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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

Nicarb®
(FAD-2019-0067; CRL/190045)

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-2019-0067 - CRL/190045**
Name of Feed Additive:	***Nicarb®***
Active Agent (s):	**Nicarbazin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report approved by: Date:	**Christoph von Holst** **29/05/2020**

EXECUTIVE SUMMARY

In the current application an authorisation is sought for *Nicarb®* (*nicarbazin*) under Article 10(2) for the category "coccidiostats and histomonostats", according to the classification system of Article 6 of Regulation (EC) No 1831/2003. The authorisation is sought for chickens for fattening.

Nicarb® is a preparation containing as active substance 250 g/kg (25 %) of *nicarbazin*. *Nicarb®* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* for chickens for fattening at the level of 125 mg *nicarbazin*/ kg *feedingstuffs*. The Applicant proposed maximum residue limits (MRLs) in chicken *tissues* ranging from 4000 to 15000 µg/kg of fresh *tissue* for *4,4-dinitrocarbanilide (DNC)*, which is the marker residue for *nicarbazin*. The proposed MRLs for *nicarbazin* (as *DNC*) are not covered by Commission Regulation (EC) No 37/2010, therefore the corresponding methods of analysis are evaluated by the EURL.

For the quantification of *nicarbazin* in the *feed additive*, the Applicant proposed a single-laboratory validated and further verified method based on high performance liquid chromatography coupled to photometric detection (HPLC-UV). For the quantification of the active substance in *premixtures* and *feedingstuffs* the Applicant applied a single-laboratory validated and further verified method based on the ring-trial validated method EN 15782.

Furthermore, the EURL is aware of another ring-trial validated method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the determination of various coccidiostats, including *nicarbazin,* in *feedingstuffs* that has been recently published as a CEN standard method (EN 17299).

For the quantification of *DNC residues* in chicken *tissues* the Applicant submitted a single-laboratory validated method based on LC-MS/MS that does not fully comply with the confirmatory requirements set by Commission Decision 2002/657/EC. However, in the frame of a previous *nicarbazin* dossiers the EURL already evaluated and recommended a similar method (AOAC 2013.07) validated for muscle, kidney, skin/fat and liver that complies with the criteria of Commission Decision 2002/657/EC.

Based on the acceptable method performance characteristics available, the EURL recommends for official control i) the single-laboratory validated and further verified method based on HPLC-UV for the quantification of *nicarbazin* in the *feed additive*; ii) the ring-trial validated method EN 15782 for the quantification of *nicarbazin* in *premixtures* and *feedingstuffs*; iii) the ring-trial validated method EN 17299 for the quantification of *nicarbazin* in *feedingstuffs* and iv) the AOAC 2013.07 method or any equivalent method,

complying with the requirements set by Commission Decision 2002/657/EC, to enforce the MRLs for *nicarbazin* (as *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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Nicarb®, nicarbazin, coccidiostats and histomonostats, chickens for fattening

1. BACKGROUND

In the current application an authorisation is sought for *Nicarb®* (*nicarbazin*) under Article 10(2) (re-evaluation of additives already authorised under the provisions of the Council Directive 70/524/EEC) for the category "coccidiostats and histomonostats", according to the classification system of Article 6 of Regulation (EC) No 1831/2003. The Authorisation is sought for chickens for fattening [1][2].

Nicarb® is a brown free flowing granular preparation containing 250 g of *nicarbazin* (active substance)/ kg of the preparation [2][3]. *Nicarbazin* is an equimolar *4,4-dinitrocarbanilide (DNC) 2-hydroxy-4,6-dimethyl-pyrimidine (HDP)* complex chemically synthesised [3].

Nicarb® is intended to be incorporated directly into *feedingstuffs* or through *premixtures* for chickens for fattening at the level of 125 mg *nicarbazin*/ kg *feedingstuffs* [2][4].

The Applicant proposed maximum residue limits (MRLs) for *4,4-dinitrocarbanilide (DNC)* (marker residue of *nicarbazin*) in chicken for fattening *tissues* (i.e. muscle, kidney, skin/fat and liver) ranging from 4000 to 15000 µg/kg (4000 µg/kg for muscle and skin/fat; 6000 µg/kg for kidney and 15000 µg/kg for liver) [2]. The MRLs for *DNC* have been already established by Commission Regulation (EC) No 875/2010 [5] and Regulation (EC) No 885/2010 [6] but are not covered by Commission Regulation (EC) No 37/2010 [7]. Therefore corresponding methods of analysis are evaluated by the EURL.

Note: The EURL previously evaluated the analytical methods for the determination of *nicarbazin* [8] in the frame of several dossiers.

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 *Nicarb®* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of *nicarbazin* in the *feed additive*, the Applicant submitted a single-laboratory validated [9] and further verified method [10] based on high performance liquid chromatography coupled to photometric detection (HPLC-UV) [11].

