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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

Vitamin B_2 / Riboflavin
produced by Eremothecium ashbyii CCTCCM 2019833
(FAD-2020-0027; CRL/200011)

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-2020-0027 - CRL/200011**
Name of Feed Additive:	***Vitamin B_2 / Riboflavin produced by Eremothecium ashbyii CCTCCM 2019833***
Active Agent:	**Riboflavin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis** **16/11/2020**
Report approved by: Date:	**Christoph von Holst** **16/11/2020**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *vitamin B_2 / Riboflavin produced by Eremothecium ashbyii CCTCCM 2019833* as *feed additive* under the category/functional group 3(a) "nutritional additive"/"vitamins, provitamins and chemically well-defined substances having a similar effect" according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all animal species.

The product presented by the Applicant contains *riboflavin* produced by fermentation with non-genetically modified *Eremothecium ashbyii CCTCC M 2019833* as *active substance* with a minimum mass fraction of 5 %. The Applicant proposes inclusion levels of the *active substance* ranging from 3 to 80 mg/kg complete *feedingstuffs*. The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*).

For the determination of *riboflavin* in the *feed additive* the Applicant proposed a method based on high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD). The method has been single-laboratory validated for the determination of *vitamin B_2* (as *riboflavin*) in *premixtures* and *feedingstuffs*. On request of the EURL, the method has been further verified by the Applicant in order to extend the scope to the analysis of the *active substance* in the *feed additive* under assessment. The following performance characteristics were reported: a relative standard deviation for *repeatability* (RSD_r) of 2.4 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) of 2.7 %; and a *recovery* rate (R_{rec}) of 98 %. Based on these performance characteristics the EURL recommends for official control the single-laboratory validated and further verified method based on HPLC-FLD to determine *riboflavin* in the *feed additive*.

For the determination of the *riboflavin* in *premixtures*, the Applicant proposed the ring-trial validated method by the Association of German Agricultural Analytical Research Institutes (VDLUFA - Bd. III, 13.9.1) based on ion-pair reversed phase HPLC coupled to UV detection (HPLC-UV). The VDLUFA method is intended for the analysis of *vitamin B_2* in the *feed additive*, *premixtures* and mineral feeds.

The following performance characteristics were reported for the quantification of *vitamin B_2* in *premixture* samples with a content ranging from 868 to 15990 mg/kg: a RSD_r ranging from 2.4 to 4.7 %; a RSD_R ranging from 4.2 to 7.3 %; and a R_{rec} ranging from 86 to 100 %. Furthermore, the following performance characteristics were reported for the quantification of *vitamin B_2* in blends of various vitamins with a content of the *feed additive* of 37.5 g/kg: a RSD_r of 1.2 % and a RSD_R of 5.4 %.

Based on these performance characteristics, the EURL recommends for official control the ring-trial validated VDLUFA method (Bd. III, 13.9.1) to determine *riboflavin* in the *feed additive* and in *premixtures* within the concentration range covered by the collaborative study.

For the determination of *riboflavin* (as total *vitamin B_2*) in *feedingstuffs* the Applicant proposed a ring-trial validated CEN method intended for foodstuffs (EN 14152). The analytical method is based on acidic hydrolysis followed by HPLC-FLD. The CEN method was ring-trial validated using milk powder and pig liver certified reference materials (CRM). The following performance characteristics for the determination of the total *vitamin B_2* content ranging from 145 to 1055 mg/kg were reported: a RSD_r ranging from 1.7 to 3.2 %; a RSD_R ranging from 7.3 to 7.9 %; and a R_{rec} of ca. 100 %. Furthermore, as described in a former EURL report, similar performance characteristics have been obtained by applying the CEN method to the analysis of total *vitamin B_2* in *feedingstuffs* samples, thus confirming the extension of scope of the CEN method to this matrix. Based on these performance characteristics, the EURL recommends for official control the ring-trial validated CEN method (EN 14152:2003) to determine *riboflavin* (as total *vitamin B_2*) in *feedingstuffs*.

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

Vitamin B_2 / Riboflavin produced by Eremothecium ashbyii CCTCCM 2019833, nutritional additives, vitamins, pro-vitamins and chemical well-defined substances having a similar effect, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4 (authorisation of a new *feed additive*) for *vitamin B_2 / Riboflavin produced by Eremothecium ashbyii CCTCCM 2019833* as *feed additive* under the category/functional group 3(a) "nutritional additive"/"vitamins, provitamins and chemically well-defined substances having a similar effect" according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all animal species [1,2].

The product presented by the Applicant is in a form of brown powder containing *riboflavin* as *active substance* with a minimum mass fraction of 5 % [3,4]. *Riboflavin* is produced by fermentation with non-genetically modified *Eremothecium ashbyii CCTCC M 2019833*. The production strain is deposited in the "China Center for Type Culture Collection" (CCTCC) under the accession number *CCTCC M 2019833* [2,4].

The Applicant proposes inclusion levels of the *active substance* ranging from 3 to 80 mg/kg complete *feedingstuffs*. The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) [5].

Vitamin B_2 (riboflavin) produced by different microorganisms is currently authorised as *feed additive* for all animal species by Commission Implementing Regulation (EU) No 2019/901 [6].

Note: The EURL has previously evaluated the analytical methods for the determination of *vitamin B_2 (riboflavin)* in the frame of several dossiers [7].

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 *vitamin B_2 (riboflavin)* 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 determination of *riboflavin* in the *feed additive* the Applicant proposed the methods presented within the European Pharmacopoeia *riboflavin* monograph [8,9]. Identification is based on specific optical rotation, thin-layer chromatography and ultraviolet/visible spectrophotometry, while quantification is based on spectrophotometry at 444 nm. Nevertheless, the abovementioned monograph is related to a product with a minimum purity (mass fraction) of *riboflavin* of 97 %. The product under assessment has a minimum mass fraction of 5 % and, without experimental evidence, the EURL cannot consider the methods proposed as fit-for-purpose [3].

As alternative, the Applicant submitted a method based on high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) [10,11]. The method has been single-laboratory validated for the determination of vitamin B_1 (as thiamine chloride) and *vitamin B_2* (as *riboflavin*) in *premixtures* and *feedingstuffs*. The method is routinely applied by the Applicant and the use in its laboratory is currently accredited by the Dutch

National Accreditation Body Raad voor Accreditatie (RVA) [12]. On request of the EURL, the method has been further verified by the Applicant in order to extend the scope to the analysis of the *active substance* in the *feed additive* under assessment [13]. The *feed additive* has been analysed following the procedure established for a validated range of *riboflavin* content ranging between 0.05 to 100 g/kg *premixtures* [11].

The sample (0.5 to 3 g), 15 g sodium chloride and 50 ml of water are added into a flask and shaken for 15 min. 50 ml of acetonitrile are added to the flask and the solution is further shaken for 15 min. 25 ml of methanol is added, the flask is shaken and the mixture is filtered. According to the range of the calibration curve, the clean solution is appropriately diluted with water and injected into the HPLC. Vitamin B_2 is then determined by FLD at 460 and 520 nm using *riboflavin* as an external standard for calibration.

The following performance characteristics for the determination of *riboflavin* in the *feed additive* have been reported by the Applicant: a relative standard deviation for *intermediate precision* (RSD_{ip}) of 2.4 %, a relative standard deviation for *reproducibility* (RSD_R) of 2.7 % and a recovery rate (R_{rec}) of 98 %.

The presented performance characteristics are in-line with the ones reported within the validation report for the determination of *vitamin B_2* (as *riboflavin*) in *premixtures* and *feedingstuffs* and, thus confirming the extension of scope of the method to the *feed additive*, which is currently under evaluation (*vitamin B_2 / Riboflavin produced by Eremothecium ashbyii CCTCCM 2019833*) [13]. Based on what is mentioned above, the EURL recommends for official control the single-laboratory validated and further verified method based on HPLC-FLD to determine *riboflavin* in the *feed additive*.

For the determination of *riboflavin* in *premixtures*, the Applicant proposed the ring-trial validated method by the Association of German Agricultural Analytical Research Institutes (VDLUFA - Bd. III, 13.9.1) based on ion-pair reversed phase HPLC coupled to UV detection (HPLC-UV). The VDLUFA method is intended for the analysis of *vitamin B_2* in the *feed additive*, *premixtures* and mineral feeds [8,14].

According to the VDLUFA method, the *active substance* is extracted with an aqueous sodium hydroxide solution from 1 to 3 g of a *premixture* sample. The extraction solution is kept in an ultrasonic bath for 1 to 2 min. Afterwards, a phosphate buffer (pH 2.75) is added and an aliquot is taken from this mixture and diluted into a titriplex solution. After filtration, the diluted extract is injected into the HPLC and *riboflavin* is measured by UV. The detector can be set at 275 nm for the simultaneous detection of *vitamin* B_1, *B_2*, B_6, nicotinic acid and nicotinamide, while 268 nm is used to detect *vitamin B_2* alone. The target analytes are quantified using an external calibration.

The following performance characteristics were reported for the quantification of *vitamin B_2* in *premixture* samples with a content ranging from 868 to 15990 mg/kg: a relative standard deviation for *repeatability* (RSD_r) from 2.4 to 4.7 % and a RSD_R from 4.2 to 7.3 %.

Likewise, in the frame of a similar dossier (FAD-2010-0304), a R_{rec} ranging from 86 to 100 % was re-calculated by the EURL based on experimental data provided in the VDLUFA document [14].

Furthermore, the following performance characteristics were reported for the quantification of *vitamin B_2* in blends of pure substances various vitamins with a content of the feed additive ranging from of 37.5 g/kg: a RSD_r of 1.2 % and a RSD_R of 5.4 % [14].

Based on these performance characteristics, the EURL recommends for official control the ring-trial validated VDLUFA method (Bd. III, 13.9.1) to determine *riboflavin* in the *feed additive* and in *premixtures* within the concentration range covered by the collaborative study.

