EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Reference Materials and Measurements (Geel)
Standards for Food Bioscience

JRC.D.5/SFB/CvH/RFO/mds/Ares

European Union Reference Laboratory

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-2010-0177; CRL/100130)

European Union Reference Laboratory

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-2010-0177 - CRL/100130**
Name of Feed Additive:	**Vitamin B_2**
Active Substance(s):	**Riboflavin**
Rapporteur Laboratory:	**European Reference Laboratory for Feed Additives (EURL-FA)**
Report prepared by:	**Rebeca Fernandez Orozco**
Report checked by:	**Piotr Robouch (EURL-FA)**
Date:	**01/09/2013**
Report approved by:	**Christoph von Holst**
Date:	**01/09/2013**

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 10(2) for *Vitamin B_2* (*Riboflavin*) 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 *feed additive* for all animal species and categories. *Riboflavin* is produced by fermentation using *Ashbya gossypii* strains. According to the Applicant the feed additive contains at least 80% of *Riboflavin*. The *feed additive* is intended to be incorporated in *feedingstuffs*, directly or through *premixtures*. The Applicant did not specify any maximum or minimum concentration of *Vitamin B_2* in *feedingstuffs,* however, typical inclusion levels range from 3 to 20 mg/kg *feedingstuffs*.

For the determination of *Riboflavin per se* (with a minimum purity of 97%) the EURL proposes the European Pharmacopoeia method (Ph. Eur. 6.0, 01/2008:0292). 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 determine *Riboflavin per se*.

For the determination of *Riboflavin* in *premixtures* and *feedingstuffs* the Applicant submitted the AOAC 940.33 microbiological method based on the titration with *Lactobacillus casei*. However, the EURL evaluated already several analytical methods in the frame of the FAD-2010-0304 dossier and recommended for official control: - 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;* and - the EN 14152 method based on acidic hydrolysis and enzymatic dephosphorylation followed by High Performance Liquid Chromatography (HPLC) with fluorescence detector, to determine *Riboflavin* (as total *Vitamin B_2*) in *feedingstuffs*.

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, nutritional additives, vitamins, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under articles 10(2) (re-evaluation of the already authorised additives under provisions of Council Directive 70/524/EEC) for *Vitamin B_2* (*Riboflavin*) 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 [1,2]. Authorisation is sought for the use of the *feed additive* for all animal species and categories [1,2].

According to the Applicant the feed grade *Vitamin B_2* is produced by fermentation using *Ashbya gossypii* strains, and contains at least 80% of *Riboflavin* in the heated and decanted fermentation broth (20%) from *Ashbya gossypii* [3].

The *feed additive* is intended to be incorporated in *feedingstuffs*, directly or through *premixtures* [3]. The Applicant did not specify any maximum or minimum concentration of *Vitamin B_2* (Riboflavin) in *feedingstuffs* [2], however, typical inclusion levels range from 3 to 20 mg/kg *feedingstuffs* [4].

The EURL has previously evaluated the methods of analysis for *Vitamin B2* in the reports FAD-2010-0049, FAD-2010-0262 and FAD-2010-0304 [8].

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 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, aflatoxins) are available from the respective European Union Reference Laboratories [5].

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

For the determination of *Riboflavin per se* (with a minimum purity of 97%) the EURL proposes 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.

For the determination of *Riboflavin* in *premixtures* and *feedingstuffs* the Applicant submitted the AOAC 940.33 method. This is a microbiological method based on titration with *Lactobacillus casei*. However, the EURL evaluated already several analytical methods in the frame of the FAD-2010-0304 dossier and recommended the following methods for official control [8]:

- the VDLUFA Bd. III, 13.9.1 method [9], using ion pair reversed phase High-Performance Liquid Chromatography coupled to UV detector (HPLC-UV), to determine *Riboflavin* in *premixtures;* and
- the EN 14152 method [10] based on acidic hydrolysis and enzymatic dephosphorylation followed by High Performance Liquid Chromatography with fluorescence detector (HPLC-FL), to determine *Riboflavin* (as total *Vitamin B_2*, endogenous and added) in *feedingstuffs*.

A detailed description of these methods is provided in the FAD-2010-0304 report [8].

Furthermore, the Applicant submitted experimental data obtained by High-Performance Liquid Chromatography (HPLC) in the frame of the stability study, for the determination of *riboflavin* in their feed grade product presented in this dossier (Lutavit B2 SG 80) [7], without providing the experimental protocol used. Therefore, the EURL cannot evaluate the suitability of this method for the determination of *Riboflavin* in the applicant's product.

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:

- the European Pharmacopoeia monograph 0292, using spectrophotometry to determine *Riboflavin per se* (with a minimum purity of 97%);

- the VDLUFA Method Book Bd. III, 13.9.1 method, using ion pair reversed phase High-Performance Liquid Chromatography with 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 Chromatography with fluorescence detector (HPLC-FL), to determine *Riboflavin* (as total *Vitamin B_2*) in *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *Riboflavin per se* (with a minimum purity of 97%):

- Spectrophotometry – European Pharmacopoeia monograph 0292

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

- High-Performance Liquid Chromatography with UV detector (HPLC-UV) – VDLUFA Method Book, Vol. III, 13.9.1

For the determination of *Riboflavin (*as total *Vitamin B_2,* both, natural and added as feed additive) in feedingstuffs:

- High Performance Liquid Chromatography with fluorescence detector (HPLC-FL) - EN 14152:2003

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*) 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/G/1 Forw. Appl. 1831/7066-2010
[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] N. Albers et al. "*Vitamins in Animal Nutrition*" Edited by the Arbeitsgemeinschaft für Wirkstoffe in der Tierernährung e.V. (AWT) ISBN 3-86037-167-3 (www.agrimedia.com)
[5] 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
[6] European Pharmacopoeia method - Ph. Eur. 6.0, 01/2008:0292

[7] Technical dossier, Section II – Identity, characterisation and conditions of use of the additive; Methods of analysis. Annex II-9- Stability-Additive.

[8] http://irmm.jrc.ec.europa.eu/EURLs/EURL_feed_additives/authorisation/evaluation_reports/
- EURL-FA report FAD-2010-0049. Ref. ARES(2010)848468
- EURL-FA report FAD-2010-0262. Ref. ARES(2013)255788
- EURL-FA report FAD-2010-0304. Ref. ARES(2011)1266605

[9] VDLUFA Methodenbuch Bd.III, 13.9.1

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

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was the European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Federaal Laboratorium voor de Voedselveiligheid Tervuren (FLVVT – FAVV), Tervuren, (BE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino, (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien, (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, (CZ)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena, (DE)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Cabrils, (ES)
- Państwowy Instytut Weterynaryjny, Puławy, (PL)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim, (DE)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes, (FR)
- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala, (SE)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana, (SI)