EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

JRC.DDG.D.6/CvH/RMO/Mdr/ARES(2010)130448

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

Dossier related to:	FAD-2008-0051 FAD-2008-0054 CRL/080029
Name of Additive:	Cygro 10G
Active Substance(s):	Maduramicin ammonium alpha
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA) Geel, Belgium
Report prepared by:	Roberto MOLTENI and Christoph von Holst (CRL-FA)
Report revised by: Date:	Piotr ROBOUCH (CRL-FA) 08/03/2010
Report approved by: Date:	Christoph von HOLST (CRL-FA) 08/03/2010

EXECUTIVE SUMMARY

Cygro (*Maduramicin ammonium alpha*) is a product already authorised as *feed additive* for chickens for fattening, and for turkeys, under the category 'coccidiostats', according to the classification system of Annex I of Regulation (EC) No 1831/2003.

Cygro consists of the active ingredient, *maduramicin ammonium alpha* and the proposed inclusion level of this compound in complete feedingstuffs, range from 5 to 7 mg/kg (min – max content) for chickens for fattening, while is set to 5 mg/kg for turkeys (up to 16 weeks of age).

Two applications are evaluated in the present report: - the re-evaluation of authorisation according to Art. 10(2) of Regulation (EC) No 1831/2003 (chickens for fattening, cf. FAD-2008-0051); - the modification of authorisation according to Art. 13(3) of the above mentioned regulation (turkeys, up to16 weeks of age, cf. FAD-2008-0054), proposing a changing of the carrier of the additive.

For the determination of *maduramicin ammonium alpha* in *premixtures* and *feedingstuffs*, the applicant submitted an in-house validated method, based on the same principle of the standard method EN 15781:2009. The performance characteristics determined by the applicant are in good agreement with those reported by the ring trial validated standard method listed hereafter (for chickens for fattening and turkeys, in *premixtures* and *feedingstuffs*):

- a relative standard deviation for *repeatability* of 3 % for premixtures and ranging from 3 and 8 % for *feedingstuffs*;
- a relative standard deviation for *reproducibility* of 11 % and 16 %, for *premixtures* and *feedingstuffs*, respectively;
- a *recovery* rate ranging from 90 to 110 %; and
- a limit of detection (LOD) and a limit of quantification (LOQ) of 0.5 and 2 mg/kg *feedingstuffs*, respectively.

The target concentrations of *maduramicin ammonium alpha* in the different *feedingstuffs* applied in this ring trial varied from 2.5 to 9.5 mg/kg, thus including the target concentrations of the applications evaluated in this report. The target concentration of *maduramicin ammonium alpha* in the *premixture* was 450 mg/kg.

Based on the satisfactory agreement of performance characteristics reported by the applicant and the CEN method, the CRL recommends for official control the standard method EN 15781:2009, for the determination of *maduramicin ammonium alpha* in *premixtures* and *feedingstuffs*.

Upon request by the CRL, the applicant provided - for the determination of the *maduramicin ammonium alpha* in the *feed additive* - experimental evidence showing that the above mentioned standard method applies also to the *feed additive* matrix. From the experimental data reported, the CRL evaluated the following performance characteristics: - a relative standard deviation for *repeatability* of 2.3 %; - a relative standard deviation for *intermediate precision* of 2.8 %. Furthermore, a recovery rate of 108 % was reported by the applicant.

Based on the above mentioned performance characteristics, the CRL recommends for official control, the standard method EN 15781:2009, for the determination of *maduramicin ammonium alpha* in *feed additive*.

Regarding **residues** of *maduramicin* in tissue, the applicant proposes maximum residues limit (MRL) for different matrices, namely (1) 120 μg/kg in liver, (2) 150 μg/kg in skin/fat, (3) 40 μg/kg in kidney, and (4) 20 μg/kg in muscle.

Based on the evaluation of available analytical methods, the CRL recommends a method developed and validated by the EU Reference Laboratory for Pharmacologically Active Substances (Berlin, Germany). This method utilises liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and allows for the enforcement of MRL – as proposed by the applicant - in muscle and liver. In addition, the method complies with the criteria established by Commission Decision 2002/657/EC[1]. However, validation data of this method are not available for the matrices kidney and skin/fat. Therefore, the CRL is not able to recommend a method for the official control of MRLs in kidney and fat.

Further testing or validation is not considered necessary.

KEYWORDS

Maduramicin ammonium alpha, coccidiostats, chickens for fattening, turkeys

[1] Maduramicin belongs to group B of Annex I of Council Directive 96/23/EC. Analytical methods for the determination of this substance in the target matrices for official control purposes have to comply with the criteria specified in Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC)

1. BACKGROUND

Cygro (*Maduramicin ammonium alpha*) is a product already authorised as *feed additive* for chickens for fattening [1], and for turkeys [2], under the category 'coccidiostats', according to the classification system of Annex I of Regulation (EC) No 1831/2003.

