EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

European Union Reference Laboratory
Feed Additives

JRC F.5/UV/SB/AS/Ares

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 Saccharomyces cerevisiae CEN.PK113-7D

(FEED-2021-1475; CRL/220059)

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:	**FEED-2021-1475 - CRL/220059**
Name of Product:	***Vitamin B2 / Riboflavin, produced by Saccharomyces cerevisiae* CEN.PK113-7D**
Active Agent:	**Riboflavin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Zigmas Ezerskis** **23/02/2024**
Report approved by: Date:	**Ursula Vincent** **23/02/2024**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *vitamin B_2* 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* as *active substance* produced by fermentation with a genetically modified *Saccharomyces cerevisiae* CEN.PK113-7D. According to the Applicant, the product under the application is a preparation containing a minimum of 80 % (w/w) of *riboflavin*. The *feed additive* preparation is intended to be added directly into *compound feed* (or through *premixtures*) and *water* for drinking. However, the Applicant did not propose any specific inclusion level of the *active substance* in the above-mentioned matrices.

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 VDLUFA method is intended for the analysis of *vitamin B_2* in the *feed additive*, *premixtures* and mineral feed. The following performance characteristics were reported for the determination of *vitamin B_2* in (i) *premixture* samples with a *vitamin B_2* 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 %; and in (ii) blends of various vitamins with a content of *vitamin B_2* in 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* preparation and in *premixtures*.

For the determination of *riboflavin* (as total *vitamin B_2*) in *compound feed* 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 *compound feed* 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 *compound feed* 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 B_2 (riboflavin), 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 Saccharomyces cerevisiae* CEN.PK113-7D 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].

Vitamin B_2 is produced by fermentation with a genetically modified *Saccharomyces cerevisiae* CEN.PK113-7D [3]. According to the Applicant, the product under the application is a preparation containing a minimum of 80 % (w/w) of the powdered orange-yellow crystalline *riboflavin* as *active substance* diluted with maltodextrin [1,4].

The *feed additive* is intended to be added directly into *feed* (or through *premixtures*) and *water* for drinking. The Applicant did not propose any specific inclusion level of the *active substance* but its recommended dosage is ranging from 3 to 80 mg/kg *compound feed*. Specifically, for ruminants and horses the recommended dosage is between 20 and 85 mg/head/day [5].

Vitamin B_2 (riboflavin) produced by different microorganisms is currently authorised as *feed additive* for all animal species by the following Commission Implementing Regulations: (EU) No 2019/901, (EU) No 2023/651 and (EU) No 2023/1705 [6-8].

Note: The EURL has previously evaluated the analytical methods for the determination of *vitamin B_2 (riboflavin)* in the frame of several dossiers [9].

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* 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*, *premixtures*, *compound feed* and *water* the Applicant proposed the methods included in the Commission Implementing Regulation (EU) 2019/901 concerning the authorisation of *riboflavin* as *feed additive* for all animal species [6].

Regarding the determination of the *active substance* in the *feed additive*, the Applicant proposed the European Pharmacopoeia *riboflavin* monograph [10,11]. Identification is based on specific optical rotation, thin-layer chromatography and ultraviolet/visible spectrophotometry, while quantification is based on spectrophotometry at 444 nm. However, the above-mentioned monograph is related to a product with a minimum purity (mass fraction) of riboflavin of 97 % while the product under assessment has a minimum purity (mass fraction) of 80 %. Furthermore, the Applicant didn't provide any specific experimental evidence demonstrating the fitness-for-purpose of the methods presented within the European Pharmacopoeia *riboflavin* monograph [10]. Therefore, the EURL cannot consider the methods proposed as fit-for-purpose in the frame of the present dossier.

However, for a similar authorised *feed additive* (3a825V, FAD-2020-0061 preparation with minimum of 80 % of riboflavin), the EURL, for the determination of *riboflavin* in the *feed additive*, evaluated and recommended the VDLUFA (Association of German Agricultural Analytical Research Institutes) method (Bd. III, 13.9.1) based on ion-pair reversed phase HPLC coupled to UV detection (HPLC-UV) [9,12]. The VDLUFA method is intended for the analysis of *vitamin B_2* in the *feed additive*, *premixtures* and mineral feeds [12]. Furthermore, the same method was proposed in the frame of the present application for the determination of *riboflavin* in *premixtures* [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 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 standard calibration. The following performance characteristics were reported for the determination 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 % and a relative standard deviation for *reproducibility* (RSD_R) from 4.2 to 7.3 %; - 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 %.

Likewise, in the frame of a similar dossier (FAD-2010-0304), an R_{rec} ranging from 86 to 100 % was re-calculated by the EURL based on experimental data provided in the VDLUFA document [9].

Based on the above mentioned data, the EURL recommends for official control the ring-trial validated VDLUFA method (Bd. III, 13.9.1) to determine *riboflavin* in the *feed additive* preparation and in *premixtures*.

For the determination of *riboflavin* (as total *vitamin B_2*) in *compound feed* 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) [10,13].

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 1 h. 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 h. 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 (CRMs). The following performance characteristics for the determination of the total vitamin B_2 content ranging from 145 to 1055 mg/kg are reported (performance characteristics were derived from the certification exercise for CRM 421 and CRM 487): RSD_r ranging from 1.7 to 3.2 %; RSD_R ranging from 7.3 to 7.9 %; and R_{rec} of 100 %.

Furthermore, the CEN method was applied in the frame of an application for an authorised feed additive (3a825ii) for the determination of total vitamin B_2 in *compound feed* and *water* samples [6,9]. The corresponding Applicant (FAD-2010-0304) reported similar performance characteristics thus confirming the extension of scope of the CEN method to *compound feed* and *water* [9]: a relative standard deviation for *repeatability* and *intermediate precision* ranging from 2.5 to 16 % measured on samples containing *vitamin B_2* between 3 and 15 mg/kg; R_{rec} ranging from 93 to 104 %; a limit of quantification (LOQ) of 0.2 mg/kg *compound feed* or *water*; and a limit of detection (LOD) of 0.05 mg/kg *compound feed* 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 *compound feed* 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 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 *premixtures*; and (ii) 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 total *vitamin B_2*) in *compound feed* and *water*.

Recommended text for the register entry (analytical method)

For the determination of *riboflavin* in the *feed additive* and *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 *compound feed* 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 B_2 / Riboflavin produced by Saccharomyces cerevisiae CEN.PK113-7D* 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] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform: https://webgate.ec.europa.eu/esfc/#/applications/2224 https://open.efsa.europa.eu/questions/EFSA-Q-2022-00846

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

[3] *Technical dossier, Section I: 1.2 Scientific summary of the dossier, section III, studies concerning safety of the additive

[4] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[5] *Technical dossier, Section II: 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 DSM23984) (sources of vitamin B2) as feed additives for all animal species, O.J. L144/41, 3.06.2019

[7] Commission Implementing Regulation (EU) 2023/651of 20 March 2023 concerning the authorisation of riboflavin (vitamin B2) produced by Bacillus subtilis KCCM 10445 and a preparation of riboflavin produced by Bacillus subtilis KCCM 10445 as feed additives for all animal species OJ L 81, 21.03.2023

[8] Commission Implementing Regulation (EU) 2023/1705 of 7 September 2023 concerning the authorisation of a preparation of riboflavin (vitamin B2) produced by Bacillus subtilis CGMCC 13326 as a feed additive for all animal species, OJ L 221, 8.9.2023

[9] EURL evaluation reports:
https://joint-research-centre.ec.europa.eu/publications/fad-2020-0027_en
https://joint-research-centre.ec.europa.eu/publications/fad-2019-0053_en
https://joint-research-centre.ec.europa.eu/publications/fad-2011-0051_en
https://joint-research-centre.ec.europa.eu/publications/fad-2010-0177_en
https://joint-research-centre.ec.europa.eu/publications/fad-2010-0262_en
https://joint-research-centre.ec.europa.eu/publications/fad-2010-0304_en
https://joint-research-centre.ec.europa.eu/publications/fad-2010-0049_en

[10] *Technical dossier, Section II: 2.6 Methods of analysis

[11] European Pharmacopoeia monograph - Ph. Eur. 6.0, monograph 0292

[12] VDLUFA Methodenbuch Bd.III, 13.9.1

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

*Refers to Dossier no: FEED-2021-1475

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)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Wageningen Food Safety Research (WFSR), Wageningen (NL)[1]
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer (DE)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)

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