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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

Stenorol®
(FAD-2010-0293; CRL/100257)

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-2010-0293 - CRL/100257**
Name of Product:	***Stenorol*** ®
Active Agent (s):	**Halofuginone hydrobromide – E764**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **28/07/2015**
Report approved by: Date:	**Christoph von Holst** **28/07/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Stenorol®*, under article 10 as a feed additive under the category "coccidiostats and histomonostats", according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for *chickens for fattening* and *turkeys*. *Stenorol®* consists of *halofuginone hydrobromide* as active substance (6 g/kg), povidone (10 g/kg) and macrogolglycerol ricinoleate as excipients (20 g/kg) in a corn cobs carrier. *Stenorol®* is intended to be incorporated directly in *feedingstuffs* or through *premixtures*. The Applicant proposed a concentration range in *feedingstuffs* (from 2 to 3 mg active substance per kg) and several Maximum Residue Limits (MRLs) for *halofuginone hydrobromide* in liver and kidney. As these MRLs are not set by Commission Regulation (EC) No 37/2010 the EURL evaluated the correspondent methods of analysis.

For the quantification of *halofuginone hydrobromide* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on High Performance Liquid Chromatography coupled with UltraViolet detection (HPLC-UV). The following performance characteristics were recalculated by the EURL based on the experimental data provided: a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.9 to 1.8 % and a *recovery rate* (R_{Rec}) ranging from 97 to 102 %. Based on these satisfactory performance characteristics, the EURL recommends for official control, this HPLC-UV method to quantify *halofuginone hydrobromide* in the *feed additive*.

While the Applicant submitted a single laboratory validated method for the quantification of *halofuginone hydrobromide* in *feedingstuffs*, the EURL identified instead the ring-trial validated Community method based on HPLC-UV. The following performance characteristics were reported: a relative standard deviation for *reproducibility* (RSD_R) ranging from 14 to 18%, R_{Rec} ranging from 74 to 88 %, and a Limit of Quantification (LOQ) equal to 1 mg/kg *feedingstuffs*. Based on the performance characteristics presented, the EURL recommends for official control the Community method to quantify *halofuginone hydrobromide* in *feedingstuffs*.

For the quantification of *halofuginone hydrobromide* in *premixtures* the Applicant submitted a single-laboratory validated and further verified HPLC-UV method based on the Community procedure for *feedingstuffs*. The following performance characteristics were recalculated by the EURL based on experimental data obtained using samples containing 100 to 600 mg/kg: a precision (relative standard deviation for *repeatability* (RSD_r) and RSD_{ip}) of 4.9 %, and R_{Rec} ranging from 88 to 100 %. Based on these satisfactory performance characteristics, the EURL recommends for official control the HPLC-UV method for the quantification of *halofuginone hydrobromide* in *premixtures*.

For the quantification of *halofuginone hydrobromide* in target tissues (liver and kidney) the Applicant submitted a single-laboratory and further verified method based on Reversed-Phase HPLC 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 *halofuginone hydrobromide* 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

Halofuginone hydrobromide, Stenorol, coccidiostat, chickens for fattening, turkeys

1. BACKGROUND

In the current application authorisation is sought for *Stenorol®*, under article 10 (authorisation of an existing product), for the category "coccidiostats and histomonostats", according to the classification system of article 6 of Regulation (EC) No 1831/2003. Authorisation is sought for *chickens for fattening* and *turkeys* [1,2].

Stenorol® is a yellow to brown granulated *feed additive* containing chemically synthesised *halofuginone hydrobromide* (6 g/kg) as active substance, povidone (10 g/kg) and macrogolglycerol ricinoleate as excipients (20 g/kg), in a corn cobs carrier [3]. The active substance contains not more than 2% of *cis-isomer of halofuginone* [2,3].

Stenorol® is intended to be incorporated directly in *feedingstuffs* or through *premixtures*, to obtain a final concentration of active substance in *feedingstuffs* ranging from 2 to 3 mg/kg [2,4].

Furthermore the Applicant proposed several Maximum Residue Limits (MRLs) for *halofuginone hydrobromide* in liver and kidney (Table 1) [2]. As these MRLs are not set up by Commission Regulation (EC) No 37/2010 [5], the EURL evaluated the correspondent methods of analysis.

Table 1. Maximum Residue Limits (MRLs) of *halofuginone hydrobromide* in the relevant foodstuffs of animal origin. All values are expressed in µg/kg of fresh material.

MRLs	Liver	Kidney
Chickens for fattening	45	30
Turkeys	20	30

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 *Stenorol®* 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 [6].

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

For the quantification of *halofuginone hydrobromide* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on High Performance Liquid Chromatography coupled with UltraViolet detection (HPLC-UV) [7-10].

An aliquot of *Stenorol®* (4g) is weighted directly into a 300 ml flask. 50 ml of acetonitrile are added for extraction. After 20 min of sonication, 200 ml of mobile phase (acetonitrile:buffer pH 4.3:water - 22:39:39) are added. A second extraction is performed by shaking the solution for 60 min. The obtained extract is filtered and directly injected (20 µl) into the chromatographic system. The active substance is then determined by reversed-phase HPLC, detected at 241 nm and quantified by single point external calibration.

