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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

Salinomax® 120G
(FAD-2014-0016; CRL/140012)

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-2014-0016 - CRL/140012**
Name of Feed Additive:	**Salinomax® 120G**
Active Agent (s):	***Salinomycin Sodium***
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA)** **08/06/2015**
Report approved by: Date:	**Christoph von Holst** **09/06/2015**

EXECUTIVE SUMMARY

Salinomax®120G is a *feed additive* - belonging to the "Coccidiostats and other medicinal substances" group listed in Directive 70/524/EEC - currently authorized for chickens for fattening by Commission Regulation (EC) No 600/2005 further amended by Commission Regulation (EC) No 496/2007. In the current applications authorisation is sought under articles 10(2) and 13(3) of the Regulation (EC) No 1831/2003. *Salinomax®120G* contains 12 % (w/w) granular *salinomycin sodium* (active substance) produced by two different *Streptomyces albus subsp. albus* strains, a calcium lignosulphonate binder, a calcium sulphate dihydrate carrier and Microtracer® FS Green Lake marker. *Salinomax®120G* is intended to be incorporated into *feedingstuffs* through *premixtures.* The Applicant proposed a concentration of *salinomycin sodium* in *feedingstuffs* ranging from 50 to 70 mg/kg for chickens for fattening. Furthermore the Applicant proposed MRLs for *salinomycin sodium* in chicken *tissues* ranging from 30 to 200 µg/kg depending on the target *tissues*.

For the quantification of *salinomycin sodium* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated method (EN ISO 14183) based on High Performance Liquid Chromatography with post-column derivatisation coupled to spectrophotometric detection (HPLC-PCD-UV-Vis). Furthermore, the Applicant applied the EN ISO 14183 to the *feed additive* (*Salinomax®120G)* and provided similar method performance characteristics. Based on the experimental evidence available the EURL recommends for official control the HPLC-PCD-UV-Vis method for the quantification of *salinomycin sodium* in the *feed additive*, *premixtures* and *feedingstuffs*.

For the quantification of *salinomycin sodium* in chicken *tissues* the Applicant submitted a single laboratory validated and further verified method based on reverse phase HPLC coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode using matrix matched standards. This method is similar to the one developed and validated by the European Union Reference Laboratory for Pharmacologically Active Substances (BVL, Berlin). The satisfactory performance characteristics provided by the Applicant for the four *tissues* of concern (i.e. muscle, kidney, skin/fat and liver) demonstrate that (i) the method proposed by the Applicant is equivalent to the BVL method, and that (ii) the Applicant method is also applicable to kidney and skin/fat *tissues*. 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 analytical methods complying with the requirements set by Commission Decision 2002/657/EC to enforce the *salinomycin sodium* MRLs in the relevant *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

Salinomycin sodium, *Salinomax®120G*, coccidiostat, chickens for fattening

1. BACKGROUND

Salinomax®120G is a *feed additive* currently authorized for chickens for fattening by Commission Regulation (EC) No 600/2005 [1] belonging to the "Coccidiostats and other medicinal substances" group listed in Directive 70/524/EEC. This regulation was further amended by Commission Regulation (EC) No 496/2007 [2]. In the current application authorisation is sought under articles 10(2) (authorisation of an existing product) and 13(3) (modification of the existing authorisation) of the Regulation (EC) No 1831/2003 [3, 4].

According to the Applicant the active agent of *Salinomax®120G* is *salinomycin sodium,* produced by fermentation of two different strains of *Streptomyces albus subsp. albus* namely ATCC21838/US 9401-06 -currently authorised [1][2]- and NCIMB 30321[3][4].

Salinomax®120G contains 12 % (w/w) granular *salinomycin sodium* (active substance), 4.0 % of calcium lignosulphonate binder, 83.8 % of calcium sulphate dihydrate carrier and 0.2% (w/w) of Microtracer® FS Green Lake marker [4]. *Salinomax®120G* is intended to be incorporated into *feedingstuffs* through *premixtures* [5]. The Applicant proposed a concentration of *salinomycin sodium* in *feedingstuffs* ranging from 50 to 70 mg/kg for chickens for fattening [4, 5].

The Applicant proposed MRLs for *salinomycin sodium* in chicken *tissues* ranging from 30 to 200 µg/kg (30 µg/kg for muscle; 100 µg/kg for kidney; 150 µg/kg for liver; and 200 µg/kg for skin/fat) [4]. These MRLs are not covered by the Commission Regulation (EC) No 37/2010 [6], and need to be evaluated by the EURL.

