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European Union Reference Laboratory for Feed Additives

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EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for the Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2010-0146 - CRL/ 100180** **FAD-2010-0156 - CRL/ 100134** **FAD-2010-0165 - CRL/ 100231**
Name of Additive:	**Vitamin D_3 - Cholecalciferol**
Active Agent (s):	**Vitamin D_3 - Cholecalciferol**
Rapporteur Laboratory:	**Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, The Czech Republic**
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Date:	**31/01/2012**
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Date:	**31/01/2012**

EXECUTIVE SUMMARY

In the current grouped application, authorisation is sought under Article 4(1)[1,3] and 10(2)[1,2,3] for *Vitamin D_3 - Cholecalciferol,* 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. Specifically, authorisation is sought for the use of *Vitamin D_3* for all animal species and categories, as requested in FAD-2010-0165.

Vitamin D_3 is produced by chemical synthesis with a minimal purity of 65% and is placed on the market as oily or powder preparations. *Vitamin D_3* is intended to be incorporated in *feedingstuffs* through *premixtures* or directly in *water* at levels ranging from 0.05 to 0.250 mg/kg (or 2 to 10 kIU/g), thus complying with Directive 70/524/EEC.

All Applicants (FAD-2010-0146, -0156, -0165) submitted a set of European Pharmacopoeia methods for the determination of *Vitamin D_3* in the *feed additives* (Eur. Ph. 6.0 01/2008:0072, 0575, 0574, 0598), where identification is based on thin-layer chromatography and ultraviolet and visible absorption spectrophotometry; while quantification is based on liquid chromatography coupled to spectrophotometry at 254 nm. Even though no performance characteristics are provided, the EURL recommends for official control the European Pharmacopoeia methods based on liquid chromatography coupled to spectrophotometry for the determination of *Vitamin D_3* in the feed additive.

For the determination of *Vitamin D3* in *premixtures* and *feedingstuffs*, Applicant (FAD-2010-0165) submitted the ring-trial validated method by the Association of German Agricultural Analytical Research Institutes (VDLUFA), based on High Performance Liquid Chromatography coupled to a UV detector (HPLC-UV). The following performance characteristics were reported by VDLUFA, for *Vitamin D_3* concentrations ranging from 0.06 to 410 mg/kg (or from 2.23 to 16 400 kIU/kg):

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.2 to 8.2%;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 5.8 to 14%; and
- a limit of quantification (LOQ) of 0.025 mg/kg *feedingstuffs* (or 1000 IU/kg).

Applicant (FAD-2010-0146) suggested for the determination of *Vitamin D_3* in *feedingstuffs* and *water* to apply the CEN ring-trial validated method intended for foodstuffs (EN 12821), claiming that this method tested for low fat milk samples should apply to a simpler water matrix without any major interfering substances. The CEN method was ring trial validated

[1] FAD-2010-0146; [2] FAD-2010-0156; [3] FAD-2010-0165

using a variety of food commodities (i.e. low lactose UHT milk, liquid infant formulas and margarine) and two Certified Reference Materials (CRM 122 and CRM 421) to cover a *Vitamin D_3* concentration range from 4 to 140 µg/kg (or 0.16 to 5.6 kIU/kg). The following performance characteristics were reported (when excluding cooking oil and fish oil data):

- RSD_r ranging from 2.4 to 6.7 %;
- RSD_R ranging from 5.5 to 12 %; and
- a limit of quantification of 0.025 mg/kg (or 1000 IU/kg).

Based on the experimental evidence and performance characteristics presented the EURL recommends for official control the ring-trial validated VDLUFA method (VDLUFA 1997, Methodenbuch, Method 13.8.1) for the determination of *Vitamin D3* in *premixtures* and *feedingstuffs*; together with the ring-trial validated EN 12821 method based on Reverse Phase High Performance Liquid Chromatography coupled with UV detection (RP-HPLC-UV) for the determination of *Vitamin D_3* in *feedingstuffs* and *water.*

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

KEYWORDS

Vitamin D_3, Cholecalciferol, nutritional additives, vitamins, all animal species and categories.

