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

**Subject:** Addendum to the EURL evaluation reports

**References:**

FAD-2007-0035 - Cycostat 66G (D08/FSQ/CvH/GS/D(2008)18855)

FAD-2008-0052 - Cycostat 66G (D08/FSQ/CVH/JP/mdr/Ares(2009)213241)

FAD-2013-0051 - Robenz® 66G (JRC.D.5/SFB/CvH/MGH /mds/Ares(2014)3534822)

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 *robenidine hydrochloride* as active substance that have been already evaluated by the EURL and/or are currently authorised by the related Regulations [3-4].

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 *robenidine hydrochloride,* 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 *robenidine hydrochloride* 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 *robenidine hydrochloride* in *compound feeds*.

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

For the determination of *robenidine hydrochloride* in the *feed additive* and *premixtures*:

- High performance liquid chromatography coupled with photometric detection (HPLC-UV)

For the determination of *robenidine hydrochloride* in *compound feed*:

- High performance liquid chromatography coupled with photometric detection (HPLC-UV) - Commission Regulation (EC) No 152/2009 or
- High performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) – EN 17299

For the determination of *robenidine hydrochloride* in *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 Implementing Regulation (EU) 2020/148 of 3 February 2020 concerning the authorisation of robenidine hydrochloride (Robenz 66G) as a feed additive forchickens for fattening and amending Regulation (EC) No 1800/2004 (holder of authorisation Zoetis OJ L 33, 5.2.2020, p. 1

[4] Commission Implementing Regulation (EU) No 532/2011of 31 May 2011concerning the authorisation of robenidine hydrochloride as a feed additive for rabbits for breeding and rabbits for fattening (holder of authorisation Alpharma Belgium BVBA) and amending Regulations (EC) No 2430/1999 and (EC) No 1800/20042020/148 OJ L 146, 1.6.2011, p. 7

Addendum
- Prepared by María José González de la Huebra
- Reviewed and approved by Stefano Bellorini and Christoph von Holst (EURL-FA), respectively, Geel, 26/01/2023

EUROPEAN COMMISSION
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**European Union Reference Laboratory for Feed Additives**

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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

**Robenz® 66G**
***(FAD-2013-0051; CRL/130036)***

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-2013-0051 - CRL/130036** |
| Name of Feed Additive: | ***Robenz® 66G*** |
| Active Agent (s): | **Robenidine hydrochloride** |
| Rapporteur Laboratory: | **European Union Reference Laboratory for Feed Additives (EURL-FA)**<br>**Geel, Belgium** |
| Report prepared by: | **María José González de la Huebra** |
| Report checked by:<br>Date: | **Piotr Robouch (EURL-FA)**<br>**21/10/2014** |
| Report approved by:<br>Date: | **Christoph von Holst**<br>**22/10/2014** |

## EXECUTIVE SUMMARY

*Robenz® 66G* is a feed additive initially authorized for rabbits for breading and chickens, rabbits and turkeys for fattening as *Cycostat 66G* by Commission Regulation (EC) No 1800/2004, belonging to the group "Coccidiostats and other medicinal substances" listed in Chapter I of Annex B of Directive 70/524/EEC. This regulation has been modified by Commission Regulations (EC) No 214/2009 and No 1014/2013. In the current application an authorisation of an existing product under article 10 (2) of the Regulation (EC) No 1831/2003 is requested. *Robenz® 66G* consists of 6.6 % (w/w) of *robenidine hydrochloride* (active substance), calcium lignosulphonate as binder and calcium sulphate dihydrate as diluent/carrier. The Applicant proposed a concentration of *robenidine* in *feedingstuffs* ranging from 30 to 36 mg/kg. Furthermore the Applicant suggested Maximum Residue Limits (MRLs) in wet *tissues* ranging from 200 to 1300 µg/kg for chicken for fattening and from 200 to 400 µg/kg for turkeys for fattening, as already established by Commission Regulation (EC) No 214/2009.

For the quantification of *robenidine* in *feedingstuffs* the Applicant submitted the ring-trial validated Community method based on High Performance Liquid Chromatography coupled to Ultraviolet detection (HPLC-UV). Furthermore the Applicant applied the Community method with minor experimental modifications to the *feed additive* (*Robenz® 66G)* and *premixtures* and obtained similar method performance characteristics. Based on the provided performance characteristics the EURL recommends for official control the HPLC-UV method for the quantification of *robenidine* in the *feed additive, premixtures* and *feedingstuffs*.

