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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

25-hydroxycholecalciferol

(FAD-2021-0057; CRL/210033)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2021-0057 - CRL/210033**
Name of Product:	***25-hydroxycholecalciferol***
Active Agent (s):	**25-hydroxycholecalciferol**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report approved by: Date:	**Christoph von Holst** **09/03/2022**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *25-hydroxycholecalciferol* under the category/ functional group (3a) "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, the authorisation is sought for the use of the *feed additive* for pigs and poultry for fattening, laying and breeding, and for ornamental birds.

According to the Applicant, the *feed additive* is intended to be marketed as a stabilised form preparation containing a minimum of 1.25 % (w/w) of *25-hydroxycholecalciferol* as an active substance.

The *feed additive* is intended to be used in *premixtures*, complete *feedingstuffs* and *water* for drinking. The Applicant proposed maximum inclusion levels of *25-hydroxycholecalciferol* in complete *feedingstuffs* and in *water* for drinking ranging from 0.05 to 0.1 mg / kg and from 0.025 to 0.05 mg / L, depending on the animal species, respectively. In addition, the Applicant indicated that the levels of *25-hydroxycholecalciferol* (in combination with cholecalciferol - vitamin D_3) should not exceed 0.05, 0.08 and 0.125 mg / kg complete *feedingstuffs* for pigs, hens for laying/breeding and poultry for fattening/ornamental birds, respectively.

For the determination of *25-hydroxycholecalciferol* in the *feed additive* preparation (Bio D® 1.25 %) and *premixtures* the Applicant submitted two single-laboratory validated and further verified methods based on reversed-phase high performance liquid chromatography (HPLC) coupled to spectrophotometric (UV) detection.

The following performance characteristics were obtained in the frame of the validation and verification studies; for the *feed additive*: a relative standard deviation for *repeatability* (RSD_r) ranging from 0.6 to 1.2 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.8 to 1.2 %; and a recovery rate (R_{Rec}) of 100 %; for *premixtures*: a RSD_r ranging from 0.5 to 2.5 %; a RSD_{ip} ranging from 1.3 to 3.3 %; and a recovery rate (R_{Rec}) ranging from 98 to 100 %.

For the determination of *25-hydroxycholecalciferol* in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified method based on reversed-phase ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS).

The following performance characteristics were obtained in the frame of the validation and verification studies for the determination of *25-hydroxycholecalciferol* in *feedingstuffs*: a RSD_r ranging from 2.1 to 5.4 %; a RSD_{ip} ranging from 2.1 to 10.0 %; a recovery rate (R_{Rec})

ranging from 96 to 108 %; and a limit of quantification (LOQ) of 0.005 mg of *25-hydroxycholecalciferol* / kg *feedingstuffs*.

Based on the performance characteristics presented the EURL recommends for the official control (i) the two above mentioned single-laboratory validated and further verified methods based on HPLC-UV for the determination of *25-hydroxycholecalciferol* in the *feed additive* and *premixtures*; and (ii) the single-laboratory validated and further verified method based on UHPLC-MS/MS for the determination of *25-hydroxycholecalciferol* in *feedingstuffs*.

For the determination of *25-hydroxycholecalciferol* in *water* the Applicant submitted a modified protocol of the above mentioned HPLC-UV method dedicated for the *feed additive*. This HPLC-UV method with a modified protocol was used in the frame of stability studies of the *feed additive* preparation in water. The Applicant analysed different type of water samples spiked with the *feed additive* preparation and RSD_r of 9.2 % was derived for a mass fraction of 10 µg of *25-hydroxycholecalciferol* / L water. In addition, a limit of detection (LOD) and a limit of quantification (LOQ) of 2.5 µg and 5.0 µg of *25-hydroxycholecalciferol* / L *water*, respectively, were reported by the Applicant. Thus, the EURL considers that the Applicant demonstrated an extension of scope of the HPLC-UV method dedicated to the *feed additive*, to *water*.

Based on the available performance characteristics presented the EURL recommends for the official control the above mentioned HPLC-UV method for the determination of *25-hydroxycholecalciferol* in *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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

25-hydroxycholecalciferol, nutritional additives, vitamins, pro-vitamins and chemically well-defined substances having similar effect, pigs, poultry for fattening, laying and breeding, and ornamental birds.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new *feed additive*) for *25-hydroxycholecalciferol* under the category/ functional group (3a) "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 [1,2]. Specifically, the authorisation is sought for the use of the *feed additive* for pigs and poultry for fattening, laying and breeding, and for ornamental birds [2].

According to the Applicant, the *feed additive* is intended to be marketed as a stabilised form of light brown-to-brown powder preparation under the trade name Bio D® 1.25 % containing a minimum of 1.25 % (w/w) of *25-hydroxycholecalciferol* as an active substance and residual amounts of cholecalciferol and of 1α,25-dihydroxycholecalciferol [3,4]. The *feed additive* is produced enzymatically from cholecalciferol (vitamin D_3) during the fermentation using the non-genetically modified strain of *Pseudonocardia autotrophica* M301 [3,4].

The *feed additive* is intended to be used in *premixtures*, complete *feedingstuffs* and *water* for drinking. The Applicant proposed maximum inclusion levels of *25-hydroxycholecalciferol* in complete *feedingstuffs* and in *water* for drinking ranging from 0.05 to 0.1 mg/kg and from 0.025 to 0.05 mg/L, depending on the animal species, respectively [5]. In addition, the Applicant indicated that the levels of *25-hydroxycholecalciferol* (in combination with cholecalciferol - vitamin D_3) should not exceed 0.05, 0.08 and 0.125 mg/kg complete *feedingstuffs* for pigs, hens for laying/breeding and poultry for fattening/ornamental birds, respectively [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *25-hydroxycholecalciferol* 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 *25-hydroxycholecalciferol* in the *feed additive* preparation (Bio D® 1.25%) the Applicant proposed [6] and submitted a single-laboratory validated and further verified method based on reversed-phase high performance liquid chromatography (HPLC) coupled to spectrophotometric (UV) detection [7].

Following the method, 400 mg of the homogenised sample is mixed with 50 ml of acetonitrile and sonicated for 20 min. The extract is filtered through a regenerated cellulose (RC) membrane filter (0.45 µm) for further chromatographic analysis. The analyte is detected at 265 nm and its quantification is performed by external standard calibration [7].

For the determination of *25-hydroxycholecalciferol* in *premixtures* the Applicant proposed [6] another single-laboratory validated and further verified method based on reversed-phase HPLC-UV [8,9].

Following the method, the homogenised sample (5 to 10 g) is mixed with 20 ml of dimethylsulfoxide and stirred on a magnetic stirrer for 45 min at room temperature. Then, 80 ml of acetonitrile are added into the mixture and stirred additionally for 75 min. The extract is filtered through a Nylon or PFTE membrane filter (0.2 µm) for further chromatographic analysis. The analyte is detected at 265 nm and its quantification is performed by external standard calibration [8,9].

For the determination of *25-hydroxycholecalciferol* in *feedingstuffs* the Applicant proposed [6] and submitted a single-laboratory validated and further verified method based on reversed-phase ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) [10].

Following the method, the homogenised sample (20 g) is mixed with 2.5 g of pyrogallol and 30 ml of water. The mixture is vortexed and left to stay for 20 min. Then, 3 g of the sample solution from the latter mixture is mixed with an internal standard (d_6-25-hydroxycholecalciferol) solution in acetonitrile (1.0 µg / ml), 3 ml aqueous sodium chloride solution (1 %, w / v), 10 ml of pyrogallol solution in ethanol (3 %, w / v), 2 ml of aqueous potassium hydroxide solution (60 %, w / v) and 3 g of potassium hydroxide flakes. The mixture is vortexed and left for 1 h at 70 °C. After the saponification, 15 ml of aqueous sodium chloride solution (1 %, w / v) is added into the reaction mixture and vortexed. Afterwards, 15 ml of the extraction mixture consisting of ethyl acetate and hexane (1:9, v / v) are added to the saponified sample mixture, shaken for 0.5 min and centrifuged. The extraction procedure of the saponified sample is repeated twice. The supernatants from the three consecutive extractions are combined and evaporated to dryness on a rotary evaporator. The residue after the evaporation is dissolved in 0.5 ml of the mixture consisting of n-hexane and 2-propanol (95:15, v / v) and the resulting solution is filtered through a PFTE membrane filter (0.2 µm). The filtrate is subjected to a semi-preparative HPLC clean-up. The fraction corresponding to the retention time of *25-hydroxycholecalciferol* is collected and evaporated till dryness under a nitrogen stream. The residue is dissolved in 0.5 ml of the mixture containing acetonitrile and water (70:30, v / v), and the resulting solution is filtered through a PFTE membrane filter (0.2 µm) for further UHPLC-MS/MS analysis. During the UHPLC-

MS/MS analysis, the analyte is ionised in positive electrospray mode (+ESI) and detected by using the following multiple reaction monitoring (MRM) transitions: m/z 401.3 > 383.3 and 401.3 > 365.2 (for the analyte); and m/z 407.3 > 389.5 (for the internal standard). The quantification is performed by using the standard calibration curve built from analysis of the standard solutions of *25-hydroxycholecalciferol* in the presence of the internal standard (d_6-25-hydroxycholecalciferol) [10].

The performance characteristics of the above described HPLC-UV [7-9] and UHPLC-MS/MS [10] methods reported by the Applicant and recalculated by the EURL [11] in the frame of the validation [12-15] and verification [16-19] studies for the quantification of *25-hydroxycholecalciferol* in the *feed additive* preparation (Bio D® 1.25%), *premixtures* and *feedingstuffs* are presented in Table 1. In addition, a limit of quantification (LOQ) of 0.005 mg *25-hydroxycholecalciferol* /kg *feedingstuffs* was reported by the Applicant [15].

Based on the performance characteristics presented the EURL recommends for the official control (i) the two above described single-laboratory validated and further verified methods based on HPLC-UV for the determination of *25-hydroxycholecalciferol* in the *feed additive* and *premixtures*; and (ii) the single-laboratory validated and further verified method based on UHPLC-MS/MS for the determination of *25-hydroxycholecalciferol* in *feedingstuffs*.

For the determination of *25-hydroxycholecalciferol* in *water* the Applicant proposed [6] and submitted a modified protocol [20] of the above mentioned HPLC-UV method dedicated for the *feed additive* [7]. This HPLC-UV method with a modified protocol was used in the frame of stability studies of the *feed additive* preparation in water [20].

Table 1 The performance characteristics of the single-laboratory validated and verified HPLC-UV [7-9] and UHPLC-MS/MS [10] methods obtained in the frame of validation [12-15] and verification [16-19] studies for the quantification of *25-hydroxycholecalciferol* in the *feed additive* preparation (Bio D® 1.25%), *premixtures* and *feedingstuffs*.

	Feed additive		**Premixtures**		**Feedingstuffs**	
	Validation	*Verification*	*Validation*	*Verification*	*Validation*	*Verification*
Mass fraction (mg / kg)	12543	12555	72.5 – 361.6	75.6 – 353.2	0.006 – 0.081	0.034 – 0.079
RSD_r (%)	1.2	0.6	0.5 – 0.6	1.2 – 2.5	3.1 – 5.4	2.1 – 3.0
RSD_{ip} (%)	1.2	0.8	1.3 – 3.3	1.5 – 2.5	3.6 – 10.0	2.1 – 3.6
R_{rec} (%)	100	100	99	98 – 100	96 – 108	102 – 105
Reference	[11,12]	[11,16]	[11,13,14]	[11,17,18]	[11,15]	[11,19]

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{rec}: *recovery* rate.

Following the modified protocol, the aqueous samples containing *25-hydroxycholecalciferol* are filtered through RC membrane filter (0.45 µm) for further chromatographic analysis. The analyte is detected at 265 nm and its quantification is performed using a calibration with a standard solution of *25-hydroxycholecalciferol* [20].

The Applicant analysed different type of water samples spiked with the *feed additive* preparation and RSD_r of 9.2 % was derived for a mass fraction of 10 µg *25-hydroxycholecalciferol* / L water [21].

Upon EURL request [22], the Applicant provided additional experimental proofs such as calibration curves, chromatograms of blank and standard solutions, including a limit of detection (LOD) and a limit of quantification (LOQ) of the method, which were 2.5 µg and 5.0 µg of *25-hydroxycholecalciferol* / L *water*, respectively [22-25]. Thus, the EURL considers that the Applicant demonstrated an extension of scope of the HPLC-UV method dedicated to the *feed additive*, to *water*.

Based on the available performance characteristics presented the EURL recommends for the official control the above mentioned HPLC-UV method (with a modified protocol) for the determination of *25-hydroxycholecalciferol* in *water*.

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

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

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For additional characterisation of the *feed additive*, the Applicant proposed the above mentioned HPLC-UV method [7] to determine cholecalciferol and 1α,25-dihydroxycholecalciferol in the *feed additive* preparation. In addition, the Applicant submitted an acceptable performance data from the validation [12] and verification [16] studies for the determination of those two substances in the preparation.

Based on the performance characteristics presented the EURL considers the HPLC-UV method suitable for the determination of cholecalciferol and 1α,25-dihydroxycholecalciferol in the frame of additional characterisation of the *feed additive*.

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 two single-laboratory validated and further verified methods based on high performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV) for the determination of *25-hydroxycholecalciferol* in the *feed additive* and *premixtures*; (ii) the single-laboratory validated and further verified method based on ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for the determination of *25-hydroxycholecalciferol* in *feedingstuffs*; and iii) the HPLC-UV method (with a modified protocol) for the determination of *25-hydroxycholecalciferol* in *water*.

Recommended text for the register entry (analytical method)

For the determination of *25-hydroxycholecalciferol* in the *feed additive*, *premixtures* and *water*:

- High performance liquid chromatography coupled to spectrophotometric detection (HPLC-UV)

For the determination of *25-hydroxycholecalciferol* in *feedingstuffs*:

- Ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *25-hydroxycholecalciferol* 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_FWD. APPL. 1831-0072-2021

[2] *Application, Annex 1 – Submission Number 1616406400451-2928

[3] *Technical dossier, Section II: 2.1.1. Name of the additive

[4] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition of the additive

[5] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition

[6] *Technical dossier, Section II: 2.6.1. Protocol of the methods of analysis for the active substance according to ISO 78:2 format

[7] *Technical dossier, Section II – Annex_II_6_1

[8] *Technical dossier, Section II – Annex_II_6_4

[9] *Technical dossier, Section II – Annex_II_6_5

[10] *Technical dossier, Section II – Annex_II_6_10

[11] *Supplementary information – EURL Calculation of performance characteristics

[12] *Technical dossier, Section II – Annex_II_6_2

[13] *Technical dossier, Section II – Annex_II_6_6

[14] *Technical dossier, Section II – Annex_II_6_7

[15] *Technical dossier, Section II – Annex_II_6_11

[16] *Technical dossier, Section II – Annex_II_6_3

[17] *Technical dossier, Section II – Annex_II_6_8

[18] *Technical dossier, Section II – Annex_II_6_9

[19] *Technical dossier, Section II – Annex_II_6_12

[20] *Technical dossier, Section II – Annex_II_4_4

[21] *Supplementary information – Performance characteristics by EURL

[22] *Supplementary information – 03-02-2022 FW Request for clarification/supplementary information FAD-2021-0057

[23] *Supplementary information – 17032021.pdf

[24] *Supplementary information – 18032021.pdf

[25] *Supplementary information – 19032021.pdf

[26] *Technical dossier, Section II: 2.6.3. Methods of analysis relating to the identity and characterisation of the additive

*Refers to Dossier no: FAD-2021-0057

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

The following National Reference Laboratories contributed to this report:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Instytut Zootechniki – Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Speyer (DE)

- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
- Istituto Superiore di Sanità. Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Roma (IT)
- RIKILT Wageningen UR, Wageningen (NL)
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