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Feed Additives

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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_{12} / Cyanocobalamin
produced by Ensifer adhaerens CGMCC 21299
(FEED -2023-15991; CRL/230018)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FEED-2023-15991 - CRL/230018**
Name of Product	***Vitamin B_{12} / Cyanocobalamin produced by Ensifer adhaerens CGMCC 21299***
Active Agent (s):	**Vitamin B_{12} / Cyanocobalamin**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Stefano Bellorini** **15/12/2023**
Report approved by: Date:	**Christoph von Holst** **18/12/2023**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *vitamin B_{12} / cyanocobalamin* under the category/functional group 3(a), "nutritional additives/vitamins, pro-vitamins and chemically well-defined substances having similar effect", according to the classification system of 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, *vitamin B_{12} / cyanocobalamin* is produced by fermentation with *Ensifer adhaerens* CGMCC 21299. The *feed additive* is a powder preparation containing a maximum *vitamin B_{12} / cyanocobalamin* content of 1 % mixed in a carrier. The *feed additive* preparation is intended to be incorporated in *compound feed* with no minimum or maximum contents proposed by the Applicant.

For the determination of *cyanocobalamin* in the *feed additive* preparation the Applicant proposed an in-house method based on high performance liquid chromatography coupled to spectrophotometry (HPLC-UV) while for the determination of *cyanocobalamin* in *compound feed*, the Applicant proposed a microbiological method according to USP 39-171. However, in the frame of a previous *vitamin B_{12}* evaluation, this microbiological method has triggered some NRLs concerns about its applicability for the quantification of *cyanocobalamin* in *compound feed.*

Furthermore, the EURL is aware of a ring-trial validated AOAC method based on immunoaffinity column clean-up and high performance liquid chromatography coupled to spectrophotometry (HPLC-UV) for the determination of *vitamin B_{12}* in food commodities. Within the frame of previous *vitamin B_{12}* dossiers, this chromatography method has been further verified in different *feed matrices* and led to acceptable performance characteristics.

Based on the performance characteristics available, the EURL recommends for official control the reversed phase liquid chromatography method coupled to spectrophotometric detection (HPLC-UV) based on the AOAC ring-trial validated method for the determination of *vitamin B_{12} / cyanocobalamin* in the *feed additive* preparation and *compound feed*.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Vitamin B_{12}, *cyanocobalamin* produced by *Ensifer adhaerens* CGMCC 21299, nutritional additives, vitamins, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new feed additive) [1] for *vitamin B_{12} / cyanocobalamin* under the category/functional group 3(a), "nutritional additives/vitamins, pro-vitamins and chemically well-defined substances having similar effect", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for all animal species and categories [1,2].

According to the Applicant, *vitamin B_{12} / cyanocobalamin* is produced by fermentation with *Ensifer adhaerens* CGMCC 21299 [3]. The *feed additive* is a powder preparation containing a maximum *vitamin B_{12} / cyanocobalamin* content of 1 % mixed in a carrier [2].

The *feed additive* preparation is intended to be incorporated in *compound feed* through premixtures [4]. Furthermore, no minimum or maximum contents in *compound feed* have been proposed by the Applicant.

Note: The EURL has previously evaluated the analytical methods for the determination of *vitamin B_{12} / cyanocobalamin* in the frame of several dossiers [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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_{12} / cyanocobalamin produced by Ensifer adhaerens* CGMCC 21299 and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the determination of *vitamin B_{12}* in the *feed additive* the Applicant proposed an in-house high performance liquid chromatography method coupled to spectrophotometry (HPLC-UV) [6].

According to the procedure, the *feed additive* preparation is extracted with water in an ultrasonic bath, further diluted, filtered through a 0.45 um membrane filter and injected into the HPLC-UV system. *Cyanocobalamin (vitamin B_{12})* is detected at 361 nm. Quantification of *cyanocobalamin* is then performed using external calibration standards [6].

However, the Applicant did not provide any validation or verification of the proposed method of analysis for the determination of *cyanocobalamin (vitamin B_{12})* in the *feed additive* preparation described above.

For the determination of *cyanocobalamin (vitamin B_{12})* in *premixtures* and in *compound feed* the Applicant proposed a microbiological method according to USP 39-171.

This method is based on the growth of the test organism in a liquid nutrient medium in the presence of *cyanocobalamin (vitamin B_{12})*. *Vitamin B_{12}* is extracted from the sample material using a suitable extraction agent. The test organism *Lactobacillus delbrueckii* ATCC 7830 is placed in the nutrient solution, which contains all the nutrients required for its growth, except *vitamin B_{12}*. A multiplication of the test microorganism is not carried out. Added calibration or sample extraction solutions with increasing *vitamin B_{12}* content caused increasing growth of the test organism. The turbidity resulted by this is measured photometrically and the *vitamin B_{12}* content (based on *cyanocobalamin (vitamin B_{12})* is determined from the standard curves. The Applicant applied the proposed method in the frame of the homogeneity and stability studies [7].

This microbiological method has been previously evaluated by the EURL in the frame of a different *vitamin B_{12}* dossier [5]. However, despite of the acceptable performance characteristics reported by the Applicant at the time, some NRLs expressed their concerns on the reliability of the proposed microbiological method for the quantification of *cyanocobalamin (vitamin B_{12})* in *compound feed* and thus the EURL was unable to recommend this method for official control [5].

Furthermore, within the frame of the evaluation of previous *vitamin B_{12}* dossiers [5], the EURL identified an alternative ring-trial validated AOAC method based on immunoaffinity column clean-up and HPLC coupled to spectrophotometry (HPLC-UV) to determine *vitamin B_{12}* in infant formulas and adult nutrition [8]. The sample preparation of this method was slightly modified and the resulting protocol was further verified on different *compound feeds* and on a different 1 % *vitamin B_{12}* preparation [5]. The acceptable performance characteristics obtained in these verifications confirmed the applicability of the modified AOAC method for the determination of *cyanocobalamin (vitamin B_{12})* in *compound feed* [5] and in the 1 % *vitamin B_{12} feed additive* preparation, which is currently authorised [9].

Based on the acceptable performance characteristics available, the EURL recommends for official control the reversed phase liquid chromatography coupled to spectrophotometric detection (HPLC-UV) method based on the AOAC ring-trial validated method for the determination of *vitamin B_{12} / cyanocobalamin* in the *feed additive* preparation and in *compound feed*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for official control the reversed phase liquid chromatography method coupled to spectrophotometric detection (HPLC-UV) based on the AOAC ring-trial validated method for the determination of *vitamin B_{12} / cyanocobalamin* in the *feed additive* preparation and in *compound feed*.

Recommended text for the register entry (analytical method)

For the quantification of *vitamin B_{12} / cyanocobalamin* in the *feed additive* preparation and in *compound feed*:

- reversed phase high performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *vitamin B_{12} / cyanocobalamin produced by Ensifer adhaerens* CGMCC 21299 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] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform – https://webgate.ec.europa.eu/esfc/#/applications/45211 https://open.efsa.europa.eu/questions/EFSA-Q-2023-00409

[2] *Application, Annex 1

[3] *Technical dossier, Section II: 2.1. Identity of the additive

[4] *Technical dossier, Section II: 2.5. Proposed mode of use in animal nutrition

[5] EURL Evaluation Reports: https://addendum_fad-2010-0199_cyanocobalamin.pdf https://finrep_fad-2021-0041_vitb12-cyanocobalamin.pdf

[6] *Technical dossier, Section II, Annex 2.28 Test method for Mixed Feed Additive 1.0% Cyanocobalamin (Vitamin B12 powder)

[7] *Technical dossier, Section II, Annex_II_25_Homogeneity-and-stability_public.pdf

[8] Campos Gimenez, E., Martin, F., Vitamin B12 (Cyanocobalamin) in Infant Formula Adult/Pediatric Nutritional Formula by Liquid Chromatography with Ultraviolet Detection: Collaborative Study, Final Action 2014.02, *Journal of AOAC International* 2018, 101, 1112-1118

[9] Commission Implementing Regulation (EU) 2022/1249 of 19 July 2022 concerning the authorisation of vitamin B12 in the form of cyanocobalamin produced by Ensifer adhaerens CNCM I-5541 as a feed additive for all animal species

*Refers to Dossier no: FEED-2023-15991

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)

- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Wageningen Food Safety Research (WFSR)[1] (NL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)

[1] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.