Cygro consists of the active ingredient, *maduramicin ammonium alpha* at concentration of 1% w/w, produced by a fermentation process by the strain *Actinomadura yumaensis* (NRRL 12515*).* Strain NRRL 12515 has been deposited with the Agricultural Research Service Culture collection, Northern Regional Research Center, Peoria, Illinos - 61604 USA [3].

Two applications are evaluated in the present report:

- the re-evaluation of authorisation according to Art. 10(2) of Regulation (EC) No 1831/2003, when using *Cygro* for chickens for fattening [4] (cf. FAD-2008-0051);

- the modification of authorisation according to Art. 13(3) of the above mentioned regulation, when using *Cygro* for turkeys (up to16 weeks of age) [5] (cf. FAD-2008-0054). In particular, the composition of the additive is proposed to be changed from a formulation containing corn cob grits as carrier to a formulation containing calcium sulphate dehydrate as carrier.

The target concentration of the active substance in complete feedingstuffs, range from 5 to 7 mg/kg (min – max content) for chickens for fattening [6], while is set to 5 mg/kg for turkeys (up to 16 weeks of age) [7].

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 *Cygro* (chickens for fattening, FAD-2008-0051; turkeys, FAD-2008-0054), 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 *maduramicin ammonium alpha* in *premixtures* and *feedingstuffs*, the applicant submitted an in-house validated method [8, 9], based on the same principle of the standard method EN 15781:2009 [10]. Minor modifications were implemented by the applicant in the operating procedure, related to sample amount and chromatographic conditions [8, 9]. *Maduramicin ammonium alpha* is extracted from the sample into acetonitrile and determined by reversed-phase high performance liquid chromatography (HPLC) using post-column derivatization with benzaldehyde, and detection at 585 nm. The performance characteristics are listed in Tab. 1. The applicant's method was linear from approximately 0.5 to 10 mg/L.

In the standard method EN 15781:2009, *maduramicin ammonium alpha* is extracted with methanol and determined by reversed-phase HPLC using post-column derivatization with vanillin and detection at 520 nm. This method was ring-trial validated and the performance characteristics are listed in Tab. 1.

The target concentrations of *maduramicin ammonium alpha* in the different *feedingstuffs* applied in this ring trial [10] varied from 2.5 to 9.5 mg/kg, thus including the target concentrations of the applications evaluated in this report. The target concentration of *maduramicin ammonium alpha* in the *premixture* was 450 mg/kg.

Based on the satisfactory agreement of performance characteristics reported by the applicant and the standard method (Tab. 1.), the CRL recommends for official control the EN 15781:2009 method [10], for the determination of *maduramicin ammonium alpha* in *premixtures* and *feedingstuffs*.

Upon request of the CRL, the applicant applied the EN 15781:2009 method on the *feed additive per se* and provided supplementary information [11]. Only minor experimental modifications were applied (i.e ultrasonication performed in a bath fill with ice water, and strict cleaning procedure were adapted). From the experimental data reported [11], the CRL evaluated the performance characteristics presented in Tab. 1.

Tab.1. Performance characteristics of analysis methods for the determination of *maduramicin ammonium alpha* in *feed additive* (FA), in *premixtures* (PM) and *feedingstuffs* (FS).

	FA	PM		FS	
	Applic.[11]	Applic. [8]	EN [10]	Applic. [9]	EN [10]
RSD_r, %	2.3 *	1.9	3	3.8	3-8
RSD_{ip}, %	2.8 *	—	—	—	—
RSD_R, %	—	—	11	—	16
Recovery, %	108	95	90-110	90 - 98	90-110
LOD, mg/kg	—	—	—	—	0.5
LOQ, mg/kg	—	—	—	—	2

* Evaluated by the CRL

Based on these acceptable performance characteristics - demonstrating the applicability of the standard method to the product - the CRL recommends for official control the standard method EN 15781:2009 for the determination of *maduramicin ammonium alpha* in the *feed additive*.

Further testing or validation is not considered necessary.

Methods of analysis for the determination of the residues of the additive or its metabolites in food.

For the determination of *maduramicin* in various tissues the applicant proposed a method from the scientific literature and which has been previously validated for nine coccidiostats including the target analyte in eggs and muscle [12]. The method is comprised of three steps, namely (1) the extraction with acetonitrile, (2) isolation of *maduramicin* by the means of silica solid phase extraction cartridges and (3) the determination by high performance liquid

chromatography (HPLC) and tandem mass spectrometry (LC-MS/MS). One precursor ion (939.8 m/z) and two products ions (877.5 m/z and 719.2 m/z) have been selected, thus fulfilling the criteria for confirmatory methods established by Commission Decision 2002/657/EC. The applicant performed additional validation experiments indicating that the method also works well when analysing all target tissues at target concentration level. However, essential data supporting this approach are missing, such as the revised version of the method protocol written in ISO 78-2 format taking into account the modifications introduced when conducting these experiments. Therefore, the CRL is not able to judge on the suitability of the method for official control to enforce the proposed MRL in target tissue.

