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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

(FAD-2021-0041; CRL/210003)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0041 - CRL/210003**
Name of Feed Additive:	***Vitamin B_{12} / Cyanocobalamin***
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 28/02/2022**
Report approved by: Date:	**Christoph von Holst 04/03/2022**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Articles 4 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 a non-genetically modified *Ensifer adhaerens* CGMCC 19596 strain and further reaction with sodium cyanide to form *cyanocobalamin (vitamin B_{12})*. The *feed additive* has a minimum *vitamin B_{12} / cyanocobalamin* purity of 96 % (w/w). *Cyanocobalamin* is intended to be incorporated in *feedingstuffs* through *premixtures*. No minimum or maximum contents in *feedingstuffs* have been proposed by the Applicant however, recommended levels range from 10 to 80 µg/kg *compound feed*, depending on the target species.

For the determination of *cyanocobalamin* in the *feed additive* the Applicant proposed the methods stated in the European Pharmacopoeia *cyanocobalamin* monograph (Eur. Ph. 0547).

For the determination of *cyanocobalamin* in *premixtures* and *feedingstuffs*, the Applicant proposed an in-house validated microbiological assay. However, in the frame of a previous *vitamin B_{12}* evaluation, this method has triggered some NRLs concerns about its applicability for the quantification of *cyanocobalamin* in *premixtures* and *feedingstuffs.*

Nevertheless, 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. The method has been further verified in different *feedingstuffs,* leading to a relative standard deviation for repeatability (RSD_r) ranging from 9.0 to 10.8 % and a relative standard deviations for intermediate precision (RSD_{ip}) ranging from 10.1 to 10.8 %.

Based on the performance characteristics available, the EURL recommends for official control (i) the European Pharmacopoeia method (Eur. Ph. 0547) based on spectrophotometry (UV/VIS) to determine *vitamin B_{12} / cyanocobalamin* in the *feed additive* and (ii) 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 *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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Vitamin B_{12}, *cyanocobalamin*, nutritional additives, vitamins, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Articles 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].

According to the Applicant, *vitamin B_{12} / cyanocobalamin* is produced by fermentation with a non-genetically modified *Ensifer adhaerens* CGMCC 19596 strain and further reaction with sodium cyanide to form *cyanocobalamin (vitamin B_{12})* [2]. The *feed additive* has a minimum *vitamin B_{12} / cyanocobalamin* content (mass fraction on dry matter basis) of 96 % [2].

Cyanocobalamin is intended to be incorporated in *feedingstuffs* through *premixtures* [3]. Furthermore, no minimum or maximum contents in *feedingstuffs* have been proposed by the Applicant. However, *vitamin B_{12} / cyanocobalamin* levels ranging from 10 to 80 µg/kg *compound feed*, depending on the target species, have been recommended [3].

Note: The EURL has previously evaluated the analytical methods for the determination of *vitamin B_{12} / cyanocobalamin* in the frame of several dossiers [4].

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* 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 *cyanocobalamin* in the *feed additive* the Applicant proposed the methods stated in the European Pharmacopoeia *cyanocobalamin* monograph (Eur. Ph. 0547).

This monograph applies to *cyanocobalamin* produced by fermentation and with a measured content from 96 to 102 % (w/w dried substance). The EURL is aware of a recent *cyanocobalamin* monograph that replaced the previous one proposed by the Applicant.

In its new version the identification and quantification of *cyanocobalamin per se* are based on spectrophotometry (UV/VIS), while purity is assessed by high performance liquid chromatography followed by spectrophotometric detection (HPLC-UV/VIS) [5].

Based on that, the EURL recommends the most recent European Pharmacopoeia *cyanocobalamin* monograph (Eur. Ph. 0547) for the determination of *vitamin B_{12} / cyanocobalamin* in the *feed additive*.

