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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

Sacox®

(FAD-2012-0041; CRL/120032)
(FAD - 2013-0029; CRL/130023)
(FAD- 2013-0053; CRL/130037)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2012-0041 - CRL/120032** **FAD-2013-0029 - CRL/130023** **FAD-2013-0053 - CRL/130027**
Name of Product:	***Sacox®***
Active Agent (s):	**Salinomycin sodium**
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)** **01/09/2014**
Report approved by: Date:	**Christoph von Holst** **02/09/2014**

EXECUTIVE SUMMARY

Sacox® 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 and reared for laying by Commission Regulations (EC) No 1463/2004 and No 1852/2003. In the current applications authorisation is sought under articles 10(2)[1,2] and under article 13(3)[3] of the Regulation (EC) No 1831/2003. *Sacox®* consists of *salinomycin sodium* (active substance) at concentrations of 120 and 200 g/kg *(Sacox® 120* and *Sacox® 200),* silica dioxide as flowability enhancer and calcium carbonate as structure-forming agent and diluent. *Sacox®* is intended to be incorporated into *feedingstuffs* directly and/or through *premixtures.* The Applicant proposed a concentration of *salinomycin sodium* in *feedingstuffs* of 50 mg/kg for chickens reared for laying or ranging from 60 to 70 mg/kg for chickens for fattening. Furthermore the Applicant proposed two sets of MRLs for *salinomycin* in chicken *tissues*: 5 µg/kg in all wet tissues[1,2] (as already established by Commission Regulation (EC) No 167/2008) or ranging from 12 to 145 µg/kg depending on the target *tissues*[3].

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-UV-Vis). Furthermore, the Applicant adapted the EN ISO 14183 with minor experimental modifications and applied it to the *feed additive* (*Sacox®*) providing similar method performance characteristics. Based on the experimental evidence available the EURL recommends for official control the HPLC-UV-Vis method for the quantification of *salinomycin* in the *feed additive*, *premixtures* and *feedingstuffs*.

For the quantification of *salinomycin* 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 in electrospray ionisation mode using matrix matched standards (RP-HPLC-MS/MS), 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 (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 single-laboratory validated and further verified 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* 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, *Sacox®*, coccidiostat, chickens for fattening & reared for laying

1. BACKGROUND

Sacox® is a *feed additive* - belonging to the group "Coccidiostats and other medicinal substances" listed in Directive 70/524/EEC - currently authorized for chickens for fattening and reared for laying by Commission Regulation (EC) No 1463/2004 [1] and No 1852/2003 [2]. In the current applications authorisation is sought under articles 10(2)[1,2] (authorisation of an existing product) [3,4] and under article 13(3)[3] (modification of the existing authorisation) [5] of the Regulation (EC) No 1831/2003.

Sacox® consists of *salinomycin sodium* (active substance) at concentrations of 120 g/kg *(Sacox® 120)* and 200 g/kg *(Sacox® 200)*, silica dioxide as flowability enhancer and calcium carbonate as structure-forming agent and diluent [6,7]. *Sacox®* is intended to be incorporated into *feedingstuffs* directly and/or through *premixtures.* The Applicant proposed a concentration of *salinomycin sodium* in *feedingstuffs* of 50 mg/kg for chickens reared for laying or ranging from 60 to 70 mg/kg for chickens for fattening [6,8].

The Applicant proposed two sets of MRLs for *salinomycin* in chicken *tissues*: 5 µg/kg in all wet tissues (i.e. muscle, kidney, skin/fat and liver) as already established by Commission Regulation (EC) No 167/2008 [9], or ranging from 12 to 145 µg/kg (12 µg/kg for muscle; 35 µg/kg for kidney; 140 µg/kg for liver; and 145 µg/kg for skin/fat) [6]. These MRLs are not covered by the Commission Regulation (EC) No 37/2010 [10], and need to be evaluated by the EURL.

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

[1] FAD 2012-0041 ; [2] FAD 2013-0029; [3] FAD 2013-0053

methods of analysis submitted in connection with *Sacox®* 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 substances 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) [12], based on High Performance Liquid Chromatography with post-column derivatisation coupled to spectrophotometric detection (HPLC-UV-Vis).

This method can distinguish between the main substances of the *narasin*, *salinomycin* and *monensin* ionophores. The detection principle of post-column derivatisation coupled to spectrophotometric detection (HPLC-UV-Vis) is specific. Potential interferences in the determination of *salinomycin*, caused by other components of the *feed additive*, *premixtures* of *feedingstuffs*, are not expected [13].

Salinomycin sodium 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 [12].

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 [12]:

- 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* (*Sacox®*) the Applicant applied the standard method mentioned above adapting the sample preparation step [14]. The performance characteristics derived from the validation and verification studies [15-19] were recalculated by the EURL leading to precisions (repeatability and intermediate precision) ranging from 0.4 to 0.8 % [20].

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 (Sacox®).*

Based on the performance characteristics available, the EURL recommends for official control the HPLC-PCD-UV-Vis method for the quantification of *salinomycin* 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* in poultry *tissues* the Applicant submitted a single-laboratory validated [21-23] and further verified method (in kidney, skin/fat, muscle and liver) [24-30] based on reverse phase HPLC coupled to a triple quadrupole mass spectrometer in electrospray ionisation mode using matrix matched standards (RP-HPLC-MS/MS).

The minced tissue is extracted with acetonitrile, vortex mixed for 30 s, placed in an ultrasonic bath for 10 min and centrifuged for another 10 min at 4 ^{o}C. The supernatant is then evaporated until dryness under a gentle stream of nitrogen at 40 ± 5 ^{o}C. The dry residue is then dissolved back in acetonitrile vortex mixed for 20 s, placed in an ultrasonic bath for 2 min and vortex mixed again for another 20 s. The resulting solution is filtered through a nylon filter before injection in the HPLC-MS/MS system [21-23].

