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CRL Evaluation Report on the Analytical Methods submitted in connection with the application for modification of authorisation as a Feed Additive

according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2008-014 FAD-2007-0051 CRL/060033
Additive name:	25-hydroxycholecalciferol
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EXECUTIVE SUMMARY

25-hydroxycholecalciferol is a product already authorised as feed additive by Regulation (EC) No 1443/2005, under the category 'Nutritional Additive', functional group '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 for chickens for fattening, laying hens and turkeys.

The current application is for the re-evaluation according to Article 10(2) of 25-hydroxycholecalciferol and for its new use for poultry and pigs according to Article 4(1) of Regulation (EC) No 1831/2003

This authorization is sought to use 25-hydroxycholecalciferol for poultry and pigs and the proposed inclusion level of active substance ranges from 0.05 to 0.100 mg/kg complete feedingstuffs, depending on the target animal species. If the 25-hydroxycholecalciferol is combined with vitamin D_3 the proposed inclusion level of the active substance ranges from 0.05 to 0.125 mg/kg complete feedingstuffs.

The active substance shall have a minimum purity of 25-hydroxycholecalciferol of 94% measured by a Reverse Phase High Performance Liquid Chromatography (RF-HPLC) with Diode Array Detection (DAD) or Ultraviolet (UV) detection at 270 nm.

For the determination of the active substance (25-hydroxycholecalciferol) in the *feed additive* the applicant proposed a Normal Phase High Performance Liquid Chromatography (NP-HPLC) method equipped with UV detection at 260 nm.

The following acceptable performance characteristics obtained using a Rovimix formulation were reported: - a relative intermediate precision standard deviation (RSD_R) of 2.6 % and – a recovery rate close to 100 %. The method is therefore considered suitable for official control.

For the determination of 25-hydroxycholecalciferol in *premixtures* the applicant proposed a NP-HPLC method with DAD or UV detection at 265 nm. A 25-hydroxyergocalciferol internal standard is used for determination of 25-hydroxycholecalciferol in premixtures with a content of 25-hydroxycholecalciferol lower than 100 mg/kg of premixtures. The method was validated with respect to selectivity, linearity, range of application, recovery, accuracy and intermediate precision. The following acceptable performance characteristics were reported : - a limit of quantification (LOQ) of 2 mg/kg premixtures; - a recovery rate close to 100 % determined at different concentration levels; - a repeatability relative standard deviations (RSD_r) ranging from 1.0 to 3.25 % and a RSD_R ranging from 1.5 to 4.3 %. The method is considered suitable for official control.

For the determination of 25-hydroxycholecalciferol in *feedingstuffs* the applicant proposes a HPLC method connected with to a mass spectrometer (MS) using a 26,27-d_6-25-hydroxycholecalciferol internal standard.

The method has been single-laboratory validated with respect to selectivity, linearity, range of application, recovery, accuracy and intermediate precision and showing an acceptable performance profile. The following acceptable performance characteristics were reported: - a LOQ of 0.005 mg/kg feedingstuffs; - a recovery rate ranging from 100 to 110 %; - a RSD_r ranging from 8.5 to 13.2 %; and - RSD_R ranging from 8.8 to 17.5 %. The method is considered suitable for official control of the content of 25-hydroxycholecalciferol in feedingstuffs.

For the analytical determination of vitamin D_3, (included as additional requirement in Annex III), the applicant suggests the CEN method (EN 12821:2000). This method was originally validated for food and the applicant provided additional data demonstrating that the method is suitable for the analysis of vitamin D_3 in feedingstuffs. The following performance characteristics were reported: - a limit of detection (LOD) and a LOQ of 0.008 and 0.02 mg/kg feedingstuffs, respectively. From the information provided by the applicant the CRL calculated a RSD_R of 17 % for concentration level of vitamin D_3 around 0.020 mg/kg feedingstuffs. The method is considered suitable for official control of vitamin D_3 content in feedingstuffs at the concentration range covered in the validation.

Further testing or validation is not considered necessary.

KEYWORDS

25-hydroxycholecalciferol, vitamin D3, vitamins, pigs, poultry.

BACKGROUND

25-hydroxycholecalciferol is a feed additive already authorised for turkeys, chickens for fattening and laying hens [1] under the category "Nutritional Additive', functional group 'Vitamins, pro-vitamins and chemically well defined substances, having a similar effect according to the classification system of Annex I of Regulation (EC) No 1831/2003.

This authorization is sought to use 25-hydroxycholecalciferol for poultry and pigs and the proposed inclusion level of active substance ranges from 0.05 to 0.100 mg/kg complete feedingstuffs, depending on the target animal species. If the 25-hydroxycholecalciferol is combined with vitamin D_3 the proposed inclusion level of the active substance ranges from 0.05 to 0.125 mg/kg complete feedingstuffs [2].

The crystalline active substance has a minimum purity of 25-hydroxycholecalciferol of 94%. The remaining constituents/impurities are listed with their corresponding maximum levels (indicated between brackets): - water (5%), - other sterols (1%); - organic solvent (1%) and – erythrosine (5 mg/kg) [2]. The methods for the determination of these impurities are submitted in the documentation [4].

In the current application submitted according to Article 4(1) and Article 10(2) of Regulation (EC) No 1831/2003 the re-evaluation of 25-hydroxycholecalciferol for turkey, chickens for fattening and laying hens and extension of the authorisation to pigs and poultry are sought [3].

TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis submitted in connection with 25-hydroxycholecalciferol (EFSA-Q-2008-014), and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

EVALUATION

Description of the methods used for the determination of the criteria listed in Annex, point 2.5.1 of Commission Directive 2001/79/EC.

Impurities in the feed additive

Methods for determination of active substance, sterol impurities, water content, content of organic solvent expressed as loss on drying, content of erythrosine in crystalline 25-hydroxycholecalciferol are submitted in documentation [4].

Active agent in the feed additive

For the determination of 25-hydroxycholecalciferol in the feed additive the applicant proposed a method based on NP-HPLC assay coupled to UV detection (at 260 nm).

The 25-hydroxycholecalciferol is extracted from 350 mg of sample with 15 ml dimethyl sulfoxide (DMSO) under sonication, diluted to 100 ml with ethyl acetate. 3 ml of extract is subsequently diluted to 100 ml with the isopropylalcohol:ethylacetat:isooctane (1:30:69) solution, filtered through a 0.45 µTF filter and injected into HPLC system. The content of 25-hydroxycholecalciferol is calculated using the external calibration.

The potential interfering compound pre25-hydroxycholecalciferol was used to test the specificity of the HPLC system [5]. The following acceptable performance characteristics were reported: a RSD_R of 2.6 % and a recovery rate close to 100 % [6]. The method is therefore considered fit for the intended purpose.

Description of the methods used for the determination of the active substance, following the criteria of the Annex point 2.5.2 of Commission Directive 2001/79/EC.

In premixtures

For the determination of 25-hydroxycholecalciferol in premixtures the applicant proposed a method based on NP-HPLC coupled to UV detection (at 265 nm).

The 25-hydroxycholecalciferol is extracted from 1.5 to 3.5 g of sample, moistened with water, by shaking with tert-butyl methyl ester (TBME). For contents lower than 100 mg/kg premixtures, an internal standard is added before extraction. After centrifugation the aliquot of TBME extract is diluted with 10 ml of a isopropanol:ethylacetate:isooctane (1:30:69) solution and later injected into a normal phase HPLC system. The 25-hydroxycholecalciferol is measured by a UV detector at 265 nm. An external standard calibration is used for the determination of 25-hydroxycholecalciferol content higher than 100 mg/kg premixtures, while for lower contents the internal standard is used [6]. The method was validated with respect to selectivity, linearity, range of application, recovery, accuracy and intermediate precision. The following acceptable performance characteristics were reported: - a recovery rate close to 100 % [8] and a LOQ of 2 mg/kg premixtures [9]. Additional data were provided by applicant [10] upon request from the CRL. RSD_R and RSD_r were estimated by the CRL to range from 1.0 to 3.3 % and from 1.5 to 4.3 %, respectively. The method is considered fit for intended purpose and therefore suitable for official control.

Description of the analytical methods for the determination of the active substance in feedingstuffs

For the determination of 25-hydroxycholecalciferol in feedingstuffs the applicant proposes a HPLC method coupled to a mass spectrometer using a 26,27-d_6-25-hydroxycholecalciferol internal standard.

10 g of sample and 500 ng of internal standard are dispersed in water and the mixture is sonicated for 10 min. The active substance is then extracted with TBME by shaking and subsequent sonication. The TBME extract is centrifuged, an aliquot of supernatant is evaporated under gentle stream of nitrogen. The residue is re-dissolved in semi-preparative mobile phase (isopropanol: ethylacetate:isooctane = 1:10:89) and cleaned-up on semi-preparative column of normal-phase HPLC. Fractions with defined substances are collected, evaporated, re-dissolved in methanol and water and analysed on reverse phase HPLC. The active substance (25-hydroxycholecalciferol) is determined by a single quadrupole MS and quantified by internal standard method [11].

The method was single-laboratory validated for the concentration range stated in Annex III [2].

The following acceptable performance characteristics were reported: - a LOQ of 0.005 mg /kg feedingstuffs [9], and - a recovery rate ranging from 100 to 110 % [12].

Additional data were provided by applicant [10] upon request from the CRL. RSD_R and RSD_r were estimated by the CRL to range from 8.5 to 13.2 % and from 8.8 to 17.5 %, respectively. The method is considered fit for intended purpose and therefore suitable for official control of content of 25-hydroxycholecalciferol in the frame of authorisation.

For the determination of **vitamin D_3** (cholecalciferol) the applicant suggests a separate method in accordance with the EN 12821:2000 standard [13] originally validated for food. The applicant used this method for determination of vitamin D_3 in feedingstuff in which 25-hydroxycholecalciferol was added [14].

2 to 10 g of sample and the corresponding amount of internal standard (vitamin D_2 = ergocalciferol) are 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 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 5 to 20 ml of supernatant 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 acetonitril and analysed on HPLC system with reverse phase analytical column. Vitamin D_3 is quantified by the internal standard method.

On request of the CRL the applicant provided additional information demonstrating the suitability of the method for the determination of vitamin D_3 in feedingstuffs samples containing 25- hydroxycholecalciferol [15]. From these additional reported analytical results the CRL derived the following performance characteristics: - a recovery rate close to 100%; - a RSD_R of 17 % at a concentration of vitamin D_3 around 0.020 mg/kg feedingstuffs; and – a LOD and LOQ of 0.008 and 0.020 mg/kg feedingstuffs, respectively.

The applicant demonstrated that the EN method is fit for the intended purpose for the determination of vitamin D3 in feedingstuffs.

Further testing or validation is not considered necessary.

CONCLUSIONS AND RECOMMENDATIONS

The analytical methods proposed for the official control of 25-hydroxycholecalciferol in the product, premixtures and feedingstuffs are suitable for official control within the frame of the authorisation sought. In addition, the applicant proposed a method for the determination of vitamin D_3 in feedingstufs suitable for the intended purpose within the concentration range stated in Annex III [2].

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

25-hydroxycholecalciferol, min. 94 % purity, $C_{27}H_{44}O_2$. H_2O

Determination of 25-hydroxycholecalciferol by high performance liquid chromatography coupled to a mass spectrometer (HPLC-MS)

Determination of vitamin D_3 in Complete feedingstuffs: reverse phase HPLC method with ultra violet (UV) detection at 265 nm [EN 12821:2000]

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of 25-hydroxycholecalciforel, have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

REFERENCES

[1] Commission Regulation (EC) No 1443/2006 of 29 September 2006 concerning the permanent authorisations of certain additives in feedingstuffs and an authorisation for 10 years for a coccidiostat

[2] * Annex III. Proposal of Register entry

[3] * SANCO/D/2 Forw. Appl. 1831/003-2008

[4] * Section II – App. 2-5-1- A, B, C, D – Determination of impurities

[5] * Section II – App. 2-3-1-A "Rovimix – Stability data and analytical method

[6] * Additional information - DSM (26/09/2008): Answer to CRL Question No 1

[7] * Section II – App. 2-5-2-A – "Determination of 25-hydroxy vitamin D_3 (HyD) in premixtures using 25-hydroxy vitamin D_2 as internal standard"

[8] * Additional information - DSM (26/09/2008): Answer to CRL Question No 8

[9] * Additional information - DSM (26/09/2008): Answer to CRL Question No 4

[10] * Additional information - DSM (26/09/2008): Answer to CRL Question No 5

[11] * Section II – App. 2-5-2-C - "Determination of 25-hydroxy vitamin D_3 (HyD) in animal feeds using d_6-25-hydroxy vitamin D_3 as internal standard".

[12] * Section II – App. 2-5-2-D - "Determination of 25-hydroxy vitamin D_3 in feed, Validation of the method"

[13] EN 12821:2000 - Determination of vitamin D by high performance liquid chromatography - Measurement of cholecalciferol (D3) and ergocalciferol (D2)

[14] * Additional information- DSM (28/11/2008): Hofman, P., Zuber, A.: DSM Report 2000839 Determination of Vitamin D_3 in Feed/Food using Vitamin D_2 as Internal Standard (2008)

[15] * Additional information- DSM (28/11/2008): Hofman, P.: DSM Analytical Phase Report on the study 3 Month Oral Toxicity Study with Rovimix D3-500 followed by a 4 Week Recovery Period in Wistar Rats, Determination of D3 in Animal Diets (2007)

* Refers to Dossier No: FAD-2007-0051

RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, 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.

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