Nicarbazin is extracted by stirring the *feed additive* in N,N-dimethylformamide (DMF) for 30 min, an aliquot of the clear liquid is then diluted with methanol, mixed and filtered before injection into the HPLC system. The target analyte is determined by reversed-phase HPLC using photometric detection at 340 nm. This method was single-laboratory validated and further verified leading to a relative standard deviation for *repeatability* (RSD_r) ranging from 0.5 to 1.3 % and a relative standard deviation for *intermediate precision* (RSD_{ip}) between 3.7 and 7.1 % [9-10]. According to the Applicant, potential interferences in the determination of *nicarbazin* are not expected [9,11].

During the systematic review process of the initial report a NRL informed the EURL about possible restrictions regarding the use of DMF due to toxic characteristics of this solvent. In addition, the NRL informed the EURL about good experiences of using an adapted version of EN 15782 for the determination of *nicarbacin* in the *feed additive*. The EURL agrees with this comment, however requires that a corresponding adaption of EN 15782 has to be carried prior its use for this purpose.

For the quantification of *nicarbazin* in *premixtures* and *feedingstuffs* the Applicant submitted another single-laboratory validated [12] and further verified [13] method using HPLC-UV [14], which is based on the EN 15782 method [15]. Upon EURL request, the Applicant confirmed the similarity between both methods.

Following the EN 15782 method, *nicarbazin* is extracted using a mixture of acetonitrile:methanol (50:50, v/v) with manual shaking, heated in a water bath at 50 ^{o}C for 15 min, further mixed and 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 reversed-phase HPLC and the *4,4'-dinitrocarbanilide (DNC)* is

detected at 350 nm. According to de Jong et al. [16], 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 [15]: 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 a limit of quantification (LOQ) of 20 mg/kg.

In addition, the EURL has developed and fully validated a multi-analyte method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the determination of all coccidiostats in *feedingstuffs* currently authorised as *feed additives* within the EU, including the one of the current application. In this method the coccidiostats are extracted with a mixture of acetonitrile:methanol:water (80:10:10, v/v/v). The extracts are centrifuged and supernatants are filtered. After a first screening analysis, the analytes are determined by reversed-phase LC-MS/MS. The quantification of the detected target analytes is performed using multi-level standard additions. This method has been ring-trial validated and recently published as a CEN standard method (EN 17299) [17].

Based on the performance characteristics available the EURL recommends for official control the single-laboratory validated and further verified HPLC-UV method [11] for the quantification of *nicarbazin* in the *feed additive*, the ring-trial validated HPLC-UV method EN 15782 for the quantification of *nicarbazin* in *premixtures* and *feedingstuffs* [15] and the ring-trial validated LC-MS/MS method EN 17299 [17] for the quantification of *nicarbazin* in *feedingstuffs*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of *DNC* (marker residue of *nicarbazin*) in target *tissues* (skin/fat, muscle, liver and kidney) the Applicant submitted a single-laboratory validated [18] method based on LC-MS/MS using electrospray ionisation in the negative mode (ESI-) [19].

However, the method proposed by the Applicant does not fully comply with the confirmatory requirements set by Commission Decision 2002/657/EC [20]. Therefore, it is not suitable for enforcing the MRLs for *nicarbazin* (as *DNC*) in the target *tissues* in the frame of official control.

Table 1. Performance characteristics for the quantification of *nicarbazin* residues (as *DNC*) in chicken tissues obtained with the AOAC 2013.07 (AOAC) method [21], compared to those reported for the European Union Reference Laboratory "Pharmacologically Active Substances" (BVL) method.

Tissue		Content (µg /kg)	RSD_r (%)	RSD_{ip} (%)
Muscle	BVL	0.75-2.75	3.4 - 8.7	8.0-11.6
	AOAC	100	0.81 - 5.3	10.0
		200	1.6 - 7.0	11.3
		400	1.9 - 4.9	4.9
		2000	1.8 - 5.0	4.5
		4000	1.4 - 4.5	5.7
		8000	1.6 - 2.2	3.0
Liver	BVL	0.75-2.75	3.4 - 8.7	8.0-11.6
	AOAC	100	2.2 - 10.4	8.2
		200	3.6 - 7.2	8.6
		400	2.2 - 4.8	4.8
		2000	3.2 - 5.4	4.8
		4000	3.2 - 6.8	5.5
		8000	2.1 - 2.6	2.5
Kidney	AOAC	100	1.3 - 10.8	6.6
		200	1.1 - 4.7	8.3
		400	1.2 - 1.9	6.9
		2000	1.9 - 4.0	5.4
		4000	0.7 - 5.7	4.4
		8000	1.2 - 6.2	8.4
Skin/Fat	AOAC	100	2.1 - 5.6	6.8
		200	2.0 - 11.6	8.2
		400	1.7 - 11.5	7.8
		2000	2.0 - 10.2	6.9
		4000	1.5 - 8.3	5.8
		8000	1.6 - 8.1	6.1

