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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

Quillaja saponaria* and *Yucca schidigera
(FAD-2021-0046; CRL/180057)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0046 - CRL/180057**
Name of Product:	***Quillaja saponaria* and *Yucca schidigera***
Active Agent:	**Quillaja saponins**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Zigmas Ezerskis** **11/11/2022**
Report approved by: Date:	**Christoph von Holst** **11/11/2022**

EXECUTIVE SUMMARY

In the current application an authorisation of a preparation of *Quillaja saponaria and Yucca schidigera* is sought under Article 4 under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for all avian species (excluding laying and breeding birds).

The *product* is intended to be marketed as preparation of *Quillaja saponaria* (85 % w/w) and *Yucca schidigera* (15 % w/w) with a guaranteed minimum *saponins* (active substance) content of 3.5 % (w/w). The preparation is meant to be incorporated directly into *feedingstuff*s in order to obtain a minimum content of 250 mg/kg in complete *feedingstuffs*.

For the determination of *saponins* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified analytical method based on reversed-phase ultra-high performance liquid chromatography coupled with ultraviolet detection (UHPLC-UV). The following method performance characteristics were derived from the validation and verification studies (partially re-calculated by the EURL): a relative standard deviation for *repeatability* (RSD_r) ranging from 1.3 to 6.5 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 2.3 to 6.3 %; and a *recovery rate* (R_{Rec}) ranging from 84 to 109 %. Based on the satisfactory performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified analytical method based on reversed-phase ultra-high performance liquid chromatography coupled with ultraviolet detection (UHPLC-UV) for the determination of *saponins* in the *feed additive.*

In the frame of supplementary information to the original dossier, the Applicant submitted for the determination of *saponins* in the *feed additive* and in *feedingstuffs* two similar single-laboratory validated and further verified analytical methods based on reversed-phase high performance liquid chromatography coupled with a mass spectrometry detector (HPLC-MS). However, due to incomplete supporting studies provided, the EURL cannot recommend for official control the HPLC-MS methods for the quantification of *saponins* in the *feed additive* and *feedingstuffs*.

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

KEYWORDS

Quillaja saponaria and Yucca schidigera;Magni-Phi®; zootechnical additives digestibility enhancer; avian species (excluding laying and breeding birds).

1. BACKGROUND

In the current application an authorisation of a preparation of *Quillaja saponaria and Yucca schidigera* is sought under Article 4(1) under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for the use of the *feed additive* for all avian species (excluding laying and breeding birds) [2].

The *product* is intended to be marketed as powdered natural preparation (Magni-Phi®) of *Quillaja saponaria* (85 % w/w) and *Yucca schidigera* (15 % w/w) with a guaranteed minimum *saponins* (active substance) content of 3.5 % (w/w) [3,4].

The preparation is intended to be incorporated directly into *feedingstuff*s in order to obtain a minimum content of 250 mg/kg in complete *feedingstuffs* [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with a preparation of *Quillaja saponaria and Yucca schidigera* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the determination of *saponins* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified analytical method based on reversed-phase ultra-high performance liquid chromatography coupled with ultraviolet detection (UHPLC-UV) [6,7].

The moisture content of the samples and of the standards (expressed as percentage) is determined by Karl Fisher's or "loss on drying" methods [7,8]. 5.0 g of sample are weighed directly in a dry bottle and 75.0 ml of MilliQ grade water are added. The bottle is placed in a warm bath (60 ± 1 ºC) and is shaken for 3 h at 80 rpm. The homogenised sample is filtrated before injection. The analysis of *saponins* is performed by a UHPLC equipped with a

reversed-phase C18 column. The samples are tested using a mobile phase containing 0.15 % formic acid in water/acetonitrile in a gradient mode at a flow rate of 0.41 ml/min. The *saponins* are detected by UV detection at 210 nm. The quantification of the *active substance* is performed using a calibration with an external standard [7,8].

The following method performance characteristics were derived from the validation and verification studies as partially re-calculated by the EURL [9,10]:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 1.3 to 6.5 %;
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 2.3 to 6.3 %; and
- a *recovery rate* (R_{Rec}) ranging from 84 to 109 %.

Based on the satisfactory performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified analytical method based on reversed-phase ultra-high performance liquid chromatography coupled with ultraviolet detection (UHPLC-UV) for the determination of *saponins* in the *feed additive.*

In the frame of supplementary information to the original dossier, the Applicant submitted for the determination of saponins in the feed additive an additional single-laboratory validated and further verified analytical method [11-14]. The method is based on reversed-phase high performance liquid chromatography coupled with a mass spectrometry detector (HPLC-MS) [12].

2.5 g of sample are weighed directly into a 500 mL glass bottle. 200 mL of deionised water are added and the solution is swirled to mix and placed in a water bath 60 ºC overnight. Once removed the bottle from the bath, the sample is mixed mechanically for 10 min with the addition of 200 ml of ethanol. The sample is left to settle for 10 min and 40 ml of extract are centrifuged for 10 min (3000 rpm). The supernatant may be further diluted using as diluent a solution 50:50 ethanol:water. 10 ml of samples and standards are transferred and mixed in a flask where 1 ml of 20 % sodium hydroxide solution and boiling stones are added. The solution is refluxed at 100 ºC for 2 h. The solution is removed and cooled at room temperature. 15 ml of phosphoric acid are added and after mixing filtered by 0.45 µm PTFE filter directly into the injection vials. 50 µL of the filtrate are analysed by reversed-phase HPLC (gradient flow at 0.3 ml/min, where the mobile phase A and B are composed respectively of 0.15 % formic acid in water and acetonitrile). Calibration standards are injected at the beginning and at the end of each analysis. The *active substance* is determined by mass spectrometry via the quantification of the QH957 and QH 971 moieties of the *saponins* (respectively measured at m/z 979.5 and 993.6).

For the determination of *saponins* in *feedingstuffs* the Applicant submitted a very similar single-laboratory validated and further verified analytical method based on HPLC-MS [6,11,15-17]. The procedure differs only in the extraction of the *saponins* from the sample.

Based on the expected content of *saponins*, a variable amount of *feed* sample is weighed directly into a 500 mL glass bottle. 150 mL of deionised water are added and the solution is swirled to mix and placed in a water bath 60 ºC overnight. Once removed the bottle from bath the procedure follows the steps as above-described for the second day of analysis.

However, the data presented by the Applicant and used for deriving the performance characteristics values within the validation study for the analysis in the *feed additive* and in the verification study for the analysis in *feedingstuffs*, are not considered fit-for-the purpose [13,17]. Nevertheless, the following method performance characteristics were presented and partially re-calculated by the EURL [10]:

- from the verification study for the analysis in the *feed additive* [14]: a RSD_r ranging from 2.5 to 7.5 %, a RSD_{ip} of 5.4 % and a R_{Rec} ranging from 75 to 93 %; and
- from the validation study for the analysis in *feedingstuffs* [16]: a RSD_r ranging from 6.0 to 9.0 % and a R_{Rec} ranging from 83 to 113 %.

Furthermore, a limit of detection (LOD) and a limit of quantification (LOQ) of 58 and 76 mg/kg of *feedingstuffs*, respectively, were presented by the Applicant [16].

However, based on the incomplete studies provided, the EURL cannot recommend for official control the HPLC-MS method for the quantification of *saponins* in the *feed additive* and *feedingstuffs*.

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

The 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: (i) the single-laboratory validated and further verified analytical method based on reversed-phase ultra-high performance liquid chromatography coupled with ultraviolet detection (UHPLC-UV) for the determination of *saponins* in the *feed additive.*

Recommended text for the register entry (analytical method)

For the determination of *saponins* in the *feed additive*:

– reversed-phase ultra-high performance liquid chromatography coupled with ultraviolet detection (UHPLC-UV)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the *Quillaja saponaria and Yucca schidigera* 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: FORW. APPL. 1831-0038-2021 & Annex I – submission number 1532346659921-2264

[2] *Technical dossier, Section II: 2.1.2 Proposal for classification

[3] *Technical dossier, Section II: 2. Introduction

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

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

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

[7] *Technical dossier, Section II: Annex_II_6_1_2_Analysis_MagniPhi_Validation

[8] *Technical dossier, Section II: Annex_II_6_1_1_Analysis_MagniPhi

[9] *Technical dossier, Section II: Annex_II_6_1_3_Analysis_MagniPhi_Verification

[10] EURL_MagniPhi_calc.xlsx

[11] *Supplementary information: 0_MagniPhi_Cover_Letter

[12] *Supplementary information: Annex_QEURL1

[13] *Supplementary information: Annex_QEURL2

[14] *Supplementary information: Annex_QEURL3

[15] *Supplementary information: Annex_II_6_1_4_updated

[16] *Technical dossier, Section II: Annex_II_6_1_5_Analysis_Feed_Validation

[17] *Technical dossier, Section II: Annex_II_6_1_6_Analysis_Feed_Verification

*Refers to Dossier no: FAD-2021-0046

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

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

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Wageningen Food Safety Research (WFSR) (NL)[1]
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)

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