JRC.F.5/CvH/MGH/AS/Ares

Subject: Addendum to the EURL evaluation report

References:

FAD-2015-0039- Coxar® (JRC.D.5/CvH/MGH /mds/Ares (2016)2861703)

Upon the publication of a new multi-analyte ring-trial validated method EN 17299 [1] for the analysis of coccidiostats the EURL, considered appropriate to include this standard method within the recommended methods of analysis for official control for the above-mentioned *feed additive* dossiers.

This addendum aims to provide an up-to-date EURL recommendations, including all the available analytical methods complying with the highest requirements as stated in Annex II of Regulation (EC) No 429/2008 [2] which will allow Member States official control laboratory full flexibility regarding the selection of method of analysis (single-analyte or multi-analyte method).

The recommendations included in this addendum apply for the *feed additives* containing *nicarbazin* as active substance that have been already evaluated by the EURL and/or are currently authorised by the related Regulations [3].

The EURL has developed and fully validated a multi-analyte method based on high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for the determination of the various coccidiostats, including *nicarbazin,* in *compound feeds.*

According to the method the coccidiostats are extracted with a mixture of acetonitrile:methanol:water. The obtained extracts are centrifuged and supernatants are filtered. The analysis of samples is conducted by reversed-phase LC-MS/MS. The quantification of the detected target analytes is performed using a multi-level standard addition approach [1].

This method has been ring-trial validated for *nicarbazin* in different feed matrices at additive and at cross-contamination levels and published as CEN standard (EN 17299) [1].

Based on the obtained performance characteristics and the scope of the method in terms of matrices, the EURL considers the multi-analyte ring-trial validated EN 17299 method based on high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) fit for purpose for the determination of *nicarbazin* in *compound feeds*.

Recommended text for the registry entry (analytical methods) (replacing the previous recommendations)

For the quantification of *nicarbazin* in the *feed additive*:

- High Performance Liquid Chromatography coupled with spectrophotometric detection (HPLC-UV)

For the quantification of *nicarbazin* in *premixtures*:

- High Performance Liquid Chromatography coupled with spectrophotometric detection (HPLC-UV) - EN ISO 15782

For the quantification of *nicarbazin* in *compound feed*:

- High Performance Liquid Chromatography coupled with spectrophotometric detection (HPLC-UV) - EN ISO 15782 or
- High Performance Liquid Chromatography coupled with tandem mass spectrometry (LC-MS/MS) – EN 17299

For the quantification of *nicarbazin (*as *4,4'-dinitrocarbanilide (DNC))* in chicken *tissues*:

- High Performance Liquid Chromatography coupled with tandem mass spectrometry (LC-MS/MS)

References

[1] EN 17299:2019 Animal feedingstuffs: Methods of sampling and analysis – Screening and determination of authorised coccidiostats at additive and 1 % and 3 % cross-contamination level, and of non-registered coccidiostats and of one antibiotic at sub-additive levels, in compound feed with High Performance Liquid Chromatography – Tandem Mass Spectrometry detection (LC-MS/MS)

[2] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisations of feed additives, OJ L 133 22.5.2008, p. 1

[3] Commission Regulation (EU) No 875/2010 of 5 October 2010 concerning the authorisation for 10 years of an additive in feedingstuffs OJ L 263, 6.10.2010, p. 4

Addendum
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- Reviewed and approved by Zigmas Ezerskis and Christoph von Holst (EURL-FA), respectively, Geel, 25/01/2023

EUROPEAN COMMISSION
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European Union Reference Laboratory for Feed Additives

JRC.D.5/CvH/MGH /mds/Ares

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

Coxar®

(FAD-2015-0039; CRL/150016)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2015-0039 - CRL/150016**
Name of Product:	***Coxar®***
Active Agent (s):	**Nicarbazin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **17/06/2016**
Report approved by: Date:	**Christoph von Holst** **17/06/2016**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Coxar®*, under article 4(1), for the category "coccidiostats and histomonostats", according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for *turkeys for fattening*. *Coxar®* consists of 250 g/kg of *nicarbazin* complemented by starch for granulation, wheat meal and calcium carbonate. *Coxar®* is intended to be incorporated in *feedingstuffs* through *premixtures* for turkeys for fattening at a concentration of *nicarbazin* of 100 mg/kg *feedingstuffs.* Furthermore the Applicant proposed Maximum Residue Limits (MRLs) in turkey tissues ranging from 4000 to 15000 µg *4,4-dinitrocarbanilide (DNC)*/kg of fresh material.

For the quantification of *nicarbazin* in the *premixtures* and *feedingstuffs* the Applicant submitted the EN ISO 15782 method, based on High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV). Precisions (relative standard deviations for *repeatability* and *reproducibility*) ranging from 2.6 to 12 % were reported, together with a limit of quantification (LOQ) of 20 mg/kg *feedingstuffs*. The Applicant submitted for the quantification of *nicarbazin* in the *feed additive* (*Coxar®*) a single-laboratory validated and further verified method based on the above mentioned EN ISO 15782 method using different extraction solvent (dimethylformamide), and reported satisfactory experimental data. Based on the performance characteristics available the EURL recommends for official control the HPLC-UV methods for the quantification of *nicarbazin* in the *feed additive*, *premixtures* and *feedingstuffs*.