Note: The EURL previously evaluated the analytical methods for the determination of *salinomycin sodium* and its residues or its metabolites in food in the frame of the FADs 2012-0041; 2013-0029 and 2013-0053 dossiers [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 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 *Salinomax®120G* 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 [8].

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

For the quantification of *salinomycin sodium* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated method (EN ISO 14183) [9], based on High Performance Liquid Chromatography with post-column derivatisation coupled to spectrophotometric detection (HPLC-PCD-UV-Vis). This specific method can distinguish between the main substances of the *narasin*, *salinomycin* and *monensin* ionophores. Potential interferences in the determination of *salinomycin*, caused by other components of the *feed additive*, *premixtures* of *feedingstuffs*, are not expected [10].

Salinomycin is extracted using methanol:water (90:10) with mechanical shaking for 1 h, filtered and subjected to analysis without further clean-up. The target analyte is determined by reverse-phase HPLC using post-column derivatisation with vanillin and detection at 520 nm [9].

This method was ring-trial validated for broiler *feedingstuffs* and *premixtures* at a mean *salinomycin* content of 68.4 and 1000 mg/kg, respectively. The following performance characteristics were reported [9]:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.5 to 3.5 %;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 2.7 to 5.5 %; and
- a limit of quantification (LOQ) of 2 mg/kg.

For the quantification of *salinomycin sodium* in the *feed additive* (*Salinomax®120G*) the Applicant applied the EN ISO 14183 method mentioned above. Precision (repeatability and intermediate precision) of 1.7 % were recalculated by the EURL based on the experimental data obtained in the frame of the verification study [11][12]. These performance characteristics are in good agreement with those reported in the EN ISO 14183 standard and

demonstrate the applicability (extension of the scope) of the EN ISO 14183 method to the *feed additive (Salinomax®120G).*

Based on the performance characteristics available, the EURL recommends for official control the HPLC-PCD-UV-Vis method for the quantification of *salinomycin sodium* in *feed additive*, *premixtures* and *feedingstuffs*.

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

For the quantification of *salinomycin sodium* in poultry *tissues* the Applicant submitted a single-laboratory validated [13] and further verified method (in kidney, skin/fat, muscle and liver) [14-17] based on reverse phase HPLC coupled to a triple quadrupole mass spectrometer in electrospray ionisation mode using matrix matched standards (RP-HPLC-MS/MS).

A similar method has been previously developed and validated by the European Union Reference Laboratory for Pharmacologically Active Substances (BVL, Berlin) for the determination of *salinomycin sodium* in two target *tissues* (muscle and liver) [18]. The EURL already evaluated and recommended a similar method in the frame of previous dossiers [7].

The minced tissue is extracted with acetonitrile, vortex mixed for a few seconds, homogenised in a homogeniser until adequate homogenisation, and centrifuged for 5 min. 5 µl of the resulting supernatant are then injected in the HPLC-MS/MS system [13].

The Applicant validated (at 1, 10, 50 and 150 µg/kg) and further verified (at 1 and 150 µg/kg) the RP-HPLC-MS/MS method in wet *tissues* (i.e. kidney, liver, skin/fat and muscle). Upon request by the EURL the Applicant submitted additional experimental evidences proving that the proposed method complies with the confirmatory requirements set by Commission Decision 2002/657/EC [19]. Four identification points were set for *salinomycin* using one parent and two daughter ions. Quantification is based on the transition m/z 773 > 431 while confirmation is based on the transition m/z 773 > 531 [20].

Table 1 presents the performance characteristics derived from the validation and verification studies [13-17] and recalculated by the EURL [21-25] together with those reported by BVL. Additionally, BVL reported a LOD of 2.3 µg/kg and recoveries ranging from 90.3 to 109 % [18].

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

Table 1. Performance characteristics for the quantification of *salinomycin* residues in chicken *tissues* derived from the validation (Val.) and verification (Ver.) studies [13-17] and recalculated by the EURL [21-25], compared to those reported by the European Union reference Laboratory Pharmacologically Active Substances (BVL).

