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Safety and efficacy of vitamin B₁₂ (in the form of cyanocobalamin) produced by *Ensifer* spp. as a feed additive for all animal species based on a dossier submitted by VITAC EEIG

EFSA Panel on Additives and Products or Substances used in Animal Feed
(EFSA FEEDAP Panel),

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Abstract

Cyanocobalamin is a synthetic form of vitamin B₁₂ used in pharmaceuticals, supplements and as a food additive. It is intended to be used in feed for all animal species and categories. The European Commission asked EFSA for an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of cyanocobalamin produced by fermentation with *Ensifer adhaerens* strains SCM 2034 or CICC 11008s or *Ensifer fredii* strain CMCC (B) 70000. Since relevant data were not provided by the applicant that would allow a proper identification and characterisation of the production strains, the Additives and Products or Substances used in Animal Feed (FEEDAP) Panel cannot conclude on the safety of the use of vitamin B₁₂ produced using *E. adhaerens* CICC 11008s and *E. fredii* CMCC (B) 70000 in animal nutrition for the target animals, the consumer, the user and the environment. Due to significant uncertainties on the identity and safety of the production strain *E. adhaerens* SCM 2034, including the presence of antibiotic resistance genes, the absence of viable cells of the production strain or their DNA in the product, the FEEDAP Panel cannot conclude on the safety of the use of vitamin B₁₂, produced by *E. adhaerens* SCM 2034 in animal nutrition for the target species, consumers and the environment. Due to high endotoxin content, potential inhalation exposure when handling premixtures and reported irritancy for skin and eyes, vitamin B₁₂ produced by *E. adhaerens* SCM2034 is considered to pose a risk to user safety. Vitamin B₁₂ additives produced by *Ensifer* spp. are regarded as effective in meeting animals' requirements.

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Summary

Following a request from European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of vitamin B₁₂ in the form of cyanocobalamin, produced by fermentation with *Ensifer adhaerens* strains SCM 2034 or CICC 11008s or *Ensifer fredii* strain CMCC (B) 70000, as a feed additive for all animal species.

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of vitamin B₁₂ in the form of cyanocobalamin was in line with the principles laid down in Regulation (EC) No. 429/2008¹ and the relevant EFSA Guidance documents. The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

Since relevant data were not provided by the applicant that would allow a proper identification and characterisation of the production strains, the FEEDAP Panel cannot conclude on the safety of the use of vitamin B₁₂ produced using *E. adhaerens* CICC 11008s and *E. fredii* CMCC (B) 70000 in animal nutrition for the target animals, the consumer, the user and the environment.

Due to significant uncertainties on the identity and safety of the production strain *E. adhaerens* SCM 2034, including the presence of antibiotic resistance genes, the absence of viable cells of the production strain or their DNA in the product, the FEEDAP Panel cannot conclude on the safety of the use of vitamin B₁₂, produced by *E. adhaerens* SCM 2034 in animal nutrition for the target species, consumers and the environment.

Due to high endotoxin content, potential inhalation exposure when handling premixtures and reported irritancy for skin and eyes, vitamin B₁₂ produced by *E. adhaerens* SCM2034 is considered to pose a risk to user safety.

Vitamin B₁₂ additives produced by *Ensifer* spp. are regarded as effective in meeting animals' requirements.

¹ Commission Regulation (EC) No. 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No. 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No. 1831/2003² establishes the rules governing the European Community authorisation of additives for use in animal nutrition. In particular, Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, at the latest 1 year before the expiry date of the authorisation given pursuant to Directive 70/524/EEC for additives with a limited authorisation period, and within a maximum of 7 years after the entry into force of this Regulation for additives authorised without a time limit or pursuant to Directive 82/471/EEC.

The European Commission received a request from VITAC EEIG Vitamins Authorisation Consortium³ for re-evaluation of the product vitamin B₁₂ in the form of cyanocobalamin, produced by *Ensifer adhaerens* (strains SCM 2034 or CICC 11008s) or *E. fredii* (strain CMC (B) 70000), when used as a feed additive for target species (category: nutritional additive; functional group: vitamins, provitamins and chemically well-defined substances having similar effect).

According to Article 7(1) of Regulation (EC) No. 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 4 May 2012.⁴

According to Article 8 of Regulation (EC) No. 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product vitamin B₁₂ in the form of cyanocobalamin, produced by *E. adhaerens* SCM 2034 or *E. adhaerens* CICC 11008s or *E. fredii* strain CMC (B) 70000, when used under the proposed conditions of use (see Section 3.3).

1.2. Additional information

Vitamin B₁₂ in the form of cyanocobalamin has been authorised without time limit under Council Directive 70/524/EEC⁵ for its use for all animal species as a nutritional additive and is currently included in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 and foreseen for re-evaluation. It is authorised as a nutritional additive for use in all animal species without maximum content.

Cyanocobalamin is described in the European Pharmacopoeia as Monograph (MG) 0547 (PhEur, 2014).

Vitamin B₁₂ in the form of cyanocobalamin is authorised for use in food⁶ and food supplements,⁷ for addition for specific nutritional purposes to foods for particular nutritional uses,⁸ to processed

² Regulation (EC) No. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

³ On 13/03/2013, EFSA was informed by the applicant that VITAC EEIG was liquidated on 19/12/2012 and their rights as applicant were transferred to FEFANA asbl (EU Association of Specialty Feed Ingredients and their Mixtures, representing the following companies: Chr. Olesen Groups, Gentofte, Denmark; DSM Nutritional Products Europe Ltd, Basel, Switzerland; Trouw Nutrition International B.V., Putten, the Netherlands. Avenue Louise, 130A, Box 1, 1050 Brussels, Belgium.

⁴ A new mandate was received in EFSA in April 2012.

⁵ Commission List of the authorised additives in feedingstuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs (2004/C 50/01). OJ C 50, 25.2.2004, p. 1.

⁶ Regulation (EC) No. 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods. OJ L 404 30.12.2006, p. 26, last amended by Commission Regulation (EC) No. 1170/2009 of 30 November 2009 amending Directive 2002/46/EC of the European Parliament and of Council and Regulation (EC) No. 1925/2006 of the European Parliament and of the Council as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements. OJ L 314 1.12.2009, p. 36.

⁷ Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. OJ L 183 12.7.2002, p. 51.

⁸ Commission Regulation (EC) 953/2009 of 13 October 2009 on substances that may added for specific nutritional purposes in foods for particular nutritional uses. OJ L 269, 14.10.2009, p. 9.

cereal-based foods and baby foods for infants and young children⁹ and to infant formulas and follow-on formulas when reconstituted as instructed by the manufacturer.¹⁰

Vitamin B₁₂ is listed as pharmacologically active substance in veterinary medicinal products and is not subject to maximum residue levels when used in food-producing animals.¹¹ Cyanocobalamin is also listed as ingredient in cosmetic products as a skin conditioning agent.¹²

The Scientific Committee on Food (SCF) issued an opinion on the tolerable upper intake level of vitamin B₁₂ (European Commission, 2000). The EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) published a scientific opinion on 5'-deoxyadenosylcobalamin and methylcobalamin as sources for Vitamin B₁₂ added as a nutritional substance in food supplements (EFSA ANS Panel, 2008) and a statement on the inability to assess the safety of vitamin B₁₂-enriched yeast added for nutritional purposes as a source of vitamin B₁₂ in food supplements and the bioavailability of vitamin B₁₂ from this source, based on the supporting dossier (EFSA ANS Panel, 2009). The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) published a scientific opinion on the use of cobalt compounds as additives in animal nutrition (EFSA FEEDAP Panel, 2009b) and another on the safety and efficacy of cyanocobalamin produced by fermentation using *E. adhaerens* CICC 11008s (EFSA FEEDAP Panel, 2015). The EFSA Panel on Dietetic Products, Nutrition and Allergies published a scientific opinion on the substantiation of several health claims related to vitamin B₁₂ pursuant to Article 13(1) of Regulation (EC) No. 1924/2006 (EFSA NDA Panel, 2009, 2010); and on dietary reference values for cobalamin (vitamin B₁₂) (EFSA NDA Panel, 2015).

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier¹³ in support of the authorisation request for the use of vitamin B₁₂ in the form of cyanocobalamin as a feed additive. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008¹⁴ and the applicable EFSA Guidance documents.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the vitamin B₁₂ in the form of cyanocobalamin in animal feed. The Executive Summary of the EURL report can be found in Annex A.¹⁵

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of vitamin B₁₂ in the form of cyanocobalamin is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance on nutritional additives (EFSA FEEDAP Panel, 2012a), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008a), Guidance for the preparation of dossiers for the re-evaluation of certain additives already authorised under Directive 70/524/EEC (EFSA, 2008b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012b), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012c), Guidance on the assessment of bacterial

⁹ Commission Directive 2006/125 EC of 5 December 2006 on processed cereal-based foods and baby-foods for infants and young children. OJ L 339 6.12.2006, p. 16.

¹⁰ Commission Directive 2006/141 EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. OJ L 401 30.12.2006, p. 1.

¹¹ Commission Regulation (EU) 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. OJ L 15, 20.1.2010, p. 1.

¹² Commission Decision 2006/257/EC of 9 February 2009 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97 5.04.2006, p. 1.

¹³ FEED dossier reference: FAD-2010-0199.

¹⁴ Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No. 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

¹⁵ The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-201-0173-0199-0326Vit_B12.pdf

susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012d), Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA GMO Panel, 2011).

3. Assessment

The current opinion deals with the re-evaluation of the use of vitamin B₁₂ (nutritional additive, functional group vitamins, provitamins and chemically well-defined substances having similar effects) in the form of cyanocobalamin produced by two strains of *E. adhaerens* or one strain of *E. fredii* for use in feed for all animal species and categories.¹⁶ Sections 3.1–3.5 describe general information common to all three additives. Specific information of every production strain and related additive follows thereafter.