For the determination of *cyanocobalamin* in *premixtures* and *feedingstuffs* the Applicant proposed an in-house validated [6] microbiological method (USP 31-171) using the test organism *Lactobacillus leichmannii* ATCC 7830.

The method is based on the growth of the test organism in a liquid nutrient medium in the presence of *Vitamin B_{12}*. The samples are extracted with the vapour-sterilised aqueous solution containing disodium hydrogen phosphate, citric acid and sodium disulfite. Standard solution is prepared by dissolving cyanocobalamin in mixture of water and ethanol and equilibrated at room temperature. Then the sample extracts as well as the standard solutions are added to nutrient medium in test tubes, autoclaved and inoculated with the test organism *Lactobacillus leichmannii* ATCC 7830. After incubation for 15 hours at 37 ± 1 ºC in water bath, the turbidity of the suspensions is measured at 540-560 nm. The quantification is done by external calibration. The method has been in-house validated for the *feed additive*, *premixtures* and *feedingstuffs*. The Applicant reported recoveries ranging from 90 to 103 % coefficients of variation for repeatability from 0.7 to 6.6 % and coefficients of variation for reproducibility ranging from 3.2 to 8.4 %. Furthermore the Applicant applied the proposed method in the frame of the homogeneity and stability studies [7].

This method has been previously evaluated by the EURL in the frame of a different *vitamin B_{12}* dossier [4]. However, despite of the performance characteristics reported by the Applicant some NRLs expressed their concerns on the reliability of the proposed microbiological

method for the quantification of *cyanocobalamin* in *premixtures* and *feedingstuffs* and thus the EURL was unable to recommend this method for official control [4].

Furthermore and within the frame of the evaluation of a previous dossier [4], the EURL identified an alternative ring-trial validated AOAC method based on immunoaffinity column clean-up and HPLC coupled to spectrophotometry (HPLC-UV) successfully applied to determine *vitamin B_{12}* in infant formulas and adult nutrition [8]. The sample preparation of this method has been slightly modified and the modified method was further verified on different *feedingstuffs* [4]. The reported performance characteristics were recalculated by the EURL leading to a relative standard deviation for repeatability (RSD_r) ranging from 9.0 to 10.8 % and a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 10.1 to 10.8 %. These values are similar than those obtained in the ring-trial validated AOAC method. In addition, the verification study reported a working range from 1 to 550 µg/kg *feedingstuffs*, a limit of quantification (LOQ) of 1 µg/kg *feedingstuffs* and *recovery* rates (R_{rec}) ranging from 85 to 108 % confirming thus the applicability of the modified AOAC method to *feedingstuffs* supplemented with *vitamin B_{12}* [4].

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 *feedingstuffs*.

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)

The 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 (i) the European Pharmacopoeia method (Eur. Ph. 0547) based on spectrophotometry (UV/VIS) to determine *vitamin B_{12}* / *cyanocobalamin* in the *feed additive* and (ii) 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 *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *vitamin B_{12} / cyanocobalamin* in the *feed additive*:

– spectrophotometry (UV/VIS) - European Pharmacopoeia monograph 0547

For the quantification of *vitamin B_{12} / cyanocobalamin* in *feedingstuffs*:

– 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* 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 SANTE/E5: FORW. APPL. 1831-0070-2021 & Annex I – submission number 1616148794583-2918

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

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

[4] EURL Evaluation Report:

https://ec.europa.eu/jrc/sites/default/files/addendum_fad-2010-0199_cyanocobalamin.pdf

[5] European Pharmacopoeia Monograph 10.3, 01/2021:0547 – Cyanocobalamin

[6] *Technical dossier, Section II, Annex 2.26 Vitamin B12 Method of analysis USP 31 171

[7] *Technical dossier, Section II, Annex 2.22 & Annex 2.23

[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

*Refers to Dossier no: FAD-2021-0041

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)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft, Nossen (DE)
- Wageningen Food Safety Research (WFSR)[1] (NL)

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