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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 produced by Bacillus subtilis CGMCC 13326

(FAD-2020-0061; CRL/200051)

European Union Reference Laboratory
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Dossier related to:	**FAD-2020-0061 - CRL/200051**
Name of Product:	***Vitamin B_2 produced by Bacillus subtilis CGMCC 13326***
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** **19/04/2021**
Report approved by: Date:	**Christoph von Holst** **19/04/2021**

EXECUTIVE SUMMARY

In the current application 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 genetically modified *Bacillus subtilis CGMCC 13326*. According to the Applicant the product is sought for authorisation formulated in a preparation containing a minimum of 80 % of *riboflavin* diluted with corn starch. The Applicant did not propose any specific inclusion level of the active substance. The *feed additive* preparation is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking.

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 feeds. The following performance characteristics were reported for the quantification of *vitamin B_2* in (i) *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 %; and in (ii) 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 preparation* and in *premixtures*.

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 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 authorisation is sought under Article 4 (authorisation of a new feed additive) for *vitamin B_2 produced by Bacillus subtilis* CGMCC 13326 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 B2 is produced by fermentation with genetically modified *Bacillus subtilis* CGMCC 13326 [3]. The production strain is deposited in the "China General Microbiological Culture Collection Center" (CGMCC) under the deposition number CGMCC 13326. According to the Applicant the fine off-white powder *feed additive* is sought for authorisation in a formulated preparation constituted by a minimum (mass fraction) of 80 % of the *active substance riboflavin* diluted with corn starch [1,4].

The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking. The Applicant did not propose any specific inclusion level of the *active substance* [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* 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 80 % and, without experimental evidence, the EURL cannot consider the methods proposed as fit-for-purpose [4]. Instead, the applicant successfully applied within the frame of the stability study of this *feed additive preparation* the VDLUFA method - Bd. III, 13.9.1 of the Association of German Agricultural Analytical Research Institutes to the determination of *vitamin B_2* in this matrix [10-12].

For the determination of *riboflavin* in *premixtures*, the Applicant proposed the ring-trial validated VDLUFA - Bd. III, 13.9.1 method 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 [9,10].

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 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 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 %;

- 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 [7,10].

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 *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) [9,13].

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 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 (CRMs). 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 for CRM 421 and CRM 487.

Furthermore, the CEN method was applied in the frame of an application for an authorised feed additive (3a825ii) to the determination of total vitamin B_2 in *feedingstuffs* and *water* 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* and *water*:

- 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 %; 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 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 *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the determination of *riboflavin* in the *feed additive preparation* 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 *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 B_2 produced by Bacillus subtilis CGMCC 13326* 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 SANTE/E5: Forw. Appl. 1831-0058-2020 & Annex I - submission number 1597738793839-2658

[2] *Technical dossier, Section II: II.5.2.3 Labelling requirements

[3] *Technical dossier, Section II: II.2.1.2 Micro-organisms

[4] *Technical dossier, Section II: II.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] EURL evaluation reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2020-0027_vitb2.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0053_vitb2.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep2_fad-2011-0051_riboflavin%20sodium%20phosphate.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] European Pharmacopoeia monograph - Ph. Eur. 6.0, 01/2008:0292

[9] *Technical dossier, Section II: II.6.1 Methods of analysis for the active substance

[10] VDLUFA Methodenbuch Bd.III, 13.9.1

[11] *Technical dossier, Section II: Annex 2.1.3.a BtB Impurities stability 106244_UK.pdf

[12] *Technical dossier, Section II: Annex 2.4.1.b Stability Premix Feed F106634_UK.pdf

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

*Refers to Dossier no: FAD-2010-0322

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)
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- Wageningen Food Safety Research (WFSR), Wageningen (NL)[1]
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Ruokavirasto, Helsinki (FI)[2]
- 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)

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[2] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors (FI).