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CRL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	FAD-2008-0053 FAD-2009-0058 CRL/080038 CRL/100011
Name of Additive:	Clinacox 0.5%
Active Substance(s):	Diclazuril
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA) Geel, Belgium
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EXECUTIVE SUMMARY

Clinacox 0.5% (*Diclazuril*) is a product already authorised as *feed additive* for (1) chickens for fattening, (2) chickens reared for laying for (3) turkeys for fattening and (4) for rabbits under the category "coccidiostats", according to the classification system of Annex I of Regulation (EC) No 1831/2003.

Two applications are evaluated in the present report, namely (1) the authorisation of an existing product (re-evaluation) according to Article 10(2) of Regulation (EC) No 1831/2003 (chicken for fattening, *cf.* FAD 2008-0053) and (2) the authorisation of a new use of a feed additive (Article 4 (1) of Regulation (EC) No 1831/2003) (guinea-fowl, *cf.* FAD 2009-0058).

The active substance of *Clinacox 0.5%* is *diclazuril* and the proposed inclusion level of this compound in complete feedingstuffs is 0.9 mg/kg for the minimum content and 1.1 mg/kg for the maximum content in both applications.

For the determination of *diclazuril* in *premixtures* and *feedingstuffs,* the applicant submitted a method based on the EU official method (Commission Regulation (EC) No 152/2009), using high-performance liquid chromatograph (HPLC) coupled to Ultraviolet detection. The performance characteristics determined by the applicant are in good agreement with those reported by the ring trial validated EU official method. The interlaboratory validation study of the EU official method included two premixtures containing diclazuril at 100 mg/kg and three complete feedingstuffs samples for poultry containing diclazuril at the target level of 1 mg/kg. The precision obtained in this study are listed hereafter:

- a relative standard deviation for *repeatability*, (1) ranging from 5.8 to 7.4 % for premixtures and (2) ranging from 1.9 to 17.3 %for feedingstuffs
- a relative standard deviation for *reproducibilty*, (1) ranging from 7.4 to 7.5 % for premixtures and (2) ranging from 9.7 to 18.6 % for feedingstuffs
- a limit of detection (LOD) of 0.1 mg/kg and a limit of quantification (LOQ) of 0.5 mg/kg

Based on the above mentioned performance characteristics the CRL recommends for official control of *diclazuril* in *premixtures* and *feedingstuffs* the EU official method (Commission Regulation (EC) No 152/2009).

Upon request by the CRL, the applicant applied the EU official method to the determination of *diclazuril* in the *feed additive,* obtaining acceptable values for the recovery rate of about 110 %. Based on these results the CRL recommends the EU official method for the determination of diclazuril in the *feed additive*.

Regarding **residues** of *diclazuril* in edible tissues, the following Maximum Residue Limits (MRLs) have been established for target poultry (chickens for fattening, turkeys for fattening and chickens reared for laying) tissues by Commission Regulation (EC) No 976/2008: (1) 1500 μg/kg in liver, (2) 1000 μg/kg in kidney, (3) 500 μg/kg in skin/fat and (4) 500 μg/kg in muscle. The same MRLs are proposed by the applicant for guinea-fowl tissues.

For the determination of *diclazuril residues* in *tissues,* the applicant submitted an in-house developed method using high-performance liquid chromatograph (HPLC) coupled to mass spectrometry detection (MS/MS). The described method has been in-house validated according to Commission Decision (2002/657/EC) and complies with the confirmatory method requirements stated in the aforementioned Commission Decision.

The relevant performance characteristics of the method determined through the in-house validation for the different chicken tissues (muscle, liver, skin/fat and kidney) demonstrated that the method is suitable for the determination of diclazuril residues at the established MRLs in the target tissues. Furthermore a verification study of the method by a second laboratory has been conducted following the recommended guidance document of the CRL. Based on the obtained results from the validation and verification study the CRL recommends this method for the official control of the above mentioned MRLs.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

KEYWORDS

Diclazuril, coccidiostat, chickens for fattening and guinea-fowls

1. BACKGROUND

Clinacox 0.5% is a product already authorised as *feed additive* for chickens for fattening [1], turkeys for fattening [2] chickens reared for laying [2] and rabbits [3] under the category 'coccidiostats' according to the classification system of Annex I of Regulation (EC) No 1831/2003.

The cream-amber colured material of *Clinacox 0.5 %* contains 0.5 % w/w of the active substance *diclazuril. Diclazuril* is a pure synthetic substance. Other components of the feed additive are protein poor soybean meal, polyvidone K30 and sodium hydroxide [4].

Two applications are evaluated in the present report:

- (1) the authorisation of an existing product according to Article 10 (2) of Regulation (EC) No 1831/2003 [5] (chicken for fattening, *cf.* FAD 2008-0053) and
- (2) the authorisation of a new use of a feed additive (Article 4 (1) of Regulation (EC) No 1831/2003) [6] (guinea-fowl, *cf*. FAD 2009-0058).