For the determination of *riboflavin* (as total *vitamin B_2*) in *feedingstuffs* the Applicant proposed a ring-trial validated CEN method intended for foodstuffs (EN 14152). The analytical method is based on acidic hydrolysis followed by HPLC-FLD [8,15].

According to the CEN method, hydrochloric or sulfuric acid is added to an appropriate amount of sample to reach a pH lower than 2. The sample is either autoclaved at 120 °C for 30 min or heated at 100 °C for an hour. After cooling to room temperature the extract is adjusted at pH 4 with a sodium acetate solution and 100 mg of taka-diastase per gram of sample is added. The mixture is incubated at 37-46 °C for 16 to 24 hours. Total vitamin B_2 is then determined by HPLC with FLD at 468 and 520 nm using *riboflavin* as an external standard for calibration.

The CEN method was ring-trial validated using milk powder and pig liver certified reference materials (CRM 421 and 487, JRC Geel - Belgium). The following performance characteristics for the determination of the total *vitamin B_2* content ranging from 145 to 1055 mg/kg have been reported: RSD_r ranging from 1.7 to 3.2 %, RSD_R ranging from 7.3 to 7.9 % and R_{rec} of ca. 100 %.

Furthermore, in the frame of an application for an authorised *feed additive* (3a825ii), the CEN method was applied for the determination of total *vitamin B_2* in *feedingstuffs* samples [6,7]. The corresponding Applicant (FAD-2010-0304) reported similar performance characteristics thus confirming the extension of scope of the CEN method to *feedingstuffs*: a relative precision (i.e. repeatability & intermediate precision) ranging from 2.5 to 16 % for *vitamin B_2* contents ranging from 3 to 15 mg/kg, R_{rec} ranging from 93 to 104 % and a limit of detection (LOD) of 0.05 mg/kg *feedingstuffs*.

Based on these performance characteristics, the EURL recommends for official control the ring-trial validated CEN method (EN 14152:2003) to determine *riboflavin* (as total *vitamin B_2*) 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)

An evaluation of corresponding methods of analysis is not relevant for the present application.

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 High Performance Liquid Chromatographic with Fluorescence detection (HPLC-FLD) to determine *riboflavin* in the *feed additive*; (ii) the VDLUFA Bd. III, 13.9.1 method based on ion-pair reversed phase High-Performance Liquid Chromatography with UV detection (HPLC-UV) to determine *riboflavin* in the *feed additive* and in *premixtures*; and (iii) the EN 14152 method based on acidic hydrolysis and enzymatic dephosphorylation followed by High Performance Liquid Chromatographic with Fluorescence detection (HPLC-FLD) to determine *riboflavin* (as <u>total</u> *vitamin B_2*) in *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *riboflavin* in the *feed additive*:

- High Performance Liquid Chromatography with Fluorescence detection (HPLC-FLD); or
- High Performance Liquid Chromatography with UV detection (HPLC-UV) - VDLUFA Bd. III, 13.9.1

For the determination of *riboflavin* in *premixtures*:

- High Performance Liquid Chromatography with UV detection (HPLC-UV) - VDLUFA Bd. III, 13.9.1

For the determination of *riboflavin (*as total *vitamin B_2)* in *feedingstuffs*:

- High Performance Liquid Chromatography with Fluorescence detection (HPLC-FLD) - EN 14152

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *vitamin B2 (riboflavin)* 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, Annex I – submission number 158633563049-2572

[2] *Technical dossier, Section II: 2.1.2 Proposal for classification

[3] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition (active substance/agent, other components, impurities, batch-to-batch variation)

[4] *Technical dossier, Section II: 2.2 Characterisation of the active substance(s)/agent(s)

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

[6] Commission Implementing Regulation (EU) 2019/901 of 29 May 2019 concerning the authorisation of riboflavin produced by Ashbya gossypii (DSM 23096), riboflavin produced by Bacillus subtilis (DSM 17339 and/or DSM 23984) and riboflavin 5′-phosphate sodium salt produced by Bacillus subtilis (DSM 17339 and/or DSM 23984) (sources of vitamin B2) as feed additives for all animal species, O.J. L144/41, 3.06.2019

[7] EURL evaluation reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0053_VitB2.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0177-Riboflavin.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0262-Riboflavin.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0304.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0049.pdf

[8] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance

[9] European Pharmacopoeia monograph - Ph. Eur. 6.0, 01/2008:0292

[10] *Supplementary information: FAD-2020-0027_SIn_Addendum_EURL_Nutreco.pdf

[11] *Supplementary information: DMS-04061 Vitamin B2 HPLC.pdf

[12] https://www.rva.nl/en: L172-sce.pdf

[13] *Supplementary information : 20200914 eurl-fa-verification-form filled final.pdf

[14] VDLUFA Methodenbuch Bd.III, 13.9.1

[15] EN 14152:2003 – Foodstuffs: Determination of vitamin B_2 by HPLC

*Refers to Dossier no: FAD-2020-0027

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

The following National Reference Laboratories contributed to this report:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Wageningen Food Safety Research[1] (WFSR), Wageningen (NL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
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- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)

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