3.1. Characterisation of the active substance

Cyanocobalamin (International Union of Pure and Applied Chemistry (IUPAC) name: cobalt(3+); [(2R,3S,4R,5S)-5-(5,6-dimethylbenzimidazol-1-yl)-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl] [(2R)-1-[3-[(1R,2R,3R,5Z,7S,10Z,12S,13S,15Z,17S,18S,19R)-2,13,18-tris(2-amino-2-oxoethyl)-7,12,17-tris(3-amino-3-oxopropyl)-3,5,8,8,13,15,18,19-octamethyl-2,7,12,17-tetrahydro-1H-corrin-24-id-3-yl] propanoylamino]propan-2-yl] phosphate; cyanide; synonyms: vitamin B₁₂, cobalamin, 5,6-dimethylbenzimidazolcyanocobalamide), is identified with the Chemical Abstracts Service (CAS) number 68-19-9 and the European Inventory of Existing Chemical Substances (EINECS) number 200-680-0. It has a molecular weight of 1355.37. Its molecular formula is C₆₃H₈₈CoN₁₄O₁₄P and its structural formula is shown in Figure 1.

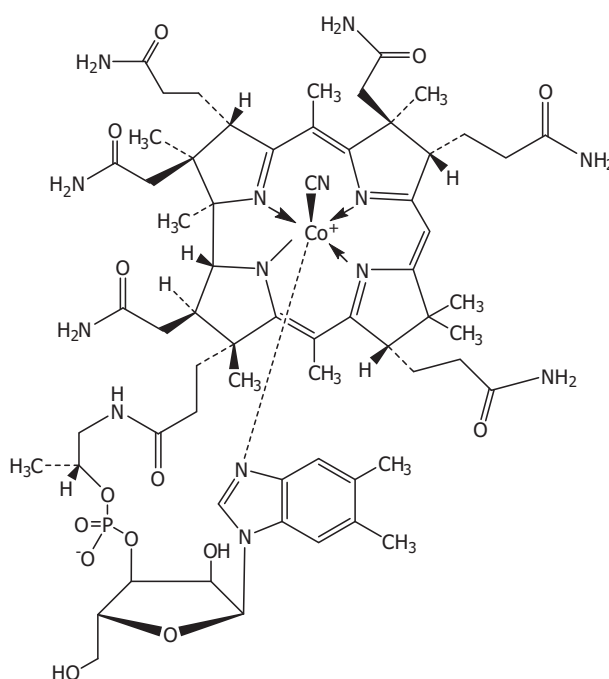


Figure 1: Structural formula of cyanocobalamin

Cyanocobalamin has a melting point > 300°C, a bulk density of 400–800 kg/m³ and is sparingly soluble in water (1.25 g dissolves in about 100 mL) and in 95% ethanol, insoluble in acetone, ether and chloroform.¹⁷ The anhydrous substance is very hygroscopic.

Vitamin B₁₂ produced by fermentation is described in the European Pharmacopoeia (PhEur 7.0, 0547) with a purity of 96.0–102.0% in the dried substance, less than 3% total related impurities, loss on drying < 12%. It is described as a dark red crystalline powder or consisting of dark red crystals and is practically odourless and tasteless.

¹⁶ Technical dossier/Section II and Supplementary information of January 2014/2014-01-08 VITAC B12 SIn reply.

¹⁷ Technical dossier/Section 2.2.2.

3.2. Manufacturing process

Vitamin B₁₂ is produced by fermentation using different strains of *E. fredii* [REDACTED] or *E. adhaerens* [REDACTED]

The fermentation process leads to cobamamide. At the end of the process, the fermentation broth is [REDACTED] to kill all possible bacteria. [REDACTED]

[REDACTED] the cyanocobalamin content in the liquid so-called 'refined B₁₂ solution' is around 1–2%. A carrier ([REDACTED]) is then added to the solution and [REDACTED] dried [REDACTED]. The concentration of cyanocobalamin in the solid 'non-standardised concentrated B₁₂' is 4–10%.²⁰ For instance, analytical data on batch to batch variation of five batches of the dry 'non-standardised concentrated B₁₂ solution' produced by *E. adhaerens* SCM2034 showed an average of 8.4% vitamin B12 (range 8–9%), loss on drying ranged from 2 to 4%, protein was 5% (in all batches) and amorphous silica ranged from 81 to 83%.²¹

The non-standardised concentrated B₁₂ is diluted with carriers ([REDACTED])²² to obtain a feed additive preparation with a fixed concentration of cyanocobalamin. Depending on the product, a concentration of 0.1, 1.0 or 2.0% is reached.

3.3. Conditions of use

Vitamin B₁₂ in the form of cyanocobalamin is intended for use in all animal species and categories without maximum limit. The active substance can be administered via feed (premixtures, complete or complementary feed).²³ The applicant proposes a use level between 10 and 100 µg/kg complete feed.

3.4. Physicochemical incompatibilities in feed

No physicochemical incompatibilities or interactions have been reported between cyanocobalamin and feed materials, carriers, other approved additives or medicinal products when the additive is added to premixtures and feed. No such incompatibilities or interactions are expected.

3.5. Characterisation of the production strains

The information received related to production strains *E. adhaerens* CICC 11008s or *E. fredii* CMC (B) 70000 was insufficient to identify and properly characterise the strains and perform an assessment.²⁴

As regards *E. adhaerens* CICC 11008s, no information was provided on the identification, toxigenic potential, antimicrobial sensitivity and history of modifications of the strain, including possible genetic modifications.