Another method which also utilised LC-MS/MS has been developed and validated by the EU Reference Laboratory for Pharmacologically Active Substances (CRL Berlin) [13]. The method uses one precursor ion (934.8 m/z) and two diagnostic product ions (431.0 and 629.5 m/z), thus fulfilling the required specificity criteria according to Commission Decision 2002/657/EC. The method has been validated on muscle and liver at concentrations ranging from 0.75 to 2.75 μg/kg obtaining acceptable values for the trueness expressed as percentage recovery ranging from 94 and 105 % and for the precision expressed as relative standard deviation for intermediate precision varying between 13 and 24 %. The target concentrations selected for the validation exercise where much lower compared to the target MRL's from this report (i.e. 20 and 120 μg/kg), since at the time when the validation has been conducted these MRL's were not known. Nevertheless, it can be assumed that the method will also show an acceptable performance profile, when using it for the control of MRL's at the higher levels of 20 and 120 μg/kg, respectively. Therefore the CRL recommends this CRL Berlin method for the official control in muscle and liver. However, since the validation experiments have not included kidney and fat, the CRL cannot conclude on the suitability of the method for official control of the target MRL's in the latter matrices.

Further testing or validation is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

The CRL recommends for official control the standard method EN 15781:2009, for the determination of *maduramicin ammonium alpha* in *feed additive, premixtures* and *feedingstuffs*.

For the determination of *maduramicin ammonium alpha* in *muscle* and *liver*, the CRL recommends for official control the method validated according to Commission Decision 2002/657/EC, by the CRL Berlin.

Recommended text for the register entry (analytical method)

For the determination of *maduramicin ammonium alpha* in *feed additive*, *premixtures* and *feedingstuffs*:

- reversed-phase high performance liquid chromatography (HPLC) using post-column derivatization with vanillin and detection at 520 nm - EN 15781:2009

For the determination of *maduramicin ammonium alpha* in muscle and liver:

- reversed-phase high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *maduramicin ammonium alpha* 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] Commission Regulation (EC) No 2380/2001 of 5 December 2001, concerning the 10 year authorisation of an additive in feedingstuffs

[3] * Technical dossier, Section II – 2.2. Characterisation of the active substance

[4] * Application, Reference SANCO/D/2 Forw. Appl. 1831/034-2008 (FAD-2008-0051)

[5] * Application, Reference SANCO/D/2 Forw. Appl. 1831/035-2008 (FAD-2008-0054)

[6] * Application, Proposal for Register Entry – Annex A (chickens for fattening)

[7] * Application, Proposal for Register Entry – Annex A (turkey)

[8] * Technical dossier, Section II – Annexes II_14 method valid premix.pdf "Quantitative analysis of Maduramicin ammonium alpha in Cygro 10G lots and in blank and medicated vitamin-mineral premix samples: method validation (V7635101)"

[9] * Technical dossier, Section II – Annexes II_14 method valid feed.pdf "Quantitative analysis of Maduramicin ammonium alpha in Cygro 10G lots and in blank and medicated broiler and turkey feed samples: method validation (V7387101)"

[10] EN 15781:2009, Animal feeding stuffs - "Determination of maduramicin ammonium by reversed-phase HPLC using post-column derivatisation"

[11] * Supplementary Information, 2009_CER_Cygro_validation_report.pdf "Validation of an analytical method for the determination of maduramicin in Cygro 10G"

[12] Dubois M. et al (2004): Efficient and sensitive detection of residues of nine coccidiostats in egg and muscle by liquid chromatography-electrospray tandem mass spectrometry. Journal of Chromatography B, 813 181-189

[13] Confirmatory method for the determination of nicarbazin, monensin, salinomycin, lasalocid, narasin and maduramicin in muscle and liver with LC-MS/MS, available for official control from EU Reference Laboratory for Pharmacologically Active Substances, Berlin

* Refers to Dossier No. FAD-2008-0051 and FAD-2008-0054

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

The following National Reference Laboratories contributed to this report:

- Plantedirektoratets Laboratorium, Lyngby, DK
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, DE
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya,Cabrils, ES
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana, SI

- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena, DE
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin, PL
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien, AT
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes, FR
- RIKILT-Instituut voor Voedselveiligheid, Wageningen, NL

The CRL for Feed Additives is also very grateful to the EU Reference Laboratory for Pharmacologically Active Substances, Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Berlin, Germany for providing details on the analytical method for the detection of *maduramicin ammonium alpha* residues in tissues.