For the quantification of *robenidine* in *tissues* (chicken kidney and turkey skin/fat) the Applicant submitted a method based on reverse phase High Performance Liquid Chromatography coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode using matrix-matched standards. This method was developed and validated according to Commission Decision 2002/657/EC by the European Union Reference Laboratory for Pharmacologically Active Substances (BVL). The satisfactory results provided by the Applicant for kidney and skin/fat demonstrate the applicability - and therefore extension of scope - of the BVL method to these two additional *tissues*. Based on the performance characteristics presented the EURL recommends for official control the RP-HPLC-MS/MS method - or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC - for the determination of *robenidine* in chicken and turkey *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**

*Robenidine hydrochloride*, *Robenz® 66G*, coccidiostat, chickens and turkeys for fattening

## 1. BACKGROUND

*Robenz® 66G* is a feed additive initially authorized for rabbits for breading and chickens, rabbits and turkeys for fattening as *Cycostat 66G* by Commission Regulation (EC) No 1800/2004, belonging to the group "Coccidiostats and other medicinal substances" listed in Chapter I of Annex B of Directive 70/524/EEC [1,2]. This regulation has been further modified according to Article 13(3) of Regulation (EC) No 1831/2003 by Commission Regulations (EC) No 214/2009 and No 1014/2013 [2,3]. In the current application an authorisation of an existing product under article 10 (2) of the Regulation (EC) No 1831/2003 is requested for chickens and turkeys for fattening [4,5].

*Robenz® 66G* consists of 6.6 % (w/w) of *robenidine hydrochloride* (active substance), calcium lignosulphonate as binder and calcium sulphate dihydrate as diluent/carrier [6]. *Robenz® 66G* is intended to be incorporated through *premixtures* into *feedingstuffs* [7]. The Applicant proposed a concentration of *robenidine* in *feedingstuffs* ranging from 30 to 36 mg/kg [5].

Furthermore the Applicant suggests Maximum Residue Limits (MRLs) in wet *tissues* ranging from 200 to 1300 µg/kg for chicken for fattening and from 200 to 400 µg/kg for turkeys for fattening, as already established by Commission Regulation (EC) No 214/2009 [2].

Note: The EURL previously evaluated the analytical methods for the determination of *robenidine hydrochloride* in the frame of the FAD 2007-0035 and FAD-2008-0052 dossiers [8].

## 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 *Robenz® 66G* 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 [9].

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

For the quantification of *robenidine* in *feedingstuffs* the Applicant submitted the ring-trial validated Community method based on High Performance Liquid Chromatography coupled to Ultraviolet detection (HPLC-UV) [10]. The scope of the Community method includes only *feedingstuffs*.

*Robenidine* is extracted with acidified methanol. An aliquot of the extract is subjected to a clean-up on an aluminium oxide column. *Robenidine* is eluted with methanol, concentrated, and made up to a suitable volume with mobile phase. The target analyte is determined by reverse-phase HPLC-UV at 317 nm. The following performance characteristics were reported for chicken *feedingstuffs* at a mean *robenidine* content of 27.5 mg/kg:

- a relative standard deviation for *repeatability* ($RSD_r$) ranging from 4.5 to 5.4 %;
- a relative standard deviation for *reproducibility* ($RSD_R$) ranging from 12.0 to 16.1 %;
- a recovery rate ($R_{rec}$) ranging from 90.0 to 93.3 %; and
- a limit of quantification (LOQ) of 5 mg/kg.

The Applicant applied the Community method adapting the sample preparation step to the *feed additive* (*Cycostat 66G)* [11] and to *premixtures* samples [12] (reducing the test portions and eliminating the purification step). Precisions (repeatability and intermediate precision) ranging from 0.5 to 1.1 % were recalculated by the EURL [13] based on the experimental data provided in the frame of the validation studies [11,12]. These performance characteristics are in good agreement with those reported in the Community method as well as with those reported in the frame of previous *robenidine* dossiers [8]. The applicability (extension of the scope) of the Community method to the *feed additive* and *premixtures* is thus demonstrated.

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

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

For the quantification of *robenidine* in *tissues* the Applicant submitted a method based on reverse phase High Performance Liquid Chromatography coupled to a triple quadrupole mass spectrometer (RP-HPLC-MS/MS) in electrospray ionisation mode using matrix-matched standards [14]. This method was developed and validated according to Commission Decision 2002/657/EC [15] by the European Union Reference Laboratory for Pharmacologically Active Substances (BVL) for the determination of *robenidine* in muscle and liver *tissues*. The EURL already evaluated and recommended this method for *robenidine* in *tissues* in the frame of the FAD-2007-0035 dossier [8].

A solution of acetonitrile/methanol (20/80 v/v) is added to the chopped tissue, mixed for 15 min and further centrifuged. An aliquot of the extract is then evaporated, reconstituted and submitted to a solid phase extraction clean-up step. The obtained extract is evaporated again, reconstituted and filtrated before injection in the RP-HPLC-MS/MS system [14].