Tissue		Conc. (µg /kg)	RSD_r (%)	RSD_{ip} (%)
Muscle	BVL	0.75-2.75	14-28	19-28
	Val.	1	2.5	3.6
		10	2.5	4.8
		50	2.2	5.2
		150	4.1	7.3
	Ver.	1	7.2	14.4
		150	4.6	6.3
Liver	BVL	0.75-2.75	14-28	19-28
	Val.	1	5.3	9.2
		10	3.0	7.3
		50	2.8	6.7
		150	3.0	3.3
	Ver.	1	12.0	12.0
		150	9.5	9.5
Kidney	Val.	1	3.8	6.0
		10	3.9	3.9
		50	4.6	5.1
		150	3.8	4.1
	Ver.	1	9.7	9.7
		150	4.3	6.9
Skin/Fat	Val.	1	2.5	8.2
		10	3.7	6.0
		50	1.9	3.5
		150	2.4	2.4
	Ver.	1	8.0	13.2
		150	7.6	8.9

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

Even though the Applicant did not provide data for some of the MRLs proposed (i.e. 30 µg/kg for muscle; 100 µg/kg for kidney; and 200 µg/kg for skin/fat [3-4]), the EURL considers the submitted HPLC-MS/MS method suitable for official control to enforce *salinomycin sodium* at the proposed MRLs in the target *tissues.*

Consequently, the EURL recommends for official control the single laboratory validated and further verified 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 [19] for the enforcement of *salinomycin sodium* levels in chicken *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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for official control (i) the HPLC-PCD-UV-Vis method for the quantification of *salinomycin sodium* 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 *salinomycin sodium* in chicken *tissues*.

Recommended text for the register entry (analytical method)

For the quantification of *salinomycin sodium* in the *feed additive*:

- High Performance Liquid Chromatography using post-column derivatisation coupled to spectrophotometric detection (HPLC-PCD-UV-Vis)

For the quantification of *salinomycin sodium* in *premixtures* and *feedingstuffs*:

- High Performance Liquid Chromatography using post-column derivatisation coupled to spectrophotometric detection (HPLC-PCD-UV-Vis) - EN ISO 14183

For the quantification of *salinomycin sodium* in *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 *Salinomax®120G* 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] Commission Regulation (EC) No 600/2005 of 18 April 2005, concerning a new authorisation for 10 years of a coccidiostat as an additive in feedingstuffs, the provisional authorisation of an additive and the permanent authorisation of certain additives in feedingstuffs.

[2] Commission Regulation (EC) No 496/2007 of 4 May 2007, amending Regulation (EC) No 600/2005 as regards the introduction of a maximum residue limit for the feed

additive 'Salinomax 120G', belonging to the group of coccidiostats and other medicinal substances.

[3] *Application, Reference SANCO/G1: Forw. Appl. 1831/0022-2014

[4] *Application, Proposal for Register Entry – Annex A

[5] *Technical dossier, Section II: II.5 Conditions of use of the additive

[6] 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

[7] EURL Evaluation Report FADs-2012-0041; 2013- 0029 and 2013-0053 https://ec.europa.eu/jrc/sites/default/files/finfep-fad-2013-0053_2013-0029_2012-0041-sacox.pdf

[8] 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

[9] EN ISO 14183:2008 *Animal feedingstuffs – Determination of monensin, narasin and salinomycin contents – Liquid chromatography method using post-column derivatisation* (ISO 14183:2005)

[10] Campbell, H., Nayeri, G. J. AOAC Int. 89 (2006) 1229-1242

[11] *Technical dossier, Section II: Annex II.6.1.1

[12] *Supplementary Information, eurl_anova_calculation.pdf

[13] *Technical dossier, Section II: Annex II.6.2.1

[14] *Technical dossier, Section II: Annexes II.6.2.2 & 6.2.3

[15] *Technical dossier, Section II: Annexes II.6.2.4 & 6.2.5

[16] *Technical dossier, Section II: Annexes II.6.2.6 & 6.2.7

[17] *Technical dossier, Section II: Annexes II.6.2.8 & 6.2.9

[18] 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

[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 result

[20] *Supplementary Information, Confirmatory_method_report.pdf

[21] *Supplementary Information, eurl_anova_kidney.pdf

[22] *Supplementary Information, eurl_anova_liver.pdf

[23] *Supplementary Information, eurl_anova_muscle.pdf

[24] *Supplementary Information, eurl_anova_skin.pdf

[25] *Supplementary Information, eurl_anova_verification.pdf

*Refers to Dossier no: FAD-2014-0016

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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya,Cabrils (ES)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Fødevarestyrelsen, Laboratorierne, Ringsted og Aarhus[1] (DK)
- Federaal Laboratorium voor de Voedselveiligheid Tervuren (FLVVT – FAVV), Tervuren (BE)
- Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors (FI)
- Istituto Superiore di Sanita' - Dipartimento di Sanita' alimentare ed animale, Roma (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- RIKILT-Instituut voor Voedselveiligheid, Wageningen (NL)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)

[1] Name and address according to Regulation (EC) No 885/2009: Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby