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

Subject: Addendum to the EURL evaluation report

Reference: FAD-2020-0094 – Vitamin B_2 / riboflavin 5'-phosphate ester monosodium salt (solid form produced after phosphorylation of riboflavin 98 % produced by Bacillus subtilis KCCM-10445), JRC F.5/CvH/SB/AS/Ares(2021)4266135

In the corresponding report related to this dossier FAD-2020-0094, the EURL could not recommend a method of analysis for the determination of the active substance *riboflavin 5'-phosphate ester monosodium salt* in *premixtures* [1]. The Applicant proposed the ring-trial validated method published by the Association of German Agricultural Analytical Research Institutes (VDLUFA - Bd. III, 13.9.1) [2]. Nevertheless, the method, based on ion-pair reversed phase High Performance Liquid Chromatography coupled to UV detection (HPLC-UV), is not intended for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures* and therefore the EURL was unable to recommend it for the official control. Subsequently, DG SANTE requested the Applicant to provide corresponding supplementary information [3]. The Applicant provided the requested information and, in the present document, the EURL evaluates the fitness for purpose [4-5].

For the determination of *riboflavin 5'-phosphate* ester monosodium salt (as total riboflavin/vitamin B2) in *premixtures* the Applicant proposes a single-laboratory validated analytical method based on liquid chromatography coupled to fluorescence detection (HPLC-FLD) [5]. The method is similar to the ring-trial validated CEN method EN14152 [6].

Vitamin B_2 is extracted from a known amount of the *premixture* sample with a solution of sulfuric acidified water. *Riboflavin 5'-phosphate ester monosodium salt* is then enzymatically hydrolysed with α-amylase to convert the *vitamin B_2* phosphate into free *vitamin B_2*. The acidic aqueous phase is then analysed using reversed-phase HPLC-FLD. Total *vitamin B_2* is then determined at 460 and 520 nm (respectively excitation and emission wavelength) using riboflavin as an external standard for calibration [5]. The Applicant reported relative standard deviations for repeatability (RSD_r) and intermediate precision (RSD_{ip}) ranging from 12.9 to 14.7 %, and recovery rates (R_{rec}) ranging from 90.8 to 102.5 % [4].

Based on the overall available performance data, the EURL recommends for official control the above-mentioned single-laboratory validated reversed-phase HPLC-FLD method for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures*.

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.

Recommended text for the registry entry (analytical method) as *addendum* to the previous recommendations

For the determination of *riboflavin 5'-phosphate ester monosodium salt (*as total *vitamin B_2)* in *premixtures*:

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

References

[1] EURL report – FAD-2020-0094 (Vitamin B2 / riboflavin 5'-phosphate ester monosodium salt - solid form produced after phosphorylation of riboflavin 98 % produced by Bacillus subtilis KCCM-10445), Ref. Ares(2021)4266135 – 30/06/2021 https://joint-research-centre.ec.europa.eu/publications/fad-2020-0094_en

[2] VDLUFA Methodenbuch Bd.III, 13.9.1

[3] 9919721 - Zhengdong Lu - 0082-2020 RQST sup info, Ref. Ares SANTE G5/AR/acg (2022) 9919721

[4] Validation report VitaminB2-phosphate, version august 2023

[5] DMS-04061 EN Vitamin B1 and B2 with HPLC-FLU

[6] EN 14152:2003 – Foodstuffs: Determination of vitamin B2 by HPLC

Addendum

- Prepared by Stefano Bellorini
- Reviewed and approved by Christoph von Holst (EURL-FA), Geel, 26/10/2023

EUROPEAN COMMISSION
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European Union Reference Laboratory
Feed Additives

JRC F.5/CvH/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 5'-phosphate ester monosodium salt (solid form produced after phosphorylation of riboflavin 98 % produced by Bacillus subtilis KCCM-10445)
(FAD-2020-0094; CRL/200030)

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-2020-0094 - CRL/200030**
Name of Product:	***Vitamin B_2 / riboflavin 5'-phosphate ester monosodium salt (solid form produced after phosphorylation of riboflavin 98 % produced by Bacillus subtilis KCCM-10445)***
Active Agent (s):	**Riboflavin 5'-phosphate ester monosodium salt**
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** **30/06/2021**
Report approved by: Date:	**Christoph von Holst** **30/06/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *vitamin B_2* (*riboflavin 5'-phosphate ester monosodium salt*) 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 5'-phosphate ester monosodium salt* as *active substance* with a purity (mass fraction) of 73 to 79 % *riboflavin* based on dry substance.

The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking. The Applicant recommended inclusion levels of the *active substance* ranging from 4 to 110 mg/kg complete compound feed and specifically for ruminants and horses between 27 and 116 mg/head/day.

For the determination of *riboflavin 5'-phosphate ester monosodium salt* in the *feed additive* the Applicant proposed the methods presented within the European Pharmacopoeia *riboflavin sodium phosphate* monograph where the quantification is based on spectrophotometry at 444 nm. The EURL recommends this method for official control to quantify *riboflavin 5'-phosphate ester monosodium salt* 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 method proposed is not intended for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures* and the EURL cannot recommend this method for official control for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures*.

For the determination of *riboflavin 5'-phosphate ester monosodium salt* (as total *riboflavin/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 enzymatic dephosphorylation and further analysis using 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 relative standard deviation for *repeatability* (RSD_r) ranging from 1.7 to 3.2 %; a relative standard deviation for *reproducibility* (RSD_R) ranging from 7.3 to 7.9 %;

and a *recovery* rate (R_{rec}) of ca. 100 %. Furthermore, as described in a former EURL report, similar performance characteristics have been obtained by applying the CEN method for the analysis of total *vitamin B_2* in samples of *feedingstuffs* and *water* 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 5'-phosphate ester monosodium salt* (as total *riboflavin/vitamin B_2*) in *feedingstuffs* and *water*.

The same method could be used for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures* by applying a proper dilution. However, as no experimental data were provided to confirm this, the EURL cannot evaluate or recommend this method for official control for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures*.

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 5'-phosphate ester monosodium salt (solid form produced after phosphorylation of riboflavin 98 % produced by Bacillus subtilis KCCM-10445), 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 5'-phosphate ester monosodium salt (solid form produced after phosphorylation of riboflavin 98 % produced by Bacillus subtilis KCCM-10445)* 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 5'-phosphate ester monosodium salt* as *active substance* with a purity (mass fraction) of 73 to 79 % *riboflavin* based on dry substance [3,4]. *Riboflavin 5'-phosphate ester monosodium salt* is synthesised after phosphorylation of *riboflavin* 98 % (w/w) produced by fermentation with Bacillus subtilis KCCM-10445 [5]. According to the Applicant, the orange-yellow crystalline powder of *vitamin B_2* is sought for authorisation in solid form [6].

The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking [2]. The Applicant did not propose maximum limits, while the recommended inclusion levels of the *active substance* are ranging from 4 to 110 mg/kg compound feed and specifically for ruminants and horses between 27 and 116 mg/head/day [2].

Vitamin B_2 / riboflavin 5'-phosphate ester monosodium salt is currently authorised as *feed additive* for all animal species by Commission Implementing Regulation (EU) No 2019/901 [7].

Note: The EURL has previously evaluated the analytical methods for the determination of *riboflavin 5'-phosphate ester monosodium salt* in the frame of several dossiers [8].

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 *riboflavin 5'-phosphate ester monosodium salt* 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 5'-phosphate ester monosodium salt* in the *feed additive* the Applicant proposed the methods presented within the European Pharmacopoeia *riboflavin sodium phosphate* monograph [9,10]. The identification is based on ultraviolet/visible spectrophotometry and liquid chromatography, while the quantification is based on spectrophotometry at 444 nm. The EURL recommends the method based on spectrophotometry at 444 nm for official control to quantify *riboflavin 5'-phosphate ester monosodium salt* 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 High Performance Liquid Chromatography coupled to UV detection (HPLC-UV) [9,11].

The method proposed is not intended for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures* and the EURL cannot recommend this method for official control for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures*.

For the determination of *riboflavin 5'-phosphate ester monosodium salt* (as total *riboflavin/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 enzymatic dephosphorylation and further analysis using HPLC coupled to fluorescence detection (FLD) [9,12].

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 to pH 4 with a sodium acetate solution and 100 mg of taka-diastase enzyme per gram of sample is added to achieve a dephosphorylation. The mixture is incubated at 37 to 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 (CRM). The following performance characteristics for the determination of the total *vitamin B_2* content in the CRMs ranging from 145 to 1055 mg/kg are reported (*): a relative standard deviation for *repeatability* (RSD_r) ranging from 1.7 to 3.2 %; a relative standard deviation for *reproducibility* (RSD_R) ranging from 7.3 to 7.9 %; and a *recovery* rate (R_{rec}) of ca. 100 %. Note: (*) 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 has been applied for the determination of total *vitamin B_2* in *feedingstuffs* and *water* samples [8]. The corresponding Applicant (FAD-2010-0304) reported similar performance characteristics thus confirming the extension of the scope of the CEN method to *feedingstuffs* and *water*: a relative standard deviations for RSD_r and an *intermediate precision* (RSD_{ip}) ranging from 2.5 to 16 % for a *vitamin B_2* content ranging from 3 to 15 mg/kg; a 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 5'-phosphate ester monosodium salt* (expressed as total *riboflavin*/*vitamin B_2*) in *feedingstuffs* and *water*.

The same method could be used for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures* by applying a proper dilution. However, as no experimental data were provided to confirm this, the EURL cannot evaluate or recommend this method for official control for the determination of *riboflavin 5'-phosphate ester monosodium salt* in *premixtures*.

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 5'-phosphate ester monosodium salt* in the *feed additive* (Ph. Eur. 10.5, 01/2017:0786) 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 5'-phosphate ester monosodium salt* (expressed as total *riboflavin/vitamin B_2*) in *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the determination of *riboflavin 5'-phosphate ester monosodium salt* in the *feed additive*:

- Spectrophotometry at 444 nm - European Pharmacopoeia Monograph 0786

For the determination of *riboflavin 5'-phosphate ester monosodium salt (*as total *vitamin B_2)* in *feedingstuffs* 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 *riboflavin 5'-phosphate ester monosodium salt* 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. 1831-0082-2020 & Annex I – submission number 1604483294297-2724

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

[3] *Technical dossier, Section II: 2.5.2.3 Labelling requirements

[4] *Technical dossier, Section II: 2.1.4 Purity

[5] *Technical dossier, section II: 2.2.1.2 Micro-organisms

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

[7] 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

[8] EURL evaluation reports:

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-2010-0304.pdf

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

[10] European Pharmacopoeia monograph - Ph. Eur. 10.5, 01/2017:0786

[11] VDLUFA Methodenbuch Bd.III, 13.9.1

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

*Refers to Dossier no: FAD-2020-0094

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)
- 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)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)