1. BACKGROUND

In the current grouped application, authorisation is sought under Articles 4(1) [1,3] (new use) and 10(2) (re-evaluation of additives already authorised under the provisions of the Council Directive 70/524/EEC) [1,2,3] for *Vitamin D_3 - Cholecalciferol* 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.

According to the Applicants *Vitamin D_3* is produced by chemical synthesis as a white to almost white crystalline powder [4,5] or as an amber to violet brown partially solidified viscous paste [6], with a minimum purity of 65 % [6]. It is placed on the market as an oily or a powder preparation.

Specifically, authorisation is sought for the use of *Vitamin D_3* for all animal species and categories as requested in FAD-2010-0165 [3]. *Vitamin D_3* is intended to be incorporated in *feedingstuffs* through *premixtures* or directly in *water* at levels ranging from 0.05 to 0.25 mg/kg (or from 2000 to 10000 IU/kg) [7,8,9], thus complying with Council Directive 70/524/EEC.

According to the Commission Regulation (EC) No 887/2009 concerning the authorisation of a stabilised form of 25-hydroxycholecalciferol as a feed additive, the content of the combination of this active substance with *Vitamin D_3* should not exceed 0.05, 0.08 and 0.125 mg/kg of complete *feedingstuffs* for pigs, other poultry and chickens/turkeys, respectively. Furthermore, the simultaneous use of Vitamin D_2 together with *Vitamin D_3* is forbidden.

Note: Applicant (FAD-2010-0156) did not submit any method nor experimental data for the determination of *Vitamin D_3* in *premixtures*, *feedingstuffs* and *water*.

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 D_3*, 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 [10].

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

All Applicants submitted a set of European Pharmacopoeia methods for the determination of *Vitamin D_3* in the *feed additives*: - a general method for *Cholecalciferol* [11]; a method for *Cholecalciferol* concentrate (oily form) [12]; a method for *Cholecalciferol* concentrate (powder form) [13]; and – a method for *Cholecalciferol* concentrate (water-dispersible form) [14]. All these methods refer to the same principles, where:

- identification is based on thin-layer chromatography and ultraviolet and visible absorption spectrophotometry; while
- quantification is based on liquid chromatography coupled to spectrophotometry at 254 nm.

Even though no performance characteristics are provided, the EURL recommends for official control the European Pharmacopoeia methods based on liquid chromatography coupled to spectrophotometry for the determination of *Vitamin D_3* in the feed additive.

Applicant (FAD-2010-0146) submitted a single-laboratory validated method (based on High Performance Liquid Chromatography (HPLC) and derived from the above mentioned European Pharmacopoeia method) for the determination of *Vitamin D3* in a specific formulation of Rovimix D_3 500 [15] and reported the following performance characteristics [16]: - a relative standard deviation for *intermediate precision* (RSD_{ip}) of 0.8 %, and - a *recovery* rate (R_{Rec}) of 101 %.

For the determination of *Vitamin D3* in *premixtures* and *feedingstuffs*, Applicant (FAD-2010-0165) submitted the ring-trial validated method by the Association of German Agricultural Analytical Research Institutes (VDLUFA) [17], based on High Performance Liquid Chromatography coupled to a UV detector (HPLC-UV).

Depending on the declared level the weight of up to 25 g of sample and corresponding amount of internal standard (vitamin D_2) is dispersed in 130 ml of ethanol together with 25 ml of 50% potassium carbonate solution, 2.0 ml sodium ascorbate solution, 2.0 ml sodium

sulphide solution and approximately 100 mg BHT. The mixture is then heated to boiling and allowed to reflux for 25 minutes. After cooling to room temperature the mixture is extracted with 250 ml of water, 25 ml of ethanol and 100 ml of petrol ether by shaking. After separation an appropriate volume of supernatant (5 to 20 ml) is taken and evaporated to dryness. The residue is re-dissolved in hexane and injected on normal phase semi-preparative column of HPLC. Fractions with Vitamin D_3 and D_2 are collected, evaporated, re-dissolved in mobile phase and analysed on HPLC system with reverse phase analytical column. The detection and determination of *Vitamin D_3* are made using UV detection at 265 nm. *Vitamin D_3* is quantified by the internal standard method.

