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Safety and efficacy of a feed additive consisting of *Solanum glaucophyllum* leaf extract for dairy cows and other dairy ruminants (Herbonis Animal Health Gmbh)

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Mojca Fašmon Durjava, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa, Ruud Woutersen, Paul Brantom, Jürgen Gropp, Kettil Svensson, Montserrat Anguita, Jaume Galobart, Fabiola Pizzo and Paola Manini

Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of *Solanum glaucophyllum* leaf extract (SGE) as a nutritional additive for dairy cows and other dairy ruminants. However, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) considered the glycosides of 1,25-dihydroxycholecalciferol (1,25 $[OH]_2D_3$) as the active substance and the bolus containing SGE-derived 1,25$[OH]_2D_3$ as the preparation of the additive. The product is intended to be administered to dairy ruminants during the pre-parturient (period from 9 days before calving to immediately before calving). The FEEDAP Panel concluded that the administration of one bolus, the preparation of the additive as applied in the animal studies evaluated, containing 500 μg of SGE-derived 1,25$[OH]_2D_3$ during the pre-parturient period is safe for cows. Owing to the lack of data, the Panel could not conclude on the safety for of a subsequent administration of a second bolus or on the safety of another SGE-derived 1,25$[OH]_2D_3$ preparation for use in dairy ruminants other than cows (*Bos taurus*). The Panel considered that, under the specified conditions of use, the product is safe for the consumer and the environment. The bolus, a preparation containing SGE, as a source of the active substance, is not irritating to skin and eyes and it is not a sensitiser. Exposure via inhalation is unlikely. The Panel concluded that the administration of the bolus, the preparation of the additive as applied in the animal studies evaluated, containing 500 μg of SGE-derived 1,25$[OH]_2D_3$ in a period from 9 days before calving to immediately before calving has the potential to prevent hypocalcaemia in dairy cows. Owing to the lack of data with another preparation, the Panel could not conclude on the efficacy in other dairy ruminants.

Keywords: nutritional additives, vitamins, pro-vitamins and chemically well-defined substances having similar effect, *Solanum glaucophyllum* extract, calcitriol, safety, efficacy

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Correspondence: feedap@efsa.europa.eu

Panel members: Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Mojca Fašmon Durjava, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa and Ruud Woutersen.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Herbonis Animal Health GmbH[2] for the authorisation of the additive consisting of *Solanum glaucophyllum* leaf extract (SGE), when used as a feed additive for dairy cows and other dairy ruminants (category: nutritional additives; functional group: vitamins, pro-vitamins and chemically well-defined substances having similar effect).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 28 May 2021.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the feed additive consisting of *S. glaucophyllum* leaf extract (SGE), when used under the proposed conditions of use (see Section 3.2.6).

1.2. Additional information

The subject of the assessment is a product that consists of SGE, intended for use as a nutritional additive (functional group: vitamins, pro-vitamins and chemically well-defined substances having similar effect) for dairy cows. SGE has not been assessed as a feed additive in the EU and is not authorised for use as a feed additive.

EFSA issued an opinion on the safety and efficacy of *S. glaucophyllum* standardised leaves when used as a feed material (EFSA FEEDAP Panel, 2015).

Waxy-leaf nightshade meal (7.15.1), a product obtained by drying and grinding the leaves of *S. glaucophyllum* is included in the catalogue of feed materials.[3] *S. glaucophyllum* is included in the list of feeds intended for particular nutritional purposes (PARNUTS), for reduction of the risk of milk fever and subclinical hypocalcaemia in dairy cows (Commission Regulation (EU) No 2020/354).[4]

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[5] in support of the authorisation request for the use of SGE as a feed additive.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the active substance in animal feed. The Executive Summary of the EURL report can be found in Annex A.[6]

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the council of 22 September 2003 on the additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] Herbonis Animal Health GmbH, Rheinstrasse 30, 4,302 augst BL, Switzerland, represented in the EU by Pen & Tec Consulting S.L.U, Pl. Ausias March, 1, 08195, Sant Cugat del Vallès, Spain.
[3] Commission Regulation (EU) 2017/1017 of 15 June 2017 amending Regulation (EU) No 68/2013 on the Catalogue of feed materials. OJ L 159, 21.6.2017, pp. 48–119.
[4] Commission Regulation (EU) No 2020/354 of 4 March 2020 establishing a list of intended uses of feed intended for particular nutritional purposes and repealing Directive 2008/38/EC.
[5] FEED dossier reference: FAD-2021-0028.
[6] The full report is available on the EURL website: https://joint-research-centre.ec.europa.eu/publications/fad-2021-0028_en

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of SGE is in line with the principles laid down in Regulation (EC) No 429/2008[7] and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012a), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017a), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018) and Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The application refers to SGE, a dry powder based on an ethanolic extract of dried *S. glaucophyllum* leaves (waxy-leaf nightshade meal). SGE is a natural source of calcitriol (1,25-dihydroxycholecalciferol; 1,25$[OH]_2D_3$), a potent metabolically active form of vitamin D_3. SGE is intended for use as a nutritional additive (functional group: vitamins, pro-vitamins and chemically well-defined substances having similar effect) in feed for dairy ruminants in the pre-parturient period to stabilise serum calcium concentration at the onset of lactation. SGE is to be used in a preparation in the form of an encapsulated, controlled-release bolus.

3.1. Introduction

Vitamin D_3 is essential for life in higher animals, it is one of the primary biological regulators of calcium homeostasis. The biological role of vitamin D is mainly exerted by calcitriol, which is a secosteroid structurally related to steroid hormones and its function is performed by interacting with its cognate vitamin D receptor.

Calcitriol (also referred to as 1,25-dihydroxycholecalciferol, 1α,25-dihydroxyvitamin D_3 or 1,25-dihydroxyvitamin D_3) is the most potent metabolite of vitamin D_3 with three hydroxyl groups. Calcitriol is synthesised in the body starting from 7-dehydrocholesterol, which is converted in a first step, to cholecalciferol (vitamin D_3) in the skin after exposure to UV light. The vitamin is transported in the blood stream bound on a vitamin D binding protein, converted into 25-hydroxyvitamin D_3 (25$[OH]D_3$) in the liver, and again hydroxylated at C1 mainly in the kidneys to form calcitriol by 1α-hydroxylase activity (a mitochondrial cytochrome P450 enzyme). Hydroxylation at C24 results in the main inactive metabolites 24,25$[OH]_2D_3$ and 1,24,25$[OH]_3D_3$.

The synthesis of calcitriol is strongly regulated by homeostatic mechanisms. The activity of the 1α-hydroxylase is regulated among others by calcitriol, which depresses the enzyme activity and promotes its own deactivation (by stimulation of 24-hydroxylase). Also, the 25$[OH]D_3$ serum concentration affects the levels of 1α-hydroxylase and 24-hydroxylase in the kidneys. If the 25$[OH]D_3$ concentration is low, the body compensates by producing more parathyroid hormone (PTH), thereby stimulating 1α-hydroxylase and depressing 24-hydroxylase. Conversely, as 25$[OH]D_3$ concentrations rise, less 1α-hydroxylase and more 24-hydroxylase are required to keep circulating calcitriol in the correct balance. Consequently, circulating calcitriol does not correlate with the 25$[OH]D_3$ concentration (Nelson and Merriman, 2014).

PTH, low serum calcium and low serum phosphate stimulate the renal production of calcitriol. Calcitriol together with PTH enhances the absorption in the gastrointestinal tract of calcium and phosphate from the diet, increases renal tubular reabsorption of calcium and stimulates the release of calcium stored in the bones.

Besides to its action on calcium homeostasis, it has been demonstrated that calcitriol promotes fatty acid synthesis, inhibits lipolysis and increases energy efficiency. Calcitriol can modulate the activity of cytokines and may regulate cell turnover/differentiation (Rodriguez et al., 2014) and affects mammary physiology in cattle. The pluripotent secosteroid 1α,25$[OH]_2D_3$ initiates physiological responses of $\geq$ 36 cell types that possess the vitamin D receptor and a paracrine production of this substance was shown for $\geq$ 10 extrarenal organs (Norman, 2008).

[7] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

3.1.1. Physiological background of the application

In the dry period of dairy cows (commonly 3–4 weeks before parturition), the calcium requirement is relatively low, only determined by the need of the growing fetus and maintenance requirement of the pregnant cow. The sudden production of milk after parturition (colostrum followed by normal milk) is an enormous challenge for the calcium metabolism. Twenty litres of milk would contain about 24 g calcium (approximately the same amount as 10 L colostrum). Feed intake is low providing about 10 g Ca/day. The total volume of circulating blood for a 650-kg cow may be 34–39 L (52–60 mL/kg) body weight (bw_), providing between 3.4 and 4.3 g total Ca. There are endogenous mechanisms to avoid Ca-blood level falling below critical concentrations (< 1.6 mmol calcium/L indicating the prevalence of milk fever), mainly skeletal release and maximising intestinal absorption. However, the mechanisms are not trained and often, particularly in multiparous cows because of a lower level of mobilisable Ca in the skeleton compared to primiparous, not sufficient to stabilise blood calcium.

