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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)

(FAD-2019-0053; CRL/190031)

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-2019-0053 - CRL/190031**
Name of Feed Additive:	***Vitamin B_2 (riboflavin)***
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:	**María José González de la Huebra** **01/09/2020**
Report approved by: Date:	**Christoph von Holst** **01/09/2020**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4 for *vitamin B_2* (*riboflavin*) 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 genetically modified Bacillus subtilis KCCM-10445 as *active substance* with a minimum purity (mass fraction) of 98 %. According to the Applicant the product is sought for authorisation containing minimum 98 % of the *active substance* or formulated in a preparation constituted by a minimum of 80 % of *riboflavin* and a maximum of 20 % of maltodextrin.

The Applicant proposes inclusion levels of the *active substance* ranging from 3 to 80 mg/kg and specifically for ruminants and horses between 20 and 85 mg/head/day complete *feedingstuffs*. The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking.

For the determination of *riboflavin* in the *feed additive* the Applicant proposed the methods presented within the European Pharmacopoeia *riboflavin* monograph where quantification is based on spectrophotometry at 444 nm. The EURL recommends this method for official control to quantify *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 High Performance Liquid Chromatography coupled to UV detection (HPLC-UV). 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) ranging from 2.4 to 4.7 %; a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.2 to 7.3 %; and a *recovery* rate (R_{rec}) ranging from 86 to 100 %. 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 *premixtures* within the concentration range covered by the collaborative study.

For the determination of *riboflavin* (as total *vitamin B_2*) in *feedingstuffs* and *water* 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 coupled to fluorescence detection (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* and *water* samples thus confirming the extension of scope of the CEN method to these matrices. 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* and *water*.

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 B2 (riboflavin), nutritional additives, vitamins, pro-vitamins and chemical well-defined substances having a similar effect, all animal species.

1. BACKGROUND

In the current application authorisation is sought under Article 4 (authorisation of a new *feed additi*ve) for *vitamin B_2 (riboflavin)* 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 [1]. The authorisation is sought for the use of the *feed additive* for all animal species [1,2].

The product presented by the Applicant contains *riboflavin* as *active substance* with a minimum purity (mass fraction) of 98 % [3,4]. *Riboflavin* is produced by fermentation with genetically modified Bacillus subtilis KCCM-10445. The production strain is deposited in the "Korean Centre of Microorganisms" (KCCM) under the accession number KCCM-10445 [4]. According to the Applicant the orange-yellow crystalline powder *vitamin B_2* is sought for authorisation in two forms: containing a minimum of 98 % of the *active substance* or formulated in a preparation constituted by a minimum (mass fraction) of 80 % of *riboflavin* and a maximum of 20 % of maltodextrin [3].

The Applicant proposes inclusion levels of the *active substance* ranging from 3 to 80 mg/kg and specifically for ruminants and horses between 20 and 85 mg/head/day complete *feedingstuffs*. The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking [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. The EURL recommends this method for official control to quantify *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 High Performance Liquid Chromatography coupled to UV detection (HPLC-UV) [8,10].

According to the VDLUFA method, the *active substance* is extracted with an aqueous sodium hydroxide solution from 1 to 3 g of *premixture* samples. The extraction solution is kept in an ultrasonic bath for 1 to 2 minutes. 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 dilute 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 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 relative standard deviation for reproducibility (RSD_R) from 4.2 to 7.3 %.

Furthermore, in the frame of a similar dossier (FAD-2010-0304), a recovery rate (R_{rec}) ranging from 86 to 100 % was recalculated by the EURL based on experimental data provided in the VDLUFA document [7].

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 *premixtures* within the concentration range covered by the collaborative study.

For the determination of *riboflavin* (as total *vitamin B_2*) in *feedingstuffs* and *water* 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 coupled to fluorescence detection (FLD) [8,11].

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). The following performance characteristics for the determination of the total *vitamin B_2* content ranging from 145 to 1055 mg/kg are 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 %.

(*) derived from the certification exercise of CRM 421 and CRM 487.

Furthermore, in the frame of an application for an authorised *feed additive*, the CEN method was applied for the determination of total *vitamin B_2* in *feedingstuffs* and *water* samples [7].

The corresponding Applicant (FAD-2010-0304) reported similar performance characteristics thus confirming the extension of scope of the CEN method to *feedingstuffs* and *water*:

- 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* or 0.04 mg/L *water*.

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* and *water*.

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 European Pharmacopoeia method using spectrophotometry at 444 nm to quantify *riboflavin* in the *feed additive* (Ph. Eur. 6.0, 01/2008:0292); (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 *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* and *water*.

Recommended text for the register entry (analytical method)

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

- Spectrophotometry at 444 nm - Ph. Eur. 6.0, monograph 01/2008:0292

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 *feedstuffs* and *water*:

- 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 1560330030054-2426

[2] *Technical dossier, Section II: 2.5.2.2 Label requirements

[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-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] VDLUFA Methodenbuch Bd.III, 13.9.1

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

*Refers to Dossier no: FAD-2019-0053

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

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
- Wageningen Food Safety Research (WFSR) (NL), Wageningen (NL)[1]
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)

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