EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

JRC.DG.D6/CvH/RMO/ag/ARES(2010)848468

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

Dossier related to:	FAD-2010-0049 CRL/100056
Name of Additive:	Vitamin B2
Active Substance(s):	Riboflavin
Rapporteur Laboratory:	Laboratorio Nacional de Investigaco Veterinaria-LNIV
Report prepared by:	Jorge Barbosa (LNIV)
Report revised by: Date:	Roberto Molteni, Piotr Robouch (CRL-FA) 22/11/2010
Report approved by: Date:	Christoph von Holst 22/11/2010

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Vitamin B_2* 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. Specifically, authorisation is sought for the use of *riboflavin (Vitamin B_2)* for all animal species and categories. The *Vitamin B_2* is produced by a strain of *Bacillus subtilis* CJKB0001. According to the Applicant the product is a fine spray-dried granules, with a minimum content of 80% of *Vitamin B_2*, a maximum of 20% of fermentation solubles. The *feed additive* is intended to be incorporated directly in *feedingstuffs* or through *premixtures*. Due to the slight solubility in *water* (0.07 g/l at 20 °C), *Vitamin B_2* can only be administered in *water* in concentrations lower than 0.07 g/l. However, the Applicant did not specify any maximum or minimum concentration of *Vitamin B_2* in *feedingstuffs* or *water*..

For the determination of *Vitamin B_2* in the *feed additive* the Applicant proposed the European Pharmacopoeia method - Ph. Eur. 5.0, method 01/2005:0292, based on spectrophotometry at wavelength of 444 nm. The CRL considers these methods suitable to be used within the frame of official control.

For the determination of the *Vitamin B_2* in *premixtures* the Applicant proposed the VDLUFA method, a ring trial validated method for *premixtures* samples. The VDLUFA method is based on ion pair reversed phase (RP) High Performance Liquid Chromatograpy (HPLC) coupled to an UV detector measuring at 268 nm and allows the simultaneous detection of vitamin B_1, B_2, B_6, Nicotinic acid and Nicotinamide.

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

- relative standard deviation for *repeatability* (RSDr) from 2.38% to 4.7%, and
- relative standard deviation for *reproducibility* (RSD_R) from 4.20% to 7.32%.

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

Based on these acceptable performance characteristics, the CRL recommends the ring-trial validated method from the Association of German Agricultural Analytical Research Institutes (VDLUFA) Bd.III, 13.9.1 for official control to determine *Vitamin B_2* in *premixtures*.

As method performance characteristics of the VDLUFA method when applied to *feedingstuffs* were not available, the CRL cannot evaluate nor recommend this method for official control to determine *Vitamin B_2* in *feedingstuffs*.

For the determination of *Vitamin B_2* in the *water*, the Applicant proposed the CEN method 14152:2003, intended for foodstuffs. However no experimental data were provided by the Applicant for the determination of the product in *water*. Therefore the CRL cannot evaluate and recommend the method for official control to determine *Vitamin B_2* in *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) is not considered necessary.

KEYWORDS

Riboflavin, Vitamin B_2, nutritional additive, 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 additive under council directive 70/524/EEC) for *riboflavin (Vitamin B_2)* 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 feed additive for all animal species and categories [2]. The *Vitamin B_2* is produced by a strain of *Bacillus subtilis* CJKB0001 [2]. The strain has been deposited at the Korean Culture Center of Microorganisms with number KCCM-10445 [3]. According to the Applicant the product is a fine spray-dried granules [4], with a minimum content of 80% of *Vitamin B_2*, a maximum of 20% of fermentation solubles [2]. The *feed additive* is intended to be incorporated directly in *feedingstuffs* or through *premixtures*. Due to the slight solubility in *water* (0.07 g/l at 20 °C), *Vitamin B_2* can only be administered in *water* in concentrations lower than 0.07 g/l. However, the Applicant did not specify any maximum or minimum concentration of *Vitamin B_2* in *feedingstuffs* or *water* [2]. Similarly to what was set in previous regulation [5].

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 Community Reference Laboratory concerning applications for authorisations of *feed additives*, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *riboflavin (Vitamin B_2)*, 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 Community Reference Laboratories [6]. Lumiflavin, a toxic photoderivative of riboflavin, does not exceed 0.025% [4], and it can be detected with thin-layer chromatography [7].

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

For the determination of *riboflavin (Vitamin B_2)* in the *feed additive* the Applicant proposed the European Pharmacopoeia method - Ph. Eur. 5.0, method 01/2005:0292 [7], based on spectrophotometry at wavelength of 444 nm. No performance characteristics of this method are provided. However, the CRL considers the method suitable to be used within the frame of official control.

For the determination of the *Vitamin B_2* in *premixtures* and *feedingstuffs* the Applicant proposed three HPLC methods:

- a method published in the literature by Rubaj *et al*. (2008) [8];
- the CEN method 14152:2003 developed for foodstuffs [9]. However, a complete method performance profile of this method when applied to *premixtures* and *feedingstuffs* is not available;

- the VDLUFA method – ring trial validated for *premixtures* [10].

When using 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 with a pH value adjusted at 2.75 is added and an aliquote 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 (RP) High Performance Liquid Chromatograpy (HPLC) coupled to an UV detector adjusted at 268 nm and allows the simultaneous detection of vitamin B_1, B_2, B_6, Nicotinic acid and Nicotinamide. The target analytes are quantified against external calibration [10].

The performance characteristics determined for the VDLUFA Method [10] using *premixtures* samples (only) with a *Vitamin B_2* content ranging from 868 to 15990 mg/kg are:

- relative standard deviation for *repeatability* (RSDr) from 2.38% to 4.7%, and

- relative standard deviation for *reproducibility* (RSD_R) from 4.20% to 7.32%.

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

Based on these acceptable performance characteristics, the CRL recommends the ring-trial validated method from the Association of German Agricultural Analytical Research Institutes (VDLUFA) Bd.III, 13.9.1 [10] for official control to determine *Vitamin B_2* in *premixtures*, within the concentration range covered by the collaborative study.

The VDLUFA method was not investigated for the *feedingstuffs*, due to potential matrix interference at the low vitamin concentrations, leading to high detection limits. A possible solution could be to use the abovementioned method using standard addition. However, this needs to be verified on a case by case basis. Therefore the CRL cannot evaluate nor recommend this method for official control to determine *Vitamin B_2* in *feedingstuffs*.

For the determination of *Vitamin B_2* in the *water*, the Applicant proposed the CEN method 14152:2003 [9], intended for foodstuffs. This method seems to be adequate to *Vitamin B_2* in *water*. However no experimental data were provided by the Applicant for the determination of the product in *water*. Therefore the CRL cannot evaluate and recommend the method for official control to determine *Vitamin B_2* in *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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

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

- the European Pharmacopeia method, using a spectrophotometry to determine *riboflavin* in *feed additive*
- the VDLUFA Bd. III, 13.9.1 method, using ion pair Reversed Phase High-Performance Liquid Chromatography coupled to UV detector (RP-HPLC-UV), to determine *riboflavin* in *premixtures*

Recommended text for the register entry (analytical method)

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

- a spectrophotometry method using a visible detector at 444 nm, from European Pharmacopeia (Ph. Eur. 5.0, method 01/2005:0292)

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

- Ion pair Reversed Phase High-Performance Liquid Chromatography coupled to UV detector (RP-HPLC-UV) - VDLUFA Bd. III, 13.9.1 method

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *riboflavin* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Application, Reference SANCO/D/2 Forw. Appl. 1831/0032-2010

[2] *Application, Proposal for Register Entry – Annex A

[3] *Technical dossier – Annex II 7 Deposition certificate

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

[5] COUNCIL DIRECTIVE 70/524/EEC of 23 November 1970 concerning additives in feedingstuffs

[6] 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

[7] European Pharmacopoeia method (Ph. Eur. 5.0)

[8] *Technical Dossier, Section II, Ref II 2.2 Rubaj Determination riboflavin

[9] EN 14152:2003. Foodstuffs - Determination of vitamin B2 by HPLC

[10] *Technical Dossier, Section II, Annex II 17 VDLUFA method of analysis

* Refers to Dossier No. FAD-2010-0049

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Laboratorio Nacional de Investigaco veterinária (LNIV), Portugal. 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)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)