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* in two target *tissues* (muscle and liver). The EURL already evaluated and recommended this method in the frame of previous *narasin* dossiers [31].

The Applicant validated and further verified the RP-HPLC-MS/MS method at the MRL level of 5 µg/kg in wet *tissues* (i.e. kidney, liver, skin/fat and muscle) thus complying with the requirements defined in "The rules governing medicinal products in the European Union" and in International Guidances (VICH GL49) [32]. Three *salinomycin* levels i.e. MRL/2; MRL and 2MRL were investigated for each target *tissue* [21-30]. 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 complying thus with the confirmatory requirements set by of Commission Decision 2002/657/EC [33].

Table 1 presents the performance characteristics reported in the frame of the validation and verification studies together with those reported by BVL. Additionally, BVL reported LOD of 2.3 µg /kg and recoveries ranging from 90.3 to 109 %.

Table 1. Performance characteristics for the quantification of *salinomycin* residues in chicken *tissues* obtained in the frame of the validation (Val.) and verification (Ver.) studies [21-30], 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.	2.5	12	18
		5.0	9.1	17
		10	9.3	12
	Ver.	5	11-15	13
Liver	BVL	0.75-2.75	14-28	19-28
	Val.	2.5	11	4.9
		5.0	3.9	10
		10	4.8	22
	Ver.	5	5-10	8.7
Kidney	Val.	2.5	4.6	7.5
		5.0	7.4	12
		10	2.2	12
	Ver.	5	5.2-13	9.3
Skin/Fat	Val.	2.5	4.9	6.6
		5.0	3.6	6.3
		10	4.0	7.7
	Ver.	5	2.9-4.4	3.7

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

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. Additionally the satisfactory results provided by the Applicant for kidney and skin/fat *tissues* further demonstrate the applicability - and therefore extension of scope - of the Applicant method to these two additional *tissues*.

Even though the Applicant did not provide data for the second set of MRLs (i.e. 145 µg/kg for skin/fat, 140 µg/kg for liver, 35 µg/kg for kidney and 12 µg/kg for muscle [5]), the EURL considers the submitted HPLC-MS/MS method suitable for official control to enforce *salinomycin* at higher 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 [33] for the enforcement of *salinomycin* 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* 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* in chicken *tissues*.

Recommended text for the register entry (analytical method)

For the quantification of *salinomycin* 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* 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* 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 *Sacox®* 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 (EU) No 1463/2004 of 17 August 2004, concerning the authorisation for 10 years of the additive 'Sacox 120 microGranulate' in feedingstuffs, belonging to the group of coccidiostats and other medicinal substances

[2] Commission Regulation (EU) No 1852/2003 of 21 October 2003, authorising the use for 10 years of a coccidiostat in feedingstuffs

[3] *Application, Reference SANCO/G1: Forw. Appl. 1831/0059-2012

[4] +Application, Reference SANCO/G1: Forw. Appl. 1831/0028-2013

[5] ×Application, Reference SANCO/G1: Forw. Appl. 1831/0048-2013

[6] *+× Application, Proposal for Register Entry - Annex A

[7] *+× Technical dossier, Section II: 2.1 Identity of the additive

[8] *+× Technical dossier, Section II: 2.5 Conditions of use

[9] Commission Regulation (EC) No 167/2008 of 22 February 2008, concerning a new authorisation for ten years of a coccidiostat as an additive in feedingstuffs

[10] 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

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

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

[14] *+× Technical dossier, Section II, References, Reference_II.43 – SOP FA

[15] *+× Technical dossier, Section II, References, Reference_II.44 – Valid FA & PM

[16] *+ Technical dossier, Section II, References, Reference_II.58 – Verif FA 120

[17] *+ Technical dossier, Section II, References, Reference_II.59 – Verif FA 200

[18] × Technical dossier, Section II, References, Reference_II.54 – Verif FA 120

[19] × Technical dossier, Section II, References, Reference_II.55 - Verif FA 200

[20] × Supplementary Information, EURL_ANOVA_calculation.pdf

[21] *+ Technical dossier, Section II, References, Reference_II.47 – Valid tissues

[22] × Technical dossier, Section II, References, Reference_II.56 – Valid tissues

[23] × Supplementary Information, validation muscle.pdf

[24] *+ Technical dossier, Section II, References, Reference_II.48 – Verif liver

[25] *+ Technical dossier, Section II, References, Reference_II.49 – Verif kidney

[26] *+ Technical dossier, Section II, References, Reference_II.50 – Verif skin/fat

[27] × Technical dossier, Section II, References, Reference_II.57 – Verif FA 120

[28] × Technical dossier, Section II, References, Reference_II.58 – Verif kidney

[29] × Technical dossier, Section II, References, Reference_II.59 - Verif skin/fat

[30] × Supplementary Information, verification muscle.pdf

[31] EURL Evaluation Reports FAD 2009-0011 & FAD 2013-0041
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2009-0011.pdf

https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2013-0041-monteban_0.pdf

[32] Guidance for Industry. Studies to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs in Food-Producing Animals: Validation of Analytical Methods used in Residue Depletion Studies. VICH GL49

[33] 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-2012-0041
+ Refers to Dossier no: FAD-2013-0029
× Refers to Dossier no: FAD-2013-0053

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:

- Sachgebiet Futtermittel des Bayrischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL). Oberschleißheim[1] (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Fødevarestyrelsen, Laboratorierne, Ringsted og Aarhus[2] (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft. Nossen[3] (DE)

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