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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

Feedlyve® AGL
(FAD-2010-0227; CRL/100244)

European Union Reference Laboratory
Feed Additives

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-2010-0227 - CRL/100244**
Name of Feed Additive:	***Feedlyve® AGL***
Active Agent (s):	**Endo 1,3 (4)-β-glucanase**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA)** **13/01/2016**
Report approved by: Date:	**Christoph von Holst** **15/01/2016**

EXECUTIVE SUMMARY

Feedlyve® AGL is currently authorised as *feed additive* for chickens for fattening by Commission Regulation (EC) No 1458/2005. In the current application authorisation is sought under article 10 (2) of Regulation (EC) No 1831/2003 under the category/functional group "zootechnical additives"/"digestibility enhancers" for chickens for fattening.

According to the Applicant, *Feedlyve® AGL* contains *endo-1,3 (4)-β-glucanase* as active agent. The Applicant expresses the *glucanase* enzymatic activity in *glucanase* units (AGL), defined as "1 AGL, the amount of enzyme which liberates 5.55 micromoles of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 4.8 and 30 °C". The product is intended to be marketed as solid and liquid formulations having a guaranteed minimum *glucanase* activity ranging from 200 to 6000 AGL/g of product. The *feed additive* formulations are intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 25 AGL/kg *feedingstuffs*. However the Applicant proposed a recommended dose ranging from 25 to 100 AGL/kg *feedingstuffs*.

For the quantification of the *glucanase* activity in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method based on the enzymatic hydrolysis of barley beta-glucan at pH 4.8 and 30 °C and the quantification of dyed oligomers released from the substrate Remazol-Brilliant-Blue-R glucan. Furthermore, the Applicant applied this method successfully for the quantification of *glucanase* in *premixtures*, in the frame of the stability studies. Based on the satisfactory performance characteristics available, the EURL recommends for official control this colorimetric method 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) is not considered necessary.

KEYWORDS

Endo-1,3(4)-β-glucanase, Feedlyve® AGL, "zootechnical additives"/"digestibility enhancers", chickens for fattening

1. BACKGROUND

Feedlyve® AGL is a *feed additive* currently authorised by Commission Regulation (EC) No 1458/2005 for chickens for fattening [1] belonging to the "Enzymes" group listed in Directive 70/524/EEC. In the current application authorisation is sought under article 10 (2) of Regulation (EC) No 1831/2003 under the category/functional group "zootechnical additives"/"digestibility enhancers" for chickens for fattening [2,3].

The product is intended to be marketed as:

i) liquid preparations (*Feedlyve® AGL 200 (L)* and *1500 (L))* with a guaranteed minimum *glucanase* activity of 200 and 1500 AGL/g respectively,
ii) solid preparations (*Feedlyve® AGL 1500 (P)* and *6000 (P))* having a guaranteed minimum *glucanase* activity of 1500 and 6000 AGL/g respectively, and
iii) a powder form (*Feedlyve® AGL BRUTE (CNS))* with a variable *glucanase* activity ranging from 15000 to 20000 AGL/g. This product is used in the formulation of more diluted enzymatic preparations mentioned above [3].

Water, sorbitol, potassium sorbate and monopotassium phosphate are used as carriers of the liquid formulations, while wheat flour and tricalcium phosphate are used for the solid formulations.

According to the Applicant, *Feedlyve® AGL* contains *endo-1,3 (4)-β-glucanase* as active agent [4]. The Applicant expresses the *glucanase* enzymatic activity in *glucanase* units (AGL), defined as "1 AGL, the amount of enzyme which liberates 5.55 micromoles of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 4.8 and 30 °C".

Feedlyve® AGL is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 25 AGL/kg *feedingstuffs*. However the Applicant proposed a recommended dose ranging from 25 to 100 AGL/kg *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 *Feedlyve® AGL* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury and mycotoxins) are available from the respective European Union Reference Laboratories [6].

Description of the analytical methods for the determination of the active substance in feed additive, premixtures and feedingstuffs

For the quantification of *endo-1,3 (4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs* [7] the Applicant submitted a single-laboratory validated [8,9] and further verified [10] colorimetric method based on the enzymatic hydrolysis of barley beta-glucan at pH 4.8 and 30 °C and the quantification of dyed oligomers released from the substrate Remazol-Brilliant-Blue-R glucan.

Feed additive samples are first diluted in 0.1 M acetate-phosphate buffer (at pH 4.6). The substrate (0.5 ml) is equilibrated in a water bath at 30 °C for 5 minutes, then 0.2 ml of the diluted and pre-equilibrated enzyme sample is added. The solution is adjusted at pH 4.8 and incubated at 30 °C for 20 minutes. The reaction is stopped by adding 2 ml of 95.5% ethanol (warmed to 30 °C). The solution is mixed and returned to the water bath for additional 10 minutes. Afterwards the solution is mixed again and the tubes are centrifuged for 10 minutes at 20 °C. The absorbance of the sample solutions are measured spectrophotometrically at 590 nm, using water as the blank. The quantification is performed against an *endo-1,3 (4)-β-glucanase* standard curve, using a reference standard with a known enzyme activity expressed in AGL (reference standard) available from the Applicant.

Feedingstuffs samples (100 g) are suspended in 0.1 M acetate-phosphate buffer (pH 3.9) up to 500 ml, stirred for 30 minutes and further centrifuged for 10 minutes and the supernatant is filtered. An aliquot of this extract (ranging from 0.5 to1.5 ml depending on the expected activity) is mixed with the same volume of the substrate solution. Both solutions i.e. extract and substrate, are pre-equilibrated for 5 minutes in the water bath at 30 ^{o}C and the pH of the mixture is checked and adjusted to 4.8. The mixture is then incubated at 30 °C for 200 minutes for samples with up to 200 AGL/kg or for 30 minutes for samples with 200-800 AGL/kg. The reaction is stopped by adding adequate volume of precipitant solution. The solution is mixed and returned to the water bath for additional 10 minutes. The tubes are then centrifuged for 10 minutes at 20°C. The absorbances of the sample solutions are measured spectrophotometrically at 590 nm, using deionised water as the blank. The quantification is performed using a feed calibration curve obtained with a reference standard.

Table 1. Performance characteristics of colorimetric method for the quantification of *glucanase* activity in the *feed additive* (FA) and *feedingstuffs* (FS) as recalculated by the EURL [11] from the validation (Val) and verification (Ver) studies.

Matrices	Conc. (AGL U/kg)	RSD_r (%)		RSD_{ip} (%)		Rrec (%)	
		Val	Ver	Val	Ver	Val	Ver
FA	205000	3.3	4.7	3.9	6.3	102	103
FS	82-419	6.5-7.2	3.7-12	6.5-8.7	5.9-12	110-109	83-90

RSD_r:and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec}: *recovery* rate (%)

The performance characteristics reported in the frame of the validation and verification studies are summarised in Table 1. Furthermore, the Applicant reported a limit of quantification (LOQ) of 50 AGL/kg *feedingstuffs* which is larger than then minimum conditions of use specified in the Annex A [4]. Instead of that the EURL estimated a more realistic LOQ by setting it equal to the lowest enzyme activity measured in *feedingstuffs* (12.5 AGL/kg) [7].

Furthermore, the Applicant applied the colorimetric method described for *feedingstuffs* in the frame of the stability studies for the quantification of *glucanase* in *premixtures* at an activity level of 25000 AGL/kg [12]. From the available experimental data the EURL derived a precision of the order of 5 % [13], which is in good agreement with the values presented in Table 1. Therefore, the EURL considers this method suitable for the analysis of *premixtures*.

Based on the performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods based on the enzymatic hydrolysis of barley beta-glucan at pH 4.8 and 30 °C and the quantification of dyed oligomers released from the substrate Remazol-Brilliant-Blue-R glucan, to quantify *endo-1,3 (4)-β-glucanase* 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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods for the quantification of *glucanase* activity in the *feed additive*, *premixtures* and *feedingstuffs.*

Recommended text for the register entry (analytical method)

For the quantification of *endo-1,3 (4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs:*

- colorimetric methods based on the quantification of dyed oligomers produced by the action of *endo-1,3 (4)-β-glucanase* on Remazol-Brilliant-Blue-R glucan at pH 4.8 and 30°C.

1 AGL is the quantity of enzyme which liberates 5.55 micromoles of reducing sugars (glucose equivalents) per minute from barley beta-glucan at pH 4.8 and 30 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Feedlyve® AGL* 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 1458/2005 of 8 September 2005, concerning the permanent and provisional authorisation of certain additives in feedingstuffs and the provisional authorisation of new uses of certain additives already authorised in feedingstuffs

[2] *Application, Reference SANCO/G1: Forw. Appl. 1831/0010-2014

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

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

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

[6] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[7] *Technical dossier, Annex II.6.1.A & Annex II.6.1.B

[8] *Technical dossier, Annex II.6.3

[9] *Technical dossier, Annex II.6.1.H

[10] *Technical dossier, Annex II.6.1.I

[11] *Supplementary information, eurl_anova_val_ver_fa_fs.pdf

[12] *Technical dossier, Annex II.4.1.E

[13] *Supplementary information, eurl_anova_pm.pdf

*Refers to Dossier no: FAD-2010-0227

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
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