The following performance characteristics were reported by VDLUFA, for *Vitamin D_3* concentrations ranging from 0.06 to 410 mg/kg (from 2.23 to 16 400 kIU/kg) [17]:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.2 to 8.2%;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 5.8 to 14%; and
- a limit of quantification (LOQ) of 0.025 mg/kg *feedingstuffs* (or 1000 IU/kg).

Alternatively, Applicant (FAD-2010-0146) submitted a single-laboratory validated [18,19] and further verified [20] method, based on Reverse-Phase High Performance Liquid Chromatography coupled to UV detection (RP-HPLC-UV) for the determination of *Vitamin D_3* in *premixtures* (only). The *active substance* is extracted from *premixtures* using acetonitrile and sonication. The extract is then injected into the HPLC system and the *Vitamin D_3* is detected by UV detector at 265 nm and quantified by external calibration method. The following performance characteristics were reported for concentrations ranging from 20 to 200 mg/kg (or 0.8 to 8 MIU/kg) [19,20]: RSD_r and RSD_{ip} ranging from 0.4 to 4%; and R_{Rec} ranging from 89 to 96%.

Furthermore, Applicant (FAD-2010-0146) suggested for the determination of *Vitamin D_3* in *feedingstuffs* and *water* to apply the CEN ring-trial validated RP-HPLC-UV method intended for foodstuffs (EN 12821) [21].

According to the CEN method, 2 to 10 g of sample and corresponding amount of internal standard (vitamin D_2) is dispersed in 60 ml of ethanol and 10 ml of 50% potassium hydroxide solution. The mixture is heated at 80 ºC in a sonication bath under reflux for 20 minutes. After cooling to room temperature the mixture is extracted with 50 ml of water and 50 ml of hexane by shaking. After separation of phases an appropriate volume of supernatant (5 to 20 ml) is taken and evaporated to dryness. The residue is re-dissolved in hexane or heptane and injected on normal phase semi-preparative column of HPLC. Fractions with Vitamin D_3 and D_2 are collected, evaporated, re-dissolved in acetonitrile and analysed on HPLC system with reverse phase analytical column. Vitamin D_3 is quantified by the internal standard method.

The CEN method was ring trial validated using a variety of food commodities (ie. low lactose UHT milk, liquid infant formulas and margarine) and two Certified Reference Materials (CRM 122 and CRM 421) to cover a *Vitamin D_3* concentration range from 4 to 140 µg/kg (or 0.16 to 5.6 kIU/kg). The following performance characteristics were reported (when excluding cooking oil and fish oil data) [21]:

- RSD_r ranging from 2.4 to 6.7 %;
- RSD_R ranging from 5.5 to 12 %; and
- R_{Rec} of *ca.* 94%.

Upon request by the EURL, the Applicant applied the CEN method to ***feedingstuff*** samples (containing from 0.018 to 0.18 mg /kg, equivalent to 0.73 to 7.2 kIU/kg) and reported similar performance characteristics [22]:

- RSD_{ip} ranging from 9 to 25(*) % (* close to LOQ),
- R_{Rec} ranging from 94 to 100%;and
- LOQ of 0.0125 mg/kg *feedingstuffs* (or 500 IU/kg).

Regarding the applicability of the CEN method (EN 12821) for the determination of *Vitamin D_3* in water the Applicant presented the following rational [23]:

> [The main component of milk is water. Furthermore, milk also contains lactose, proteins, fats and various vitamins, including *Vitamin D_3*. Hence, water enriched with *Vitamin D_3* can also be analysed with the method EN 12821, as water is less complex than the milk used for the ring-test.
>
> From an analytical perspective, water is the simplest matrix to work with (as it does not contain e.g. any proteins or fat soluble compounds like in milk). Certainly the analyses of milk would have been more challenging as its fat soluble ingredients (like other vitamins) might have had an influence on the chromatographic separation which was not the case for water enriched with *Vitamin D_3*. Therefore it is reasoned that if the method works for milk, then it also works for water enriched with *Vitamin D_3*.]

Based on the experimental evidences and performance characteristics presented, the EURL recommends for official control

- the dedicated ring trial validated VDLUFA method based on HPLC-UV for the determination of *Vitamin D_3* in *premixtures* and *feedingstuffs*;

- the ring-trial validated EN 12821 method based on RP-HPLC-UV for the determination of *Vitamin D_3* in *feedingstuffs* and *water.*

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- The internationally accepted European Pharmacopoeia methods (01/2008: 0072;0575;0574;0598 to determine *Vitamin D_3* in the *feed additive*
- The ring trial validated VDLUFA method (1997, Methodenbuch, Method 13.8.1) based on HPLC-UV to determine *Vitamin D_3* in *premixtures* and *feedingstuffs.*
- The ring-trial validated method EN 12821 based on RP-HPLC-UV to determine *Vitamin D_3* in *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the determination of *Vitamin D_3* in the *feed additive*:

- High Performance Liquid Chromatography coupled to UV detection (HPLC-UV, 254 nm) - European Pharmacopoeia method 01/2008:0574, 0575, 0598

For the determination of *Vitamin D_3* in *premixtures*:

- High Performance Liquid Chromatography coupled to UV detection at 265 nm (HPLC-UV) - VDLUFA 1997, Methodenbuch, Method 13.8.1

For the determination of *Vitamin D_3* in *feedingstuffs*:

- High Performance Liquid Chromatography coupled to UV detection at 265 nm (HPLC-UV) - VDLUFA 1997, Methodenbuch, Method 13.8.1; or
- Reverse-Phase High Performance Liquid Chromatography coupled to UV detection at 265 nm (RP-HPLC-UV), EN 12821.

For the determination of *Vitamin D_3* in *water*

- Reverse-Phase High Performance Liquid Chromatography coupled to UV detection at 265 nm (RP-HPLC-UV), EN 12821.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Vitamin D_3* 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] [a]Application/Ref:SANCO/D/2: Forw.Appl. 1831/0102/(9919)/2010
[2] [b]Application/Ref:SANCO/D/2: Forw.Appl. 1831/7057
[3] [c]Application/Ref:SANCO/D/2: Forw.Appl. 1831/0103-(9995)/2010
[4] [a] Technical dossier, Section II: 2.2. Characterisation of the active substance
[5] [b] Technical dossier, Section II: 2.2. Characterisation of the active substance
[6] [c] Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[7] [a] Technical dossier, Section II: 2.5 Conditions of use
[8] [b] Technical dossier, Section II: 2.5 Conditions of use
[9] [c] Technical dossier, Section II: 2.5 Conditions of use
[10] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories
[11] European Pharmacopoeia 6.0 01/2008:0072
[12] European Pharmacopoeia 6.0 01/2008:0575
[13] European Pharmacopoeia 6.0 01/2008:0574
[14] European Pharmacopoeia 6.0 01/2008:0598
[15] [a] Technical dossier, Section II: Annex 2-30
[16] [a] Technical dossier, Section II: Annex 2-31
[17] [c] Technical dossier, Section II: Annex_II_06_VDLUFA_1997
[18] [a] Technical dossier, Section II, Annex 2.46
[19] [a] Technical dossier, Section II, Annex 2.47
[20] [a] Technical dossier, Section II, Annex 2.48
[21] EN 12821:2009 – *Foodstuffs – Determination of Vitamin D by high performance liquid chromatography – Measurement of cholecalciferol (D3) or ergocalciferol (D2)*
[22] [a] Supplementary information – Report_00004215.pdf
[23] [a] Technical dossier, Section II, Annex 2.34

[a] Refers to Dossier no: FAD-2010-0146
[b] Refers to Dossier no: FAD-2010-0156
[c] Refers to Dossier no: FAD-2010-0165

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, The Czech Republic. 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:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Federaal Laboratorium voor de Voedselveiligheid Tervuren (FLVVT – FAVV), Tervuren (BE)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)