In relation to *E. fredii* CMC (B) 70000, reports on the identification and antimicrobial sensitivity tests of a different strain [REDACTED] were provided, [REDACTED] although its relationship with the production strain could not be established. No information was provided on the history of modifications including possible genetic modifications.

Those production strains and their related products were not further considered in the assessment.

²⁰ Technical dossier/Supplementary information January 2014/2014-01-08 VITAC B12 Sin reply, response to Qi.

²¹ Technical dossier/Supplementary information January 2014/Annexes/Annex Table II.2b data.

²² Technical dossier/Section 2.1.4.2.

²³ Technical dossier/Section 2.5.1.

²⁴ Technical dossier/Supplementary information January 2014/Annex E.

3.5.1. Characterisation of *Ensifer adhaerens* SCM2034

The product Vitamin B₁₂ under assessment is produced by a genetically modified strain stated to belong to *E. adhaerens* species, identified as SCM2034. The strain is not deposited in a culture collection.

The production strain was not identified. [REDACTED]

[REDACTED] Details of the relationship between this strain and the production strain were not provided.

Ensifer adhaerens is a Gram-negative bacterium. Colonisation by *Ensifer* enables plants to utilise nitrogen from the environment. The applicant conducted a literature search on the safety of *E. adhaerens*, using the following key words: *Ensifer adhaerans*, toxin and virulence factor plus other key words that were not specified. The search produced no results.²⁷ Former names of the species were not included in the search and no further details on the search (e.g. the databases searched, the complete set of key words used) were provided. Therefore, uncertainties remain on the safety of this production strain.

Some *Ensifer* species have been reported to have resistance to multiple antibiotics (Bromfield et al., 2010; Rathore et al., 2015).²⁸ No data on the sensitivity of the production strain to antimicrobials relevant for human or veterinary medicine were provided except for [REDACTED]

Information related to the genetically modified microorganism³⁰

Characteristics of the recipient or parental microorganism

Characteristics of the donor organisms

Description of the genetic modification process

As a consequence of the genetic modification [REDACTED], leading to increased vitamin B₁₂ production, and [REDACTED].

3.6. Characterisation of the additive produced by *E. adhaerens* SCM2034

Standardised vitamin B₁₂ feed additive

Analytical data on batch to batch variation of five batches of the dry product formulated at 0.1% vitamin B₁₂ showed an average of 0.1% vitamin B₁₂ (the same value in all batches).²¹

Analytical data on batch to batch variation of 10 batches of the product formulated at 1% vitamin B₁₂ showed an average of 1% vitamin B₁₂ (range 1.00–1.01%).³² The remainder of the additive is

²⁷ [REDACTED] Technical dossier/Supplementary information January 2017/20170112 Sin reply/reply to question viii.

²⁸ [REDACTED] Technical dossier/Section 3.1.2.

³⁰ [REDACTED] Technical dossier/Supplementary information January 2017.

³² [REDACTED] Technical dossier/Supplementary information January 2014/Anexes/Annex Table II.2b data and supplementary information January 2017/Annex Q3 addendum CoA B12 feed grade.

stated to contain of calcium carbonate, silica and loss on drying were ~ 90%, ~ 8% and < 1%, respectively.³³

Heavy metals and arsenic were determined in three batches of the additive standardised at 1% vitamin B₁₂. Cadmium and lead ranged from 0.040 to 0.043 mg/kg, mercury from < 0.005 to 0.005 mg/kg and arsenic from 0.20 to 0.22 mg/kg.³⁴ Dioxins, sum of dioxins and dioxin-like polychlorinated biphenols (PCBs) and non-dioxin-like PCBs were determined in three batches and were 0.2 ng/kg, 0.32 ng/kg and 1.17–1.19 ng/kg, respectively. These analytical results are considered not to raise safety concerns. No data were provided on microbial contamination or mycotoxin content in the final product.

Ensifer adhaerens is a Gram-negative bacterium able to produce endotoxins. Three batches were analysed for endotoxin activity [REDACTED]

The applicant declared that no antibiotics are used during the manufacturing process of vitamin B₁₂ 1%.³⁶

The absence of viable cells of the production strain was tested on three batches of the fermentation product [REDACTED]

The FEEDAP Panel considers that this [REDACTED] methodology is not sufficiently reliable for demonstrating the absence of viable cells of the production strain in the product. Therefore, uncertainty remains on the potential presence of viable cells of the production strain in the final product.

No recombinant DNA was found in five batches of the formulated additive containing 1% vitamin B₁₂ [REDACTED]

However, the target sequence exceeded the size of the [REDACTED] gene, which is the gene of concern. Uncertainty remains on the possible presence of recombinant DNA corresponding to the [REDACTED] gene in the product.

Physical characteristics

The formulated feed additive, either at 0.1 or 1% vitamin B₁₂, is described as a solid pale red–brown fine powder, odourless. It has a density of 1.16 g/cm³. It is insoluble in water.³⁹

The particle size distribution of three batches of the formulation containing 1% vitamin B₁₂ was measured by laser diffraction. The fractions of particles having a diameter < 107, < 51 and < 11 µm ranged from 89 to 91%, from 57 to 64% and from 21 to 25%, respectively.⁴⁰

The dusting potential (Stauber–Heubach) was measured in three batches of the formulated additive containing 1% vitamin B₁₂.⁴¹ The values ranged from 2.4 to 3.0 g/m³.

Stability and homogeneity

The applicant provided data on the shelf-life of the additive formulated at 1% vitamin B₁₂. The samples were stored in the commercial packaging at room temperature for 36 months. Losses observed ranged from 3 to 6%.⁴²

No data on the stability of the additive in premixtures and feedingstuffs were provided

The capacity of the additive (one batch) to distribute homogeneously in three different feedingstuffs (two for chicken for fattening and one for piglets) was studied (10 subsamples each) when supplemented at 4 mg/kg feed (corresponding to 40 µg vitamin B₁₂/kg feed).⁴³ The additive was added via premixture to the feedingstuffs. The coefficient of variation was 6 and 9% for the chicken complete feed, respectively, and 9% for the piglet feed.

³³ Technical dossier/Supplementary information January 2017/20170112 Sin reply/Question 3

³⁴ Technical dossier/Supplementary information January 2017/Annex Q5 addendum CoA dioxin HM.

³⁶ Technical dossier/Supplementary information January 2017/20170112 Sin reply EFSA/Response Sin letter Question iv.

³⁹ Technical dossier/Supplementary information January 2014/Annex D.

⁴⁰ Technical dossier/Supplementary information January 2017/Annex Q15 add granulo laser.

⁴¹ Technical dossier/Supplementary information January 2017/Annex Q15 add dusting.

⁴² Technical dossier/Supplementary information January 2017/20170112 Sin reply EFSA, last question.

⁴³ Technical dossier/Supplementary information January 2017/Annex Q 10 add homogeneity.

3.7. Safety of cyanocobalamin produced by *Ensifer adhaerens* SCM2034

3.7.1. Safety aspects of the production strain

The identity of the production strain was not available. The literature search performed by the applicant did not make it possible to conclude on the capacity of the production strain to produce toxins or to harbour virulence factors.

As regards the genetic modification, the traits introduced in the production strain *E. adhaerens* SCM2034 include [REDACTED]. Possible resistance to [REDACTED] relevant antimicrobials is unknown. The strain carries [REDACTED]. The potential presence in the product of the gene [REDACTED] and [REDACTED] possible antibiotic resistance genes is considered a concern. Uncertainties remain on the possible presence of the production strain and its recombinant DNA in the product. Therefore, the EFSA FEEDAP Panel cannot conclude on the safety of the product Vitamin B₁₂, manufactured with *E. adhaerens* SCM2034, with regard to the genetically modified production strain.

3.7.2. Toxicological studies

Owing to the characteristics of the production process and its resulting products (e.g. in the concentrated non-standardised vitamin B₁₂, the amount of active substance is low and the unidentified material exceeds 1%), toxicological studies were provided.

The applicant stated that all toxicological studies were performed with non-standardised concentrated vitamin B₁₂ produced by *E. adhaerens* SCM2034. ■

Genotoxicity studies

Bacterial reverse mutation tests

Bacterial reverse mutation tests (OECD Guideline 471) were performed on the non-standardised concentrate vitamin B₁₂ using *Salmonella Typhimurium* strains TA98, TA1535, TA1537, TA100 and TA102, with and without metabolic activation.⁴⁵ Significant increases or revertant colonies occurred in *Salmonella* strain TA98 with and without metabolic activation. These increases were not abolished when the 'treat and wash' method was applied, suggesting that the effect is not due to the presence of proteins and amino acids in the test item.

Gene mutation test in mammalian cells

The non-standardised concentrated vitamin B₁₂ did not induce any biologically significant increase in mutant frequency in the *in vitro* gene mutation tests in mammalian cells (TK gene in L5178Y mouse lymphoma cells) (OECD Guideline 490) when tested in the absence of metabolic activation, either using 3 h or 24 h treatments.⁴⁶ In the 3-h treatment, an apparent dose–response trend was observed both in the presence and in the absence of metabolic activation (linear trend test ($b^2/\text{var}(b)$): $p < 0.001$); however, only in the presence of metabolic activation (3 h treatment), a slight biologically significant increase in the mutant frequency was observed at 2,000 µg/mL, a concentration inducing a cytotoxicity level close to the limit recommended by OECD (12.2 vs. 10% relative total growth). The observed increase was mainly due to the induction of small colonies, indicating a possible clastogenic activity.

In vitro micronucleus test

The possible clastogenic activity mentioned above was not confirmed when non-standardised concentrate vitamin B₁₂ was tested in the *in vitro* micronucleus assay conducted on TK6 cells (according to OECD Guideline 487).⁴⁷ No induction of micronucleated cells was reported in the absence (short-term and continuous treatments) and in the presence (short-term treatment) of metabolic activation when tested up to 5,000 µg/mL of the product. Significant cytotoxicity was observed at the two highest concentrations tested (2,500 and 5,000 µg/mL).

⁴⁵ Technical dossier/Supplementary information January 2017/SIn 12Jan17/Annexes 20 and 21.

