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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-2010-0139 EURL/0100182
Product Name:	Vitamin B_6
Active Substance(s):	Pyridoxine Hydrochloride
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
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EXECUTIVE SUMMARY

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 *pyridoxine hydrochloride (Vitamin B_6)* 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. According to the Applicant the product is a white to yellowish powder containing a minimum of 98.5 % pyridoxine hydrochloride. The *feed additive* is intended to be incorporated in *feedingstuffs* (including complete *feedingstuffs* or complementary *feedingstuffs*) through *premixtures* or directly in *water*. However, the Applicant did not specify any maximum or minimum concentration of *Vitamin B_6* in *feedingstuffs* or *water*, similarly to what was set in previous regulation.

For the determination of the *Vitamin B_6* in the *feed additive* the Applicant proposes the titration method described in the European Pharmacopoeia (Ph.Eur.6th, monograph 0245). The EURL considers this method suitable to be used within the frame of official control.

For the determination of *Vitamin B_6* in *premixtures* the Applicant proposes a single-laboratory validated method, based on ion pair reversed phase High Performance Liquid Chromatograpy (RP-HPLC) coupled to an UV detector. This method provided satisfactory results when compared to the collaborative trial organised by the Association of German Agricultural Analytical and Research Institutes (VDLUFA, Germany). The following performance characteristics were reported for the VDLUFA ring-trial validated UV-HPLC method on *premixtures* containing from 627 to 11530 mg Vitamin B6 /kg of *premixture*:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.6 to 3.4 %, and
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.0 to 5.1 %.

Based on these acceptable performance characteristics, the EURL recommends for official control the ring-trial validated VDLUFA method based on UV-HPLC for the determination of *Vitamin B_6* in *premixtures* within the concentration range covered by the collaborative study.

For the determination of *Vitamin B_6* in *feedingstuffs* and in *water*, the Applicant submitted a *simplified* method based on the European Standard (EN 14164:2008, "Foodstuffs – Determination of vitamin B_6 by HPLC"). The Applicant demonstrated the suitability of this method for the determination of *Vitamin B_6* in *feedingstuffs* and *water*, for *Vitamin B_6* concentrations ranging from 0.6 to 400 mg/kg *feedingstuffs* and *water*.

The Applicant determined the following performance characteristics, which are in agreement with those reported in the standard method:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 1.3 to 7.3 %, and

- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 5.0 to 17.8 %.

Based on these acceptable performance characteristics the EURL recommends for official control the *simplified* method based on the European Standard method (EN 14164:2008) to determine *Vitamin B_6* in *feedingstuffs* and *water,* within the concentration range covered by the experimental data.

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

Pyridoxine hydrochloride, vitamin B_6, nutritional additive, vitamins, all animal species, all 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 *pyridoxine hydrochloride (Vitamin B_6)* 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 [1]. According to the Applicant the product is a white to yellowish powder containing a minimum of 98.5 % pyridoxine hydrochloride [3]. The *feed additive* is intended to be incorporated in *feedingstuffs* (including complete *feedingstuffs* or complementary *feedingstuffs*) through *premixtures* or directly in *water*. However, the Applicant did not specify any maximum or minimum concentration of *Vitamin B_6* in *feedingstuffs* or *water* [2], similarly to what was set in previous regulation [4].

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 EURL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application. For this dossier, the methods of analysis submitted in connection with *Vitamin B_6*, 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 B_1 and dioxins) are available from the respective Community 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 *Vitamin B_6* in the *feed additive*, the Applicant proposes the internationally recognised European Pharmacopoeia method (Ph.Eur. 6th Edition, monograph 0245) [6], based on a potentiometric titration with 0.1 M perchloric acid. In this assay 1 mL of 0.1 M perchloric acid is equivalent to 20.56 mg of $C_8H_{12}ClNO_3$. Even though no performance characteristics are provided, the EURL recommends for official control the European Pharmacopoeia method to determine *Vitamin B_6* in the *feed additive*.

For the determination of *Vitamin B_6* in *premixtures* the Applicant proposed a single-laboratory validated method [7], based on ion pair reversed phase (RP) High Performance Liquid Chromatograpy (HPLC) coupled to an UV detector measuring at 280 and 245 nm. The sample is extracted with a mixture of acetic acid, acetonitrile and water and subjected without further clean-up to HPLC. The target analytes are quantified against external calibration.

The Applicant participated with this method to an inter-laboratory comparison [8] organised by the VDLUFA and achieved similar performance characteristics as the other laboratories participating with a preliminary version of the VDLUFA method. The VDLUFA method

allows for the simultaneous determination of vitamin B_1, B_2, B_6, Nicotinic acid and Nicotinamide and is based on ion pair reversed phase High Performance Liquid Chromatograpy (RP-HPLC) coupled to an UV detector measuring at 291 nm. The sample is extracted with a mixture of diethylenetriaminepentaacetic acid (titriplex V) and methanol and subjected without further clean-up to HPLC. The target analytes are quantified against external calibration.

The VDLUFA method was further ring-trial validated [9] for *premixtures* containing *Vitamin B_6* at concentration ranging from 627 to 11530 mg/kg. The following performance characteristics were reported:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.6 to 3.4 %, and
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.0 to 5.1%.

Based on the above considerations and the performance characteristics presented, the EURL recommends for official control the VDLUFA method (Bd.III, 13.9.1), to determine *Vitamin B_6* in *premixtures*, within the concentration range covered by the experimental data.

For the determination of *Vitamin B_6* in *feedingstuffs* and in *water,* the Applicant proposes a method based on the European Standard (EN 14164:2008, "Foodstuffs – Determination of vitamin B_6 by HPLC") [10]. The method allows the determination of total *Vitamin B_6* (as the sum of pyridoxine, pyridoxal, pyridoxamine and their phoshorylated derivatives determined as pyridoxine). The sample is extracted with 0.1 mol/L hydrochloric acid. Thereafter the phosphorylated forms are transformed enzymatically and the pyridoxamine is catalytically transformed with glyoxylic acid to pyridoxal which is thereafter reduced to pyridoxine with sodium borohydrid in alkaline medium. The total pyridoxine is then quantified via external calibration employing RP-HPLC coupled to a fluorescence detector. The Applicant submitted a *simplified* experimental protocol based on the EN 14164:2008 method, omitting the enzymatic treatment and reduction step [11] as specified in the corresponding method protocol [12]. The Applicants feed additive only contains pyridoxine hydrochloride therefore the endogenouse amount of pyridoxal, pyridoxamine and the phosphorylated derivatives in the feed are not of interest. Consequently the enzymatic treatment and reduction step can be omitted. Further the Applicant verified the suitability of the *simplified* method in the range from 0.6 to 400 mg/kg *Vitamin B_6* for *feedingstuffs* and *water* [12].

The performance characteristics determined by the Applicant are in agreement with those reported in the standard method (see Table 1).

Table 1. Comparison of performance characteristics of EN 14164:2008 and the *simplified* protocol

	RSD_r	RSD_R / RSD_{ip}
EN 14164:2008	3 % - 18 %	12 % - 35 %
Simplified protocol	1.3 % – 7.3 %	5.0 % - 17.8 %

Based on the acceptable performance characteristics presented, the EURL recommends for official control the *simplified* protocol of the ring trial validated RP-HPLC-FLD European Standard method (EN 14164:2008) to determine *Vitamin B_6* in *feedingstuffs* and *water,* within the concentration range covered by the experimental data.

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 method (Ph. Eur. 6th edition, monograph 0245) using titration with perchloric acid for the determination of *Vitamin B_6* in the *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) for the determination of *Vitamin B_6* in *premixtures*; and

- the Applicant's *simplified* method based on CEN EN14164:2008, using reversed phase High Performance Liquid Chromatography coupled to fluorescence detector (RP-HPLC-FLD) for determination *Vitamin B_6* in *feedingstuffs* and *water*

Recommended text for the register entry (analytical method)

For the determination of *pyridoxine hydrochloride (Vitamin B_6)* in the *feed additive:*

- Titration with perchloric acid (Ph. Eur. 6th edition, monograph 0245)

For the determination of *pyridoxine hydrochloride (Vitamin B_6)* in *premixtures*:

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

For the determination of *pyridoxine hydrochloride (Vitamin B_6)* in *feedingstuffs* and *water*:

- Reversed phase High Performance Liquid Chromatography coupled to fluorescence detector (RP-HPLC-FLD) – method based on EN14164:2008

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Vitamin B_6* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application/Ref: SANCO/D/2: Forw. Appl. 1831/0087-2010

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

[3] *Technical dossier, Section II: Identity

[4] COUNCIL DIRECTIVE 70/524/EEC of 23 November 1970 concerning additives in feeding-stuffs

[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] *Technical Dossier, Section II, Annex 2.07

[7] *Technical Dossier, Section II, Annex 2.22

[8] *Technical Dossier, Section II, Annex 2.23

[9] Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch) Bd. III 13.9.1 Bestimmung der B-Vitamine einschl. Nicotinsäure

[10] *Technical Dossier, Section II, Annex 2.25

[11] *Technical Dossier, Section II, Annex 2.26

[12] *Technical Dossier, Section II, Annex 2.28

* Refers to Dossier No. FAD-2010-0139

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was 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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- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
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