The target concentrations of the active substance in complete feedingstuffs range from 0.9 to 1.1 mg/kg (min – max content) for both applications [7] and [8].

The following MRLs have been established for target poultry (chickens for fattening, turkeys for fattening and chickens reared for laying) tissues by Commission Regulation (EC) No 976/2008 [9]: (1) 1500 μg/kg in liver, (2) 1000 μg/kg in kidney, (3) 500 μg/kg in skin/fat and (4) 500 μg/kg in muscle. The same MRLs are proposed by the applicant for guinea-fowl tissues.

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Clinacox 0.5 %* (chicken for fattening,

EFSA-Q-2008-749; guinea-fowl, EFSA-Q-2009-00968), and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, mercury and lead) are available at the respective Community Reference Laboratories (Commission Regulation (EC) No 776/2006).

Description of the analytical methods for the determination of the active substance in feed additive, premixtures and feedingstuffs.

For the determination of *diclazuril* in *feedingstuffs,* the applicant submitted a method [10] based on the EU official method [11], using high-performance liquid chromatograph (HPLC) coupled to Ultraviolet (UV) detection. The applicant introduced only minor modifications in the method protocol compared to the EU official method. The validation study of the applicant's method reveals a standard deviation for *repeatability* (RSD_r) of 2.1 % and a *recovery* rate ranging between 88 and 97% [10].

In the EU official method [11] the sample is extracted with acidified methanol after addition of an internal standard. When analysing feed samples, the extract is purified by using a C18 solid phase extraction cartridge. Diclazuril is eluted from the cartridge with a mixture of acidified methanol and water. Then, the extract is evaporated and the residues are dissolved in a mixture of dimethylformamide and water. Finally the solution is subjected to HPLC/UV detection measuring at 280 nm. When analysing premixtures, the extract is measured with the HPLC/UV system without any clean-up of the extract.

The interlaboratory validation study of the EU official method included two premixtures containing diclazuril at 100 mg/kg and three complete feedingstuffs samples for poultry containing diclazuril at the target level of 1 mg/kg. The precision obtained in this study are listed hereafter:

- a relative standard deviation for *repeatability*, (1) ranging from 5.8 to 7.4 % for premixtures and (2) ranging from 1.9 to 17.3 % for feedingstuffs
- a relative standard deviation for *reproducibilty*, (1) ranging from 7.4 to 7.5 % for premixtures and (2) ranging from 9.7 to 18.6 % for feedingstuffs

– a limit of detection (LOD) of 0.1 mg/kg and a limit of quantification (LOQ) of 0.5 mg/kg

Based on the above mentioned performance characteristics the CRL recommends for official control of *diclazuril* in *premixtures* and *feedingstuffs* within the frame of authorisation and within the concentration range of the target analyte in premixtures covered by the interlaboratory study the EU official method (Commission Regulation (EC) No 152/2009).

Upon request by the CRL, the applicant applied the EU official method to the determination of *diclazuril* in the *feed additive,* obtaining acceptable values for the recovery rate of about 110 %. Based on these results the CRL recommends the EU official method for the determination of diclazuril in the *feed additive* [12] .

Methods of analysis for the determination of the residues of the additive in food.

For the determination of *diclazuril* in target tissues the applicant proposed an in-house developed method [13] which is comprised of three steps, namely (1) extraction with acetonitrile, (2) centrifugation of the combined extracts, followed by the evaporation of an aliquot of the supernatant to dryness and re-dissolution of the dry residue in a mixture of acetonitrile/water and (3) the determination by reversed-phase HPLC coupled to a triple quadrupole mass spectrometer (MS/MS) in electrospray ionisation (ESI) mode using matrix matched standards. The method has been in-house validated on all target matrices (i.e. kidney, muscle, liver and skin/fat) at three concentrations, namely 250, 500 and 1000 µg/kg (muscle); 750, 1500 and 3000 µg/kg (liver); 250, 500 and 1000 µg/kg (skin/fat); 500, 1000 and 2000 µg/kg (kidney).

The most relevant performance characteristics of the method determined by the applicant for the different chicken tissues (muscle, liver, skin/fat and kidney) are detailed in the table below and concerns the precision expressed as *percentage relative standard deviation for repeatability* (RDS_r) and *percentage relative intermediate standard deviation* ($RDS_{intermdediate}$) and trueness expressed as percentage recovery. The CRL considers the validation study conducted on chicken tissue valid for both applications. The obtained values for the precision and the recovery – the latter one calculated by the CRL from the obtained results - were taken from appendix 5a to 6d in the corresponding documentation [13] and are summarised in Table 1:

Table 1: Performance profile of the method for the determination of residues in different matrices expressed as percentage recovery rate, percentage relative standard deviation for repeatability (RSDr) and percentage relative intermediate standard deviation ($RSD_{intermediate}$)

