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Feed Additives

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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

Solanum glaucophyllum leaf extract (SGE)

(FAD-2021-0028; CRL/210011)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0028 - CRL/210011**
Name of Product:	***Solanum glaucophyllum leaf extract***
Active Agent (s):	**Glycosylated 1,25-dihydroxycholecalciferol**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**Stefano Bellorini** **20/09/2021**
Report approved by: Date:	**Christoph von Holst** **21/09/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *solanum glaucophyllum leaf extract (SGE)* 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 dairy cows for milk production and other dairy ruminants.

According to the Applicant, the *feed additive (SGE)* is a powder preparation of ethanol extract of dried *solanum glaucophyllum* leaves. The *active substance* of the *feed additive* is *glycosylated 1,25-dihydroxycholecalciferol* with its content ranging from 50 to 160 mg/kg *feed additive*.

The *feed additive* is to be used as a *complementary feed* in the form of controlled release *bolus* given to dairy cows or other dairy ruminants prior calving. The intended dose for dairy cows is 1 or 2 *bolus* corresponding to 500 µg of *1,25-dihydroxycholecalciferol* per animal, while the dose for other dairy ruminants is 1 or 2 *bolus* corresponding to 1.25 µg of *1,25-dihydroxycholecalciferol*/kg body weight of the animal. The *feed additive* is not intended to be used in *premixtures* or complete *feedingstuffs*.

For the quantification of *glycosylated 1,25-dihydroxycholecalciferol* in the *feed additive* and *complementary feed* (*bolus*) the Applicant submitted a single-laboratory validated and further verified method based on analysis by high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) after an enzymatic hydrolysis with beta-glucosidase enzyme followed by derivatisation with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD).

The following performance characteristics were obtained for the quantification of the *glycosylated 1,25-dihydroxycholecalciferol* content in the *feed additive* and *complementary feed* (*bolus*) in the frame of the validation and verification studies:

- for the *feed additive* with *glycosylated 1,25-dihydroxycholecalciferol* content ranging from 115.4 to 140.0 mg/kg: a relative standard deviation for *repeatability* (RSD_r) ranging from 5.2 to 6.3 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 8.0 to 8.2 %; and a *recovery* rate (R_{Rec}) ranging from 95 to 103 %.
- For *bolus* with *glycosylated 1,25-dihydroxycholecalciferol* content ranging from 28.3 to 99.3 mg/kg: RSD_r ranging from 3.3 to 4.8 %; RSD_{ip} ranging from 5.8 to 18.4 %; R_{Rec} ranging from 94 to 101 %; and a limit of quantification (LOQ) of 12.4 mg *glycosylated 1,25-dihydroxycholecalciferol*/kg of *bolus*.

Based on the acceptable performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified method based on LC-MS/MS for the quantification of the *glycosylated 1,25-dihydroxycholecalciferol* content in the *feed additive* and *complementary feed* (*bolus*).

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

Glycosylated 1,25-dihydroxycholecalciferol, solanum glaucophyllum leaf extract (SGE), nutritional additives, vitamins, pro-vitamins and chemically well-defined substances having similar effect, dairy cows for milk production and other dairy ruminants.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new *feed additive*) for *solanum glaucophyllum leaf extract (SGE)* 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 dairy cows for milk production and other dairy ruminants [2].

According to the Applicant, the *feed additive (SGE)* is as a beige or brown powder preparation of ethanol extract of dried *solanum glaucophyllum* leaves which may be standardised with maltodextrin or other suitable carriers or used without any excipient [3]. The *active substance* of the *feed additive* is *glycosylated 1,25-dihydroxycholecalciferol* with its content ranging from 50 to 160 mg/kg *feed additive* [3].

The *feed additive* is to be used as a *complementary feed* in the form of controlled release *bolus* given to dairy cows or other dairy ruminants prior calving [2,4]. The intended dose for dairy cows is 1 or 2 *bolus* corresponding to 500 µg of *1,25-dihydroxycholecalciferol* per animal, while the dose for other dairy ruminants is 1 or 2 *bolus* corresponding to 1.25 µg of *1,25-dihydroxycholecalciferol*/kg body weight of the animal [2,4]. The *feed additive* is not intended to be used in *premixtures* or complete *feedingstuffs* [4].

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 *solanum glaucophyllum leaf extract (SGE)* 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 quantification of *glycosylated 1,25-dihydroxycholecalciferol* in the *feed additive* and *complementary feed* (*bolus*) the Applicant submitted a single-laboratory validated and further verified method based on analysis by high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) after an enzymatic hydrolysis with beta-glucosidase enzyme followed by derivatisation with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). However, the analytical protocols for the two matrices are slightly different [5-8].

According to the standard operating procedures, the sample of 50 mg of the *feed additive* is mixed with water, sonicated and the extract is centrifuged [5]. In case of *bolus* the homogenised sample (100 mg) is mixed with aqueous ethanol solution (65 %, v/v) (for immediate release *bolus*) or with 10 ml of disodium hydrophosphate buffer (0.2 M, pH 8) (for slow release *bolus*). The samples are shaken, sonicated and the extracts are centrifuged [8]. The aliquots of the supernatants from the extraction of *feed additive* and *bolus* samples after a dilution are mixed with aqueous Triton X-100 solution (2.5 %, w/w) and with the internal standard (1α, 25-dihydroxy vitamin D_2) solution in ethanol (100 ng/ml). The mixtures are evaporated to dryness under a vacuum at 35 ^{o}C. The residues are dissolved in water and citrate-phosphate buffer (0.1 M, pH 5), aqueous ascorbic acid solution (10 %, w/v) and beta-glucosidase enzyme solution in the citrate-phosphate buffer (10 %, w/v) are added. The mixtures are incubated under a shaking at 37 ^{o}C for 18 h. After the enzymatic reaction, the aliquots are mixed with tert-butylmethyl ether and shaken for 17 min. The aliquots of the extracts (upper organic phase) are evaporated under vacuum at 35 ^{o}C until dryness [6,8]. The residues are mixed thoroughly with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) solution in acetonitrile (0.5 mg/ml) and kept in darkness at room temperature for 1 to 2 h. The reaction is

stopped by adding the mixture of two mobile phases containing water and methanol (1:1, v/v) with the traces of methylamine and formic acid. For the standard samples of *1,25-dihydroxycholecalciferol* the extraction and the enzymatic hydrolysis steps are omitted, however the samples undergo the spiking with the internal standard followed by further derivatisation with PTAD. The samples and the calibration standards are analysed by LC-MS/MS in multiple reaction monitoring mode (MRM) using a positive electrospray ionisation (+ESI). The quantification is performed by using an internal standard calibration curve [7-8].

The performance characteristics presented by the Applicant [9-13] (or recalculated by the EURL [14]) for the quantification of the *glycosylated 1,25-dihydroxycholecalciferol* content in the *feed additive* and *complementary feed* (*bolus*) in the frame of the validation [9,10,12] and verification [11,13] studies are summarised in Table 1.

In addition, a limit of quantification (LOQ) of 12.4 mg *glycosylated 1,25-dihydroxycholecalciferol*/kg of *bolus* was reported by the Applicant [12].

Based on the acceptable performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified method based on LC-MS/MS for the quantification of the *glycosylated 1,25-dihydroxycholecalciferol* content in the *feed additive* and *complementary feed* (*bolus*).

Table 1: The performance characteristics obtained for the quantification of the *glycosylated 1,25-dihydroxycholecalciferol* content in the *feed additive* and *complementary feed* (*bolus*) in the frame of the validation [9,10,12] and verification [11,13] studies

	Feed additive		**Complementary feed (bolus)**	
	Validation	Verification	Validation	Verification
Method	LC-MS/MS		LC-MS/MS	
Mass fraction (mg/kg)	115.4	140.0	28.3 – 99.3	29.8 – 93.4(*)
RSD_r (%)	6.3	5.2	3.3 – 4.8	3.4 – 3.9(*)
RSD_{ip} (%)	8.2	8.0	5.8 – 7.8	6.2 – 18.4(*)
R_{rec} (%)	95	103	94 – 101	101(**)
Reference	[10]	[11]	[12]	[13,14]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and for *intermediate precision;* R_{rec}: recovery rate; (*) re-calculated by the EURL [14]; (**) based on expected content of the analyte in the known sample (slow release *bolus*).

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 a single-laboratory validated and further verified method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the quantification of *glycosylated 1,25-dihydroxycholecalciferol* content in the *feed additive* and *complementary feed (bolus*).

Recommended text for the register entry (analytical method)

For the quantification of *glycosylated 1,25-dihydroxycholecalciferol* in the *feed additive* and *complementary feed (bolus*):

– Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *solanum glaucophyllum leaf extract (SGE)* 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. 1831-0029-2021

[2] *Application, Annex 1 – Submission Number 1613058034199-2828

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

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

[5] *Supplementary information – SOP_01-11_Herbonis

[6] *Supplementary information – SOP_01-14_Herbonis

[7] *Supplementary information – SOP_01-15_Herbonis

[8] *Technical dossier, Section II – Annex_II_6_1_4

[9] *Technical dossier, Section II – Annex_II_6_1_1_Part1

[10] *Technical dossier, Section II – Annex_II_6_1_1_Part2

[11] *Technical dossier, Section II – Annex_II_6_1_2

[12] *Technical dossier, Section II – Annex_II_6_1_3

[13] *Technical dossier, Section II – Annex_II_6_1_4

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

*Refers to Dossier no: FAD-2021-0028

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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- [1]Wageningen Food Safety Research (WFSR) (NL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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[1] Name and address according to COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.