
Vitamin B₁₂ / Cyanocobalamin

FAD-2010-0173 - CRL/100070
FAD-2010-0199 - CRL/100076
FAD-2010-0326 - CRL/100318

Dossier related to: FAD-2010-0173 - CRL/100070  
FAD-2010-0199 - CRL/100076  
FAD-2010-0326 - CRL/100318

Name of Feed Additive: Vitamin B₁₂ / Cyanocobalamin

Active Agent (s): Vitamin B₁₂ / Cyanocobalamin

Rapporteur Laboratory: University of Ljubljana, Veterinary Faculty, National Veterinary Institute (VF-NVI), Ljubljana, Slovenia

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Date: 29/05/2013

Report approved by: Christoph von Holst  
Date: 31/05/2013
EXECUTIVE SUMMARY

In the current three applications (FAD-2010-0173, 0199 and 0326), authorisation is sought under Articles 4(1) and 10(2) for Vitamin B₁₂ / 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 Applicants, Vitamin B₁₂/cyanocobalamin is produced by fermentation, using different bacterial strains and later reaction with cyanide to form a dark red crystals or crystalline powder of cyanocobalamin, with a minimum purity of 96%. The active substance (Cyanocobalamin) will be marketed in several preparations: - for Applicant FAD-2010-0173 the feed additive consists of the pure active substance containing a minimum of 96% cyanocobalamin; - Applicant FAD-2010-0199 describes a "crude Vitamin B₁₂" preparation as organic or inorganic carriers including from 0.1 to 5 % cyanocobalamin, while -Applicant FAD-2010-0326 refers to a "feed grade Vitamin B₁₂" preparation containing from 30 to 40% cyanocobalamin. Vitamin B₁₂ is intended to be incorporated in feedingstuffs through premixtures or directly in water. No minimum or maximum concentrations in feedingstuffs or in water are specified, however the typical concentration ranges from 10 to 80 μg/kg compound feed, depending on the target species.

For the characterisation of cyanocobalamin per se, Applicants FAD-2010-0173 submitted the European Pharmacopoeia method (Eur. Ph. 6.0, 01/2008:0547), where identification is based on spectrophotometry or thin-layer chromatography (TLC); quantification is based on spectrophotometry (UV/VIS), while purity is assessed by liquid chromatography followed by spectrophotometry (LC-UV/VIS). Even though no performance characteristics of the method are provided, the EURL recommends for official control the European Pharmacopoeia method for the characterisation of cyanocobalamin per se.

For the determination of cyanocobalamin in water, the Applicant (FAD-2010-0173) proposed the European Pharmacopoeia UV/VIS method mentioned above without providing any experimental data to support such a claim. Therefore, the EURL can neither evaluate nor recommend this method to determine Vitamin B₁₂ in water.

For the determination of cyanocobalamin in premixtures and feedingstuffs, the Applicants submitted several microbiological essays, such as the AOAC and US Pharmacopoeia methods. Even though the validation and verification data provided by Applicants FAD-2010-0173 and FAD-2010-0199 seems to be acceptable some NRLs expressed their concern about
the applicability of the proposed microbiological method for the quantification of cyanocobalamin in premixtures and feedingstuffs. Alternative HPLC methods have been published in the scientific literature, related to the determination of Vitamin B₁₂ in food commodities. As they were not tested on feed samples, they are not recommended by the EURL for official control.

**KEYWORDS**

Vitamin B₁₂, cyanocobalamin, nutritional additives, vitamins, all animal species and categories.

**1. BACKGROUND**

In the current three applications (FAD-2010-0173, 0199 and 0326), authorisation is sought under Articles 4(1) (new use in water) [1] and 10(2) (re-evaluation of additives already authorised under the provisions of the Council Directive 70/524/EEC) [1-3] for Vitamin B₁₂/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 Applicants, Vitamin B₁₂/cyanocobalamin is produced by fermentation, using different bacterial strains, and later reaction with a cyanide to form cyanocobalamin [4-6]. It is a dark red crystals or crystalline powder with a minimum purity of 95 % [4].

For Applicant FAD-2010-0173 the feed additive consists of the pure active substance containing a minimum of 96% cyanocobalamin [4]. Applicant FAD-2010-0199 describes a "crude Vitamin B₁₂" preparation as organic or inorganic carriers including from 0.1 to 5 % cyanocobalamin [5], while Applicant FAD-2010-0326 refers to a "feed grade Vitamin B₁₂" preparation containing from 30 to 40% cyanocobalamin [6].

Cyanocobalamin is intended to be incorporated in feedingstuffs through premixtures or directly in water [7]. No minimum or maximum concentrations in feedingstuffs or in water are specified, however the typical concentration ranges from 10 to 80 μg/kg compound feed [7,8], depending on the target species.
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 (EFSA) for each application or group of applications. For these dossiers, the methods of analysis submitted in connection with Vitamin B\textsubscript{12} / cyanocobalamin 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) are available from the respective European Union Reference Laboratories [9].

