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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**

Dry Grape Extract
(FAD-2021-0069; CRL/210019)



**Evaluation Report on the Analytical Methods submitted
in connection with the Application for Authorisation of a
Feed Additive according to Regulation (EC) No 1831/2003**

Dossier related to: **FAD-2021-0069 - CRL/210019**

Name of Feed Additive: ***Dry Grape Extract***

Active Agent (s): ***Total polyphenols***

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
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Date: **27/06/2022**

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Date: **27/06/2022**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *Dry Grape Extract* under the category / functional group (4 e) "zootechnical additives" / "physiological condition stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the *feed additive* is sought to be used for all poultry species.

The *feed additive (Dry Grape Extract)* is a dry grape extract of *Vitis vinifera* spp. *Vinifera* with a minimum contents of 80 % (w/w) of *polyphenols* (active substance) and 0.7 g / kg of *malvidin-3-O-glucoside* (phytochemical marker), respectively. The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* at a recommended minimum and maximum content of *Dry Grape Extract (feed additive)* of 30 and 100 mg / kg *feedingstuffs*, respectively. This *feed additive* is currently authorised as sensory additive by Commission Implementing Regulation (EU) No 2017/307.

For the determination of the *total polyphenols* (active substance) content in *Dry Grape Extract (feed additive)*, the Applicant submitted a method based on spectrophotometry at 280 nm using external calibration with catechin standards. This method has been single-laboratory validated and further ring-trial validated leading to relative standard deviation for *reproducibility* (RSD_R) ranging from 1.2 to 4.5 %, and recovery rates (R_{rec}) up to 130 %.

Based on the acceptable performance characteristics the EURL recommends for official control the above-mentioned method based on spectrophotometry for the determination of the *total polyphenols* in the *feed additive (Dry Grape Extract)*.

The Applicant did not provide any experimental data or an analytical method for the determination of the *total polyphenols* in *premixtures* and *feedingstuffs*.

The Applicant also proposed three chromatographic methods for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in the *feed additive*, *premixtures* and *feedingstuffs*.

Based on the acceptable performance characteristics presented, the EURL considers the proposed chromatographic methods suitable for official control for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in the *feed additive*, *premixtures* and *feedingstuffs*.

Moreover, as the unambiguous determination of the *feed additive (Dry Grape Extract)* added to *premixtures* and *feedingstuffs* is not achievable experimentally, the EURL cannot evaluate nor recommend any method for official control for the determination of *Dry Grape Extract* in *premixtures* and *feedingstuffs*.

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

KEYWORDS

Dry Grape Extract, Vitis vinifera L., total polyphenols, malvidin-3-O-glucoside, zootechnical additives, physiological condition stabilisers, all poultry species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (authorisation of a new use of a feed additive) for *Dry Grape Extract* under the category / functional group (4 e) "zootechnical additives"/" physiological condition stabilisers", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. Specifically, the *feed additive* is sought to be used for all poultry species [1,2].

The *feed additive (Dry Grape Extract)* is a purple to brown free-flowing powder obtained from dry grape extract of *Vitis vinifera* spp. *Vinifera*. According to the Applicant, the *feed additive* contains 100 % (w/w) of dry grape extract from seed and skin of *Vitis vinifera extract* [3], a minimum of 80 % (w/w) of *polyphenols* (active substance) and 0.7 g / kg of *malvidin-3-O-glucoside*, which is the phytochemical marker of *Dry Grape Extract* as specified by the Applicant. This *feed additive* is currently authorised as sensory additive by Commission Implementing Regulation (EU) No 2017/307 [4].

According to the Applicant the *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures* at a recommended minimum and maximum content of *Dry Grape Extract (feed additive)* of 30 and 100 mg / kg *feedingstuffs*, respectively [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 *Dry Grape Extract* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

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

For the determination of the *total polyphenols* (active substance) content in *Dry Grape Extract* (feed additive), the Applicant submitted a method based on spectrophotometry at 280 nm using an external calibration with catechin standards [6].

The *feed additive* sample (1 g) is sonicated with 100 ml of deionised water for 5 min. After decantation, an aliquot (0.5 ml) is diluted with water by factor of 50 or 100. The absorbance of this solution is measured by spectrophotometry at 280 nm. The *total polyphenols* content is quantified by external calibration using catechin standard aqueous solutions [6].

This method has been single-laboratory validated and further ring-trial validated in the frame of a previous *Dry Grape Extract* dossier leading to relative standard deviation for *reproducibility* (RSD_R) ranging from 1.2 to 4.5 % and recovery rates (R_{rec}) up to 130 % [7].

Based on the acceptable performance characteristics presented in the frame of the previous *Dry Grape Extract* dossier the EURL recommends for official control the above-mentioned method based on spectrophotometry for the determination of the *total polyphenols* in the *feed additive* (*Dry Grape Extract*).

The Applicant did not provide any experimental data or an analytical method for the determination of the *total polyphenols* in *premixtures* and *feedingstuffs*.

Instead, the Applicant submitted another two single-laboratory validated [8-9] and further verified chromatographic methods [10-11] for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in *premixtures* (for ruminants) and *feedingstuffs* (for broilers).

For the *premixtures*, two sample aliquots (1.0 g) are treated with a mixture of methanol / water / trifluoroacetic acid (TFA). One of the aliquots is further fortified with a standard solution of *malvidin-3-O-glucoside*. Both aliquots are further diluted with the methanol / water / TFA mixture and extracted in an ultrasonic bath at room temperature. Then, the mixtures are centrifuged and the supernatants are filtered before being injected in the ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) system. The quantification of *malvidin-3-O-glucoside* is performed by standard addition [12].

