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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 (3a670a)

(FAD-2018-0074; CRL/180048)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2018-0074 - CRL/180048**
Name of Feed Additive:	***25-hydroxycholecalciferol (3a670a)***
Active Agent:	**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** **17/09/2020**

EXECUTIVE SUMMARY

In the current application a renewal of authorisation is sought under Article 14 for *25-hydroxycholecalciferol* (*25-OH-D_3*) as *feed additive* under the category/functional group 3(a) "nutritional additive"/"vitamins, provitamins and chemically well-defined substances having a similar effect" according to the classification system of Annex I of Regulation (EC) No 1831/2003. *25-OH-D_3* is currently authorised with identification number 3a670a as *feed additive* for chickens for fattening, turkeys for fattening, other poultry and pigs by Regulation (EC) No 887/2009.

The *feed additive* has a minimum purity of the *active substance* (*25-OH-D_3*) corresponding to 94 % (w/w). *25-OH-D_3* is sensitive to oxidation, light and heat and requires to be stabilised in a formulation in order to be used in animal nutrition. The Applicant proposes inclusion levels of the *active substance* ranging from 50 to 100 μg/kg complete *feedingstuffs*. According to the present authorisation "*the additive shall be incorporated in feedingstuffs via the use of a premixture*".

For the determination of *25-OH-D_3* in stabilised forms of the *feed additive* the Applicant proposed a single-laboratory validated and further verified method based on reversed-phase Ultra Performance Liquid Chromatography (UPLC) coupled to spectrophotometric (UV) detection. For the determination of *25-OH-D_3* in *premixtures* the Applicant proposed a single-laboratory validated and further verified method based on reversed-phase High Performance Liquid Chromatography (HPLC) coupled to spectrophotometric (UV) detection. The method is applicable for samples containing *25-OH-D_3* at a minimum of 1500 μg/kg of the *feed additive*. For the determination of *25-OH-D_3* in low concentrated *premixtures* and *feedingstuffs* the Applicant proposed a single-laboratory validated and further verified method based on HPLC coupled to tandem mass spectrometry (MS/MS). The method is applicable for samples containing *25-OH-D_3* between 10 and 3000 μg/kg of the *feed additive*. The following performance characteristics were reported by the Applicant from the validation and verification studies for the quantification of *25-OH-D_3* in the *feed additive* (stabilised products), *premixtures*, low concentrated *premixtures* and *feedingstuffs*:

- in the *feed additive*: a relative standard deviation for *repeatability* (RSD_r) and a relative standard deviation for *intermediate precision* (RSD_{ip}) of 1.2 %; a *recovery* rate (R_{rec}) of 101 %;
- in *premixtures* (minimum 1500 μg/kg): a RSD_r ranging from 1.5 to 4.4 %; a RSD_{ip} ranging from 1.6 to 5.0 %; a R_{rec} of 102 %; and
- in low concentrated *premixtures* and *feedingstuffs* (10 to 3000 μg/kg): a RSD_r ranging from 3.0 to 5.3 %; a RSD_{ip} ranging from 3.1 to 5.9 %; a R_{rec} of 92 %; and a limit of quantification (LOQ) of 2 μg of *25-OH-D_3*/kg *feedingstuffs*.

Based on the performance characteristics presented the EURL recommends for the official control the single-laboratory validated and further verified analytical method mentioned above based on UPLC-UV for the determination of *25-OH-D_3* in stabilised forms of the *feed additive*; the single-laboratory validated and further verified analytical method based on HPLC-UV for the determination of *25-OH-D_3* in *premixtures*; and the single-laboratory validated and further verified analytical method based on HPLC-MS/MS for the determination of *25-OH-D_3* in low concentrated *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

25-hydroxycholecalciferol (3a670a), nutritional additives, vitamins, pro-vitamins and chemical well-defined substances having a similar effect, chickens for fattening, turkeys for fattening, other poultry pigs.

1. BACKGROUND

In the current application a renewal of authorisation is sought under Article 14 for *25-hydroxycholecalciferol* (*25-OH-D_3*) as *feed additive* under the category/functional group 3(a) "nutritional additive"/"vitamins, provitamins and chemically well-defined substances having a similar effect" according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. *25-OH-D_3* is currently authorised with identification number 3a670a as *feed additive* for chickens for fattening, turkeys for fattening, other poultry and pigs by Regulation (EC) No 887/2009 [3].

The white crystalline powdered *feed additive* has a minimum purity of the *active substance* (*25-OH-D_3*) corresponding to 94 % (w/w) [4]. *25-OH-D_3* is sensitive to oxidation, light and heat and requires to be stabilised in a formulation in order to be used in animal nutrition [5]. Manufacturers could use different formulation to preserve the activity of *25-OH-D_3*. In the frame of the current dossier the Applicant presented studies carried out on a product containing 12500 mg of *25-OH-D_3*/kg of *feed additive* (i.e. Rovimix® Hy·D® 1.25 %) [5].

The Applicant proposes inclusion levels of the *active substance* ranging from 50 to 100 µg/kg complete *feedingstuffs* [6]. According to the present authorisation "*the additive shall be incorporated in feedingstuffs via the use of a premixture*" [3].

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 the *active substance* in the *feed additive*, stabilised products, *premixtures* and *feedingstuffs* the Applicant proposed several analytical methods based on liquid chromatography [7]. However, within the original dossier, the Applicant did not provide the corresponding data related to the validation and the verification studies for the determination of *25-OH-D_3* in the various matrices of interest [7]. In order to be able to evaluate the fitness-for-purpose of the analytical methods for official control, the EURL requested additional information from the Applicant [8]. Updated standard operating procedures, validation and verification studies have then been provided by the Applicant [9-17].

For the determination of *25-OH-D_3* in stabilised forms of the *feed additive* the Applicant proposed a single-laboratory validated and further verified method based on reversed-phase Ultra Performance Liquid Chromatography (UPLC) coupled to spectrophotometric (UV) detection [9,12,15]. As above mentioned, the method has been developed, validated and verified for testing *25-OH-D_3* in a representative Applicant's product (i.e. Rovimix® Hy·D® 1.25 % spray dried form) [5].

100 mg of the product are weighted into a 25 mL volumetric flask. 2.5 mL of Milli-Q water are added, the flask is swirled and the solution is warmed in a water bath set to 45-50 °C for 2 min. Once the solution is sonicated, 20 mL of methanol are added, the flask is cooled at room temperature and diluted to volume with additional methanol. The solution is transferred into an amber 50 mL tube and centrifuged. 5 mL of the supernatant is transferred to a 10 mL volumetric flask and further diluted to volume with methanol. The extract is filtered (0.2 µm PTFE filter) and placed into the vial for the UPLC analysis. *25-OH-D_3* is detected at 265 nm and its quantification is performed by an external calibration.

For the determination of *25-OH-D_3* in *premixtures* the Applicant proposed a single-laboratory validated and further verified method based on reversed-phase High Performance Liquid Chromatography (HPLC) coupled to spectrophotometric (UV) detection. The method is applicable for samples containing *25-OH-D_3* at a minimum of 1500 µg/kg of the *feed additive* [10,13,16].

Depending on the target concentration, 0.5 to 2 g of the sample are weighed into a brown 50 mL volumetric flask. 0.5 mL of a commercially formulated enzyme (i.e. Multifect® PR6L) and 5 to 10 mL of extraction solution (ammonium hydroxide solution/water 8/92, v/v) are added. The solution is swirled and warmed in a water bath set to 40-45 °C for 30 min. The flask is cooled to room temperature and the content is diluted to volume with additional methanol. After shaking, an aliquot is centrifuged at room temperature. The clear supernatant is transferred into a HPLC vial. *25-OH-D_3* is detected at 265 nm and its quantification is performed by an external calibration.

For the determination of *25-OH-D_3* in low concentrated *premixtures* and *feedingstuffs* the Applicant proposed a single-laboratory validated and further verified method based on HPLC coupled to tandem mass spectrometry (MS/MS). The method is applicable for samples containing *25-OH-D_3* between 10 and 3000 µg/kg of the *feed additive* [11,14,17].

Depending on the target concentration, 1 to 10 g of sample are weighted directly into a brown 250 mL glass bottle. 30 mL of deionised water are added and the solution is soaked on a stirring plate for 5 min. 1 mL of d_6-25-hydroxycholecalciferol internal standard solution is added. 30 mL of methanol and 15 mL of aqueous potassium hydroxide solution (47 %, w/w) are placed into the reaction flask and stirred. The bottle is incubated under a temperature controlled shaking bath (130-150 rpm for 30 min at 80 ± 5 °C). After the reaction of saponification is complete, the flask is cooled down at room temperature, its content is further diluted with 60 mL of deionised water and mixed. In order to extract *25-OH-D_3*, 40 mL of methyl tert-butyl ether (TBME) are added and the bottle is shaken (180-220 rpm for 15 min). After the separation of the organic and the aqueous phases, 1 mL of the upper TBME phase is transferred to a test tube and evaporated to dryness at 45 °C (± 5 °C). The residue is dissolved in 0.25 mL injection solvent (methanol/acetonitrile + 0.05 % formic acid), transferred to an Eppendorf tube and centrifuged at 4 °C (± 2 °C). The clear supernatant is placed into an HPLC vial and 10 µL are analysed by reversed-phase HPLC. *25-OH-D_3* is detected by tandem mass spectrometry (m/z 383.4 > 211.3) and the quantification is carried out by using d_6-25-hydroxy cholecalciferol as internal standard.

The performance characteristics reported by the Applicant in the frame of the validation and verification studies for the quantification of *25-OH-D_3* in the *feed additive* (stabilised products), *premixtures* and *feedingstuffs* are presented in Table 1.

Table 1 The performance characteristics of the single-laboratory validated and verified methods [9-11], respectively, for the quantification of *25-OH-D3* in the *feed additive* (stabilised product), *premixtures*, low concentrated *premixtures* and *feedingstuffs*

	Feed additive		Premixtures		Feedingstuffs and low concentrated premixtures	
	Validation [12]	Verification [15]	Validation [13]	Verification [16]	Validation [14]	Verification [17]
Content	12500 mg/kg		55-113 mg/kg		10-3000 µg/kg	
RSD_r %	1.2	1.2	1.5	4.4	5.3	3.0
RSD_{ip} %	1.2	1.2	1.6	5.0	5.9	3.1
R_{rec} %	n.a.	101	n.a.	102	n.a.	92

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

In addition a limit of quantification (LOQ) of 2 µg/kg *feedingstuffs* was reported by the Applicant [17].

Based on the performance characteristics presented the EURL recommends for the official control (i) the above described single-laboratory validated and further verified analytical method based on UPLC-UV for the determination of *25-OH-D3* in stabilised forms of the *feed additive*; (ii) the single-laboratory validated and further verified analytical method based on HPLC-UV for the determination of *25-OH-D3* in *premixtures*; and (iii) the single-laboratory validated and further verified analytical method based on HPLC-MS/MS for the determination of *25-OH-D3* in low concentrated *premixtures* and *feedingstuffs*.

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

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

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single-laboratory validated and further verified analytical methods based on (i) Ultra Performance Liquid Chromatography coupled to spectrophotometric detection (UPLC-UV) for the determination of *25-OH-D3* in stabilised forms of the *feed additive*; (ii) High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV) for the determination of *25-OH-D3* in *premixtures*; and (iii) High Performance Liquid Chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) for the determination of *25-OH-D3* in low concentrated *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *25-hydroxycholecalciferol* in the *feed additive*:

– Ultra Performance Liquid Chromatography coupled to spectrophotometric detection (UPLC-UV)

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

– High Performance Liquid Chromatography coupled to spectrophotometric detection (HPLC-UV)

For the determination of *25-hydroxycholecalciferol* in low concentrated *premixtures* and *feedingstuffs*:

– High Performance Liquid Chromatography coupled to tandem mass spectrometry (HPLC-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. 1831-0008-2019

[2] *Application, Annex I – submission number 1539701894129-2306

[3] Commission Regulation (EC) No 887/2009 of 25 September 2009 concerning the authorisation of a stabilised form of 25-hydroxycholecalciferol as a feed additive for chickens for fattening, turkeys for fattening, other poultry and pigs

[4] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition (active substance/agent, other components, impurities, batch-to-batch variation)

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

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

[7] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance

[8] *Supplementary information: EURL-EFSA Letters_signed_091219.pdf

[9] *Supplementary information: Appendix_1_SOP_Rovimix.pdf

[10] *Supplementary information: Appendix_2_SOP_Premix.pdf

[11] *Supplementary information: Appendix_3_SOP_Feed.pdf

[12] *Supplementary information: Appendix_4_Validation_Rovimix.pdf

[13] *Supplementary information: Appendix_5_Validation_Premix.pdf

[14] *Supplementary information: Appendix_6_Validation_Feed.pdf

[15] *Supplementary information: Appendix_7_Verification_Rovimix.pdf

[16] *Supplementary information: Appendix_8_Verification_Premix.pdf

[17] *Supplementary information: Appendix_9_Verification_Feed.pdf

*Refers to Dossier no: FAD-2018-0074

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)

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