⁴⁶ Technical dossier/Supplementary information January 2017/SIn 12Jan17/Annex *In vitro* mammalian cell gene mutation test.

⁴⁷ Technical dossier/Supplementary information January 2017/SIn 12Jan17/Annex 23.

In vivo comet assay

Oral administration of non-standardised concentrated vitamin B₁₂ at 500, 1,000 or 2,000 mg/kg body weight (bw)/day for 2 days in male rats did not induce any statistically significant increases in the incidence of DNA strand breaks (either pairwise or trend tests and values within the intervals of negative historical control data) in the comet assay conducted in liver and stomach in accordance with OECD Guideline 489.⁴⁸ Therefore, the product tested showed no genotoxic activity *in vivo* in these organs when tested up to 2,000 mg/kg.

Subchronic oral toxicity study

A repeated-dose oral toxicity study was performed with non-standardised concentrated vitamin B₁₂ according to the OECD Guideline 408 at the doses of 250, 500 and 750 mg/kg bw and day for 91 days (10 animals per sex).⁴⁹ A withdrawal period of 28 days followed for the control and the highest dose level (additional 10 animals/sex). Although some mortality was observed, it was not related to the test substance and appeared to be caused by the frequency of gavage. A full examination of haematological, biochemical, urinalysis, ophthalmological and histopathological parameters was carried out.

There were some statistically significant changes in haematological parameters: e.g. red cell volume increased (from 12.3 to 13.0%), prothrombin time increased (from 14.4 to 15.0 s) in males at the highest dose on day 119. Potassium levels in plasma were reduced (4.2 vs 3.9 mmol/L) at the highest dose in males at the end of the period on day 119.

Histopathological examination at day 91 revealed a mild subcortical plasma cell hyperplasia in the examined (submaxillary) lymph node. This finding was observed in 5 of 16 (1/8 males and 4/8 females) at the highest dose level; it was present only in a single animal in control and the other treatment groups (250 and 500 mg/kg bw and day). This finding is considered as potentially adverse and related to the test compound. The study provided a no observed adverse effects level (NOAEL) of 500 mg/kg bw and day.

Conclusions on toxicological studies

Non-standardised concentrated vitamin B₁₂ increased the rate of revertants in *Salmonella* Typhimurium strains TA98 both in the presence and in the absence of metabolic activation. In a gene mutation assay in mammalian cells, an apparent dose–response trend was observed both in the presence and in the absence of metabolic activation, but a slight biologically significant increase in the mutant frequency was observed only with metabolic activation at a concentration inducing a cytotoxicity level close to the limit recommended by OECD. The test item did not induce any statistically significant increase in the incidence of micronuclei in mammalian cells in any experimental condition.

The positive findings observed *in vitro* were not confirmed in an *in vivo* comet assay conducted in the stomach (first site of contact) and in the liver, therefore, a genotoxic potential of the product is considered unlikely.

A subchronic study in rats showed a NOAEL of 500 mg/kg bw and day, based on haematological effects.

3.7.3. Safety for the target species and for the consumer

The requirements of the different animal species, the commercial use levels and the effects of excess supplementation were addressed in a previous opinion (EFSA FEEDAP Panel, 2015). Vitamin B₁₂ itself is considered safe for the target species.

Absorption, distribution, metabolism and excretion; deposition in eggs; vitamin B₁₂ contents in food; average vitamin B₁₂ human intake in the EU, were described in a previous opinion (EFSA FEEDAP Panel, 2015). No concerns for the consumer arise from the use of the cyanocobalamin in animal nutrition. The use of vitamin B₁₂ as feed additive is not expected to modify substantially the content of vitamin B₁₂ in food of animal origin.

For nutritional additives produced by fermentation, the risk associated with the residues of the fermentation process in the final product needs to be assessed. The production strain *E. adhaerens* has not been properly characterised in terms of identity, virulence, toxigenic potential and antibiotic

⁴⁸ Technical dossier/Supplementary information January 2017/SIn 12Jan17/Annex 22.

⁴⁹ Technical dossier/Supplementary information January 2017/SIn 12Jan17/Annex 14.

resistance. The absence of the production strain or its DNA in the final product has not been demonstrated. The strain carries [REDACTED]. Possible resistance of the strain to [REDACTED] antimicrobials relevant for human or veterinary medicine is unknown.

Therefore, the FEEDAP Panel cannot conclude on the safety of the use of the product under assessment, produced by *E. adhaerens* SCM2034 in animal nutrition for the target species and for the consumer.

3.7.4. Safety for the user

There is lack of information on identity, antibiotic resistance as well as the toxigenic and virulence potentials of the strain under assessment.

Effects on the respiratory system

In relation to the additive formulated at 1% vitamin B12, the fractions of particles having a diameter < 107, < 51 and < 11 µm ranged from 89 to 91%, from 57 to 64% and from 21 to 25%, respectively (see Section 3.6). The measured dusting potential ranged from 2.4 to 3.0 g/m³. Exposure of the users by inhalation is likely.

The endotoxin content of the additive is high (the values ranged [REDACTED]). The scenario used to estimate the exposure of persons handling the additive to endotoxins in the dust, based on the EFSA Guidance on user safety (2012c), is described in Appendix A. The health-based recommended threshold for the quantity of inhaled endotoxins per working day is 900 IU, derived from provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (DECOS) (Health Council of the Netherlands, 2010) and the UK Health and Safety Executive (HSE, 2013). Based on the calculation of the potential endotoxin content in dust (Wallace et al., 2016), based on the dusting potential and respirable particle fraction, the inhalation exposure could be up to [REDACTED] endotoxin IU per 8-h working day, indicating a risk from the exposure to endotoxins for people handling the additive.

Effects on skin and eyes

No studies on skin and eye irritation and dermal sensitisation were provided by the applicant. The applicant conducted a literature search to find data on the potential of vitamin B₁₂/cyanocobalamin to be irritant to skin and eyes and/or to be a skin sensitiser.⁵⁰ Four databases were searched (Biological abstracts, CAB abstracts, Scopus and Web of Science). The period of the search was not reported. The search strings consisted different combinations of 'vitamin B₁₂ OR cyanocobalamin'; 'skin OR eye*'; and 'irrita* OR sensit*' using the 'AND' Boolean operator. Title, abstract and keywords were the object of the search. The total number of hits retrieved, inclusion and exclusion criteria and the number of reports rejected was not indicated. Abstracts were screened and a summary of the findings in a tabulated form was provided.⁵¹ From CAB abstracts, eight papers were selected, three from Biological abstracts, six from Web of Science and 15 from Scopus. The most relevant one was a case report of occupational contact dermatitis from vitamin B₁₂ in a worker through handling of animal feed (Rodriguez et al., 1994). In addition, the safety data sheet (SDS) provided by the applicant indicates that the additive (1% vitamin B₁₂) may be irritating to human skin and eyes and it may cause rare hypersensitivity reactions.⁵²

Conclusion on user safety

Due to high endotoxin content, the additive is considered to pose a risk to user upon inhalation.

No conclusion can be taken in regard to antibiotic resistance, toxigenic and virulence potentials of the additive under assessment.

No studies on skin and eye irritation or dermal sensitisation were performed. The additive is reported to be irritant to human skin and eyes and it may cause rare hypersensitivity reactions.

3.7.5. Safety for the environment

Vitamin B₁₂ occurs in nature, in bacteria and the animal kingdom. Its use in animal nutrition is not expected to substantially increase its concentration in the environment.

⁵⁰ Technical dossier/Supplementary information January 2014/2014-01-08 VITAC B12 SIn repl/Qii.

⁵¹ Technical dossier/Supplementary information January 2014/Annexes/Vitamin B12 Literature search.xlsx.

⁵² Technical dossier/Supplementary information January 2014/Annexes SIn 16-05-2012/Annex D MSDS for Vitamin B12 (cyanocobalamin).pdf

However, uncertainty remains on the absence of the production strain and on the possible presence of recombinant DNA and/or antibiotic resistance genes in the product. Therefore, the FEEDAP Panel cannot conclude on the environmental safety of the product with regard to the genetically modified production strain.

3.8. Efficacy

Vitamin B₁₂ has been globally used in animal nutrition for decades. Owing to the long history of use and its established nutritional role in domestic animals, cyanocobalamin is regarded as effective in covering the animal's requirement when administered via feed. Data on requirement, allowances and recommendations for feed supplementation are easily accessible as standard literature for animal nutrition experts.

The additive is considered as an effective source of vitamin B₁₂ in animal nutrition.

3.9. Post marketing monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation⁵³ and Good Manufacturing Practice.

4. General Conclusions

Since relevant data were not provided by the applicant that would allow a proper identification and characterisation of the production strains, the FEEDAP Panel cannot conclude on the safety of the use of vitamin B₁₂ produced using *E. adhaerens* CICC 11008s and *E. fredii* CMCC (B) 70000 in animal nutrition for the target animals, the consumer, the user and the environment.

Due to significant uncertainties on the identity and safety of the production strain *E. adhaerens* SCM 2034, including the presence of antibiotic resistance genes, the absence of viable cells of the production strain or their DNA in the product, the FEEDAP Panel cannot conclude on the safety of the use of vitamin B₁₂, produced by *E. adhaerens* SCM 2034 in animal nutrition for the target species, consumers and the environment.

Due to high endotoxin content, potential inhalation exposure when handling premixtures and reported irritancy for skin and eyes, vitamin B₁₂ produced by *E. adhaerens* SCM2034 is considered to pose a risk to user safety.

Vitamin B₁₂ additives produced by *Ensifer* spp. are regarded as effective in meeting animals' requirements.

Documentation provided to EFSA

- 1) Vitamin B₁₂/cyanocobalamin as a feed additive for all animal species. 2010. Submitted by VITAC EEIG Vitamins Authorisation Consortium.
- 2) Vitamin B₁₂/cyanocobalamin as a feed additive for all animal species. Supplementary information. January 2014. Submitted by VITAC EEIG Vitamins Authorisation Consortium.
- 3) Vitamin B₁₂/cyanocobalamin as a feed additive for all animal species. Supplementary information. January 2017. Submitted by FEFANA (EU Association of Specialty Feed Ingredients and their Mixtures) asbl.
- 4) Evaluation report of the European Union Reference Laboratory for Feed Additives on the Methods(s) of Analysis for vitamin B₁₂ (cyanocobalmin).
- 5) Comments from Member States.