For the quantification of *DNC* (target compound for *nicarbazin*) in target turkey tissues (skin/fat, muscle, liver and kidney) the Applicant submitted a single-laboratory and further verified method based on Reversed-Phase High Performance Liquid Chromatography coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode (ESI) using matrix matched standards. Based on the performance characteristics presented, the EURL recommends for official control the RP-HPLC-MS/MS method proposed by the Applicant or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs of *DNC* in the target *tissues*.

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

Nicarbazin, *Coxar®*, coccidiostat, turkeys for fattening

1. BACKGROUND

In the current application authorisation is sought for *Coxar®*, under article 4(1), for the category "coccidiostats and histomonostats", according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for *turkeys for fattening* [1][2].

Coxar® consists of 250 g/kg of *nicarbazin* (active substance), starch for granulation as binding agent and wheat meal and calcium carbonate [4][2][5]. *Nicarbazin* is chemically synthesised from 4,4-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethyl-pyrimidine (HDP) with a minimum purity of 95 % [5][6] and consists of an equimolecular crystalline complex of 4,4-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethyl-pyrimidine (HDP) [7].

Coxar® is intended to be incorporated in *feedingstuffs* through *premixtures* for turkeys for fattening at a concentration of *nicarbazin* of 100 mg/kg *feedingstuffs* [2][8].

Furthermore the Applicant proposed Maximum Residue Limits (MRLs) in turkey skin/fat, muscle, liver and kidney ranging from 4000 to 15000 µg DNC/kg of fresh material [2][3]. As these MRLs are not set up by Commission Regulation (EC) No 37/2010 [9], the correspondent methods of analysis have to be evaluated by the EURL.

Note: The EURL previously evaluated the analytical methods for the determination of *nicarbazin* in the frame of the several dossiers [10].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL 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 *Coxar®* 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, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [11].

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

For the quantification of *nicarbazin* in the *premixtures* and *feedingstuffs* the Applicant submitted the EN ISO 15782 method [12][13] based on High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV).

Nicarbazin is extracted using acetonitrile:methanol (50:50) with manual shaking, heated in a water bath at 50^{o}C for 15 min, further mixing and then sonicated for another 15 min. After appropriate dilution with the eluent, an aliquot is filtered and subjected to analysis without further clean-up. The target analyte is determined by reverse-phase HPLC and the *4,4'-dinitrocarbanilide (DNC)* moiety is detected at 350 nm. According to *Jacob de Jong et al.* [15] potential interferences in the determination of *nicarbazin* cannot be expected. This method was ring-trial validated for broiler *feedingstuffs* and *premixtures* at a mean *nicarbazin* content ranging from 22 to 7308 mg/kg leading to the following performance characteristics [12]: - a relative standard deviation for *repeatability* (RSD_r) ranging from 2.6 to 10.2 %; - a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.8 to 12.3 %; and - limits of detection (LOD) and quantification (LOQ) of 0.5 and 20 mg/kg , respectively.

For the quantification of *nicarbazin* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method [14] based on the EN ISO 15782 [12], but using a different extraction solvent (dimethylformamide). Based on the experimental data provided in the frame of the validation study [14], the EURL recalculated the relative standard deviations for *repeatability* and *intermediate precision:* RSD_r = 0.6 % and RSD_{ip} = 1.9 % [16].

Based on the performance characteristics available the EURL recommends for official control the HPLC-UV methods for the quantification of *nicarbazin* in the *feed additive* [14] *premixtures* and *feedingstuffs* [12].

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

For the quantification of *DNC* (target compound for *nicarbazin*) in target tissues (skin/fat, muscle, liver and kidney) the Applicant submitted in the frame of previous dossier (FAD-2012-0027) [10] a single-laboratory validated (in turkey and chicken) [10][17] and further verified (in chicken) [18] method based on Reversed Phase High Performance Liquid Chromatography coupled to a triple quadrupole mass spectrometer in electrospray ionisation mode using matrix matched standards (RP-HPLC-MS/MS).

The homogenised tissue sample is fortified with the isotopically labelled dinitrocarbanilide-d8 as internal standard and vortex-mixed with acetonitrile, placed in an ultrasonic bath for 10 minutes and further centrifuged. An aliquot of the supernatant is then transferred to an Eppendorf cup and again diluted with acetonitrile. After vortex –mixing another aliquot is further diluted with acetonitrile, vortex-mixed and quantified by RP-HPLC-MS/MS. Quantification is based on the transition m/z 301.0 > 136.9 while confirmation is based on the transition m/z 301.0 > 106.7 [10][17][18] thus complying with the confirmatory requirements set by of Commission Decision 2002/657/EC [19].

The target turkey and chicken tissues (skin/fat, muscle, liver and kidney) were investigated at different *DNC* concentrations [17][10]. The method was further verified by a second independent laboratory in chicken tissues [18]. Three *DNC* levels (i.e. MRL/2; MRL and 2MRL) were investigated for each target *tissue* and satisfactory performance characteristics were reported (Table 1) [10]. Furthermore the Applicant reported a LOQ of 1000 µg/kg for muscle, liver, kidney and skin/fat *tissues* [17].

The satisfactory performance characteristics provided by the Applicant for the target chicken *tissues* [10] demonstrate the applicability of the Applicant method to the chicken *tissues*. Additionally the acceptable results provided by the Applicant for the turkey *tissues* [17] demonstrate the suitability – and therefore the extension of the scope – of the Applicant method to turkey.

Based on the performance characteristics presented, the EURL recommends for official control the RP-HPLC-MS/MS method proposed by the Applicant or any equivalent other analytical methods complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs for *DNC* in the target *tissues*.

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.

Table 1. Performance characteristics of analytical method for the determination of *DNC residues* in food [10] .

Tissues		µg/kg (#)	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
			Valid	Verif	Valid	Verif	Valid(*)	Verif
Chicken	Muscle	2000	3.0	---	2.6	---	99.6	---
		4000	5.0	5.6-6.4	2.2	6.2	100.8	106.7
		8000	2.2	---	4.3	---	97.9	---
	Liver	7500	2.6	---	2.6	---	105.3	---
		15000	3.8	6.1	3.5	6.9	99.9	95.8
		30000	4.6	---	6.8	---	92.2	---
	Skin/fat	2000	1.8	---	2.6	---	96.4	---
		4000	2.5	3.1-6.2	1.4	4.7	98.2	99.9
		8000	1.6	---	1.0	---	98.9	---
	Kidney	3000	2.2	---	2.3	---	99.4	---
		6000	0.9	7.0-7.8	2.4	7.4	98.6	93.8
		12000	1.3	---	2.4	---	99.3	---
Turkey	Muscle	2000	2.2	---	4.2	---	101.6	---
		4000	2.3	---	4.6	---	100.9	---
		8000	1.7	---	1.1	---	102.3	---
	Liver	7500	0.9	---	2.3	---	98.9	---
		15000	2.7	---	3.6	---	95.0	---
		30000	3.9	---	1.0	---	106.4	---
	Skin/fat	2000	2.1	---	1.2	---	101.4	---
		4000	1.1	---	1.2	---	102.3	---
		8000	0.8	---	0.9	---	103.6	---
	Kidney	3000	1.3	---	1.6	---	98.7	---
		6000	1.0	---	1.7	---	99.3	---
		12000	1.5	---	1.8	---	99.3	---

RSD_r; RSD_R and RSD_{ip}: relative standard deviation for *repeatability*, *reproducibility* and *intermediate precision*, respectively; R_{Rec}: *recovery* rate;
Fortified level; (*) Recalculated by the EURL.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control (i) the HPLC-UV methods for the quantification of *nicarbazin* in the *feed additive*, *premixtures* and *feedingstuffs* and (ii) the RP-HPLC-MS/MS single laboratory validated and further verified method proposed by the Applicant - or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC - for the quantification of *4,4'-dinitrocarbanilide (DNC)* in turkey *tissues.*

Recommended text for the register entry (analytical method)

For the quantification of *nicarbazin* in the *feed additive*:

– High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV)

For the quantification of *nicarbazin* in *premixtures* and *feedingstuffs*:

– High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV) - EN ISO 15782

For the quantification of *4,4'-dinitrocarbanilide (DNC)* in turkey *tissues*:

– Reversed-Phase High Performance Liquid Chromatography coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Coxar*® have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANCO/G1: Forw. Appl. 1831/0027-2015
[2] *Application, Proposal of Registry Entry – Annex A
[3] *Application, Application Form – Annex I
[4] *Technical dossier, Section II: 2.1 Identity of the additive
[5] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[6] *Technical dossier, Section II: 2.1.4 Purity
[7] *Technical dossier, Section II: 2.2.1 Description
[8] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[9] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin
[10] EURL Evaluation Reports FAD 2007-0016, FAD 2008-0037, FAD 2012-0027 and FAD 2014-0036/0045
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2007-0016.pdf
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2008-0037.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2012-0027-monimax.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2014-0036_0045_maxiban160.pdf

[11] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[12] EN ISO 15782:2009 Animal feedingstuffs – Determination of nicarbazin – High performance liquid chromatography method

[13] *Technical dossier, Section II, Annexes, Reference II.22

[14] *Technical dossier, Section II, Annexes, Reference II.26, II.27 & II.28

[15] Jacob de Jong et al. Liquid Chromatographic Method for Nicarbazin in Broiler Feeds and Premixtures: Development, Validation, and Interlaboratory Study, J. of AOAC Int., 87, 6, 1269 – 1277, 2004

[16] *Supplementary Information, eurl_anova_coxar_fa.pdf

[17] *Technical dossier, Section II, Annexes, Reference II.34

[18] *Technical dossier, Section II, Annexes, Reference II.32

[19] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results

*Refers to Dossier no: FAD-2015-0039

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Fødevarestyrelsens Laboratorie Ringsted (DK)
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- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Istituto Superiore di Sanità. Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Roma (IT)
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