/v)

Add 65 ml of extraction solvent (4.2.8) into glass graduated cylinder of 100 ml (4.5.18). Add 35 ml of water (4.2.1) into glass graduated cylinder of 50 ml (4.5.17). Transfer the contents of both cylinders into conical flask of 250 ml with stopper (4.5.20) and mix.

4.2.10 Mixture of extraction solvent and water (80%:20%, v/v)

Add 80 ml of extraction solvent (4.2.8) into glass graduated cylinder of 100 ml (4.5.18). Add 20 ml of water (4.2.1) into glass graduated cylinder of 25 ml (4.5.16). Transfer the contents of both cylinders into conical flask of 250 ml with stopper (4.5.20) and mix.

4.2.11 Mobile phase for HPLC:

4.2.11.1 Eluent A: ammonium acetate - tetrabutylammonium hydrogen sulphate (TBHS) aqueous solution

Add 5 g ammonium acetate (4.2.5) and 3.4 g TBHS (4.2.6) in graduated flask of 1000 ml (4.5.15), dissolve, make up to the mark with water (4.2.1) and mix. Prepare fresh solutions monthly.

4.2.11.2 Eluent B: acetonitrile (4.2.2)

4.2.11.3 Eluent C: methanol (4.2.3)

4.3 Reference standards

Purity required for each lot of reference and internal standards:

4.3.1 Diclazuril, minimum 98% purity

4.3.2 Bis-Diclazuril, minimum 97% purity, to be used as internal standard (I.S.) for Diclazuril (4.3.1)

4.4 Standard solutions

4.4.1 Stock standard solutions, ca. 0.5 mg ml^{-1}

4.4.1.1 Diclazuril

Accurately weigh 25.0 mg of Diclazuril standard substance (4.3.1) in a 50 ml graduated flask (4.5.14) (or any other appropriate amount of Diclazuril standard substance (4.3.1) in appropriate volume of graduated flask). Dissolve in DMFA (4.2.4), make up to the mark

with DMFA (4.2.4) 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.

Determine the accurate concentration of the Diclazuril stock standard solution using the reference standard purity value provided by the supplier using equation [1].

$$C_{DICL} = \frac{m}{V} \times \frac{P}{100} \qquad \text{eq. [1]}$$

where

C_{DICL} is the concentration of Diclazuril standard substance (4.3.1) in the stock standard solution in milligrams per millilitre,

P is the purity of the Diclazuril standard substance (4.3.1) given by the supplier in percent (e.g. 98%)

m is the weighed mass of Diclazuril standard substance (4.3.1) in milligrams

V is the volume of the stock standard solution (4.4.1.1) in millilitres

4.4.1.2 Bis-Diclazuril (I.S.)[1]

Accurately weigh 25.0 mg of internal standard substance (4.3.2) in a 50 ml graduated flask (4.5.14) (or any other appropriate amount of the internal standard substance (4.3.2) in appropriate volume of graduated flask). Dissolve in DMFA (4.2.4), make up to the mark with DMFA (4.2.4) 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.

Determine the accurate concentration of the bis-Diclazuril stock standard solution using the reference standard purity value provided by the supplier using equation [2].

$$C_{bis-DICL} = \frac{m}{V} \times \frac{P}{100} \qquad \text{eq. [2]}$$

where

$C_{bis-DICL}$ is the concentration of internal standard substance (4.3.2) in the stock standard solution in milligrams per millilitre,

P is the purity of internal standard substance (4.3.2) given by the supplier in percent (e.g. 96.5%)

m is the weighed mass of internal standard substance (4.3.2) in milligrams

V is the volume of the stock internal standard solution (4.4.1.2) in millilitres

4.4.2 Intermediate standard solutions, ca. 0.05 mg ml^{-1}

4.4.2.1 Diclazuril

Transfer 5.00 ml of the stock standard solution (4.4.1.1) into a 50 ml graduated flask (4.5.14) (or any other appropriate volume of Diclazuril stock standard solution (4.4.1.1) in appropriate volume of graduated flask[2]), make up to the mark with DMFA (4.2.4) and mix.

[1] In the frame of the collaborative study, please skip 4.4.1.2 and proceed with 4.4.2.2 using the 0.5 mg ml^{-1} stock solution of bis-Diclazuril (nominal concentration after purity correction is 0.48 mg ml^{-1}) delivered from EURL-FA.

[2] For instance, transfer 2.5 ml of the stock solution of bis-Diclazuril (4.4.1.2) delivered from EURL-FA into 25 ml graduated flask, make up to the mark with DMFA (4.2.4) and mix.

Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 ^{o}C the solution is stable for 1 month.

4.4.2.2 Bis-Diclazuril (I.S.)

Transfer 5.00 ml of the stock internal standard solution (4.4.1.2) into a 50 ml graduated flask (4.5.14) (or any other appropriate volume of stock internal standard solution (4.4.1.2) in appropriate volume of graduated flask), make up to the mark with DMFA (4.2.4) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 ^{o}C the solution is stable for 1 month.

4.4.3 Calibration solution, **ca. 0.001 mg ml^{-1}** (Diclazuril) and ca. **0.002 mg ml^{-1}** (I.S.)

Pipet 1.00 ml Diclazuril intermediate standard solution (4.4.2.1) and 2.00 ml intermediate internal standard solution (4.4.2.2) into a 50 ml graduated flask (4.5.14). Add 17 ml DMFA (4.2.4), make up to the mark with water (4.2.1) and mix. This solution must be prepared freshly before use.

4.4.4 Calibration solution, **ca. 0.002 mg ml^{-1}** (Diclazuril) and ca. **0.002 mg ml^{-1}** (I.S.)

Pipet 2.00 ml Diclazuril intermediate standard solution (4.4.2.1) and 2.00 ml intermediate internal standard solution (4.4.2.2) into a 50 ml graduated flask (4.5.14). Add 16 ml DMFA (4.2.4), make up to the mark with water (4.2.1) and mix. This solution must be prepared freshly before use.

4.4.5 Calibration solution**, ca. 0.003 mg ml^{-1}** (Diclazuril) and **ca. 0.002 mg ml^{-1} (**I.S.)

Pipet 3.00 ml Diclazuril intermediate standard solution (4.4.2.1) and 2.00 ml intermediate internal standard solution (4.4.2.2) into a 50 ml graduated flask (4.5.14). Add 15 ml DMFA (4.2.4), make up to the mark with water (4.2.1) and mix. This solution must be prepared freshly before use.

4.4.6 Calibration solution**, ca. 0.004 mg ml^{-1}** (Diclazuril) and **ca. 0.002 mg ml^{-1} (**I.S.)

Pipet 4.00 ml Diclazuril intermediate standard solution (4.4.2.1) and 2.00 ml intermediate internal standard solution (4.4.2.2) into a 50 ml graduated flask (4.5.14). Add 14 ml DMFA (4.2.4), make up to the mark with water (4.2.1) and mix. This solution must be prepared freshly before use.

4.4.7 Calibration solution**, ca. 0.005 mg ml^{-1}** (Diclazuril) and **ca. 0.002 mg ml^{-1} (**I.S.)

Pipet 5.00 ml Diclazuril intermediate standard solution (4.4.2.1) and 2.00 ml intermediate internal standard solution (4.4.2.2) into a 50 ml graduated flask (4.5.14). Add 13 ml DMFA (4.2.4), make up to the mark with water (4.2.1) and mix. This solution must be prepared freshly before use.

NOTE: the calibration solutions (4.4.3, 4.4.4, 4.4.5, 4.4.6 and 4.4.7) are covering Diclazuril concentration in feed ranging from 0.5 to 2.5 mg kg^{-1} when using the current protocol.

4.5 Apparatus

Usual laboratory apparatus and, in particular, the following,

4.5.1 HPLC system consisting of the following:

4.5.1.1 Ternary or quaternary pump, pulse-free, able of maintaining a flow rate up to 2 ml min^{-1}

4.5.1.2 Injection system, with a loop suitable for 20 µl volume injections

4.5.1.3 Column heater, set at 25°C

4.5.1.4 UV detector, with variable wavelength adjustment or diode array detection

4.5.1.5 Computer with a software, for HPLC system control and data processing

4.5.2 HPLC column: Hypersil ODS, 3 µm packing, 100 mm x 4.6 mm ID or equivalent

4.5.3 HPLC column guard: Hypersil ODS, 3 µm packing, 10 mm x 4.0/4.6 mm ID or equivalent

4.5.4 Mechanical shaker

4.5.5 Rotary evaporator

4.5.6 Ultrasonic bath

4.5.7 Balances, one analytical of 10 g capacity or greater with 0.1 mg readability

4.5.8 Vacuum pump, for solid phase extraction (SPE) applications

4.5.9 Vacuum manifold, for SPE applications

4.5.10 Membrane filter, 0.45 µm

4.5.11 Disposable syringe, 5 ml

4.5.12 SPE cartridge, C18 phase, size - 20 cc, sorbent weight - 5000 mg

4.5.13 Variable-volume air or positive displacement piston pipettes, suitable for pipetting volumes ranged from 10 to 5000 µl

4.5.14 Graduated flask, of 50 ml

4.5.15 Graduated flask, of 1000 ml

4.5.16 Glass graduated cylinder, of 25 ml

4.5.17 Glass graduated cylinder, of 50 ml

4.5.18 Glass graduated cylinder, of 100 ml

4.5.19 Conical flask, of 100 ml, with stopper

4.5.20 Conical flask, of 250 ml, with stopper

4.5.21 Conical flask, of 500 ml, with stopper

4.5.22 Graduated glass pipette, of 20 ml

4.5.23 Round bottom flask, of 50 ml

4.5.24 Storage vial, of 5 ml with cap

4.5.25 Glass vial, of 1.5 ml for HPLC with cap

4.6 Procedure

4.6.1 General

4.6.1.1 Blank feed

It is recommended to analyse a blank feed using the same procedure as for the unknown samples in order to check that neither Diclazuril nor any interfering substances are present. The blank feed should be of a similar type to that of the sample subjected to analysis.

4.6.2 Extraction

4.6.2.1 Feed

Prepare 2 replicate test portions from each unknown sample. Weigh to the nearest 0.01 g approximately 50 g of the sample. Transfer to a 500 ml conical flask with stopper (4.5.21), add 1.00 ml intermediate internal standard solution (4.4.2.2), 200 ml extraction solvent (4.2.8) and stopper the flask. Shake the mixture on the shaker (4.5.4) overnight. Allow to settle the extract for 10 minutes. Take 20 ml aliquot of the supernatant with graduated glass pippete of 20 ml (4.5.22), transfer into conical flask of 100 ml with stopper (4.5.19), add 20 ml of water (4.2.1) and mix. Transfer this solution on the SPE extraction cartridge (4.5.12) mounted on vacuum manifold (4.5.9) and pass through using vacuum pump (4.5.8) connected to the vacuum manifold (4.5.9). Wash the cartridge (4.5.12) with 25 ml of a mixture (65%:35%, v/v) (4.2.9) of extraction solvent (4.2.8) and water (4.2.1). Discard the collected fraction and elute the compounds with 25 ml of a mixture (80%:20%, v/v) (4.2.10) of extraction solvent (4.2.8) and water (4.2.1). Collect the latter fraction into round bottom flask of 50 ml (4.5.23). Evaporate this fraction until it had just reached dryness by means of the rotary evaporator (4.5.5) at 60 ^{o}C. Dissolve the residue in 1.0 ml DMFA (4.2.4), add 1.5 ml of water (4.2.1), transfer the solution in storage vial of 5 ml (4.5.24) and mix. Filter the solution through a membrane filter (4.5.10) mounted on disposable syringe (4.5.11) into storage vial of 5 ml (4.5.24). Transfer 1.5 ml of the latter filtrate into the glass vial for HPLC (4.5.25), cap and proceed with the HPLC determination (4.6.3).

4.6.3 HPLC determination

The conditions are following,

4.6.3.1 HPLC column (4.5.2)

4.6.3.2 HPLC Column guard (4.5.3)

4.6.3.3 Mobile phase:

- Eluent A (4.2.11.1)
- Eluent B (4.2.11.2)
- Eluent C (4.2.11.3)

4.6.3.4 Elution mode - linear gradient:

- **At 0 min:** Eluent (A+B+C) = 60%+20%+20% (v+v+v)
- **At 10 min:** Eluent (A+B+C) = 60%+20%+20% (v+v+v)
- **At 40 min:** Eluent (A+B+C) = 45%+20%+35% (v+v+v)
- **At 40.01 min:** Eluent B = 100% (v)
- **At 50 min:** Eluent B = 100% (v)

- **At 50.01 min:** Eluent (A+B+C) = 60%+20%+20% (v+v+v)
- **At 55 min:** Eluent (A+B+C) = 60%+20%+20% (v+v+v)

4.6.3.5 Column temperature: 25 °C

4.6.3.6 Flow rate: 1.5 ml min^{-1}

4.6.3.7 Injection volume: 20 µl

4.6.3.8 Detector wavelength: 280 nm

4.6.3.9 Calibration solutions

Inject 20 µl of the calibration solutions (4.4.3, 4.4.4, 4.4.5, 4.4.6, 4.4.7) twice each, identify and integrate Diclazuril and bis-Diclazuril peaks, and draw the calibration curve based on ratio of the mean peak area of Diclazuril to the mean peak area of internal standard versus Diclazuril concentration in calibration solution (in milligrams per millilitre).

4.6.3.10 Sample solution

Inject 20 µl of the unknown sample solution of each test portion (4.6.2.1) once, identify and integrate Diclazuril and bis-Diclazuril peaks; determine the peak area of Diclazuril and the peak area of the internal standard from each replicate of the same sample.

4.7 Calculation of the results

The Diclazuril content ***w*** (mg kg^{-1}) in each replicate is given by the following formula:

$$\boldsymbol{w} = \frac{\frac{\boldsymbol{Area(d,s)}}{\boldsymbol{Area(i,s)}} - \boldsymbol{b}}{\boldsymbol{a}} \times \frac{\boldsymbol{10000V}}{\boldsymbol{m}} \qquad \text{eq. [3]}$$

where:

Area(d,s) is the peak area of Diclazuril in the unknown sample solution replicate (4.6.2.1, 4.6.3.10)

Area(i,s) is the peak area of the internal standard in the unknown sample solution replicate (4.6.2.1, 4.6.3.10)

b is intercept of calibration curve derived from analysis of calibration solutions (4.4.3, 4.4.4, 4.4.5, 4.4.6, 4.4.7)

a is slope of calibration curve derived from analysis of calibration solutions (4.4.3, 4.4.4, 4.4.5, 4.4.6, 4.4.7)

m is weight of the test portion in grams

V is final volume in millilitres of the sample extract after re-dissolving according to 4.6.2.1 (i.e. 2.5 ml)

The final Diclazuril content ***W*** (mg kg^{-1}) in the sample is the average of the two contents ***w*** (mg kg^{-1}) determined for the two replicates of the same sample.

4.8 Normative references

The following referenced documents are indispensable for the application of this protocol. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

- COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed
- ISO 6498, Animal feeding stuffs – Preparation of test samples

Annex 3 : Electronic reporting forms

This information will assist you in reporting your results

This excel workbook contains four worksheets and should be sent back when filled in.

In all worksheets, the accessible areas for writing are the dedicated empty **couloured areas**.

In the worksheet "**Method questionnaire**", enter the information related to the method used for the identification and quantification of diclazuril. The protocol of the method used should be the one provided by the EURL-FA Control in the frame of this study. However, if you modified one or the other parameter in the protocol provided, please **indicate** it in the "comments" text box as well as any additional information you wish to provide. Please be as precise and accurate as possible.

"**Calibration"** form: please fill in the results from each of the 5 unknown samples sent to your laboratory. Remember that only the empty **coloured areas** of the sheets are reserved for inserting results and other requested information.

"**Report**" form: please fill in the information and results requested from each of the 5 calibration solutions. Remember that only the empty **coloured areas** of the sheets are reserved for inserting results and other requested information.
Report the result obtained for the concentration for each sample and each replicate, with 3 decimals, in **mg kg^{-1}**.

"**Results to be faxed or mailed**" sheet: this sheet is automatically filled in and not accessible for writing. Please check that all results you reported are correctly transferred; contact the EURL-FA Control if this would not be the case. When your reporting is complete, print out the form, date and sign, and return to the EURL-FA Control as indicated.

Please note:
- use the "." as **decimal point** (and not the ",");
- if you detect diclazuril but at a concentration lower than LOQ, then report the concentration as <**LOQ**;
- if you detect diclazuril but at a concentration lower than LOD or if you do not detect diclazuril, then report the concentration as< **LOD**". **Do not leave any result cell as blank**.

Results should be reported together with their related uncertainty. Report the **expanded** uncertainty for **each** result. The expanded uncertainty is obtained by multiplying the combined standard uncertainty by a coverage factor *k;* please **report** the expanded uncertainty with **k = 2**, to give a level of confidence of 95%. Please specify in the comments text box ("Method questionnaire" sheet) how the reported uncertainty was calculated. **Do not leave any blank**.

Reporting the results

The analysis should be conducted within 2 months from the date of reception of the samples. The **deadline** for reporting the results is **29 May 2015**.

Please modify the name of the file by inserting the labcode. For instance: For laboratory with the labcode L04 the filename should be:

L04_DICL_study_2015_Reporting_sheet.xlm

What to report to the EURL-FA Control:
- the complete current file
- the signed and dated "**results to be faxed or emailed**" sheet

How to report to the EURL-FA control:
- by e-mail to jrc-irmm-eurl-feed-additives-control@ec.europa.e

In addition, please send to us the chromatograms from all analyses either by e-mail or by normal mail.

The information collected will be kept confidential and only used for the evaluation of results and discussion with the related laboratory if necessary/relevant.

Thanks and kind regards,

Dr Ursula Vincent

Observed concentrations

Results reporting form for the determination of diclazuril in compound poultry feed

Name of laboratory:	
Lab code:	

	Sample Code	Mean concentration of 2 replicates in mg kg^{-1}	expanded uncertainty (k=2) in mg kg^{-1}
Sample 1			
Sample 2			
Sample 3			
Sample 4			
Sample 5			

Calibration form:

Diclazuril stock solution

Weighed mass of diclazuril pure standard (mg)	0.0
Purity of diclazuril pure standard (%)	0.0
Exact concentration of the diclazuril stock solution (mg mL^{-1})	**0.00**

Calibration

Lab code:

	EXACT volume pipetted from the diclazuril intermediate solution (ml)	EXACT concentration of the calibration point (mg mL^{-1})	Measured Area of diclazuril	Measured Area of bis-diclazuril (I.S.)	Measured Area of diclazuril	Measured Area of bis-diclazuril (I.S.)
			injection 1	injection 1	injection 2	injection 2
HPLC standard 1 c.a. 0.001 mg ml^{-1}						
HPLC standard 2 c.a. 0.002 mg ml^{-1}						
HPLC standard 3 c.a. 0.003 mg ml^{-1}						
HPLC standard 4 c.a. 0.004 mg ml^{-1}						
HPLC standard 5 c.a. 0.005 mg ml^{-1}						
CALIBRATION SLOPE						
Y INTERCEPT						
determination coefficient R^2						

Please build up the calibration curve with the averaged areas over the 2 injections as described in 4.6.3.9 of the SOP provided by the EURL-FA Control

Please report the determination coefficient (R^2) **NOT** the correlation coefficient (r)

The final Diclazuril content W (mg kg^{-1}) in the sample is the average of the two contents w (mg kg^{-1}) determined for the two replicates of the same sample.

Regression mathematics model

Y=Ax+B	(Y/N)	
Y=Ax	(Y/N)	

Signed reported results

Print this area for reporting the results

Date, sign and return to EURL-FA Control

by fax:+32 14 571 787

by email: jrc-irmm-eurl-feed-additives-control@ec.europa.eu

Method Validation study 2015: Diclazuril in poultry feed

Laboratory name:

Laboratory code:

	Sample Code	Mean concentration (2 replicates) mg kg^{-1}	expanded uncertainty (k=2) mg kg^{-1}
Sample 1	0	0.000	0.000
Sample 2	0	0.000	0.000
Sample 3	0	0.000	0.000
Sample 4	0	0.000	0.000
Sample 5	0	0.000	0.000

---------- ----------

Signature (responsible of analysis): Date

Annex 4 : **Method questionnaire form**

Questionnaire to the participants

General information on the laboratory and the method used

Laboratory name		
Laboratory code		You will find the labcode on the document accompanying the shipment of the samples
Quality programme for the laboratory		Example: ISO 17025 accredited or certifications
Is your lab accredited in feed field? (Y/N)		
	From	To
Period during which the analysis was conducted (dd/mm/yyyy - dd/mm/yyyy)		
I confirm that I have read the information in the worksheet "Important information"	☐	

Specific information on the method used

Sample preparation

Extraction procedure

Apparatus type (Trademark)		Example: head-over-head (Roto-Shake Genie)

	Sample Code	Weighed mass (g)	
Sample 1, replicate 1 weighed mass (in g)			*e.g. 50.05*
Sample 1, replicate 2 weighed mass (in g)			*e.g. 49.10*
Sample 2, replicate 1 weighed mass (in g)			*e.g. 50.07*
Sample 2, replicate 2 weighed mass (in g)			*e.g. 48.56*
Sample 3, replicate 1 weighed mass (in g)			*e.g. 50.10*
Sample 3, replicate 2 weighed mass (in g)			*e.g. 49.90*
Sample 4, replicate 1 weighed mass (in g)			*e.g. 50.11*
Sample 4, replicate 2 weigted mass (in g)			*e.g. 48.80*
Sample 5, replicate 1 weighed mass (in g)			*e.g. 50.12*
Sample 5, replicate 2 weighed mass (in g)			*e.g. 50.14*
Extraction duration (h)			e.g. 8.0 h
Membrane Filter size, type and trademark			

Clean-up Procedure

SPE cartridge

C phase	
Size cc	
Sorbent weight mg	
Trademark and reference	

LC-chromatography

	HPLC column	Guard column	
Column phase			e.g. C18
Length (mm)			e.g 100, 10
Internal diameter (mm)			e.g. 4.6 or 4.0
Particule diameter (µm)			e.g. 3
Trademark, exact commercial name			e.g. Hypersil ODS

Injection volume (µl)		e.g. 20
Flow rate (ml min^{-1})		e.g. 1.5
Column temperature (°C)		e.g. 25
Average Diclazuril retention time (min)		e.g. 29

Detector

Instrument type		
Supplier		Example: Shimadzu
Detection wavelenght (nm)		**e.g. 280**

Standards

Diclazuril pure standard weighed mass (mg)		e.g. 25.2
Purity of the diclazuril pure standard (%)		e.g. 98
I used the bis-diclazuril standard solution provided by the EURL-FA Control (Y/N)		e.g. Y

Quality control - interferences check

Did you analyse a blank feed as recommended in 4.6.1.1 of the SOP provided by the EURL-FA Control? (Y/N)		e.g. Y

Space for comments or description of deviation(s) from the method description (even **slight** modifications): *indicate any deviation to the protocol received (e.g. different column, different sample reparation, etc.); additional information?*

Annex 5 : Homogeneity of the materials

10 bags of each test item were randomly selected (except for the blank material). Two aliquots from each bag were extracted and further analysed in duplicate by LC-MS/MS according to the multi-analyte method implemented at the EURL-FA Control. The precision of the EURL-FA Control analytical method has been demonstrated to be suitable for the assessment of the homogeneity of the materials. The obtained concentrations were corrected for purity. The mean concentration values of the sub-sample duplicates were subjected to analysis of variance and the results evaluated according to the ISO 13528 and to the IUPAC protocol.

The results obtained for each of the test items showed that the homogeneity of the test items was sufficient to proceed with the inter-comparison exercise.

MAT 1

	variances		
	0.0012	s_x =	0.034
MSW =	0.0002	$s_{an}=s_u$ =	0.016
s^2_{sam} =	0.0010	s_r =	0.032
		s_r =	0.032

m =	10
mean =	0.889

1

16.1%	= σ-trg(%)
0.143	= σ-trg
0.043	= 0,3* s-trg

Homogeneity Tests

1) Cochran test 0.6768 $C=D_{max}^2/SDD$

outlier	no outlier	
0.6020	0.7175	= Crit
@ 95%	@ 99%	

2) ISO-13528 Ss < 0,3*strg => passed

3) IUPAC 0.001 0.00 = Crit = F1*(0,3*s)²+F2*MSW

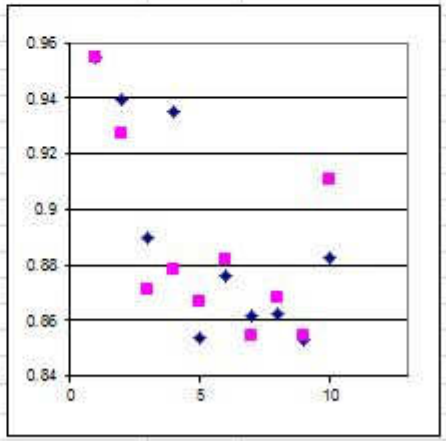
Ss2 < Crit => passed

2

Bottle	Result_a	Result_b	diff	sum	avg
1	0.954796519	0.954383834	0.000412685	1.909180353	0.954590177
2	0.939842891	0.927588986	0.012253905	1.867431877	0.933715939
3	0.889879921	0.87105314	0.018826781	1.760933061	0.88046653
4	0.935583479	0.878070963	0.057512516	1.813654441	0.906827221
5	0.854007207	0.866773881	-0.012766674	1.720781088	0.860390544
6	0.876047802	0.881874674	-0.005826872	1.757922476	0.878961238
minimum 7	0.861720243	0.854388101	0.007332142	1.716108344	0.858054172
8	0.86215724	0.868590435	-0.006433194	1.730747675	0.865373837
9	0.85342148	0.854487887	-0.001066406	1.707909367	0.853954684
10	0.882799149	0.910759264	-0.027960115	1.793558413	0.896779206
11					
12					

SDD=∑(diff)²= 0.004887458

MSB = var(sum)/2 = 0.0023



IUPAC

m	Tab1 Crit-95%	Cochran Crit-99%
3	0.9669	0.9933
4	0.9065	0.9676
5	0.8412	0.9279
6	0.7808	0.8828
7	0.7271	0.8376
8	0.6789	0.7945
9	0.6385	0.7544
10	0.6020	0.7175
11	0.5700	0.684
12	0.5410	0.6528

Tab2

m	F1	F2
3	2.996	4.276
4	2.605	2.796
5	2.372	2.096
6	2.214	1.694
7	2.099	1.433
8	2.010	1.250
9	1.938	1.115
10	1.880	1.010
11	1.831	0.927
12	1.789	0.859

MAT 2

	variances		
	0.0019	s_x =	0.044
MSW =	0.0008	s_{an}=s_u =	0.028
s^2_{rem} =	0.0015	s_r =	0.039
		s_r =	0.039

m = 10
mean = 1.445

1

14.9%	= σ-trg(%)
0.216	= σ-trg
0.065	= 0,3* s-trg

Homogeneity Tests

1) Cochran test 0.3074 $C=D_{max}^2/ISOD$

no outlier	no outlier	
0.6020	0.7175	= Crit
@ 95%	@ 99%	

2) ISO-13528 Ss < 0,3*strg => passed

3) IUPAC 0.002 0.01 = Crit = F1*(0,3*s)²+F2*MSW

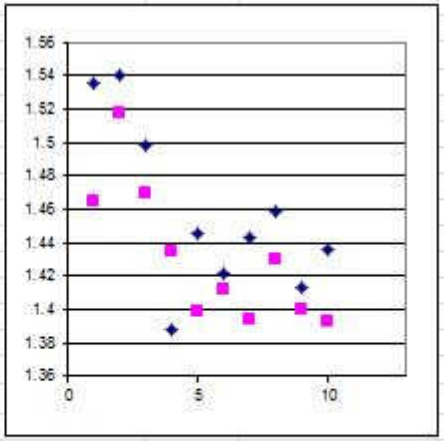
Ss2 < Crit => passed

Bottle	Result_a	Result_b	diff	sum	avg
1	1.535705108	1.46510112	0.070603988	3.000806228	1.500403114
2	1.540960411	1.516996292	0.023964119	3.057956703	1.528978351
3	1.498806867	1.469873158	0.028933709	2.968680025	1.484340013
4	1.387339178	1.434841801	-0.047502623	2.82218098	1.41109049
5	1.445440166	1.399127427	0.046312739	2.844567593	1.422283796
6	1.42135583	1.411559957	0.009795874	2.832915787	1.416457894
minimum 7	1.443318441	1.39380331	0.049515131	2.837121751	1.418560876
8	1.459056045	1.430302825	0.028753219	2.88935887	1.444679435
9	1.413219224	1.400252262	0.012966962	2.813471485	1.406735743
10	1.436344532	1.393009752	0.04333478	2.829354284	1.414677142
11					
12					

2

SDD=∑(diff)²= 0.016218231

MSB = var(sum)/2 = 0.0038



IUPAC

Tab1 Cochran

m	Crit-95%	Crit-99%
3	0.9669	0.9933
4	0.9065	0.9676
5	0.8412	0.9279
6	0.7808	0.8828
7	0.7271	0.8376
8	0.6789	0.7945
9	0.6385	0.7544
10	0.6020	0.7175
11	0.5700	0.684
12	0.5410	0.6528

Tab2

m	F1	F2
3	2.996	4.276
4	2.605	2.796
5	2.372	2.096
6	2.214	1.694
7	2.099	1.433
8	2.010	1.250
9	1.938	1.115
10	1.880	1.010
11	1.831	0.927
12	1.789	0.859

Annex 6 : Accompanying letter and receipt form

18 May 2016

Collaborative study 2015

Determination of diclazuril at additive level in compound poultry feed

INSTRUCTIONS

The materials you received should be analysed utilizing strictly the accompanying protocol for analysis.
Please check the content of the package, fill in and send back the 'List of Contents of the Package' form as specified below.

All materials have been grinded and homogenised; no further pre-treatment is necessary before the analysis. The samples have to be left at room temperature for 1 h before the start of the analysis.
Analyse all materials for the presence and the content of diclazuril. The content should be reported as the concentration of the detected diclazuril in **mg kg^{-1}** of feed given with 3 decimals. This concentration is the mean value obtained for the measurement of 2 aliquots of the same sample bag. When present, the concentration of diclazuril is close to the authorized concentration as feed additive as defined in the Regulation (EC) No 1831/2003.
Instructions on how to report your results and information on the method used will be sent to you in due time in the format of a digital form via an electronic message.

Please note that the ultimate deadline for reporting the results is **29 May 2015**.

Please remember that the major objective of this study is to verify a modified standard operational procedure of the current Community Method (Regulation (EC) 152/2009) for the determination of diclazuril in feed. Depending on the outcome of the exercise, a modification of the current Community method may be formally requested to the European Commission.

Major deviation as regards the non-respect of the protocol, the deadline and/or the specified units for reporting will lead to the exclusion of your results from the statistical evaluation.

Latest in Autumn 2015, a report including the statistical evaluation of all valid participant results will be issued and distributed to all reporting participants. The report will be confidential. The list of participating laboratories will be included but any result will be strictly linked only to the laboratory code. Each participant will be able to retrieve its results using its individual confidential laboratory code communicated on the list of contents of the package form included in this letter.

If you have any question, please contact the EURL-FA (Control), jrc-irmm-eurl-feed-additives-control@ec.europa.eu.

Kind regards,

Dr Ursula VINCENT (Collaborative study Coordinator)
On behalf of the EURL for Feed Additives

LIST OF CONTENTS OF THE PACKAGE[1]

ACKNOWLEDGEMENT OF RECEPTION

Dear «TITLE» «FirstNAME»«SURNAME»,

please find below the list of contents for the collaborative study related to the determination of diclazuril at additive level, organised by the EURL-Feed Additives (Control) with the aim of assessing a revision of the Community method.
Please check that the sample codes of the samples you received correspond to those declared on this list. The samples should **be stored at -18°C / -20°C** upon reception and until one hour before the analysis.

Your laboratory code is given below. This code will be applied to your laboratory for the whole exercise.

Your laboratory code is: **«Lab_code»**

Number of samples in the package: 4

Sample code	Present Y/N
«SampleCode»	
«SampleCode»	
«SampleCode»	
«SampleCode»	
«SampleCode»	
IS diclazuril	

Additionally you receive a 3 ml solution (0.5 mg ml^{-1}) of internal standard for diclazuril.

Samples received on (dd/mm/yyyy) :
Content checked on (dd/mm/yyyy) :
Comments (if applicable) :

Date: Signature:

[1] Form to fill in, sign and send back to the EURL-Feed additives (control) by electronic mail (jrc-irmm-eurl-feed-additives-control@ec.europa.eu) or by fax (+32 14 571 787)

Annex 7 : Additional information extracted from the questionnaire and the analysis of the chromatograms

Participant	Have you introduced any modification to the protocol?
L01	Filtration (0.2µm) of final extract; chromatographic column Hypersil C18 100mm, 4.6mm, 5µm; column temperature of 20 C; and 1 ml/min flow rate were used.
L02	Chromatographic column ACE 5C 18-HL 150 mm, 4.6 mm, 5µm; column temperature of 30 C; Mobile phase composition - A: (2.5g ammonium acetate + 1,7g TBHS) in 500 ml water + 250 ml of acetonitrile, B: Acetonitrile, C: MeOH; Chromatographic programme - 0 min: A+ B+ C = 75% + 0% + 25%, 20 min: A+ B + C = 32% + 30% + 38%, 21 min: A + B + C = 0% + 50% + 50%, 23.1 min: A + B + C = 0% + 50% + 50%, 24 min: A + B + C = 75% + 0% + 25%, 30min: A+B+C = 75%+ 0% + 25%; and 1.2 ml/min flow rate were used.
L03	Chromatographic column Phenomenex/Kinetex C18 150, 4.6 mm, 5µm; chromatographic programme - 0 min: A + B + C = 60% + 20% + 20%, 10 min: A + B + C = 60% + 20% + 20%, 50 min: A + B + C = 45% + 20% + 35%; and stock solution of Diclazuril at 40 mg/50 ml DMFA were used.
L04	Filtration (0.2µm) of final extract; 10 µl injection volume; Chromatographic column Phenomenex C18, 50 mm, 3.2 mm, 3 µm; 0.5 ml/min flow rate; and gravimetric preparation of stock standard solution were used.
L05	5 g of sample; 25 ml of extraction solvent; 2h extraction time; 5 ml of extract + 5 ml of water for SPE clean-up; mass of SPE sorbent - 500 mg; none washing step of the SPE (note: the results were presented with the washing step excluded); column Hypersil C18, 100 mm, 4.6mm, 5µm; mobile phase composition and programme - C: water 65% + acetonitrile 35% + 0.1 ml phosphoric acid; B: water 10% + acetonitrile 90%; 0.5 ml/min flow rate; and 4 calibration points were used.
L08	Mass of SPE sorbent - 360 mg; injection volume - 50 µl; column Symmetry C18, 150 mm, 3.9 mm, 5 µm; and 1 ml/min flow rate were used.
L09	Filtration before SPE clean-up; chromatographic column Spherisorb ODS, 150 mm, 4.6 mm, 5 µm; and 2.5 ml/min flow rate were used.
L10	Centrifugation and filtration before SPE clean-up; filtration (0.2µm) of final extract; and Chromatographic column Phenomenex/Kinetex C18, 150 mm, 4.6 mm, 5 µm were used.
L11	25 g of sample; Colum MZ aqua perfect C18, 150 mm, 4.6 mm, 5 µm; mobile phase composition: methanol + ammonium acetate = 80% + 20%; isocratic programme and 1ml/min flow rate were used.
L12	Chromatographic column Hypersil C18, 100 mm, 4.0mm, 3µm was used.
L13	None filtration of final extract; column Hypersil C18, 250 mm, 4.0mm, 5µm; ambient column temperature; flow rate at 1ml/min; three calibration points; and quantification based on peak height (not area) were used.
L14	2ml/min flow rate was used.
L15	Filtration (0.2µm) of final extract; and column RP-18e, 100 mm, 4.6mm, N/A µm were used.
L16	None filtration of final extract; column Hypersil C18, 250 mm, 4.6mm, 5µm; ambient column temperature; chromatographic programme - 0 min: A + B + C = 60% + 20% + 20%, 10 min: A + B + C = 60% + 20% + 20%, 40 min: A + B + C = 45% +20%+ 35%, 50 min: B=100%; and stock solution of Diclazuril at 20 mg/50 ml DMFA were used.

Annex 8 : Proposal for the revised method

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. Lower limits of quantification are achievable but this is to be validated by the user.

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 C18 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: (+)-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

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 less than or equal to 4 °C the solution is stable for 1 month[1].

3.8.2. Diclazuril standard solution, 50 µg/ml

Transfer 5,00 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 less than or equal to 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 (methyl diclazuril)

3.9.1. Internal standard stock 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 less than or equal to 4 °C the solution is stable for 1 month.

3.9.2. Internal standard solution, 50 µg/ml

[1] Longer stability (up to 1 year) might be possible but it has to be confirmed by the individual laboratory.

Transfer 5,00 ml of the internal standard stock 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 less than or equal to 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 (4.7), 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 less than or equal to 4 °C the solution is stable for 1 month.

3.10. Calibration solutions

3.10.1. Calibration solution, 1 µg/ml (diclazuril)

Pipette 1,00 ml diclazuril standard solution (3.8.2) and 2.00 ml internal standard solution (3.9.2) into a 50 ml graduated flask. Add 17 ml DMFA (3.6), make up to the mark with water (3.1) and mix. This solution must be prepared freshly before use.

3.10.2. Calibration solution, 2 µg/ml (diclazuril)

Pipette 2,00 ml diclazuril standard solution (3.8.2) and 2,00 ml internal standard solution (3.9.2) into a 50 ml graduated flask. Add 16 ml DMFA (3.6), make up to the mark with water (3.1) and mix. This solution must be prepared freshly before use.

3.10.3. Calibration solution, 3 µg/ml (diclazuril)

Pipette 3,00 ml diclazuril standard solution (3.8.2) and 2,00 ml internal standard solution (3.9.2) into a 50 ml graduated flask. Add 15 ml DMFA (3.6), make up to the mark with water (3.1) and mix. This solution must be prepared freshly before use.

3.10.4. Calibration solution, 4 µg/ml (diclazuril)

Pipette 4,00 ml diclazuril standard solution (3.8.2) and 2,00 ml internal

standard solution (3.9.2) into a 50 ml graduated flask. Add 14 ml DMFA (3.6), make up to the mark with water (3.1) and mix. This solution must be prepared freshly before use.

3.10.5. Calibration solution, 5 µg/ml (diclazuril)

Pipette 5,00 ml diclazuril standard solution (3.8.2) and 2,00 ml internal standard solution (3.9.2) into a 50 ml graduated flask. Add 13 ml DMFA (3.6), make up to the mark with water (3.1) and mix. This solution must be prepared freshly before use.

NOTE: the calibration solutions (3.10.1, 3.10.2, 3.10.3, 3.10.4 and 3.10.5) are covering diclazuril concentration in feed ranging from 0,5 to 2,5 mg/kg when using the current protocol.

3.11. C_{18} solid phase extraction cartridge, e.g. Mega Bond Elut, size: 20 cc, sorbent weight: 5 000 mg (pre-conditioning following the supplier guidelines).