Tissue	Fortified level (µg/kg)	% recovery rate	RSDr (%)	$RSD_{intermediate}$ (%)
Muscle	250	98-108	2.3-5.4	5.3
	500	96-108	1.3-5.0	6.5
	1000	94-103	1.3-4.5	5.4
Liver	750	101-105	4.0-4.9	4.7
	1500	102-104	2.9-3.3	3.1
	3000	103-105	2.4-4.5	3.6
Skin/fat	250	103-104	2.4-3.4	3.7
	500	102-107	2.3-3.9	5.0
	1000	98-106	2.1-2.6	5.0
Kidney	500	96-105	6.1-8.8	7.8
	1000	95-100	5.5-7.3	6.5
	2000	99-105	2.7-5.1	4.7

The validation [13] has been conducted according to Commission Decision 2002/657/EC and complies with the confirmatory method requirements stated in this Commission Decision:

- number of identification points
- tolerance limit for the relative retention time between the samples and the calibrants
- relative intensities of the detected ions
- specificity requirements
- precision and recovery determined through spiked samples at the MRL, below and above the MRL
- Fulfilling the criteria established for
    - Trueness: for all concentrations included in the current study the obtained values for the percentage recovery need to be above 80 % and below 110%.

- Precision: All values for the relative standard deviation for within-laboratory reproducibility need to be equal or below the corresponding value calculated by the Horwitz equation

The evaluation of the results from the validation study confirmed compliance with these criteria.

Furthermore, the method performance characteristics obtained in the applicant's laboratory have been verified by a second laboratory, which performed experiments using the applicant's protocol on all matrices concerned. The experiments have been conducted according to the CRL-FA verification guidance and the results confirmed successful transferability of the method [14].

Based on the results of validation and verification study the CRL considers this method suitable to be used within the frame of official control to enforce the MRLs for diclazuril in target matrices.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

The CRL recommends for official control the EU official method for the determination of *diclazuril* in *feed additive*, *premixtures* and *feedingstuffs*.

For the determination of *diclazuril* in poultry *tissues,* the CRL recommends for official control the method proposed by the applicant. This method has been validated by the Animal and Human Health department of the CER group (Belgium) according to Commission Decision 2002/657/EC.

Recommended text for the register entry (analytical method)

For the determination of *diclazuril* in *feed additive*, *premixtures* and *feedingstuffs*:

- reversed-phase high performance liquid chromatography (HPLC) using Ultraviolet detection at 280 nm - EU official method (Regulation (EC) No 152/2009.

For the determination of *diclazuril* in poultry *tissues*:

- HPLC coupled to triple quadrupole mass spectrometer (MS/MS) using one precursor ion and two diagnostic product ions.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Clinacox* have been sent to the Community Reference Laboratory for Feed Additives.

The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Commission Regulation (EC) No 2430/1999 of 16 November 1999, linking the authorisation of certain additives belonging to the group of coccidiostats and other medicinal substances in feedingstuffs to persons responsible for putting them into circulation

[2] List of the authorised additives in feedingstuffs (1) published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs (2004/C 50/01)

[3] Commission Regulation (EC) No 971/2008 of 3 October 2008 concerning a new use of a coccidiostat as additive in feedingstuffs

[4] * Technical dossier, Section II – 2.. Characterisation of the active substance

[5] * Application, Reference SANCO/D/2 Forw. Appl. 1831/036-2008 (FAD 2008-53)

[6] * Application, Reference SANCO/D/2 Forw. Appl. 1831/036-2009 (FAD 2009-58)

[7] * Application, Proposal for Register Entry (chickens for fattening)

[8] * Application, Proposal for Register Entry Annex A (guinea-fowl)

[9] Commission Regulation (EC) No 976/2008 of 6 October 2008, amending Regulations (EC) No 2430/1999, (EC) No 418/2001 and (EC) No 162/2003 as regards the terms of the authorisation of the feed additive "Clinacox", belonging to the group of coccidiostats and other medicinal substances.

[10] *Annex II 10.pdf of section II

[11] Commission Regulation (EC) No 152/2009 of 29 January 2009 laying down the methods for sampling and analysis for the official control in feed.

[12] * CRL Responses - Appendix 1 "Determination of *diclazuril* in Clinacox 0.5% oral premix and premixtures containing Clinacox 0.5% oral premix based on the Community method", supplementary provided by the applicant on request of the CRL

[13] * CRL Responses - "Validation of an analytical method for the determination of *diclazuril (Clinacox)* in chicken tissues (muscle, liver, kidney and skin/fat)

[14] * CRL Responses - CRL-FA verification forms for chicken muscle, liver, kidney and skin/fat

*Refers to Dossier No. FAD-2008-0053 and FAD 2009-0058

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SLO).
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- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (D)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
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