RSD_r; RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*

The EURL is aware of another multi-residue method also based on LC-MS/MS and previously validated by the European Union Reference Laboratory "Pharmacologically Active Substances" (BVL) for muscle and liver *tissues*. Additionally, the EURL and the Applicant are also aware of a similar method (AOAC 2013.07) [21] based on LC-MS/MS using electrospray ionisation in the negative mode (ESI-) and validated for muscle, kidney, skin/fat and liver according to Commission Decision 2002/657/EC [20]. This method has been already evaluated and recommended by the EURL in the frame of previous *nicarbazin* dossiers [8].

The method performance characteristics of the AOAC method (validated for muscle, kidney, skin/fat and liver) together with those reported for the BVL method (validated for muscle and

liver) are presented in Table 1. Furthermore, a limit of quantification (LOQ) of 20 µg/kg was reported for the AOAC method for muscle, liver, kidney and skin/fat *tissues* [21].

The satisfactory performance characteristics provided by the AOAC method for muscle and liver *tissues* demonstrate that the AOAC method was equivalent to the BVL method. Additionally the results provided by the AOAC method for kidney and skin/fat [21] further demonstrate the applicability - and therefore the extension of scope - of the AOAC method to these two additional *tissues.*

Based on the performance characteristics presented, the EURL recommends for official control the AOAC 2013.07 method based on LC-MS/MS or any equivalent analytical method complying with the requirements set by Commission Decision 2002/657/EC to enforce the MRLs for *nicarbazin* (as *DNC*) in the target *tissues*.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of the corresponding methods of analysis is not considered necessary by the EURL.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control: i) the single-laboratory validated and further verified method based on HPLC-UV for the quantification of *nicarbazin* in the *feed additive*; ii) the ring-trial validated method EN 15782 for the quantification of *nicarbazin* in *premixtures* and *feedingstuffs;* iii) the ring-trial validated method EN 17299 for the quantification of *nicarbazin* in *feedingstuffs*; and iv) the AOAC 2013.07 method based on LC-MS/MS - or any equivalent method complying with the requirements set by Commission Decision 2002/657/EC - for the quantification of *nicarbazin* (as *DNC*) in chicken *tissues*.

Recommended text for the register entry (analytical method)

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

– high performance liquid chromatography coupled to photometric detection (HPLC-UV)

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

– high performance liquid chromatography coupled to photometric detection (HPLC-UV) – EN ISO 15782

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

– high performance liquid chromatography coupled to photometric detection (HPLC-UV) – EN ISO 15782 or

– liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) – EN 17299

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

– liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) – AOAC 2013.07 or any equivalent method 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 *Nicarb®* 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 SANTE/E5: FORW. APPL. 1831-0068-2019

[2] *Application. Application Form - Annex I. Submission number 1571842218865-2466

[3] *Technical dossier, Section II: 2.1 Identity of the additive

[4] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[5] Commission Regulation (EU) No 875/2010 of 5 October 2010 concerning the authorisation for 10 years of an additive in feedingstuffs.

[6] Commission Regulation (EU) No 885/2010 of 7 October 2010 concerning the authorisation of the preparation of narasin and nicarbazin as a feed additive for chickens for fattening (holder of authorisation Eli Lilly and Company Ltd) and amending regulation (EC) No 2430/1999.

[7] 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

[8] EURL Evaluation Reports:
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
https://ec.europa.eu/jrc/sites/jrcsh/files/finirep-fad-2015-0039-coxar-turkeys.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0056-nilablend.pdf

[9] *Technical dossier, Section II: Annex II.40

[10] *Supplementary information Annex II.47 & EURL_ANOVA_FA.pdf

[11] *Technical dossier, Section II: Annex II.16

[12] *Technical dossier, Section II: Annex II.35

[13] *Supplementary information Annex II.46

[14] *Technical dossier, Section II: Annex II.44

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

[16] 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

[17] 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)

[18] *Technical dossier, Section II: Annex II.39

[19] *Technical dossier, Section II: Annex II.42

[20] 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

[21] Coleman et al. Determination and Confirmation of Nicarbazin, Measured as 4,4-Dinitrocarbanilide (DNC), in Chicken Tissues by Liquid Chromatography with Tandem Mass Spectrometry: First Action 2013.07, J of AOAC Int., 97, 2, 630 - 640, 2014

[22] *Refers to Dossier no: FAD-2019-0067

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)

- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Wageningen Food Safety Research[1] (WFSR) (NL)
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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Ruokavirasto Helsinki[2] (FI)

[1] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.

[2] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors