EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
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European Union Reference Laboratory for Feed Additives

JRC.DG.D.6/CvH/ZE/mds/ARES(2011)1266605

EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for the Authorisation of Feed Additives according to Regulation (EC) No 1831/2003

Dossier related to:	FAD-2010-0304 - CRL/100183
Feed Additive Name:	Vitamin B_2
Active Substance(s):	Riboflavin Riboflavin 5'-phosphate ester monosodium salt
Rapporteur Laboratory:	Austrian Agency for Health and Food Safety (AGES)
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Report approved by: Date:	Christoph von Holst 25/11/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Vitamin B_2* (*Riboflavin* and *Riboflavin 5'-phosphate ester monosodium salt*) under the category/functional group 3(a) 'nutritional additives'/'vitamins, pro-vitamins and chemically well defined substances having similar effect' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the two *feed additives* for all animal species and categories. According to the Applicant the products are powders, containing at least 80% of *Riboflavin* and 65% of *Riboflavin 5'-phosphate ester monosodium salt*. The *feed additives* are intended to be incorporated in *feedingstuffs* (directly or through *premixtures*) and *water*. However, the Applicant did not specify any maximum or minimum concentration of *Vitamin B_2* in *feedingstuffs* or *water*.

For the determination of *Riboflavin* in the *feed additives* the Applicant proposed the European Pharmacopoeia method (Ph. Eur. 6.0, method 01/2008:0292). Even though no performance characteristics are provided, the EURL recommends this method for official control to determine *Riboflavin* in the *feed additive*.

For the determination of the *Riboflavin* in *premixtures*, the Applicant proposed a single-laboratory validated method, similar to the official ring-trial validated VDLUFA method, based on ion pair reverse phase High Performance Liquid Chromatography couple to an UV detector (HPLC-UV). The performance characteristics determined using *premixtures* samples (only) with a *Vitamin B_2* content ranging from 868 to 15990 mg/kg are: - a relative standard deviation for *repeatability* (RSDr) from 2.4 to 4.7 %, and - a relative standard deviation for *reproducibility* (RSD_R) from 4.2 to 7.3 %. Furthermore, the *recovery* rate (R_{Rec}) ranging from 86 to 100% was calculated by the EURL based on experimental data provided in the VDLUFA document. 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* in *feedingstuffs* and *water* the Applicant proposed a single-laboratory validated method, similar to the ring trial validated CEN method (EN 14152) intended for foodstuffs, based on acid hydrolysis followed enzymatic dephosphorylation and using High Performance Liquid Chromatography (HPLC) with fluorimetric detection. The CEN method was ring trial validated using milk powder and pig liver certified reference materials (CRM). The following performance characteristics for 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%. Furthermore, the Applicant applied the CEN method for determination of total *Vitamin B_2* to *feedingstuffs* and *water*

samples and reported similar performance characteristics, thus confirming the extension of scope of the CEN method to *feedingstuffs* and *water.* Based on these performance characteristics, the EURL recommends for official control the ring-trial validated CEN method (EN 14152:2003) based on the enzymatic dephosphorylation followed by HPLC with fluorimetric detection to determine total *Vitamin B_2* (expressed as *Riboflavin)* in *feedingstuffs* and *water*.

For the determination of *Riboflavin 5'-phosphate ester monosodium salt* in all the matrices under investigation the Applicant proposed the US Pharmacopoeia method, without providing any experimental data proving the applicability of this method to *premixtures*, *feedingstuffs* and *water*. Therefore the EURL could not evaluate nor recommend this method for official control of the above mentioned matrices. Additionally the Applicant submitted for the determination of *Riboflavin 5'-phosphate ester monosodium salt* in the *feed additive* the European Pharmacopoeia method (Ph. Eur. 6.0, method 01/2008:0786). Even though no performance characteristics are provided, the EURL recommends this method for official control to determine *Riboflavin 5'-phosphate ester monosodium salt* in the *feed additive*.

Furthermore, due to the enzymatic dephosphorylation step included in the evaluated earlier ring-trial validated CEN method (EN 14152) [11] the EURL considers this method suitable for the determination of *Riboflavin 5'-phosphate ester monosodium salt* (expressed as total *Riboflavin*) in *feedingstuffs* and *water*.

The same method could be used for the determination of *Riboflavin 5'-phosphate ester monosodium salt* in *premixtures* by applying proper dilution. However, as no experimental data were provided to confirm this, the EURL cannot evaluate nor recommend this method for official control 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) is not considered necessary.

KEYWORDS

Vitamin B_2, Riboflavin, Riboflavin 5'-phosphate ester monosodium salt, nutritional additives, vitamins, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use in water) and 10(2) (re-evaluation of the already authorised additives under provisions of Council Directive 70/524/EEC) for *Vitamin B_2* (*Riboflavin* and *Riboflavin 5'-phosphate ester monosodium salt*) under the category/functional group 3(a) 'nutritional additives'/'vitamins, pro-vitamins and chemically well defined substances having similar effect' according to Annex I of Regulation (EC) No 1831/2003 [1].

Authorisation is sought for the use of the two *feed additives* for all animal species and categories [2]. Both feed additives submitted by the Applicant (*Riboflavin* and *Riboflavin 5'-phosphate ester monosodium salt*) are produced using genetically modified *Bacillus subtilis* strains [3]. According to the Applicant the products (Riboflavin Universal, ROVIMIX® B_2 80-SD and Riboflavin 5'-Phosphate Sodium) are powders, containing at least 96 % & 80 % of *Riboflavin* and 65% of *Riboflavin 5'-phosphate ester monosodium salt*, respectively [3].

The *feed additives* are intended to be incorporated in *feedingstuffs* (directly or through *premixtures*) and *water* [2, 3]. However, the Applicant did not specify any maximum or minimum concentration of *Vitamin B_2* in *feedingstuffs* or *water* [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 *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

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [4].

Lumiflavin, a toxic photoderivative of riboflavin, does not exceed 0.025% in Riboflavin Universal formulation [3], and it can be detected with liquid chromatography [5].

Description of the analytical methods for the determination of the active substance in feed additive, premixtures, feedingstuffs and water

Riboflavin

For the determination of *Riboflavin* in the *feed additives* the Applicant proposed the European Pharmacopoeia method [6]. Identification is based on specific optical rotation, thin-layer chromatography and ultraviolet/visible spectrophotometry while quantification is based on spectrophotometry at 444 nm. Even though no performance characteristics are provided, the EURL recommends this method for official control to determine *Riboflavin* in the *feed additive*.

For the determination of the *Riboflavin* in *premixtures*, the Applicant proposed a single-laboratory validated method [7, 8], similar to the official ring-trial validated method by the Association of German Agricultural Analytical Research Institutes (VDLUFA) [9].

According to the VDLUFA method, between 1 and 3 g of the *premixtures* are extracted with an aqueous sodium hydroxide solution. 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 titriplex solution. After passing through a membrane filter the dilute extract is submitted to liquid chromatographic analysis. The measurement is based on ion pair reversed phase High Performance Liquid Chromatography coupled to an UV detector (HPLC-UV). When adjusted at 275 nm it allows 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 against external calibration [9].

The performance characteristics determined for the VDLUFA Method [9] using *premixtures* samples (only) with a *Vitamin B_2* content ranging from 868 to 15990 mg/kg are:

- 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, the *recovery* rate (R_{Rec}) ranging from 86 to 100 % was recalculated by the EURL based on experimental data provided in the VDLUFA document.

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* in *feedingstuffs* and *water* the Applicant proposed a single-laboratory validated method [10], similar to the ring trial validated CEN method intended for foodstuffs (EN 14152) [11], based on acid hydrolysis followed by enzymatic dephosphorylation and using HPLC with fluorimetric detection.

According to the CEN method, hydrochloric or sulphuric acid is added to an appropriate amount of sample to reach a pH less than 2. The sample is either autoclaved at 120 ^{o}C for 30 minutes or heated at 100 ^{o}C for an hour. After cooling to room temperature the extract is adjusted at pH 4 with sodium acetate solution and 100 mg Taka-Diatase per gram of sample is added to achieve dephosphorylation. The mixture is incubated at 37-46 ^{o}C for 16 to 24 hours. Total *Vitamin B_2* is then determined by HPLC with fluorimetric detection 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 total *Vitamin B_2* content ranging from 145 to 1055 mg/kg are reported [11]:

- 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, the Applicant applied the CEN method for determination of total *Vitamin B_2* to *feedingstuffs* and *water* samples and reported similar performance characteristics [12], 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* content 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) based on the enzymatic dephosphorylation

followed by HPLC with fluorimetric detection to determine total *Vitamin B_2* (expressed as *Riboflavin)* in *feedingstuffs* and *water*.

Riboflavin 5'-phosphate ester monosodium salt

For the determination of *Riboflavin 5'-phosphate ester monosodium salt* in all matrices under investigation the Applicant proposed the US Pharmacopoeia method [13], without providing any experimental data proving the applicability of this method to *premixtures*, *feedingstuffs* and *water*. Therefore the EURL could not evaluate nor recommend this method for official control of these matrices.

Additionally the Applicant submitted for the determination of *Riboflavin 5'-phosphate ester monosodium salt* in the *feed additive* the European Pharmacopoeia method [14]. Identification is based on ultraviolet/visible spectrophotometry and liquid chromatography, while quantification is based on spectrophotometry at 444 nm. Even though no performance characteristics are provided, the EURL recommends this method for official control to determine *Riboflavin 5'-phosphate ester monosodium salt* in the *feed additive.*

Furthermore, due to the enzymatic dephosphorylation step included in the evaluated earlier ring-trial validated CEN method (EN 14152) [11], the EURL considers this method suitable for the determination of *Riboflavin 5'-phosphate ester monosodium salt* (expressed as total *Riboflavin*) in *feedingstuffs* and *water*.

The same method could be used for the determination of *Riboflavin 5'-phosphate ester monosodium salt* in *premixtures* by applying proper dilution. However, as no experimental data were provided to confirm this, the EURL cannot evaluate nor recommend this method for official control 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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- two European Pharmacopoeia methods, using a spectrophotometry at 444 nm to determine *Riboflavin* (Ph. Eur. 6.0, 01/2008:0292) and *Riboflavin-5'-phosphate ester monosodium salt* (Ph. Eur. 6.0, 01/2008:0786) in the *feed additives*;
- the VDLUFA Bd. III, 13.9.1 method, using ion pair reversed phase High-Performance Liquid Chromatography coupled to UV detector (HPLC-UV), to determine *Riboflavin* in *premixtures;*
- the EN 14152 method based on acidic hydrolysis and enzymatic dephosphorylation followed by High Performance Liquid Chromatographic with a FLuorimetric detection (HPLC-FL), to determine *Riboflavin* and *Riboflavin-5'-phosphate ester monosodium salt* (as total *Vitamin B_2*) in *feedingstuffs* and *water*.

The CEN method (EN 14152) method could be used for the determination of *Riboflavin 5'-phosphate ester monosodium salt* in *premixtures* applying proper dilution. However, as no experimental data were provided to confirm this, the EURL cannot evaluate nor recommend this method for official control in *premixtures*.

Recommended text for the register entry (analytical method)

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

- Spectrophotometry method at 444 nm (Ph. Eur. 6.0, method 01/2008:0292)

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

- Spectrophotometry method at 444 nm (Ph. Eur. 6.0, method 01/2008:0786)

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

- High-Performance Liquid Chromatograpy coupled to UV detector, HPLC-UV (VDLUFA Bd. III, 13.9.1)

For the determination of *Riboflavin* and *Riboflavin5'-phosphate ester monosodium salt (*as total *Vitamin B_2)* in *feedstuffs* and *water*:

- High Performance Liquid Chromatography with Fluorescence detection, HPLC-FL (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* and *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 SANCO/D/2 Forw. Appl. 1831/(00167) (10317)-2010
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II – Identity, characterisation and conditions of use of the additive; Methods of analysis
[4] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories
[5] European Pharmacopoeia method - Ph. Eur. 6.0, 2.2.29
[6] European Pharmacopoeia method - Ph. Eur. 6.0, 01/2008:0292
[7] *Technical dossier, Section II – Annex 2-37: Analytical method for the determination of B vitamins (B_1, B_2 and B_6) in premixes
[8] *Technical dossier, Section II – Annex 2-38: Determination of B vitamins (B_1, B_2 and B_6) in premixes - validation of analytical method
[9] VDLUFA Methodenbuch Bd.III, 13.9.1
[10] *Technical dossier, Section II – Annex 2-39: Determination of vitamin B_2 in food, feed and water
[11] EN 14152:2003 – Foodstuffs: Determination of vitamin B_2 by HPLC
[12] *Technical dossier, Section II – Annex 2-40: Determination of vitamin B_2 in feeds and water – applicability of EN 14152 to the matrices feed and water/verification of analytical method Determination of vitamin B_2 in food, feed and water
[13] US Pharmacopoeia method – USP29-NF24, page 1914
[14] European Pharmacopoeia method - Ph. Eur. 6.0, 01/2008:0786

*Refers to Dossier No. FAD-2010-0304

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Austrian Agency for Health and Food Safety (AGES), Austria. 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Cabrils (ES)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)