The Applicant verified the RP-HPLC-MS/MS method in chicken kidney (at MRL/2 level) and turkey skin/fat (at MRL level) (Table 1). Additionally the Applicant reported recovery rates of 97 and 92 % and a detection limits (LOD) of 2.5 and 9.4 µg /kg for the chicken and turkey *tissues*, respectively [16].

Table 1 presents the performance characteristics reported in the frame of the verification studies together with those reported by BVL. Additionally, BVL reported a LOD of 0.56 and 0.71 µg /kg and $R_{rec}$ ranging from 100 to 108 % for muscle and liver *tissues* [14].

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

Consequently, the EURL recommends for official control the RP-HPLC-MS/MS method - or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC - for the determination of *robenidine* in chicken and turkey *tissues*.

**Table 1.** Performance characteristics for the quantification of *robenidine* residues in chicken tissues obtained in the frame of the verification (Ver.) studies, compared to those reported by the European Union reference Laboratory Pharmacologically Active Substances (BVL).

| Tissue | | Conc. (µg /kg) | $RSD_r$ (%) | $RSD_{ip}$ (%) | Ref |
|---|---|---|---|---|---|
| Muscle | BVL | 0.23-1.08 | 10.8-45.8 | 21.7-75.4 | [14] P.17/25 |
| | | 0.5-10 | 4.35 | 9.51 | [14] P.23/25 |
| Liver | BVL | 0.23-1.08 | 10.8-45.8 | 21.7-75.4 | [14] P.17/25 |
| | | 1.0-10 | 12.8 | 16.5 | [14] P.23/25 |
| Kidney (chicken) | Ver | 175 | 3.9-6.3 | 7.4 | [16] |
| Skin/Fat (turkey) | Ver | 400 | 3.6-5.4 | 5.3 | [16] |

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

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-UV method for the quantification of *robenidine* in the *feed additive, premixtures* and *feedingstuffs* and (ii) the RP-HPLC-MS/MS method or any equivalent methods complying with the requirements set by Commission Decision 2002/657/EC for the quantification of *robenidine* in chicken and turkey *tissues*.

***Recommended text for the register entry (analytical method)***

For the quantification of *robenidine* in *feed additive* and *premixtures*:

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

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

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

For the quantification of *robenidine* 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 *Robenz® 66G* 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 1800/2004 of 15 October 2004, concerning the authorisation for 10 years of the additive Cycostat 66G in feedingstuffs, belonging to the group of coccidiostats and other medicinal substances

[2] Commission Regulation (EC) No 214/2009 of 18 March 2009, amending Regulation (EC) No 1800/2004 as regards the terms of the authorisation of the additive Cycostat 66G

[3] Commission Regulation (EU) No 1014/2013 of 22 October 2013, amending Regulations (EC) No 2380/2001, (EC) No 1289/2004, (EC) No 1455/2004, (EC) No 1800/2004, (EC) No 600/2005, (EC) No 874/2010, Implementing Regulations (EU) No 388/2011, (EU) No 532/2011 and (EU) No 900/2011 as regards the name of the holder of the authorisation of certain additives in animal feed

[4] *Application, Reference SANCO/G1: Forw. Appl. 1831/0046-2013

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

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

[7] *Technical dossier, Section II: 2.5 Conditions of use of the additive

[8] EURL Evaluation Reports FAD 2007-0035 & FAD 2008-0052
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2007-0035.pdf
https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2008-0052.pdf

[9] 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

[10] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, Annex IV, part E

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

[12] *Technical dossier, Section II: Annex II.6.1.2

[13] *Supplementary Information, EURL_ANOVA_calculation.pdf

[14] *Technical dossier, Section II: Annex II.6.2.3

[15] 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

[16] *Technical dossier, Section II: Annex II.6.2.4 & 6.2.23

*Refers to Dossier no: FAD-2013-0051

## 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:

- Fødevarestyrelsen, Laboratorierne, Ringsted og Aarhus[1] (DK)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Cabrils (ES)
- Istituto Superiore di Sanita' - Dipartimento di Sanita' alimentare ed animale, Roma (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Federaal Laboratorium voor de Voedselveiligheid Tervuren (FLVVT – FAVV), Tervuren (BE)
- Sachgebiet Futtermittel des Bayrischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL). Oberschleißheim[2] (DE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft. Nossen[3] (DE)
- Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid[4] (ES)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)

[1] Name and address according to Regulation (EC) No 885/2009: Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby
[2] Name and address according to Regulation (EC) No 885/2009: Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim
[3] Name and address according to Regulation (EC) No 885/2009: Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Labore Landwirtschaft, Leipzig
[4] Name and address according to Regulation (EC) No 885/2009: Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid