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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

Ronozyme® VP

(FAD-2010-0194; CRL/100185)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2010-0194 - CRL/100185**
Name of Feed Additive:	***Ronozyme® VP***
Active Agent (s):	**Endo-1,3(4)-beta-glucanase (EC 3.2.1.6)**
Rapporteur Laboratory:	**Austrian Agency for Health and Food Safety (AGES), Austria**
Report prepared by:	**Irmengard Strnad**
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Report approved by: Date:	**Christoph von Holst** **13/02/2023**

Corrected version: corrections are highlighted in yellow

EXECUTIVE SUMMARY

Ronozyme® VP is currently authorised as *feed additive* for chickens for fattening and piglets (weaned) by Commission Regulation (EC) No 1259/2004 and Commission Regulation (EC) No 1811/2005 respectively. In the current application authorisation is sought under Article 10 (2) for *Ronozyme® VP* under the category/functional "zootechnical additives"/"digestibility enhancers". The authorisation is sought for the use of the *feed additive* for chickens for fattening and piglets (weaned).

According to the Applicant, the active agent of *Ronozyme® VP* is *endo-1,3(4)-beta-glucanase* (*glucanase*) produced by *Aspergillus aculeatus*. The Applicant expressed the *glucanase* enzymatic activity in fungal beta-glucanase units (FBG), where one FBG is defined as "the amount of enzyme which under standard conditions (pH 5.0 and 30 °C) liberates glucose or other reducing carbohydrates at a rate corresponding to 1 µmol glucose per minute".

The product is intended to be marketed as solid (CT) and liquid (L) formulations having a guaranteed minimum *glucanase* activity of 50 FBG / g and 120 FBG / ml respectively. The *feed additive* formulations are intended to be included through *premixtures* (solid) or directly in *feedingstuffs* (solid and liquid) to obtain a minimum activity of 10 FBG / kg *feedingstuffs*.

For the quantification of the *glucanase* activity in the *feed additive* the Applicant provided a single-laboratory validated and further verified colorimetric method. *Glucanase* cleaves non-starch polysaccharides (NSP) releasing glycosylic moieties with reducing ends from beta-glucan. The reducing moieties are oxidized in an alkaline milieu by forming orange-yellow compounds with the 2-hydroxy-3,5-dinitrobenzoic acid. These orange-yellow compounds are measured at a wavelength of 530 nm and quantified against a validated *Ronozyme® VP* standard available from the Applicant upon request.

For the quantification of the *glucanase* activity in *premixtures* and *feedingstuffs* the Applicant proposes a single-laboratory validated and further verified colorimetric method based on the quantification of the water soluble dyed fragments produced by the action of *endo-1,3(4)-beta-glucanase* on azo-barley beta-glucan substrate. The quantification of the *glucanase* activity is determined by using a standard curve of a certified *Ronozyme® VP glucanase* standard available from the Applicant upon request.

Based on the satisfactory performance characteristics the EURL recommends for official control the colorimetric methods mentioned above for the quantification of the *glucanase* activity in the *feed additive, 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

Endo-1,3(4)-beta-glucanase, Ronozyme® VP, "zootechnical additives/digestibility enhancers", chickens for fattening, piglets (weaned)

1. BACKGROUND

Ronozyme® VP is a *feed additive* already authorised by Commission Regulation (EC) No 1259/2004 for chickens for fattening and by Commission Regulation (EC) No 1811/2005 for piglets (weaned) [1-2]. In the current application authorisation is sought under Article 10(2) (re-evaluation) of the Regulation (EC) No 1831/2003 [3] for *Ronozyme® VP* under the category/functional group "zootechnical additives"/"digestibility enhancers". The authorisation is sought for the use of the *feed additive* for chickens for fattening and piglets (weaned) [4].

According to the Applicant, the active agent of *Ronozyme® VP* is *endo-1,3(4)-beta-glucanase* (*glucanase*) produced by *Aspergillus aculeatus* (CBS 589.94) [5,6].

The Applicant expresses the *glucanase* enzymatic activity in fungal beta-glucanase units (FBG), where one FBG is defined as "the amount of enzyme which under standard conditions (pH 5.0 and 30 °C) liberates glucose or other reducing carbohydrates at a rate corresponding to 1 µmol glucose per minute"[7][1].

The product is intended to be marketed as solid (*Ronozyme® VP* (CT)) and liquid (*Ronozyme® VP* (L)) formulations having a guaranteed minimum *glucanase* activity of 50 FBG / g and 120 FBG / ml respectively. The *feed additive* formulations are intended to be included through *premixtures* (solid) or directly in feedingstuffs (solid and liquid) to obtain a minimum activity of 10 FBG / kg *feedingstuffs* [8].

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 *Ronozyme® VP* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

[1] In the text of the original EURL report (Ref. Ares(2021)7175138 - 22/11/2021) was present a typo in the definition of the FBG units (i.e. 50 °C).

3. EVALUATION

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

For the quantification of the *glucanase* activity in the *feed additive* the Applicant provided an automatic single-laboratory validated and further verified colorimetric method [9] based on the quantification of the reducing sugars released by the hydrolysis of the beta-glucan by *endo-1,3(4)-beta-glucanase* using a reference standard with a known enzyme activity. This method requires the use of an "automatic device" (Konelab Analyzer), which is hardly available for official feed laboratories. Upon request of the EURL, the Applicant submitted a method description which can be performed manually [10]. Beta-glucanase cleaves non-starch polysaccharides (NSP) releasing glycosylic moieties with reducing ends. The reducing moieties are oxidized in an alkaline milieu by forming orange-yellow compounds with the 2-hydroxy-3,5-dinitrobenzoic acid. These orange-yellow compounds are measured at a wavelength of 530 nm and quantified against a validated *Ronozyme® VP* standard available from the Applicant upon request.

The *feed additive* samples (1 g), are dissolved by stirring for 45 min in 100 ml of 0.1 M acetate buffer with 20 mM calcium chloride and 0.04 % Tween 20 at pH 5.0. 2 ml of the extract is then centrifuged and appropriately diluted with the buffer. An aliquot (30 µl) of the diluted extract is pre-incubated for 5 min at 50 °C. 0.3 ml of pre-incubated 1.5 % beta-glucan substrate is added. Sample extracts are mixed and incubated at 50 °C for 20 minutes. After the incubation time, the reaction is stopped by adding 0.15 ml dinitro salicylic acid (DNSA) reagent, mixed and centrifuged for 20 s. Then, the mixture is boiled for 12 min and transferred to an ice bath for another 5 min. The sample is further centrifuged for another 20 s and diluted with 1.5 ml of distilled water. Finally the absorbance of the solution is measured against the blank at 530 nm. Quantification is performed against external calibration curve of a *Ronozyme® VP* standard with a known certified activity expressed in FBG [11].

The single-laboratory validated method was further verified by a second independent laboratory. The reported performance characteristics are summarized in Table 1.

Upon request of the EURL, the Applicant proposed for the quantification of glucanase activity in *premixtures* and *feedingstuffs* a single-laboratory validated and further verified colorimetric method based on the quantification of the water soluble dyed fragments produced by the action of *endo-1,3(4)-beta-glucanase* on Azo-barley beta-glucan substrate. The quantification of the *glucanase* activity is determined by using a standard curve of a certified *Ronozyme® VP glucanase* standard available from the Applicant upon request [14].

Table 1: Performance characteristics of analytical method for determination of the activity of *endo-1,3(4)-beta-glucanase* in *feed additive*s (FA) based on the experimental data provided by the Applicant [12,13].

Matrix	Activity (FBG/g)	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)
		Val.[1]	*Ver.*[1]	*Val.*[1]	*Ver.*[1]	*Ver*
FA	50-118	1.5-1.9	5.5	2.8-3.5	12.5	125

RSD_r; RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{Rec}: *Recovery rate; Val.: validation; Ver.: verification;* [1]Recalculated by EURL [17]

Two 1.0 g portions of a *premixture* sample are diluted with around 50 g milled wheat and are extracted in 500 ml of 0.1 M sodium acetate buffer (containing 0.02 % Tween, pH 4.5) for 60 minutes. A 2 ml aliquot of the extract are centrifuged for 3 minutes.

Two 50.0 g aliquots of a *feedingstuffs* sample are extracted in 500 ml of 0.1 M sodium acetate buffer (containing 0.02 % Tween, pH 4.5) for 60 minutes. 2 ml of the extract are centrifuged for 3 minutes.

If necessary, extracts have to be diluted with wheat extract. A wheat blank has to be included in case of endogenous wheat *glucanase* activity.

Triple determinations and two blanks are performed for each weighing. 100 µl (diluted) extract of samples and standard are pre-incubated for 5 minutes at 50°C. 300 µl pre-heated substrate ae added. The samples are mixed and incubated for exactly 60 min at 50 °C. After 60 min, 1.2 ml "stop and precipitation" solution are added. After mixing samples are kept for 45 min at room temperature. The samples are mixed again and then centrifuged for 3 minutes. The clear supernatant is measured at 590 nm. Blanks are not incubated and the "stop and precipitation" solution is added prior the substrate. The measured optical density (OD) of blanks and wheat blanks are substracted from the OD of samples and standards. Quantification is performed against external calibration curve of a *Ronozyme® VP* standard with a known certified activity expressed in FBG.

The single-laboratory validated method was further verified by a second independent laboratory. Furthermore the Applicant reported a limit of quantification (LOQ) of 2.6 FBG / kg *feedingstuffs* [15].

The single-laboratory validated method was further verified by a second independent laboratory. The reported performance characteristics are summarized in Table 2.

Table 2: Performance characteristics of analytical method for determination of the activity of *endo-1,3(4)-beta-glucanase* in *feedingstuffs* (FS) and *premixtures* (PM) based on the experimental data provided by the Applicant [15, 16]

Matrix	Activity (FBG/kg)	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)
		Val.	*Ver.*[1]	*Val.*	*Ver.*[1]	*Ver*
PM	1000 - 6000	5.0 – 6.1	17.6	5.3 – 8.5	18.5	94.4 – 96.3
FS	5 - 60	3.8 – 8.1	6.0-12.2	6.0 – 12.5	8.3-18.4	97.7 – 108.3

RSD_r; RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{Rec}: *Recovery rate; Val.: validation; Ver.: verification;* [1]Recalculated by EURL [18-19]

Based on the performance characteristics available, the EURL recommends for official control the colorimetric methods mentioned above for the quantification of the *glucanase* activity in the *feed additive*, *premixtures* 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)

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

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

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric for the quantification of *1,3(4)-beta-glucanase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *1,3(4)-beta-glucanase* activity in the *feed additive*,

- colorimetric method measuring coloured compound produced by the dinitro salicylic acid (DNSA) based on the enzymatic hydrolysis of beta-glucan at pH 5.0 and 50°C.

For the quantification of *1,3(4)-beta-glucanase* activity in *premixtures* and *feedingstuffs*,

- colorimetric method measuring water soluble dyed fragments based on the enzymatic hydrolysis of cross-linked azo-barley-glucan at pH 4.5 and 50 °C.

One glucanase units (FBG), corresponds to the amount of enzyme which under standard conditions (pH 5.0 and 30 °C) liberates glucose or other reducing carbohydrates at a rate corresponding to 1 µmol glucose per minute.[2]

[2] In the text of the original EURL report (Ref. Ares(2021)7175138 - 22/11/2021) was present a typo in the definition of the FBG units (i.e. 50 °C).

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ronozyme® VP* 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] Commission Regulation (EC) No 1259/2004 of 8 July 2004 concerning the permanent authorisation of certain additives already authorised in feedingstuffs

[2] Commission Regulation (EC) No 1811/2005 of November 2005 concerning the provisional and permanent authorisation of certain additives in feedingstuffs and the provisional authorisation of a new use of an additive already authorised in feedingstuffs

[3] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition

[4] *Application, Reference SANTE_E5_FWD. APPL. 1831-0056-2019

[5] *Application, Proposal for Register Entry – Annex A

[6] *Technical dossier, Section II: II.2 Characterisation of the active substance

[7] *Technical dossier, Section II: II.1 Identity of the additive

[8] *Technical dossier, Section II: II.5 Condition of use of the additive

[9] *Technical dossier, Appendix 2.41

[10] *Supplementary information: Method Ronozyme VP_02E_per se

[11] *Technical dossier, Appendix 2.45

[12] *Supplementary information: Appendix 1a - VP_Validation_PerSe_VP02E

[13] *Supplementary information: Appendix 1b - VP_Verification_PerSe_VP02E

[14] *Supplementary information: Method Ronozyme VP GLU-101_02E_feed_PM

[15] *Supplementary information: VALIDATION of the Method Ronozyme VP-Glu-101-02E

[16] *Supplementary information: VP_Verification_Feed_GLU-101-02E

[17] *Supplementary information: eurl-anova-fa.pdf

[18] *Supplementary information: eurl-anova-pm.pdf

[19] *Supplementary information: eurl-anova-fs.pdf

*Refers to Dossier no: FAD-2010-0194

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the Austrian Agency for Health and Food Safety (AGES), Vienna, Austria. 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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- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)