The following performance characteristics were recalculated by the EURL based on the experimental data provided by the Applicant [11]:

- - a relative standard deviation for intermediate precision (RSDip) ranging from 0.9 to 1.8 %; and
- - a recovery rate (RRec) ranging from 97.6 to 102 %.

Based on these satisfactory performance characteristics, the EURL recommends for official control the HPLC-UV method for the quantification of *halofuginone hydrobromide* in the *feed additive*.

A slightly modified version of the above described HPLC-UV method was validated by the Applicant for the quantification of *cis-isomer of halofuginone hydrobromide* in the *feed additive* [7,12]. Satisfactory performance characteristics were reported [13].

For the quantification of *halofuginone hydrobromide* in *feedingstuffs*, the Applicant presented a single laboratory validated method based on the Community method [14-16] where several modifications were implemented. The procedure involves the use of an internal standard (Cebrazolone), solid-phase extraction (SPE) and the quantification via internal calibration curve [15]. The following performance characteristics were obtained in the frame of the validation study [16]: - a relative standard deviation for *repeatability* (RSD_r) ranging from 4.7 to 6.6%; - RSD_{ip} of 5.7% (as recalculated by the EURL) [11]; - R_{Rec} ranging from 86.9 to 92.8 %; and - LOQ of 1 mg/kg [16].

The EURL recommends instead, for the quantification of *halofuginone hydrobromide* in *feedingstuffs,* the ring-trial validated Community method based on HPLC-UV [14]. A sample (10g) is weighted in a test tube. Water, EDTA and sodium ascorbate are added and the solution is mixed and heated in a water bath to 80ºC. After cooling, 10 ml of sodium carbonate solution 10% is added. *Halofuginone hydrobromide* is first extracted as a base in ethyl acetate, then as hydrochloride in an acidic aqueous solution. The extract is purified by ion-exchange chromatography. An aliquot is injected into the HPLC system, the *active substance* detected at 243 nm and quantified by external calibration curve.

The following performance characteristics were reported for the ring-trial validated Community method [14]:

- a relative standard deviation for *reproducibility* (RSD_R) ranging from 14 to 18%;
- R_{Rec} ranging from 74 to 88 %; and
- a Limit of Quantification (LOQ) equal to 1 mg/kg *feedingstuffs.*

For the quantification of *halofuginone hydrobromide* in *premixtures* the Applicant submitted a single-laboratory validated and further verified HPLC-UV method based on the Community method mentioned above [7,14,17-19]. 1g of sample is weighted in a test tube. Water, EDTA and sodium ascorbate are added and the solution is mixed and heated in a water bath to 80ºC. After cooling, 10 ml of sodium carbonate solution 10% is added. *Halofuginone hydrobromide* is first extracted as a base in ethyl acetate, then as hydrochloride in an acidic aqueous solution. The acidic extract obtained is transferred into a 100 ml graduated flask. The internal standard (Cebrazolone) is added, the solution is mixed and made up to the mark. An aliquot is injected into the HPLC system, the *active substance* detected at 243 nm and quantified by calibration curve (plotting the ratio between *halofuginone hydrobromide* peak areas and the

ones of the internal standard) [17]. The following performance characteristics were recalculated by the EURL [11] based on experimental data obtained using samples containing 100 to 600 mg/kg [18-19]:

- RSD_r and RSD_{ip} of 4.9 %;
- R_{Rec} ranging from 88.1 to 100.1 %; and
- LOQ of 10 mg/kg.

Based on these satisfactory performance characteristics, the EURL recommends for official control the HPLC-UV method for the quantification of *halofuginone hydrobromide* in *premixtures*.

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

For the quantification of *halofuginone hydrobromide* in target tissues (liver and kidney) the Applicant submitted a single-laboratory and further verified method based on Reversed-Phase HPLC coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode (ESI) using matrix matched standards [20-25].

Minced tissue is spiked with an appropriate amount of nigericin sodium (internal standard) and *halofuginone hydrobromide* to prepare the matrix-matched calibrators. The sample is vortex mixed. Acetonitrile is added to the tissue samples for a second vortex-homogenisation followed by ultrasonic bath. The sample is then centrifuged for 10 min at 4ºC. The supernatant is transferred into a clean tube and evaporated under nitrogen. The dried residue is reconstituted in 1ml acetonitrile, vortex mixed, sonicated and vortex mixed once again. Finally the extract is filtered and transferred into a vial for injection in the RP-HPLC-MS/MS. *Halofuginone hydrobromide* is detected by MS/MS (positive mode) after ionisation by ESI [20,23].

Target tissues (liver and kidney) of chicken and turkeys were investigated at different *halofuginone hydrobromide* concentrations [20,23]. The method was further verified by a second independent laboratory [21,24]. Three *halofuginone hydrobromide* levels (i.e. MRL/2; MRL and 2MRL) were examined for each target *tissue*. Four identification points were set for *halofuginone hydrobromide* using one parent and two daughter ions [22,25]. Quantification is based on the transition m/z 416.1 > 100.05 while confirmation is based on the transition m/z 416.1 > 138.15 to comply with the confirmatory requirements set by Commission Decision 2002/657/EC [23].

The performance characteristics derived from the validation and verification studies are presented in Table 2. Furthermore the Applicant reported an LOQ of 5 µg/kg for all the target tissues.

Table 2. Performance characteristics of analytical method for the quantification of *halofuginone hydrobromide* in tissues [20-21,23,24].

Tissues		µg/kg (#)	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
			Valid	Verif	Valid	Verif	Valid	Verif
Chicken [20,21]	Liver	22.5	9.0	---	23.9	---	101.1	---
		45	13.3	5.0-6.7	14.1	8.5	93.8	94.1
		90	11.2	---	22.1	---	96.1	---
	Kidney	15	5.0	---	3.8	---	99.6	---
		30	4.0	11.9-14.4	8.3	12.7	92.2	88.2
		60	6.6	---	8.6	---	84.5	---
Turkey [23,24]	Liver	10	14.4	---	26.2	---	90.3	---
		20	11.8	4.8-13.6	22.9	9.9	109.2	106.3
		40	10.2	---	14.5	---	104.4	---
	Kidney	15	5.6	---	22.6	---	93.8	---
		30	7.9	10.7-13.4	14.9	11.8	93.2	102.7
		60	4.4	---	16.4	---	109.7	---

RSD_r: relative standard deviation for *repeatability*; RSD_{ip}: relative standard deviation for *intermediate precision*; R_{Rec}: *recovery* rate; # Fortified level.

Based on these performance characteristics, 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 *halofuginone hydrobromide* in the target tissues.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

− High Performance Liquid Chromatography coupled with UltraViolet detection (HPLC-UV) for the quantification of *halofuginone hydrobromide* in the *feed additive*, *premixtures* and *feedingstuffs*; and

− Reversed-Phase HPLC coupled to triple quadrupole mass spectrometer (RP-HPLC-MS/MS) - or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC - for the quantification of *halofuginone hydrobromide* in chicken and turkey *tissues.*

Recommended text for the register entry (analytical method)

For the quantification of *halofuginone hydrobromide* in the *feed additive* and *premixtures*:

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

For the quantification of *halofuginone hydrobromide* in the *feedingstuff*:

- High Performance Liquid Chromatography coupled with UltraViolet detection (HPLC-UV) - Commission Regulation (EC) No 152/2009

For the quantification of *halofuginone hydrobromide* 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 *Stenorol®* 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. 1831/0020-2012
[2] *Application, Proposal of Registry Entry – Annex A
[3] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[4] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[5] 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
[6] 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
[7] *Technical dossier, Section II, 2.6 Methods of analysis and reference samples
[8] *Technical dossier, Section II, Annexes, Reference II.28
[9] *Technical dossier, Section II, Annexes, Reference II.21
[10] *Technical dossier, Section II, Annexes, Reference II.34
[11] EURL FA data recalculated.xlsx
[12] *Technical dossier, Section II, Annexes, Reference II.29
[13] *Technical dossier, Section II, Annexes, Reference II.22

[14] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, O.J. L 54, 26.02.2009 (Annex IV, D)
[15] *Technical dossier, Section II, Annexes, Reference II.31
[16] *Technical dossier, Section II, Annexes, Reference II.26
[17] *Technical dossier, Section II, Annexes, Reference II.30
[18] *Technical dossier, Section II, Annexes, Reference II.25
[19] *Technical dossier, Section II, Annexes, Reference II.35
[20] *Supplementary information, validation broiler tissues.pdf
[21] *Supplementary information, verification broiler tissues.pdf
[22] *Supplementary information, additional validation chicken tissues.pdf
[23] *Supplementary information, validation turkey tissues.pdf
[24] *Supplementary information, verification turkey tissues.pdf
[25] *Supplementary information, additional validation turkey tissues.pdf
[26] 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-2010-0293

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:

- Fødevarestyrelsens Laboratorie Ringsted (kemisk og mikrobiologisk) (DK)[1]
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Avdelningen för kemi, miljö och fodersäkerhet, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)[2]
- RIKILT Wageningen UR, Wageningen (NL)[3]
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Istituto Superiore di Sanità. Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Roma (IT)[4]
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft, Nossen (DE)[5]
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Ministerio de Agricultura, Alimentación y Medio Ambiente. Madrid (ES)[6]
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)[7]

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[2] Name and address according to Regulation (EC) No 885/2009: Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)
[3] Name and address according to Regulation (EC) No 885/2009: RIKILT-Instituut voor Voedselveiligheid, Wageningen (NL)
[4] Name and address according to Regulation (EC) No 885/2009: Istituto Superiore di Sanita' - Dipartimento di Sanita' alimentare ed animale, Roma (IT)
[5] Name and address according to Regulation (EC) No 885/2009: Sächsische Landesanstalt für Landwirtschaft. Fachbereich 8 – Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
[6] Name and address according to Regulation (EC) No 885/2009: Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid (ES)
[7] Name and address according to Regulation (EC) No 885/2009: Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)