Description of the analytical methods for the determination of the active substances in feed additive, premixtures and feedingstuffs.

For the characterisation of cyanocobalamin in the feed additive, Applicant (FAD-2010-0173) submitted the European Pharmacopoeia method (Eur. Ph. 6.0, 01/2008:0547) [10], where:

- **identification** is based on UV/VIS spectrophotometry at 278, 361 and 547-559 nm or on thin-layer chromatography (TLC);

- **quantification** is based on spectrophotometry (UV/VIS) at 361 nm. The content of cyanocobalamin is calculated taking into account the specific absorbance ($E_{1\%}^{1\text{ cm}} = 207$);

- while purity control is performed using liquid chromatography followed by spectrophotometry at 361 nm (LC-UV/VIS).

Even though no performance characteristics of the method are provided, the EURL recommends for official control the above mentioned European Pharmacopoeia method for the characterisation of cyanocobalamin per se.
For the determination of cyanocobalamin in water, the Applicant (FAD-2010-0173) proposed the European Pharmacopoeia spectrophotometric method mentioned above [10], without providing any experimental data to support such a claim. Therefore the EURL can neither evaluate nor recommend this method to determine Vitamin B$_{12}$ in water.

For the determination of cyanocobalamin in premixtures and feedingstuffs Applicants (FAD-2010-0173 and FAD-2010-00326) proposed two similar microbiological methods (USP 31-171 [11] and AOAC 952.20 [12]), using the test organism Lactobacillus leichmannii ATCC 7830. However, these two methods were developed for the determination of pure substance and for determination of Vitamin B$_{12}$ in vitamin preparations. At first none of the two Applicants provided experimental evidence proving the applicability of these methods to premixture or feedingstuffs samples. Both Applicants were then requested by the EURL to submit supplementary experimental data. Applicant FAD-2010-0173 suggested using the microbiological method submitted by Applicant FAD-2010-0199 and discussed hereafter [13].

Applicant FAD-2010-0326 reported additional experimental data [14] related to Vitamin B$_{12}$ results obtained by only two official control laboratories using the AOAC method 952.20. The scattered data reported do not clearly demonstrate the suitability of the AOAC method to determine Vitamin B$_{12}$ in premixtures and/or feedingstuffs.

Applicant (FAD-2010-0199) proposed a single-laboratory validated microbiological method [15], based on the USP 31-171 method [11] for the determination of Vitamin B$_{12}$ in food, feed, vitamin premixes for feed and vitamin B$_{12}$ concentrates. This method was further verified by a second independent laboratory [16].

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 (0.5 to 10 g for solids or 5 to 10 mL for liquid samples) are extracted with the vapour-sterilised aqueous solution containing disodium hydrogen phosphate, citric acid and sodium disulfite. Standard solution is prepared by dissolving 1 mg cyanocobalamin in 25 mL of water; 12.5 mL of ethanol are added, equilibrated at room temperature and filled with water to 50 mL. Then the sample extracts as well as the standard solutions are added to nutrient medium (commercially available MRS Bouillon from Merck) in test tubes, autoclaved and inoculated with the test organism Lactobacillus leichmannii ATCC 7830. After incubation for 15 hours at 37 ± 1 °C in water bath, the turbidity of the suspensions is measured in 2 cm cuvettes using a spectrophotometer at 540-560 nm. The quantification is done by external calibration.
The Applicant provided four sets of experimental results, consisting of the validation study [15], two consecutive supplementary information data sets [17, 18], and the verification study performed by a second independent expert laboratory [16]. The reported method performance characteristics related to the quantification of cyanocobalamin in the "crude Vitamin B12" preparation, premixtures and feedingstuffs are summarised in Table 1. Furthermore, the Applicant reported limits of detection (LOD) and quantification (LOQ) of 1 and 2 μg/kg feedingstuffs, respectively [15].

Satisfactory recovery rates (Rrec) and precisions (e.g. repeatability and intermediate precision relative standard deviations, RSDr and RSDip) are reported for the feed additive and feedingstuffs; only one reported recoveries for feedingstuffs (Rrec = 142% [17]) seems relatively high. As for premixture samples, large dispersions of Rrec (from 52 to 103 %) and of RSDip (from 4 to 21 %) are observed.

Even though the validation and verification data provided by Applicants FAD-2010-0173 and FAD-2010-0199 seems to be acceptable, some NRLs expressed their concern on the reliability of the proposed microbiological method for the quantification of cyanocobalamin in premixtures and feedingstuffs. In the recent years, few NRLs applied the microbiological method mentioned above and observed significant differences between the analytical results and the vitamin B12 contents indicated on the labels. Therefore the EURL is unable to recommend this method for official control.

<table>
<thead>
<tr>
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<th>CVB (mg/kg)</th>
<th>PM (mg/kg)</th>
<th>FS (mg/kg)</th>
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<tr>
<td><strong>RSDr (%)</strong></td>
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<tr>
<td>Validation</td>
<td>[15]</td>
<td>2,4</td>
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<tr>
<td>Verification</td>
<td>[16]</td>
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<td><strong>RSDip (%)</strong></td>
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<tr>
<td>Validation</td>
<td>[15]</td>
<td>4,1</td>
<td>3,2</td>
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<td>[17]</td>
<td>6</td>
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<tr>
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<td>[16]</td>
<td>12</td>
<td>21,6</td>
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<tr>
<td><strong>Rrec (%)</strong></td>
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<td>[15]</td>
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<tr>
<td>Verification</td>
<td>[16]</td>
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</table>

RSDr and RSDip: relative standard deviation for repeatability and intermediate precision, respectively. 
Rrec: recovery rate
The EURL identified alternative liquid chromatography methods successfully applied to determine Vitamin $B_{12}$ in food commodities. High performance liquid chromatography coupled to supercritical fluid extraction (SFE-HPLC) provided results comparable to those obtained when using the AOAC microbiological method 952.20 for the analysis of infant formulas and adult nutritionals [19]. Similarly, reverse-phase HPLC after immunoaffinity extraction proved to be successful when analyzing Vitamin B12 in food supplements and premixes [20, 21]. As no experimental data is available to prove the applicability of these methods to determine cyanocobalamin in feed samples, the EURL cannot recommend them for official control. However, laboratories may consider implementing these methods and perform proper validation.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for official control the European Pharmacopoeia method (Eur. Ph. 6.0, 01/2008:0547) based on thin-layer chromatography (TLC) and spectrophotometry (UV/VIS) to determine cyanocobalamin per se.

For the determination of cyanocobalamin in water, the Applicant (FAD-2010-0173) proposed the European Pharmacopoeia UV/VIS method mentioned above but did not provide any experimental data to support his claim. Therefore the EURL can neither evaluate nor recommend this method to determine Vitamin $B_{12}$ in water.

For the determination of cyanocobalamin in premixtures and feedingstuffs, the Applicants submitted several microbiological essays, such as the AOAC and US Pharmacopoeia methods. Even though the validation and verification data provided by Applicants FAD-2010-0173 and FAD-2010-0199 seems to be acceptable, some NRLs expressed their concern on the applicability of the proposed microbiological method for the quantification of cyanocobalamin in premixtures and feedingstuffs. Alternative HPLC methods have been published in the scientific literature, related to the determination of Vitamin $B_{12}$ in food commodities. As they were not tested on feed samples, they are not recommended by the EURL for official control.

**Recommended text for the register entry (analytical method)**

For the determination of Vitamin $B_{12}$/ cyanocobalamin in the feed additive:

- thin-layer chromatography (TLC) and spectrophotometry (UV/VIS) - European Pharmacopoeia monograph 0547
5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Vitamin B12 / 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] aApplication/Ref: SANCO/D/2:0111-2010
[4] cTechnical dossier, Section II, 2.1.3 Qualitative and quantitative composition
[5] aTechnical dossier, Section II, 2.1.3 Qualitative and quantitative composition
[6] bTechnical dossier, Section II, 2.1.3 Qualitative and quantitative composition
[7] a,b,cTechnical dossier, Section II, 2.5 Conditions of use
[8] aTechnical dossier, Section II, Ref 2.5.01 FEFANA vitamin supplementation
[12] cTechnical dossier, Section II, Annex II 16 AOAC Microbiological assay
[13] bSupplementary Information, SIN-2013, Lohmann_FAD-2010-0173_Sln Letter_26022013.pdf
[16] bSupplementary Information, SIN-2012, Verification Report Vitamin B12_Final_040211
[17] bSupplementary Information, SIN-2012, Vitamin B12 LUFA-ITL Kiel-official verification report format.pdf
[18] bSupplementary Information, SIN-2013, Annex 2_Lohmann.pdf

a Refers to Dossier FAD-2010-0173
b Refers to Dossier FAD-2010-0199
c Refers to Dossier FAD-2010-0326
7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was University of Ljubljana, Veterinary Faculty, National Veterinary Institute (VF-NVI), Ljubljana, Slovenia. 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:

- Laboratori Agroalimentari, Departament d’Agricultura, Ramaderia, Pesca, Alimentacio i Medi Natural Generalitat de Catalunya, Cabrils (ES)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Danish Veterinary and Food Administration, Ringsted (DK)
- Federaal Laboratorium voor de Voedselveiligheid Tervuren (FLVVT – FAVV), Tervuren (BE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Istituto Superiore di Sanita’ - Dipartimento di Sanita' alimentare ed animale, Roma (IT)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Jena (DE)
- Schwerpunktltabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)