Chronology

Date	Event
11/04/2012	Dossier received by EFSA
03/04/2012	Reception mandate from the European Commission
04/05/2012	Application validated by EFSA – Start of the scientific assessment

⁵³ Regulation (EC) No. 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

Date	Event
16/05/2012	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation of the production strain, characterisation of the additive, safety for the user</i>
04/08/2012	Comments received from Member States
22/08/2012	Request of supplementary information to the in line with Article 8(1)(2) of Regulation (EC) No 1831/2003. <i>Issues: method of analysis of the active substance in premixtures and feedingstuffs.</i>
13/06/2013	Reception of supplementary information from the applicant
03/06/2013	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
10/01/2014	Reception of supplementary information from the applicant - Scientific assessment re-started
20/02/2014	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: characterisation, safety for target species, safety for the consumer, safety for the user and efficacy</i>
22/04/2016	Complementary request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – <i>Issues: characterisation of the production strains and of the additives, safety for the consumer</i>
13/01/2017	Reception of supplementary information from the applicant - Scientific assessment re-started
12/06/2018	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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- EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food), 2008. Opinion of the Scientific Panel on Food Additives and Nutrient Sources added to Food on 5'-deoxyadenosylcobalamin and methylcobalamin as sources for Vitamin B₁₂ added as a nutritional substance in food supplements. *EFSA Journal*, 2008;6(10):815, 21 pp. <https://doi.org/10.2903/j.efsa.2008.815>
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Abbreviations

ANS	EFSA Scientific Panel on Additives and Nutrient Sources added to Food
CAS	Chemical Abstracts Service
EINECS	European Inventory of Existing Chemical Substances
EURL	European Union Reference Laboratory
FEEDAP	Additives and Products or Substances used in Animal Feed
NOAEL	no observed adverse effect level
PCB	polychlorinated biphenols
SCF	Scientific Committee on Food
SDS	safety data sheet

Appendix A – Safety for the user

Calculation of maximum acceptable levels of exposure from feed additives: The probable exposure time according to EFSA Guidance (EFSA FEEDAP Panel, 2012c) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 seconds = $40 \times 20 = 800$ seconds/day. With an uncertainty factor of 2, maximum inhalation exposure would occur for $2 \times 800 = 1,600$ seconds = 0.444 h/day. Again, assuming a respiration volume of 1.25 m³/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be $0.444 \times 1.25 = 0.556$ m³/day. This volume should contain no more than 900 IU endotoxin, so the dust formed from the product should contain no more than $900/0.556 = 1,619$ IU/m³.

Calculation of endotoxin content of dust: Two key measurements are required to evaluate the potential respiratory hazard associated with the endotoxin content of the product (the dusting potential of the product, expressed in g/m³, and the endotoxin activity of the dust, determined by the Limulus amoebocyte lysate assay (expressed in IU/g)). If data for the dust are not available, the content of endotoxins of the product can be taken instead. If the content of endotoxins of the relevant additive is: a IU/g and the dusting potential is b g/m³, then the content of endotoxins of the dust, c IU/m³, is obtained by simple multiplication, $a \times b$. This resulting value is further used for calculation of the potential inhalatory exposure of users to endotoxins from the additives under assessment (Table A.1) (EFSA FEEDAP Panel, 2012c).

Table A.1: Estimation of user exposure to endotoxins from the additive vitamin B₁₂ formulated at 1% produced by *Ensifer adhaerens* SCM 2034, including consideration of using a filter mask FF P2 or FF P3 as a preventative measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content IU/g product	■	Technical dossier
	b	Dusting potential (g/m ³)	■	Technical dossier
$a \times b$	c	Endotoxin content in the air (IU/m ³)	■	
	d	No of premixture batches made/working day	■	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
	e	Time of exposure (s) per production of one batch	■	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
$d \times e$	f	Total duration of daily exposure/worker (s)	■	
	g	Uncertainty factor	■	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
$f \times g$	h	Refined total duration of daily exposure/worker (s)	■	
$h/3,600$	i	Refined total duration of daily exposure (h)	■	
	j	Inhaled air (m ³) per eight-hour working day	■	EFSA Guidance on user safety (EFSA FEEDAP Panel, 2012c)
$j/8 \times i$	k	Inhaled air during exposure (m ³)	■	
$c \times k$	l	Endotoxin inhaled (IU) during exposure per eight-hour working day	■	
	m	Health-based recommended exposure limit of endotoxin (IU/m ³) per eight-hour working day	■	Health Council of the Netherlands, 2010
$m \times j$	n	Health-based recommended exposure limit of total endotoxin exposure (IU) per eight-hour working day	■	
$l/10$		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P2 (reduction factor 10)	■	
$l/20$		Endotoxins inhaled (IU) per eight-hour working day reduced by filter mask FF P3 (reduction factor 20)	■	

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for Vitamin B12 (Cyanocobalamin)

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 g/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 Association of Official Agricultural Chemists (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.