Administration of calcitriol before parturition is expected to stabilise blood calcium and to provide enough Ca for milk production. The FEEDAP Panel already concluded in 2015 (EFSA FEEDAP Panel, 2015) that *S. glaucophyllum* leaves have 'the potential to be efficacious in the prevention of milk fever in dairy cows. This conclusion is extended to other dairy ruminants'. However, the Panel reported data from Horst et al. (2003) who showed in a study that all cows (five in total) receiving calcitriol from *Solanum* leaves suffered from hypocalcaemia between 6 and 8 days after *S. glaucophyllum* treatment was ended, and one of them developed milk fever. High exogenous supply of calcitriol would have blocked the endogenous synthesis of calcitriol. The Panel interpreted the findings as an indication of the necessity of a phased withdrawal after treatment with calcitriol from *Solanum* leaves before parturition (successful prevention of milk fever) to avoid life-threatening acute calcium deficiency in the period after cessation of calcitriol treatment. The Panel consequently mentioned that 'Efficacy and tolerance of $1,25[OH]_2D_3$ from *S. glaucophyllum* obviously depends on the conditions of use (dose, duration)'.

The current application refers to a preparation of calcitriol in the form of a bolus which is expected to release enough calcitriol in the period before parturition to prevent a critical decline of blood-Ca and to provide later lower amounts of calcitriol for a longer period to allow the endogenous synthesis of calcitriol to recover (to reach the physiologically necessary level). The bolus – a controlled delayed release preparation of calcitriol from SGE – is applied to ensure an efficient and safe supplementation to dairy cows around parturition.

3.2. Characterisation

3.2.1. Manufacturing process

SGE is a dried aqueous ethanol extract of *S. glaucophyllum* leaves, a feed material naturally rich in glycosides of $1,25[OH]_2D_3$.[8] SGE may be used as such or standardised by dilution with maltodextrin or other excipients (granulated or not) to contain the glycosides in a concentration corresponding to 50–160 mg $1,25[OH]_2D_3$/kg (here and hereinafter given concentrations refer to the aglycone $1,25[OH]_2D_3$). Dry leaves are extracted [redacted]

The SGE containing the active substance calcitriol in a glycosylated form is used for formulation of standardised preparations providing a controlled delayed release of calcitriol in the gastrointestinal tract of dairy ruminants. Such a preparation is described in the application as a bolus consisting of mini-boli.

3.2.2. Characterisation of the active substance

The main active components responsible for the vitamin D-like activity of *S. glaucophyllum* are the glycosides of calcitriol. According to the previous opinion of the FEEDAP Panel (EFSA FEEDAP Panel, 2015) 'Glycoside conjugation to hydroxy groups of the aglycone occurs at C1, C3 and C25. The molecular distribution of glycosyl moieties has been found to be 1–12 hexose units per aglycone; a mono-glycoside as well as di- and tri-glycosides have been identified'.

[8] Technical dossier/Section II/ Annex II.3.1.3. Conf and Annex II.3.1.4.Conf.

Calcitriol (synonym: 1,25-dihydroxycholecalciferol; 1,25[OH]$_2$D$_3$) is defined by the International Pure and Applied Chemistry (IUPAC) name 9,10-secocholesta-5,7,11(19)-triene-1,3,25-triol, the Chemical Abstract Service (CAS) number 32222–06-3, the European Inventory of Existing Chemical Substances (EINECS) number 250–963-8. The molecular formula of 1,25[OH]$_2$D$_3$ is $C_{27}H_{44}O_3$ and its molecular weight is 416.6 g/mol. The structural formula of the calcitriol is presented in Figure 1.

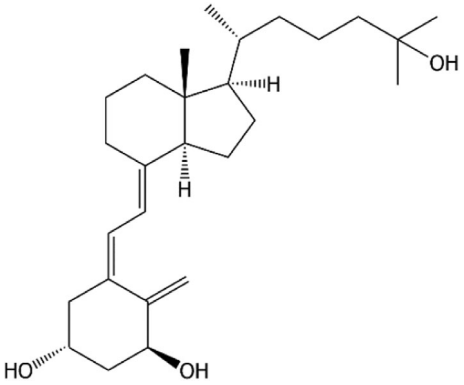

Figure 1: Structural formula of calcitriol (1,25[OH]$_2$D$_3$) as aglycone

3.2.3. Characterisation of SGE, the source of calcitriol

The analysis of eight batches of the raw extract by ultraperformance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) showed an average 1,25[OH]$_2$D$_3$ content of 150 (range 129–178) mg 1,25[OH]$_2$D$_3$/kg (analysed as free 1,25[OH]$_2$D$_3$).[9] In four of the batches, the content of 25-hydroxycholecalciferol (ranging 0.0282–0.0557 mg/kg) and cholecalciferol (ranging 0.0091–0.0133 mg/kg, corresponding to 364–531 IU vitamin D$_3$/kg) were also measured.

For standardisation, the raw SGE is diluted with a suitable carrier, e.g. maltodextrin. The standardised SGE is specified to contain 50–160 mg 1,25[OH]$_2$D$_3$/kg (analysed as free 1,25[OH]$_2$D$_3$). Analytical data of seven batches (two as dry powder, five as dry granulate) were submitted. Values of the dry powder were 55 and 64 mg 1,25[OH]$_2$D$_3$/kg, and those of the granulate between 50 and 54 mg 1,25[OH]$_2$D$_3$/kg (mean 52 mg).[10]

The content of alkaloids (e.g. α-solanine and α-chaconine) was < 10 mg/kg each in four batches of raw SGE.[11] Three batches of raw SGE were analysed for undesirable substances,[12] i.e. ethanol (< 0.3–0.5%), arsenic (0.029–0.10 mg/kg), cadmium (0.0015–0.10 mg/kg), mercury (0.009–0.08 mg/kg), lead (0.026–0.10 mg/kg), aflatoxins B1, B2, G1 and G2 (< 1 μg/kg each, their sum < 4 μg/kg), ochratoxin A (2–5 μg/kg), zearalenone (≤ 0.01 μg/kg) and deoxynivalenol (0.015–0.03 μg/kg). Pesticides were not detected in a multiresidue analysis. Total aerobes were ≤ 120 colony forming units (CFU)/g, yeasts and moulds ≤ 100 CFU/g, bile-tolerant Gram-negative strains ≤ 10 CFU/g, *Escherichia coli* and coliforms not detected in 1 g, and *Salmonella* spp. not detected in 25 g. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) were analysed in four batches of raw SGE. The calculated (upper bond) level of dioxins and the sum of dioxins and dioxin-like PCBs were 0.0574–0.0609 ng WHO-PCDD/F-TEQ/kg and 0.0919–0.0976 ng WHO-PCDD/F-PCB-TEQ/kg.[13] All values were of no concern.

SGE is a dry, beige or brown powder, soluble in water. The applicant submitted also data on bulk density (300–600 kg/m^3), dusting potential (135 mg/m^3 air for one batch by Stauber–Heubach)[14] and particle size (two batches, mean 0.1 μm, 80% of particles between 0.06 and 0.2 mm; sieve method)[15] for a standardised SGE (with maltodextrin).

[9] Technical dossier/Section II/ Annex_II_1_3 and Supplementary Information February 2022/Annexes 1 to 4.
[10] Technical dossier/Section II/ Annex_II_1_3.
[11] Technical dossier/Section II/Annex_II_1_4_1_CoC and Supplementary information February 2022.
[12] Technical dossier/Section II/Annex_II_1_4_1_CoC and Supplementary information February 2022/Annexes 1–4.
[13] Technical dossier/Supplementary information February 2022/Annexes 1–4.
[14] Technical dossier/Section_II/Annex_II_1_5_1.
[15] Technical dossier/Section_II/Annex_II_1_5_2.

3.2.4. Characterisation of the additive

The applicant submitted information on one example, Solbovine® Once (SBO), formulated for dairy cows or small ruminants.[16]

The bolus for dairy cows ([redacted] of [redacted]) contains 500 µg 1,25$[OH]_2D_3$ from either raw SGE, or standardised SGE with maltodextrin or other suitable carriers. SBO is composed of [redacted] mini-boli (specified composition given in Section 3.2.4.1) [redacted] of which are intended for immediate release (IR-mini-bolus; [redacted] for slow release (SR-mini-bolus; [redacted]). The applicant provided a typical composition of SBO indicating a SGE content of [redacted] per IR-mini bolus [redacted] and [redacted] per SR-mini bolus [redacted]. Data on the batch-to-batch variation was provided for seven batches of SBO and showed an average 1,25 $[OH]_2D_3$ content of 559 (range: 469–745) µg per bolus (concentration levels given as measured for free 1,25$[OH]_2D_3$).[17] The bolus is reported to contain [redacted]

One bolus for dairy cows (a composite sample of seven batches) was analysed for undesirable substances. The contents of lead (0.23 mg/kg bolus), cadmium (0.07 mg/kg), mercury (< 0.005 mg/kg), arsenic (2.5 mg/kg), nickel (4.6 mg/kg), fluorine (137 mg/kg), dioxins (0.0612 ng WHO-PCCD/F-TEQ/kg) and PCBs (0.0376 ng WHO-PCB TEQ/kg)[18] were of no concern.

The bolus formulated for small ruminants is basically modified to account for the total amount of 1,25$[OH]_2D_3$. It has also a mass of [redacted] and contains [redacted], derived from a proposed dose of 1 µg/kg body weight and a default value of 60 kg body weight for a small dairy ruminant. The applicant provided a typical composition of SBO bolus for small ruminants indicating a SGE concentration of [redacted] 1,25$[OH]_2D_3$) per IR-mini bolus [redacted] and [redacted] per SR-mini bolus [redacted] 1,25$[OH]_2D_3$). The composition in terms of other formulating ingredients is similar but adapted to compensate for the lower content of SGE.

3.2.4.1. Characterisation of the mini-boli as parts of the large bolus

The mini bolus consists of SGE and different feed materials and technological feed additives as carriers. The content of SGE and the carriers varies according to weight and the intended 1,25$[OH]_2D_3$ content of the mini bolus. Table 1 illustrates the composition of the different mini boli when SGE contains 150 mg 1,25$[OH]_2D_3$/kg.[19]

Table 1: Quantitative specified composition of mini boli for dairy cows or for small dairy ruminants. The example is based on a *Solanum glaucophyllum* leaf extract (SGE) containing 150 mg 1,25$[OH]_2D_3$/kg

Release characteristics	Dairy cows		Small dairy ruminants	
	IR	SR	IR	SR
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]		[redacted]	
[redacted]		[redacted]		[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]		[redacted]	
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]

IR: immediate release; SR: slow release.

[16] Technical dossier/Section II and Supplementary information February 2022.
[17] Technical dossier/Supplementary information February 2022/Annex_9.
[18] Technical dossier/Supplementary information February 2022/Annex_11.
[19] Technical dossier/Supplementary information February 2022, Tables 9–12.

3.2.5. Stability and homogeneity

The applicant proposed a shelf-life of 2 years for SGE stored in a cool and dry environment in original, closed, light-tight packaging. To support this shelf-life the applicant submitted data on two batches of a standardised SGE, which were stored at 25°C/60% RH (relative humidity) and 40°C/75% RH. No losses were found on the content of 1,25[OH]$_2$D$_3$ after 12 months.[20] One batch of raw SGE was stored under dry conditions at 25°C for 36 and 48 months. The recovery of 1,25[OH]$_2$D$_3$ was 96% and 115% of time zero, respectively.[21]

SGE is supplied to the animals in the form of a controlled released bolus (SBO), and it is not used in premixtures. Stability and homogeneity of SGE in the mini-boluses present in SBO (SR and IR, from two batches) was assessed in mini-boluses produced in 2017, stored under warehouse conditions at ca. 25°C and retested after 3 years storage. Data showed a mean recovery of [redacted] and [redacted] for SR- and IR-mini boli, respectively.[22]

Homogeneity was examined by calcitriol analysis of 10 IR- and 10 SR-mini-boli. The coefficient of variation (CV) was 2% for IR-mini-boli (mean: [redacted]) and 6% for SR-mini-boli (mean: [redacted]).

3.2.6. Conditions of use

The applicant proposes to administer the active substance in a preparation to form a bolus, the recommended application form of the additive. It should be given directly to pre-parturient dairy ruminants.

The bolus formulated for dairy cows, containing about 500 μg 1,25[OH]$_2$D$_3$, is recommended to be administered once in a period from 9 days before calving to immediately before calving, ideally 1–2 days before calving. If the cow has not calved within 9 days after bolus administration, the supply of a second bolus is recommended.[23]

The same administration scheme is proposed for other dairy ruminants. The bolus is formulated to deliver 1 μg 1,25[OH]$_2$D$_3$ per kg body weight, from 9 days before parturition to immediately before parturition, ideally 1–2 days prior to parturition.

3.3. Safety

Natural occurrence of poisoning in cattle by consumption of *Solanum malacoxylon*/*glaucophyllum* and the toxicity symptoms were described in detail in the former opinion of the FEEDAP Panel (EFSA FEEDAP Panel, 2015).

3.3.1. Toxicological studies

The applicant submitted two *in vitro* and one *in vivo* genotoxicity studies, two repeated dose toxicity studies, which have been already evaluated in the previous assessment (EFSA FEEDAP Panel, 2015). In addition, a new 90-day study and two additional studies, one *in vitro* and another *in vivo*, on the oestrogenic activity of SGE, were submitted.

In all studies, the test item was a standardised water-soluble dried powder of a water ethanol SGE containing 64.8 mg 1,25[OH]$_2$D$_3$/kg. The FEEDAP Panel notes that the 1,25[OH]$_2$D$_3$ content of test item complies with the specification of the additive under evaluation.

3.3.1.1. Genotoxicity studies

Based on the data provided[24] (a gene mutation test in five *Salmonella* Typhimurium strains, TA 98, TA 100, TA 102, TA 1535 and TA 1537), a mouse lymphoma gene mutation assay in cultured mammalian cells (L5178Y TK +/−) and an *in vivo* bone marrow micronucleus test in mice, the FEEDAP Panel considered that 'the water-soluble extract of *S. glaucophyllum* containing 64.8 μg 1,25(OH)$_2$D$_3$/g is non-genotoxic' (EFSA FEEDAP Panel, 2015).

The studies were newly evaluated also for their compliance with the current EFSA Guidance documents. The FEEDAP Panel confirmed its previous conclusions.

[20] Technical dossier/Section II/Annex_II_4_1_1.
[21] Technical dossier/Section II/Annex_II_4_1_2.
[22] Technical dossier/Section II/Annex_II_4_2.
[23] Technical dossier/Section II.
[24] Technical dossier/Section III/Annex_III_2_2_2_1, Annex_III_2_2_2_2 and Annex_III_2_2_2_3.

3.3.1.2. Repeated dose oral toxicity studies

The two repeated dose toxicity study in rats with a duration of 2 and 4 weeks[25] were already assessed in 2015. The FEEDAP Panel concluded that 'The identification of a safe level of exposure is limited by the absence of repeated dose studies of duration longer than four weeks and the absence of any reproduction toxicity studies other than the developmental toxicity studies' (EFSA FEEDAP Panel, 2015).

The applicant submitted in the current dossier a 90-day repeated oral dose study in rats.[26]

Groups of 10 Crl:CD(SD) rats received the test item, a SGE preparation containing 64.8 mg $1,25[OH]_2D_3$/kg in 0.8% hydroxypropylmethyl cellulose, by gavage for 91 days at doses of 0, 5, 15 and 35 mg/kg bw per day. An additional group received 1.75 mg $1,25[OH]_2D_3$/kg bw per day. Additional groups of five rats of each sex were included for 4-week reversal studies at control, high dose and positive control. Satellite groups of additional nine rats of each sex at each treatment except for the control (only 3 per sex were included for the control group) were included for blood sampling only. The study was conducted in compliance with Good Laboratory Practice (GLP) but made no claim to comply with Organisation for Economic Co-operation and Development (OECD) Technical Guidance (TG). The protocol included full clinical observation of animals throughout the study including ophthalmoscopy, haematology, clinical chemistry and urine measurements. A simple auditory test was performed but not a complete functional observational battery. All animals from the main and reversal groups were subject to full necropsy with organ weights, bone marrow samples and tissues retained for possible histopathological examination. Histopathology was initially confined to control, high-dose and positive control groups. Following findings in femur and kidney these tissues were examined microscopically in all the other groups.

There was one death during the study, which was considered unrelated to treatment, otherwise there were no treatment-related effects on behaviour and appearance of the animals. Body weight was slightly lower than that of controls in groups receiving 15 or 35 mg SGE/kg bw per day from about week 11 onwards but terminal body weight was only statistically different from controls for the male groups. The group receiving 5 mg SGE/kg bw per day showed no differences from controls. Body weight of positive controls was lower than that of controls from around week 6 onwards. The highest dose group showed no difference from control at the end of the reversal period while body weight of the positive control was still lower than that of controls after that period. Food and water intake, and results of ophthalmoscopy and auditory examinations were not affected by treatment with SGE or the positive control material.

Haematological data showed some statistically significant differences between treated and control groups (elevated haemoglobin in high-dose females after reversal; elevated reticulocytes in high-dose males after reversal; reduced haematocrit in high-dose males and reduced thromboplastin time and mean corpuscular volume (MCV) in high-dose females at 90 days), but these were not considered to be related to treatment. Haematological data showed no differences between the treated and positive control groups which could be attributed to treatment. Serum calcium levels were increased in all male treated groups and in the highest two female groups compared with controls. Serum potassium levels were increased in both sexes at the highest dose. The positive control group males showed similar increases, but these were not seen in the females at the same treatment. These differences were not found after the recovery period. Urine pH was lower than that of controls for all treated groups and for the positive control group. These differences were not present after the recovery period.

At necropsy, there were not relevant differences between treated and control groups. Although some differences were present in organ weights between treated and control groups (90-days: increased relative kidney weight in females; decreased relative heart weight in females), these were not considered to be related to treatment. Bone marrow examinations showed no difference between treated groups, including positive control and untreated controls. Histopathological examination showed treatment-related hyperostosis of the femur and calcium deposits in the kidneys in both sexes at all doses and in the positive control group. The femur hyperostosis was not present in the lowest dose group after the recovery period.

Although after recovery, there were no signs of adverse effects at the lowest dose tested, the Panel agreed that a NOAEL could not be derived from this study for a substance influencing the calcium metabolism. In addition, considering the species differences, the study in rat was considered not relevant for the safety assessment in ruminants and other target species.

[25] Technical dossier/Section III/Annex_III_2_2_3_1 and Annex_III_2_2_3_2.
[26] Technical dossier/Section III/Annex_III_2_2_3_3.

3.3.1.3. Other toxicity studies

Two studies on the oestrogenic activity of SGE (containing 64.8 mg 1,25$[OH]_2D_3$/kg) were submitted by the applicant. The first study[27] consisted of an *in vitro* E-Screen test on the MCF7 breast cancer cell line. The positive control compound (17β-oestradiol) exerted the expected effects. SGE did not show oestrogenic activity up to a concentration of 5,000 μg/mL (0.5, 5, 50, 500 and 5,000 μg/mL were tested). It was not possible to test a higher concentration (50,000 μg/mL), because the compound formed precipitates at the surface of the test plates.

The second study[28] consisted of an *in vivo* Allen Doily study (stated as non-GLP, not according to any OECD TG). The study used a total of 48 spayed female rats (ca. 10–14 weeks of age) and included 8 experimental groups (6 animals/group): 1 – Negative Control (vehicle, oral administration) 2 – SGE at 104 mg/kg bw with oral administration (= 6.74 μg 1,25$[OH]_2D_3$/kg bw), and Groups 3–8: positive controls (with doses between 0.02 up to 25 μg/animal of oestradiol, administered either orally or subcutaneously). Animals received the corresponding dose twice daily for 2 consecutive days. On each of the 3 following days after the last administration, a vaginal smear was taken from each animal to assess the oestrogenic activity. The positive controls receiving subcutaneous injections of 2.5, 7.5 or 25 μg oestradiol/animal, respectively, resulted in the expected oestrogenic activity. SGE did not reveal any oestrogenic activity. Vaginal smears of groups 3, 4 and 5 (positive controls with 0.02, 0.1 or 0.5 μg oestradiol administered orally) did not reveal any oestrogenic activity.

3.3.1.4. Conclusions on toxicology

The FEEDAP Panel confirms the conclusion of the previous assessment that the water-soluble extract of *S. glaucophyllum* leaves containing 64.8 mg 1,25$[OH]_2D_3$/kg is non-genotoxic. From the available data set, a NOAEL cannot be identified. These conclusions are extended to the additive under assessment.

3.3.2. Safety and efficacy for the target species

Two studies on dairy cows were submitted. One study (study 1) investigated the effect of 1,25$[OH]_2D_3$ glycosides in different doses and preparations on blood pharmacokinetics and pharmacodynamics in pregnant dry Holstein and Red Holstein dairy cows. The other study (study 2) examined the effect of 1,25$[OH]_2D_3$ glycosides released from a bolus on mineral status in periparturient primiparous and multiparous dairy cows and their newborn calves. Extended summaries of both studies can be found in the Appendices A and B, respectively. The text below reports to the findings which are immediately relevant for the assessment of the application. No data were provided on small ruminants.

3.3.2.1. Study 1[29]

The study was done with dry pregnant dairy cows (Holstein and Red Holstein,[30] 220–257 days post-insemination[31]) which were allocated to 6 treatment groups. Group size was 5 cows and the study duration was 14 days. The experiment ended circa 24 days prior to calving but some parameters were followed up until 28 days post-partum. Cows had ad libitum access to hay and were fed daily 300 g/cow of a mineralised concentrate, providing 3,245 IU vitamin D_3/cow and day. The calcitriol doses from SGE given in bolus once at start were 200, 300, 500 and 1,000 μg (two 500 μg boli). The preparations contained mini-boli, prepared for immediate (IR) and/or slow release (SR, both [redacted]) of the active substance.

The results showed that the mean dry matter intake (13.2 kg DM/day), water intake (78.6 L/day) and body weight (732 kg at day 4; 741 kg at day 14) as well as body condition score (3.0 at days −4 and 14) were not affected by the treatments ($p > 0.10$).

No treatment effects were observed for the pharmacokinetic parameters C_{max} (peak observed for 1,25$[OH]_2D_3$ or mineral concentration), T_{max} (time of peak concentration), and AUC (area under the concentration–time curve) of plasma 1,25$[OH]_2D_3$ and Ca (also for plasma P), except that the C_{max} and AUC of Ca were 5% higher ($p < 0.05$) for the group receiving 1,000 μg 1,25

[27] Technical dossier/Section III/ Annex III.2.2.6.1.
[28] Technical dossier/Section III/ Annex III.2.2.6.2.
[29] Technical dossier/Section III/Annex III.1.1.1 Part 1 to Part 3.
[30] Age: 4.4 ± 2 years, body weight at start: 732 ± 98 kg
[31] To finalise the pharmacokinetic study at least 2 weeks before expected calving.

$[OH]_2D_3$ (2 boli with 500 μg each) than for a group receiving 300 μg from [redacted] slow release mini-boli.

Bolus application (calcitriol treatment) increased blood serum concentrations of

- $1,25[OH]_2D_3$ from the initial baseline ($p < 0.001$) between experimental day (d) d0.5 and d5 with a peak from d1.25 to d2, before reaching values comparable ($p > 0.10$) to the initial baseline at d9
- Ca from the initial baseline ($p < 0.001$) between d0.5 and d11 with a peak from d2 to d4, before reaching values comparable ($p > 0.10$) to the initial baseline at d14
- P from the initial baseline between d1 ($p < 0.001$) and d11 ($p < 0.05$) with a peak from d3 to d5, before reaching values comparable ($p > 0.10$) to the initial baseline at d14
- but decreased serum Mg from the initial baseline between d1 and d9 ($p < 0.001$) with minima from d2 to d4, before reaching values comparable ($p > 0.10$) to the initial baseline at d11

Although the boluses containing higher amounts of $1,25[OH]_2D_3$ (500 μg) were also more effective in producing a response in blood serum $1,25[OH]_2D_3$, there was no clear difference between dosages in the response of serum Ca, P and Mg.

No time×treatment interaction for the responses of Ca, P and Mg was found ($p > 0.10$). Finally, contrast analysis revealed that blood serum Ca, P and Mg response did not differ ($p > 0.10$) between [redacted] boluses. Serum Ca response tended ($p < 0.10$) to be higher in 2 × 500 compared to 500, whereas serum P and Mg response did not differ ($p > 0.10$) between 500 and 2 × 500.

The end of the experiment was 24.4 ± 11.2 days before the effective calving, without differences between treatments ($p > 0.10$). Also, no differences were seen in the endpoints measured post-calving, including dry matter intake, milk production, and serum concentrations of Ca, P and Mg in cows, as well as the body weight of newborn calves.

3.3.2.2. Study 2[32]

In study 2, a 2 × 2 factorial design was applied, 2 factors for treatment (control - bolus) and 2 factors for parity (primiparous - multiparous), resulting in 4 experimental groups (Control-Primi, Bolus-Primi, Control-Multi, Bolus-Multi). The additive was administered in the form of a bolus (500 μg 1,25 $[OH]_2D_3$ from SGE provided by [redacted] IR mini-boli and [redacted] SR mini-boli) 3–4 days prior to the expected calving date. Animals remained in the study until 21 days after actual calving (day 0), groups size was 6 cows. Three cows (Control-Multi) showed clinical signs of hypocalcaemia with blood serum Ca below the defined threshold of 1.6 mmol/L, they were excluded from the study and replaced by other cows of the same parity.

A basal diet consisting of 75% hay and 25% maize silage (DM basis) was fed ad libitum. In addition, different protein concentrates and mineral feeds were given for the pre-calving and the lactation period. Dietary cation anion difference (DCAD) was 582 and 499 meq/kg DM for the two periods. The high DCAD values, which reflect typical situations occurring in herbage-based diets, are expected to increase the risk of Ca deficiency after calving.

A multitude of parameters were examined. The main endpoints were the concentrations of Ca, followed by $1,25[OH]_2D_3$, PTH, P and Mg in blood at several days of the study.

Multiparous cows had a higher ($p < 0.001$) feed intake on a weekly basis (+53% pre-partum, +37% in lactation). Multiparous cows were about 135 kg heavier ($p < 0.001$) than primiparous cows before and after calving. Milk yield was higher ($p < 0.001$) in multiparous cows (40 kg/day as a mean of weeks 2 and 3) than in primiparous cows (26 kg/day). Bolus administration did not have a significant effect ($p > 0.10$) on any of the cows' performance data during the study.

The relevant endpoint serum Ca remained stable (mean 2.25 ± 0.15 mmol/L) over the entire experimental period in Control-Primi and Bolus-Primi cows but varied significantly ($p < 0.001$) in Control-Multi and Bolus-Multi cows. From d0.5 to d2 after calving, the Ca concentrations of Control-Multi cows dropped by 40% compared to the initial value and was lower compared to those of the other groups within d1 and d1.5. In Control-Multi, Ca declined from 2.19 mmol/L on d-2 to 1.66 on d0.5, 1.39 at d1 and to 1.35 mmol Ca/L at d1.5 and returned to a physiological level (around 2 mmol/L) at d4. In the Bolus-Multi, a peak was seen on d-2, and after this peak, the Ca concentrations returned to values similar to the initial values until the end of the experiment. Control-Primi, Bolus-Primi and Bolus-Multi cows did not have, at any time, mean blood serum Ca concentrations below

[32] Technical dossier/Section III/Annex III.1.1.2.

2.00 mmol/L and none had values higher than 3.00 mmol/L. Serum P concentrations behaved similar to those of Ca.

Whereas 1,25$[OH]_2D_3$ concentration remained constant in Control-Primi and Bolus-Multi cows between d − 4 and d15, there was a temporary increase in Bolus-Primi around calving (from d − 2 until d1) and in Control-Multi after calving (from d1 until d2) compared to d − 4 and d8 − d15, respectively ($p < 0.01$).

The PTH serum concentration remained stable over time in all groups, except in Control-Multi, in which PTH significantly increased from 12 pg/mL at d-4 to 69 pg at d0.5 and 72 pg at d1.5.

The bolus administration had no effect ($p > 0.10$) on calf blood serum minerals, 1,25$[OH]_2D_3$ and IGG concentrations. All serum Ca concentrations of calves were within the normal range (2.0–3.0 mmol/L).

3.3.3. Synopsis of the two studies with dairy cows

In the first study, oral application of a bolus containing 1,25$[OH]_2D_3$ glycosides resulted in a rapid increase of blood serum 1,25$[OH]_2D_3$, followed by increased Ca and P and decreased Mg. Although higher doses of 1,25$[OH]_2D_3$ glycosides tended to give a more pronounced response in blood serum 1,25$[OH]_2D_3$, there was no clear difference between dosages (200–500 and 2 × 500 μg cow) in the response of Ca, P and Mg. The mode of action of 1,25$[OH]_2D_3$ remains unclear (bone mineral mobilisation, intestinal Ca absorption or both).

Under the conditions of this study, no adverse effects of 1,25$[OH]_2D_3$ at any dose were observed. However, the study ended about 3 weeks before parturition and the strong challenge of the Ca-metabolism and its regulating factors in the close period at and after parturition could not be simulated in this study. Therefore, no conclusions on the safety of the additive when administered at the conditions of use (administration 9 or less days before parturition) can be drawn.

In the second study and under the experimental conditions applied, primiparous cows did not show any sign of subclinical hypocalcaemia (< 2.0 mmol Ca/L) following calving. An effect of bolus application on stabilising blood calcium could therefore not be expected. This is confirmed by the experimental data; however, absorption of calcitriol was shown by a response of blood 1,25$[OH]_2D_3$ after bolus administration.

The situation was different in multiparous cows. Their higher daily milk yield compared to primiparous cows induced a 57% higher Ca export via milk (36.8 vs. 23.5 g/day). In addition to the 3 discarded Control-Multi cows with clinical signs of hypocalcaemia, the Control-Multi cows of the study showed blood Ca and P evolution profiles and increasing blood PTH and 1,25$[OH]_2D_3$ concentrations, all indicative for an activation of Ca metabolism of these cows to improve intestinal Ca absorption and bone mineral resorption. Under these conditions, the application of the calcitriol bolus to multiparous cows was successful as blood Ca, P and PTH concentrations remained stable and similar to primiparous cows around calving. Between d4 and d22, blood Ca never felt below 2 mmol/L in the bolus-treated cows. This suggests that the continuous release of 1,25$[OH]_2D_3$ glycosides from the bolus prevented the potential delayed occurrence of hypocalcaemia associated with abrupt withdrawal of vitamin D and its metabolites supply, such as described in the previous FEEDAP opinion.

Calving process, mineral concentrations in colostrum and milk and blood serum mineral concentrations of the offspring remained generally unaffected in primiparous or in multiparous cows by the administration of the calcitriol bolus before calving.

In both studies, elevated blood levels of Ca (and of 1,25$[OH]_2D_3$) as a result of bolus administration returned back to initial levels after 14 days. Toxicity symptoms of exogenous 1,25$[OH]_2D_3$ in target animals are characterised mainly as calcinosis, a calcification of tissues (vessels) and organs, as described (EFSA FEEDAP Panel, 2015), after long term exposure to 1,25$[OH]_2D_3$ by consumption of plants containing the metabolite (e.g. *S. malacoxylon*/*glaucophyllum*, *Cestrum diurnum* and *Trisetum flavescens*). Taking all together and considering that acute effects were not seen also at a double dose, it appears reasonable to consider the singular administration of a 1,25$[OH]_2D_3$ bolus in one lactation as safe for dairy cows. However, no data were provided with the subsequent administration of a second bolus as recommended in the conditions of use, if the cow has not calved within 9 days after bolus administration. The lack of such data on the effect of a prolonged administration of calcitriol around calving on the endogenous synthesis of calcitriol and its likely impact on the calcium homeostasis prevents a conclusion on the safety of this treatment.

3.3.4. Conclusions on the safety and efficacy for the target species

The administration of the bolus containing 500 μg of SGE-derived $1,25[OH]_2D_3$ as glycosides at 3–4 days prior expected calving has the potential to prevent hypocalcaemia in multiparous cows fed a high DCAD diet. It prevents the critical depression of blood Ca and P following parturition particularly in high yielding multiparous cows. Primiparous cows were less sensitive to a potential depression of blood Ca and P after calving. Consequently, an effect of calcitriol bolus administration as seen in multiparous cows could not be demonstrated in primiparous cows.

No potentially adverse effects of the administration of the $1,25[OH]_2D_3$ bolus were seen within the first 3 weeks of lactation. Since vitamin D toxicity is generally observed after long time application, it can also be concluded that the singular application of the calcitriol bolus containing 500 μg of SGE-derived $1,25[OH]_2D_3$ is safe for periparturient cows, when administered $\leq$ 9 days before calving. Owing to the lack of data in the relevant physiological period, the Panel is not in the position to conclude on the safety of the subsequent administration of two boluses to the periparturient cows.

The conclusions on the safety and efficacy apply to the bolus, the preparation of the additive as applied in the animal studies evaluated.

No data were provided on other dairy ruminants. Therefore, no conclusion on safety and efficacy of SGE derived $1,25[OH]_2D_3$ can be drawn.

3.3.5. Safety for the consumer

In its previous assessments on vitamin D_3 supplementation of feedingstuffs (EFSA FEEDAP Panel, 2012b, 2013, 2014), the FEEDAP Panel concluded that the use of vitamin D_3 in animal nutrition at the currently authorised maximum dietary content is of no concern to consumer safety. The FEEDAP Panel also concluded that the use of *S. glaucophyllum* standardised leaves as a feed material did not increase the concentration of $1,25[OH]_2D_3$ in animal tissues compared with feed supplementation with vitamin D_3. Therefore, the FEEDAP Panel concluded that the use of *S. glaucophyllum* in animal nutrition is safe for consumers (EFSA FEEDAP Panel, 2015). Furthermore, consumers will have limited exposure to $1,25[OH]_2D_3$ via dairy foods/milk originating from dairy ruminants, bearing in mind that the SGE bolus is given to dry cows and colostrum produced immediately post-partum is not intended for human consumption. Therefore, under the proposed conditions of use, the additive is considered safe for consumers.

3.3.6. Safety for the user

The applicant made reference to the data assessed by the Panel on the dried *S. glaucophyllum* leaves (EFSA FEEDAP Panel, 2015). The effects of dried *S. glaucophyllum* leaves containing minimum 10 mg $1,25[OH]_2D_3$/kg on skin/eyes were evaluated in an acute dermal irritation/corrosion test, an acute eye irritation/corrosion test and a skin-sensitising test compliant with OECD guidelines 404, 405 and 429, respectively. The studies showed that dried *S. glaucophyllum* leaves are not irritant to skin/eyes nor a dermal sensitiser. Dried *S. glaucophyllum* leaves constitute the raw material used for the manufacture of SGE, hence these data may be representative of SGE.

The preparation intended to be used prevents the user to inhale the dust and/or be in contact with the active substance. Therefore, the active substance used in the preparations described in the application is of no concern for user safety.

The FEEDAP Panel concludes that the bolus, a preparation containing SGE, as a source of the active substance, is not irritating to skin and eyes and it is not a sensitiser. Exposure via inhalation is unlikely.

3.3.7. Safety for the environment

In ruminants, $1,25[OH]_2D_3$-glycosides are deglycosylated in the rumen to $1,25[OH]_2D_3$. Therefore, only negligibly amounts of glycosylated $1,25[OH]_2D_3$ are excreted. This fraction is hydrolysed and oxidised by light and air (EFSA FEEDAP Panel, 2015). The aglycone $1,25[OH]_2D_3$ is extensively metabolised in the animal and excretion products (mainly calcitroic acid) are devoid of vitamin activity. Considering the above, and the fact that vitamin D is widely distributed in plants and animals as a result of endogenous synthesis; it is susceptible to oxidation by light and oxygen (EFSA FEEDAP Panel, 2012a, 2013, 2014), the use of SGE in animal nutrition at the proposed conditions of use will not pose a risk to the environment.

The bolus described by the applicant, SBO, contains SGE, feed materials and feed additives as carriers, therefore no safety concerns for the environment would raise from that formulation.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[33] and Good Manufacturing Practice.

4. Conclusions

The active substance, 1,25$[OH]_2D_3$ in form of glycosides from an extract of *Solanum glaucophyllum leaves*, is contained in a preparation, a bolus ensuring a controlled and delayed release of the active substance in the gastrointestinal tract of ruminants.

The administration of one bolus, the preparation of the additive as applied in the animal studies evaluated, containing 500 μg of SGE-derived 1,25$[OH]_2D_3$ during the pre-parturient period (from 9 days before calving to immediately before calving) is safe for dairy cows. Owing to the lack of data the Panel is not in the position to conclude on the safety (i) of the subsequent administration of a second bolus as recommended by the applicant, if the cow has not calved within 9 days after bolus administration and (ii) of another SGE-derived 1,25$[OH]_2D_3$ preparation intended for use in dairy ruminants other than cows (*Bos taurus*).

The Panel considers the use of the product to be safe for the consumer.

The bolus, a preparation containing SGE, as a source of the active substance, is not irritating to skin and eyes and it is not a sensitiser. Exposure via inhalation is unlikely.

The preparations intended for use in ruminants as described in the application are considered not to raise safety concerns for the environment.

The administration of the bolus, the preparation of the additive as applied in the animal studies evaluated, containing 500 μg of SGE-derived 1,25$[OH]_2D_3$ in a period from 9 days before calving to immediately before calving has the potential to prevent hypocalcaemia in dairy cows. Owing to the lack of data, the Panel cannot conclude on the efficacy in other dairy ruminants.

5. Recommendation

Considering that 25-hydroxycholecalciferol depresses the activity of 1α-hydroxylase in the kidney, the simultaneous use of SGE and 25-hydroxycholecalciferol should be avoided.

6. Documentation provided to EFSA/Chronology

Date	Event
09/04/2021	Dossier received by EFSA. SGE (*Solanum glaucophyllum* leaf extract) for dairy cows for milk production and other dairy ruminants. Submitted by Herbonis Animal Health Pen & Tec Consulting S.L.U.
09/04/2021	Reception mandate from the European Commission
28/05/2021	Application validated by EFSA – Start of the scientific assessment
30/09/2021	Comments received from Member States
21/09/2021	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
27/10/2021	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation*
04/02/2022	Reception of supplementary information from the applicant - Scientific assessment re-started
29/06/2022	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

[33] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

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Abbreviations

AUC	area under the curve
BW	body weight
CAS	Chemical Abstracts Service
CFU	colony forming unit
C_{max}	maximum plasma concentration
CV	coefficient of variation
DCAD	dietary cation anion difference
DM	dry matter
EINECS	European Inventory of Existing Chemical Substances
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GLP	Good Laboratory Practice
IR	immediate release
IUPAC	International Union of Pure and Applied Chemistry
LOQ	limit of quantification
MCV	mean corpuscular volume
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
RH	relative humidity
PARNUTS	feeds intended for particular nutritional purposes
PCBs	dioxin-like polychlorinated biphenyls
PCDD	polychlorinated dibenzo-*p*-dioxins
PCDF	polychlorinated dibenzofurans
PTH	parathyroid hormone
SG	*Solanum glaucophyllum*
SGE	*Solanum glaucophyllum* leaf extract
SR	slow release
TG	Technical guidance
T_{max}	time of highest peak concentration
UPLC–MS/MS	ultraperformance liquid chromatography–tandem mass spectrometry
WHO	World Health Organization

Appendix A – Study on the effects of the additive on the pharmacokinetics and pharmacodynamics in pregnant dry dairy cows

The applicant submitted a research report entitled 'Effect of 1,25-dihydroxycholecalciferol-glycosides given as a rumen bolus on blood pharmacokinetics and pharmacodynamics in dry dairy cows'. The aim of this study was to determine the time response of blood parameters following the application of a bolus filled with tablets containing 1,25$[OH]_2D_3$-glycosides. The source of 1,25$[OH]_2D_3$ was an extract of glycosides from *Solanum glaucophyllum* leaves (SGE).

The experimental research protocol was approved by the Office for Food Safety and Veterinary Affairs and all procedures were conducted in accordance with the Swiss Ordinance on Animal Protection and the Ordinance on Animal Experimentation.

Thirty pregnant dry dairy cows (Holstein and Red Holstein,[30] 220–257 days post insemination[31]) were allocated 4 days prior experimental start within each bloc to 5 blocs with 6 treatments each. Three groups were treated with one bolus/cow containing 200 μg 1,25$[OH]_2D$ from [redacted] IR-mini-boli (group 200(2)), 300 μg from [redacted] SR-mini-boli (group 300(8)) and 500 μg 1,25$[OH]_2D_3$ from [redacted] IR- + [redacted] SR-mini-boli, respectively (group 500([redacted])). The boli of 2 other groups contained [redacted] SR-mini boli, one bolus providing 300 μg 1,25$[OH]_2D_3$ from [redacted] SR-mini-boli (group 300(8)c), and one bolus providing 500 mg 1,25$[OH]_2D_3$ from [redacted] IR- + [redacted] SR-mini-boli (group 500([redacted])c). An overdose group received 2 boli each containing 500 μg 1,25$[OH]_2D_3$ from [redacted] IR- + [redacted] SR-mini-boli (group 2 × 500 ([redacted])). The animals were allocated to five blocks according to parity (primiparous, multiparous) and to expected calving date.

Cows had ad libitum access to hay[34] (2nd seasonal harvest; botanical composition: 78% graminea, 19% legumes, 3% herbs) and water and were fed 300 g per cow per day a pelleted mineral concentrate[35] from at least 10 days before oral bolus application (day 0) until day 14 after bolus application.

Hay intake was recorded on experimental days 2, 3, 4, 9, 10 and 11. Individual water consumption was recorded between days −4 to 0, 0 to 7 and 7 to 14. Body condition score was evaluated and body weight was recorded for each cow on days −4 and 14. Blood samples were taken from each cow; blood pharmacokinetics were determined for Ca, P, Mg and 1,25$[OH]_2D_3$ at d0 (mean of d-4, d-3, d0) and at regular intervals after d0 bolus administration (1 h, 3 h, 6 h, d0.5, d1.25, d1, d1.125, d1.25, d2, d3, d4, d5, d7, d9, d11 and d14). Blood haematology[36]/biochemistry[37] was determined at d0, d1, d1.125, d1.25, d14 for groups 500([redacted]) and 2 × 500([redacted]).

Following the end of the pharmacokinetic experiment, cows were handled as usual and additional data were recorded until 28 days post-partum. The data collected around calving were: calving date, calf birth weight, calf gender, score for assistance (unassisted, light pull, hard pull), preventive and curative treatments against hypocalcemia and hypocalcemia diagnostic. During lactation daily individual dry matter intake, milk yield and body weight were recorded automatically.

The individual cow was considered as the experimental unit. The repeated blood serum and plasma concentrations were used to calculate by noncompartmental analysis the pharmacokinetic parameters of the orally administered 1,25$[OH]_2D_3$ bolus. The pharmacokinetic parameters were area under the curve (AUC), maximum plasma concentration for a mineral or vitamin (C_{max}), time of highest peak concentration (T_{max}) and terminal half-life. The calculated residence time is the average time that molecules of the dosed compound spend in the body. Non-repeated performance data were analysed using the general linear model including block and treatment. Repeated blood serum and plasma concentrations were analysed with the mixed model including block, treatment, time and treatment × time. The cow was included as fixed random factor. Comparisons among means were calculated using Tukey's contrasts. One cow (group 2 × 500([redacted])) was finally not pregnant and was

[34] Analysed values of hay in g/kg DM: crude protein 149; crude fibre 287; fat 31; ash 99; Ca 6.2; P 4.4; Mg, 1.8; K 38.9; Na 0.6.

[35] The mineral concentrate consisted of wheat bran, apple pomace, oats, sodium chloride, magnesium oxide, molasses, trace mineral and vitamin premix, beta-carotene and biotin. It was supplemented with 10.816 IU vitamin D_3/kg. Analysed values of mineral feed concentrate in g/kg DM: crude protein 119; crude fibre 118: fat: 37: ash 181: Ca 7.8; P 6; Mg. 22; K 8.5; Na 2.32.

[36] Haematocrit %, Red blood cells Mio/μL, White blood cells k/μL, Platelet k/μL, Haemoglobin g/dL, Mean corpuscular haemoglobin pg/cell, Mean corpuscular concentration g/dL, Mean corpuscular volume fL, Large cell count k/μL, Middle cell count g/dL, Small cell count k/μL.

[37] Total protein g/L, Albumin g/L, Globulin g/L, Glucose mmol/L, Urea mmol/L, Cholesterol mmol/L, Creatinine μmol/L, Bilirubin μmol/L, Alanine aminotransferase IU/L, Aspartate aminotransferase IU/L, Gamma-Glutamyl-Transferase IU/L, Creatine kinase IU/L, Lactate dehydrogenase IU/L, Amyloid A ng/mL, Haptoglobin ng/mL, Sodium mmol/L, Potassium mmol/L, Chloride mmol/L.

removed from the dataset. Differences were considered as significant when $p < 0.05$ and tendencies were noted at $p < 0.10$.

Dry matter intake (13.2 ± 1.6 kg DM/day), water intake (78.6 ± 14.9 L/day), body weight (732 ± 98 kg at d-4; 741 ± 95 kg at d14) as well as body condition score (3.0 ± 0.3 at days −4 and 14) were similar between treatments ($p > 0.10$).

The pharmacokinetic parameters C_{max}, T_{max}, and AUC of plasma $1,25[OH]_2D_3$ and Ca are presented in Table A1. No treatment effects were observed (also for plasma P), except that the C_{max} and AUC of Ca were 5% higher ($p < 0.05$) for the group 2 × 500(2+8) compared to 300(8)c. Tendencies were observed for $1,25[OH]_2D_3$: C_{max} from 500([redacted]) doubled ($p = 0.09$) compared to 200(2) and AUC from 500([redacted]) and 500([redacted])c doubled ($p = 0.08$) compared to 200(2).

Table A.1: Pharmacokinetic parameters of plasma $1,25[OH]_2D_3$ and calcium

Group	$1,25[OH]_2D_3$ in bolus	Plasma $1,25[OH]_2D_3$			Plasma calcium		
	Analysed (µg)	C_{max}	T_{max}	AUC	C_{max}	T_{max}	AUC
200[redacted]	198	57	19	5,962	2.79	96	842
300[redacted]	273	111	42	10,671	2.79	149	856
500[redacted]	512	123	37	11,934	2.79	110	853
300[redacted]c	292	61	44	7,311	2.77	139	844
500[redacted]c	517	116	37	11,941	2.89	82	876
2 × 500[redacted]	(2 × 512)	99	36	10,501	2.94	61	884

C_{max} (pg/mL): peak observed for vitamin or mineral concentration; T_{max} (h): time of peak concentration; AUC (pg/mL and h): area under the concentration-time curve.

[redacted]

Following bolus application, blood serum $1,25[OH]_2D_3$ (Table A.2) increased from the initial baseline ($p < 0.001$) between d0.5 and d5 with a peak from d1.25 to d2, before reaching values comparable ($p > 0.10$) to the initial baseline at d9. The time dependent response was more pronounced in 300(8), 500[redacted], 500[redacted]c and 2 × 500[redacted] than in 200[redacted] and 300[redacted]c (time × treatment interaction, $p < 0.05$). Blood serum $1,25[OH]_2D_3$ response was not different ($p > 0.10$) between uncoated and coated boluses and did not differ between the group receiving 500[redacted] and 2 × 500 [redacted].

Table A.2: Plasma $1,25[OH]_2D_3$ (pg/mL) at different times after administration

Group	$1,25[OH]_2D_3$ in bolus	Time after bolus application						
	Analysed (µg)	0 h	d0.5	d1.25	d2	d3	d5	d9
200[redacted]	198	9.9	53.4	53.6	42.1	34.2	23.3	3.4
300[redacted]	273	14.1	61.6	104.2	87.0	66.1	38.7	16.6
500[redacted]	512	13.6	95.2	98.5	103.0	65.7	48.2	13.6
300[redacted]c	292	13.8	31.2	51.6	59.8	51.8	29.5	12.3
500[redacted]c	517	14.2	80.5	115.3	97.4	75.3	43.9	14.2
2 × 500[redacted]	(2 × 512)	10.4	50.8	93.1a	77.8	67.9	46.4	9.1

Blood serum Ca (Table A.3) increased from the initial baseline ($p < 0.001$) between d0.5 and d11 with a peak from d2 to d4, before reaching values comparable ($p > 0.10$) to the initial baseline at d14.

Table A.3: Serum Calcium (mmol/L) at different times after administration

Group	$1,25[OH]_2D_3$ in bolus	Time after bolus application							
	Analysed (µg)	0 h	d0.5	d1.25	d2	d3	d5	d9	d14
200[redacted]	198	2.21	2.36	2.64	2.67	2.68	2.55	2.61	2.30
300[redacted]	273	2.22	2.35	2.61	2.66	2.70	2.65	2.65	2.23
500[redacted]	512	2.25	2.34	2.59	2.66	2.69	2.60	2.61	2.27
300[redacted]c	292	2.22	2.24	2.47	2.67	2.68	2.63	2.61	2.15
500[redacted]c	517	2.25	2.46	2.70	2.85	2.82	2.69	2.69	2.22
2 × 500[redacted]	(2 × 512)	2.23	2.43	2.63	2.79	2.79	2.80	2.63	2.34

Following bolus application, blood serum P increased from the initial baseline between d1 ($p < 0.001$) and d11 ($p < 0.05$) with a peak from d3 to d5, before reaching values comparable ($p > 0.10$) to the initial baseline at d14. Blood serum Mg decreased from the initial baseline between d1 and d9 with minima from d2 to d4, before reaching values comparable ($p > 0.10$) to the initial baseline at d11.

The overall response in blood serum Ca from 300[redacted]c was lower ($p < 0.001$) than from 500[redacted]c and 2×500[redacted], whereas the response of blood serum P and Mg was similar between treatments ($p > 0.10$). No interaction of time × treatment ($p > 0.10$) was identified for the responses of Ca, P and Mg. Blood serum Ca, P and Mg response did not differ ($p > 0.10$) between [redacted] boluses. Serum Ca response tended ($p < 0.10$) to be higher in 2 × 500[redacted] compared to 500 [redacted], whereas serum P and Mg response was not different between these two groups.

The measurements done in blood, haematology and biochemistry, in the groups 500[redacted] and 2 × 500[redacted] did not reveal major differences between the treatments and there was no time × treatment interaction. These results indicate that double bolus did not present a risk different from that of a single bolus. Thus, none of the measured haematology and biochemistry parameters suggested that the overdose would result in any relevant difference to a single application.

The end of the experiment (pharmacokinetics of d14) was 24.4 ± 11.2 days before the effective calving, without differing between treatments ($p > 0.10$). Before calving (1–7 days before calving), 13 cows received a preventive therapy against hypocalcaemia. Despite the treatment, two of these cows showed clinical signs of hypocalcemia following calving and were treated with intravenous and oral calcium. One these cows (group 300[redacted]) with a prolapsed uterus, whereas the other cow (group 500 [redacted]) recovered well. Twenty-nine cows gave birth to a healthy calf (birth body weight 41.5 ± 6 kg). During the first 28 days post-partum dry matter intake (19 ± 3.5 kg/day) and milk production (38.2 ± 8.4 kg/day) of cows were similar between treatments ($p > 0.10$) and were comparable with cows at similar lactation stages of the same dairy operation. The initial and final blood serum concentrations of $1,25[OH]_2D_3$, Ca, P and Mg were within published ranges considered as normal (5–20 pg $1,25[OH]_2D_3$/L, 2.0–3-0 mmol Ca/L, 1.4–2.6 mmol P/L and 0.7–1.5 mmol Mg/L).

Appendix B – Effect of 1,25 dihydroxycholecalciferol glycosides released from a rumen bolus on mineral status in periparturient primiparous and multiparous dairy cows and their newborn calves

The study started with a total of 36 pregnant dairy cows to have 24 cows[38] meeting the conditions of the study.

The experimental research protocol was approved by the Office for Food Safety and Veterinary Affairs and all procedures were conducted in accordance with the Swiss Ordinance on Animal protection and the Ordinance on Animal Experimentation.

The study was set up as a 2 × 2 factorial design with factors treatment (Control (C) or bolus (B)) and parity (primiparous (P) or multiparous with ≥ 3 lactations (M)) to obtain four groups: Control-Primi (CP), Bolus-Primi (BP), Control-Multi (CM) and Bolus-Multi (BM) (with 6 cows each). The 6 replicates (cows) per group were defined according to a power analysis (a difference in blood Ca plasma of 0.5 mmol/L between C and B cows should identified).

Animals were selected for the study 21 days prior to the expected calving date,[39] and the bolus was administered 3–4 days prior to the expected calving date.[40] Animals remained in the study until 21 days after actual calving (day 0). If clinical signs suggesting hypocalcaemia were observed after calving and a blood Ca analysis showed < 1.6 mmol/L, the cow was excluded from the experiment and replaced by another animal of the same parity.

The basal diet consisted of hay and maize silage (75/25% of DM) and was fed ad libitum. In addition, two pelleted cereal-based concentrates differing in protein content and two pelleted mineral feeds were given for the pre-calving and the lactation period, respectively.[41] The total daily ration contained per kg DM by analysis for the prepartum and the lactation period 127 and 138 g crude protein, 217 and 181 g crude fibre, and 5.7 and 6.1 MJ NEL, respectively. Dietary cation anion difference (DCAD) was 582 and 499 meq/kg DM for the two periods. The high DCAD values, which reflect typical situations occurring in herbage-based diets, are expected to increase the risk of Ca deficiency after calving. The calves received twice a day colostrum and milk from their mothers until d5. Then, they were fed twice a day milk enriched with milk powder. Calves had free access to hay and water.

A multitude of parameters were examined.[42] The main endpoints were the concentrations of Ca, followed by $1,25[OH]_2D_3$, PTH, P and Mg in blood at several days of the study.

The data were statistically analysed[43] for repeated blood values by a mixed model with group, day and group × day and the cow as fixed random effect. When the day, the group or the interaction

[38] The cows (Holstein & Red Holstein) had an age of 4.7 years and a body weight at start of 753 ± 94 kg.

[39] In order to obtain calving's of 6 primiparous and 6 multiparous cows within the time-response after bolus application, 18 pregnant heifers and 18 pregnant dry cows were selected from the dairy herd 260 days after successful insemination, corresponding to 21 days prior to expected calving. None of the selected multiparous cows had previous cases of hypocalcaemia. At 277–278 days after insemination (3–4 days prior to expected calving), a bolus (a placebo for C or the calcitriol containing bolus for B) was administered. Within 3 consecutively expected calving's of heifers or cows, a placebo bolus was provided to the first animal (C) and a bolus containing the additive to the two next animals (B). All C-cows which calved after placebo-bolus application and the B-cows which calved within 1 and 9 days after bolus application remained in the experiment until 3 weeks post-partum.

[40] According to the pharmacological study (see Appendix A) the optimal time-window for a response to bolus treatment on blood serum Ca was between 9 and 0.5 days before actual calving. In the dairy herd used in the study, the mean actual calving date is at 281 days pregnancy with a standard deviation of 5 days. Considering these 5 days of variability, the recommended application of the bolus should be 3 to 4 days before the expected calving day to meet the recommendation of the applicant, the bolus should be administered once from 9 days prior to calving to just before calving.

[41] Analysed values: Concentrates: 122 and 544 g crude protein, 9.4 and 4.9 g Ca, 3.5 and 6.1 g P, 1.2 and 3.3 g Mg; Mineral feed: 9.7 and 148 g Ca, 5.2 and 23.5 g P, 48 and 83 g Mg g per kg DM, respectively.

[42] Examinations: Daily health check, cow performance data (daily feed intake, daily milk yield, body weight (and body condition score) 4 days prior to the expected calving date and on d2). Blood (Ca, P, Mg and $1,25(OH)_2D_3$) at bolus administration (3 and 4 days prior to the expected calving date), then every second day prior to calving; d0.5, d1, d1.5, d2 after the actual calving (which is d0), then twice a week until 21 days in lactation (corresponding to standardised days: d4, d8, d11, d15, d18 and d22); urine sample at bolus administration and on d0.5 and d2. Feed analysis (basal diet: sampled once a week, pooled every 2nd week for analysis; concentrates: collected once a week and pooled every 4 weeks).Calving process, gender, birth weight and health observation of calves; colostrum sample (first milking, analysis of Ca, P, Mg, IGG); milk sample (weekly for analysis of fat, protein, lactose, urea, somatic cell count (SCC), Ca, P, Mg, IGG); calf blood sample 36-48 h after birth and d21 (for analysis of Ca, P, Mg and $1,25(OH)_2D_3$, IGG).

[43] As blood sampling days were not equal between cows when related to actual calving day, sampling days were standardised in reference to actual calving day (d0) as follows: d-4, d-2, d0.5, d1, d1.5, d2, d4, d8, d11, d15, d18 and d22. Data from cows for blood $1,25(OH)_2D_3$, PTH and carboxyterminal telopeptide of type I collagen (CTx), urinary Ca and Ca/creatinine ratio, colostrum IGG and milk SCC were previously log transformed to obtain normally distributed data.

between group and day or parity and bolus were significant, the least square means were compared using Tukey's contrasts. For non-repeated data, a general linear model (GLM) was applied including parity, bolus and parity × bolus.

To collect data from 24 cows, 35 cows had to be included in the experiment. Thus, 11 cows were excluded from the experiment. Five cows (2 BP, 3 BM) calved outside of the defined time frame of 1–9 days after bolus application. Three CM-cows showed clinical signs of hypocalcaemia with blood serum Ca below the defined threshold of 1.6 mmol/L (1.54, 0.73 and 0.72 mmol/L at 32 h, 17 h and 24 h post-partum, respectively). Another 3 cows (2 BP and 1 CM) had to be treated with antibiotics after calving.

Multiparous cows had a higher ($p < 0.001$) feed intake on a weekly basis (+53% pre-partum, +37% in lactation) and a higher intake of each separate diet component than primiparous cows. The bolus administration did not have any effect on feed intake of the cows. Multiparous cows were heavier ($p < 0.001$) than primiparous cows before (818 vs. 687 kg) and after calving (766 vs. 628 kg). The body condition score varied between 3.0–3.3 and was similar among groups ($p > 0.10$). Milk yield was higher ($p < 0.001$) in multiparous cows (39.2 and 40.8 kg/day in week 2 and 3, respectively) than in primiparous cows (24.9 and 26.6 kg/day in week 2 and 3, respectively). Bolus administration did not affect ($p > 0.10$) any of the cows' performance data.

Considering calving criteria (calving without human help, birthweight, intervals bolus to calving, calving to placenta expulsion and calving to calf first stand-up), no effect ($p > 0.10$) was observed for parity, bolus and their interaction.

The concentration of $1,25[OH]_2D_3$ and calcium in blood serum of cows is presented in Table B.1. Whereas $1,25[OH]_2D_3$ concentration remained constant in CP and BM cows between d-4 and d15, it increased in BP around calving (d-2, d0.5) and in CM after calving (d1 to d2) compared to d4 and d8 to d15, respectively ($p < 0.01$, day to group interaction).

Table B.1: $1,25[OH]_2D_3$ and calcium in cow blood serum in days from calving

Day from calving	Control-Primi		Bolus-Primi		Control-Multi		Bolus-Multi	
	$1,25[OH]_2D_3$ pmol/L	Ca mmol/L	$1,25[OH]_2D_3$ pmol/L	Ca mmol/L	$1,25[OH]_2D_3$ pmol/L	Ca mmol/L	$1,25[OH]_2D_3$ pmol/L	Ca mmol/L
–4	53	2.09	48	2.34	37	2.25	34	2.38
–2	57	2.13^{a}	169	2.59^{ab}	53	2.19^{b}	69	2.97^{a}
0.5	56	2.13^{ab}	101	2.42^{b}	89	1.66^{a}	115	2.15^{ab}
1	68	2.02^{b}	115	2.39^{b}	108	1.39^{a}	89	2.03^{b}
1.5	64	2.03^{b}	74	2.52^{b}	120	1.35^{a}	83	2.00^{b}
2	74	2.10^{ab}	73	2.42^{b}	139	1.73^{a}	97	2.02^{ab}
4	75	2.19	25	2.15	68	2.32	66	2.19
8	51	2.36	33	2.09	32	2.29	61	2.17
11	51	2.33	36	2.21	35	2.35	58	2.25
16/15	47	2.38	39	2.20	31	2.27	48	2.24

a,b: Values within a row with different superscript are significantly different ($p < 0.10$).

Serum Ca remained stable (mean of 2.25 ± 0.15 mmol/L) over the entire experimental period in CP and BP cows, but varied in CM and BM cows ($p < 0.001$ day to group interaction). After calving (from d0.5 to d2), the Ca concentrations of CM cows dropped by 40% compared to the initial value (d – 4) and was lower compared to those of the other groups. In the BM cows, Ca peaked before calving (d – 2) at a higher concentration compared to the initial values on d – 4 and to CP and CM cows. After that peak, the serum Ca concentrations of BM cows returned to values similar to the initial values until the end of the experiment. CP, BP and BM cows did not have, at any time, mean blood serum Ca concentrations below 2.00 mmol/L and none had values higher than 3.00 mmol/L.

The P concentration in cows' blood serum remained stable (mean of 1.30 ± 0.33 mmol/L) over the entire experimental period for CP and CM cows. However, P serum levels in ControlMulit cows reached values below 0.8 mmol/L between d0.5-d2, which were lower than those of BP cows ($p < 0.001$ day to group interaction). The P concentrations peaked in BP on d-2 (2.43 mmol/L), d0.5 (2.19 mmol) and d1.5 (2.16 mmol) and in BM on d-2 (2.48 mmol/L) compared to values after d8 (1.28–1.39 mmol/L for BP, 1.16–0.90 mmol for BM; $p < 0.001$, day to group interaction).

For CP, BP and CM cows, the Ca/P ratio remained stable over the entire experimental period. This ratio was increased in CM cows (2.25) compared to CP (1.50) and BP cows (1.48).

The Mg concentration in blood serum remained stable (mean of 0.98 ± 0.05 mmol/L) over the entire experimental period for BP and BM cows. In CM cows, the Mg concentration increased first and peaked on d0.5 (1.12 mmol/L), then dropped to its lowest value on d4 (0.88 mmol) before stabilising until the end of the experimental period (0.95–0.96 mmol/L). No differences were found in CP except on d4 (0.83 mmol/L) which was significantly lower than the other Mg values (0.87–0.97 mmol/L)

The parathyroid hormone (PTH) serum concentration remained stable over time in all groups, except in CM. The average of all groups was higher on d1.5 (48 pg/mL) compared to the initial PTH value (29 pg). In CM PTH increased significantly from 12 pg/mL at d-4 to 69 pg at d0.5 and 72 pg at d1.5.

Blood serum bone resorption marker CTx (carboxyterminal telopeptide of type I collagen) in cows blood serum increased constantly over time in all groups, except CP ($p < 0.001$, day × group interaction). Primiparous cows had higher CTx concentrations than BM over the whole experimental phase, but the difference was higher in the phase d-4 to d0.5 ($p < 0.001$ day to group interaction).

The bolus administration had no effect ($p > 0.10$) on calf blood serum minerals, $1,25[OH]_2D_3$ and IGG concentrations. All serum Ca concentrations of calves were within the normal range (2.0–3.0 mmol/L).

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of the Analysis for glycosylated 1,25-dihydroxycholcalciferol in *Solanum glaucophyllum* extract

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* (RSDr) ranging from 5.2% to 6.3%; a relative standard deviation for *intermediate precision* (RSDip) ranging from 8.0% to 8.2%; and a *recovery* rate (RRec) ranging from 95% to 103%.
- For *bolus* with *glycosylated 1,25-dihydroxycholecalciferol* content ranging from 28.3 to 99.3 mg/kg: RSDr ranging from 3.3% to 4.8%; RSDip ranging from 5.8% to 18.4%; RRec 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.