European Union Reference Laboratory


Vitamin B$_2$ - Riboflavin
(FAD-2010-0177; CRL/100130)
European Union Reference Laboratory


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)
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EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 10(2) for Vitamin B₂ (Riboflavin) under the category/functional group 3(a) ‘nutritional additives’/‘vitamins, provitamins 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 B₂ 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 B₂) 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 B₂, 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 B2 (Riboflavin) under the category/functional group 3(a) ‘nutritional additives’/‘vitamins, provitamins 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 B2 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 B2 (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 B2 (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 B₂*, 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 B2) 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 B2, 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 B2 (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**

[3] Technical dossier, Section II – Identity, characterisation and conditions of use of the additive; Methods of analysis

- 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

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

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- 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)
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