3.12. Extraction solvent: acidified methanol.
Pipette 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 (e.g. chemically resistant Nylon), 0,45 µm

4.5. Disposable syringe, 5 ml

4.6. Vacuum manifold

4.7. 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 1,00 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 (3.1). Transfer this solution on an extraction cartridge (3.11), and pass through by applying vacuum (4.6). Wash the cartridge with 25 ml of a mixture of extraction solvent (3.12) and water (3.1), 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 °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) mounted on a disposable syringe (4.5). 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 1,00 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 °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 or better 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);
  - then 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 (3.10.2), containing 2 µg/ml of diclazuril and of internal standard, until constant peak heights and retention times are achieved.

5.3.2. Chromatographic analysis of calibration solutions

Inject 20 µl of the calibration solutions (3.10.1, 3.10.2, 3.10.3, 3.10.4 and 3.10.5) twice each, identify and integrate the diclazuril and internal standard peaks, and draw the calibration curve based on the ratio of the mean peak height or area of diclazuril to the mean peak height or area of internal standard versus diclazuril concentration in the calibration solutions (µg/ml).

5.3.3. Chromatographic analysis of sample solutions

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

6. **Calculation of the results**

6.1. *Feed*

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

$$w = \frac{\frac{Height(d,s)}{Height(i,s)} - b}{a} \times \frac{10V}{m} \qquad \text{or} \qquad w = \frac{\frac{Area(d,s)}{Area(i,s)} - b}{a} \times \frac{10V}{m}$$

where:

Height(d,s) is the peak height of diclazuril in the unknown sample solution (5.2.1)

Area(d,s) is the peak area of diclazuril in the unknown sample solution (5.2.1)

Height(i,s) is the peak height of the internal standard in the unknown sample solution (5.2.1)

Area(i,s) is the peak area of the internal standard in the unknown sample solution (5.2.1)

b is the intercept of the calibration curve plotted from the calibration solutions (3.10.1, 3.10.2, 3.10.3, 3.10.4 and 3.10.5) according to 5.3.2

a is the slope of the calibration curve plotted from the calibration solutions (3.10.1, 3.10.2, 3.10.3, 3.10.4 and 3.10.5) according to 5.3.2

m is the mass of the test portion in grams

V is the final volume in millilitres of the sample extract after re-dissolving 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:

$$w = \frac{\frac{Height(d,s)}{Height(i,s)} - b}{a} \times \frac{0.02V}{m} \times p \qquad \text{or} \qquad w = \frac{\frac{Area(d,s)}{Area(i,s)} - b}{a} \times \frac{0.02V}{m} \times p$$

where:

Height(d,s) is the peak height of diclazuril in the unknown sample solution (5.2.2)

Area(d,s) is the peak area of diclazuril in the unknown sample solution (5.2.2)

Height(i,s) is the peak height of the internal standard in the unknown sample solution (5.2.2)

Area(i,s) is the peak area of the internal standard in the unknown sample solution (5.2.2)

b is the intercept of the calibration curve plotted from the calibration solutions (3.10.1, 3.10. 2, 3.10.3, 3.10.4 and 3.10.5) according to 5.3.2

a is the slope of the calibration curve plotted from the calibration solutions (3.10.1, 3.10.2, 3.10.3, 3.10.4 and 3.10.5) according to 5.3.2

m is the mass of the test portion in grams

V is the final volume in millilitres of the sample extract after re-

dissolving according to 5.2.2 (i.e. 25 ml)

p is the 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 (3.10.2) 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 (3.10.2). 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 independent measurements carried out on two sub-samples must not exceed:

— 30 % relative, to the higher value for diclazuril contents from 0,5 mg/kg to 2,5 mg/kg,

— 30 % to 15 % relative for diclazuril contents between 2,5 mg/kg and 5 mg/kg respectively,

— 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**

Two collaborative studies were organised. In the first one, carried out by another group in 1994, among the samples analysed were two premixtures. One sample 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 laboratories were instructed to analyse each of the samples once or in duplicate. (More detailed information on the first collaborative study can be found in the *Journal of AOAC International, Volume 77, No 6, 1994, p. 1359-1361*).

In the second collaborative study, three compound feeds for poultry, containing diclazuril at concentrations of 0,9 mg/kg (MAT 1), 1,5 mg/kg (MAT 2) and blank feed (MAT 3) were analysed. Detailed information on

the second study can be found in the JRC Technical report (2016). The results of the two collaborative studies are given in the following table.

	Sample 1 A 100	Sample 2 O 100	Sample 3 MAT 1	Sample4 MAT 2	Sample5 MAT 3
L	11	11	10	9	10
n	19	18	20	18	10
Mean (mg/kg)	100,8	103,5	1,0	1,5	<LOQ
S_r (mg/kg)	5,88	7,64	0,11	0,07	-
CV_r (%)	5,83	7,38	11,2	4,5	-
S_R (mg/kg)	7,59	7,64	0,18	0,21	-
CV_R (%)	7,53	7,38	18,1	14,3	-
Nominal content (mg/ kg)	100	100	0,9	1,5	-
Reference[2]	First study from 1994	First study from 1994	Second study from 2015	Second study from 2015	Second study from 2015

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

LOQ = Limit of quantification

9. General comments

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

At least for the analysis of diclazuril in feed with a high fat content, the analytical method may be substituted by other HPLC based methods, e.g. a high performance liquid chromatography coupled to mass spectrometry (HPLC-MS) based method, provided that the alternative method has equivalent performance characteristics (recovery rate, precision at repeatability and reproducibility conditions).

[2] First study from 1994: Journal of AOAC International, Volume 77, No 6, 1994, p. 1359-1361;
Second study from 2015: JRC Technical report "*Re-validation of a method for the determination of diclazuril by collaborative study*" (2016).

This page is blank.

This page is blank.

LA-NA-27954-EN-N

JRC Mission

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Serving society
Stimulating innovation
Supporting legislation

doi:10.2787/973816

ISBN 978-92-79-58903-4