In the case of *feedingstuffs*, the sample is spiked with cyaniding-3-O-sambubioside (for recovery calculation) and submitted to a two-step defatting process with dichloromethane and ethyl acetate. The obtained residue is then extracted with a mixture of methanol / formic acid and filtered under vacuum. The obtained filtrate is evaporated and the obtained residue is

re-solubilised in a mixture of methanol / water / TFA. The solution is then centrifuged and the supernatant is further filtrated. One aliquot of the supernatant is further spiked with known amounts of *malvidin-3-O-glucoside* and cyaniding-3-O-sambubioside, and finally injected in the ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) system. The content of *malvidin-3-O-glucoside* is determined by standard addition [13].

The performance characteristics reported in the frame of the validation [8-9] and verification [10-11] studies for the determination of *malvidin-3-O-glucoside* in *premixtures* and *feedingstuffs* are presented in Table 1.

Furthermore, the Applicant reported for the UHPLC-MS/MS method a limit of detection (LOD) of 0.29 ng *malvidin-3-O-glucoside* / g *feedingstuffs* and a limit of quantification (LOQ) of 0.96 ng *malvidin-3-O-glucoside* / g *feedingstuffs* [9].

Based on the acceptable performance characteristics presented, the EURL considers the single-laboratory validated and further verified chromatographic methods submitted by the Applicant suitable for official control for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in *premixtures* and *feedingstuffs*.

However, as the unambiguous determination of the *feed additive (Dry Grape Extract)* added to *premixtures* and *feedingstuffs* is not achievable experimentally the EURL cannot evaluate nor recommend any method for official control for the determination of *Dry grape Extract* in *premixtures* and *feedingstuffs*.

Table 1 Performance characteristics of the single-laboratory validated and verified chromatographic methods for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in *premixtures* and *feedingstuffs* (UHPLC-MS/MS).

	<i>Malvidin-3-O-glucoside</i>			
	Premixtures		Feedingstuffs	
	validation	verification	validation	verification
Mass fraction, ng/g	10 000		60	
RSD _r , %	4.2	6.9	6.6	5.7
RSD _{ip} , %	5.1	8.4	7.0	6.0
R _{rec} , %	99	89	127	109
Reference	[8]	[10]	[9]	[11]

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

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

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

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the further characterisation of the *feed additive* the Applicant provided an additional chromatographic method for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in the *feed additive (Dry Grape Extract)* [14].

The *feed additive* sample (30 mg) is mixed with methanol in an ultrasonic bath, then the mixture is centrifuged and the supernatant is directly injected in the high performance liquid chromatography coupled to a photometric detection (HPLC-UV) system. The quantification of *malvidin-3-O-glucoside* is performed by an external standard calibration curve [14].

The Applicant stated that this method is used as an internal control test for some of their products, including the one which is subject of this dossier, and provided data from five different batches of the *feed additive (Dry Grape Extract)* leading to precision values below 10 %. Additionally, the Applicant reported a recovery rate of 99 % [14].

Based on the experimental evidences provided, the EURL considers the proposed HPLC-UV method suitable for official control for the determination of the *malvidin-3-O-glucoside* (phytochemical marker) content in the *feed additive*.

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 ring-trial validated method based on spectrophotometry for the determination of the *total polyphenols* in *Dry Grape Extract* (the *feed additive*); (ii) the method based on high performance liquid chromatography coupled to a photometric detection (HPLC-UV) for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in *Dry Grape Extract* (the *feed additive*); and (iii) the single-laboratory validated and further verified methods based on ultra-high performance liquid chromatography coupled to mass spectrometry detection (UHPLC-MS/MS) for the determination of *malvidin-3-O-glucoside* (phytochemical marker) in *premixtures* and *feedingstuffs*.

As the unambiguous determination of the *feed additive (Dry Grape Extract)* added to *premixtures* and *feedingstuffs* is not achievable experimentally the EURL cannot evaluate nor

recommend any method for official control for the determination of *Dry Grape Extract* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *total polyphenols* in the *feed additive*:

- spectrophotometry at 280 nm expressing the *total polyphenols* content as catechin equivalents

For the determination of *malvidin-3-O-glucoside* (phytochemical marker) in the *feed additive*:

- high performance liquid chromatography coupled to a photometric detection (HPLC-UV)

For the determination of *malvidin-3-O-glucoside* (phytochemical marker) in *premixtures* and *feedingstuffs*:

- ultra-high performance liquid chromatography coupled to mass spectrometry detection (UHPLC-MS/MS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Dry Grape Extract* 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. 1831-0049-2021
- [2] *Application, Annex I - Submission number 1616660754422-2966
- [3] *Technical dossier, Section II: 2.1 Identity of the additive
- [4] Commission Implementing Regulation (EU) 2017/307 of 21 February 2017 concerning the authorisation of dry grape extract of *Vitis vinifera* spp. *vinifera* as a feed additive for all animal species except for dogs
- [5] *Technical dossier, Section II: 2.5 Conditions of use
- [6] *Technical dossier, Section II – Annex_II.57
- [7] EURL Evaluation Report:
https://joint-research-centre.ec.europa.eu/document/download/aa1d5b08-830b-4a53-b12c-a3481af1fbec_en?filename=finrep-fad-2010-0077-dry-grape-extract.pdf
- [8] *Technical dossier, Section II – Annex_II.60
- [9] *Technical dossier, Section II – Annex_II.62
- [10] *Technical dossier, Section II – Annex_II.61

[11] *Technical dossier, Section II – Annex_II.62-1

[12] *Technical dossier, Section II – Annex_II.38

[13] *Technical dossier, Section II – Annex_II.48

[14] *Technical dossier, Section II – Annex_II.58

*Refers to Dossier no: FAD-2021-0069

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

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

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Wageningen Food Safety Research (WFSR)¹ (NL)
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- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)

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