EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
European Union Reference Laboratory for Feed Additives

JRC.D.5/FSQ/CvH/DM/ag/ARES(2012)667103

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

Dossier related to:	**FAD-2011-0033**
	CRL/110012
Product Name:	**Econase GT**
Active Substance(s):	**Endo-1,3(4)-β-glucanase (E.C. 3.2.1.6)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Dijana Mitić (EURL-FA)**
Report revised by: Date:	**Piotr Robouch (EURL-FA)** **05/06/2012**
Report approved by: Date:	**Christoph von Holst** **05/06/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) (new authorisation) for *Econase GT,* under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Econase GT* for chickens for fattening and weaned piglets. According to the Applicant, the *feed additive* contains *endo-1,3(4)-β-glucanase* (EC 3.2.1.6) as the active agent, produced by the strain *Trichoderma reesei* (CBS 126896). It is intended to be marketed as different formulations: (i) solid *Econase GT 200 P* and *Econase GT P*, with a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 200 and 2000 BU/mg, respectively; and (ii) liquid *Econase GT 400 L,* with a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 400 BU/mL.

The activity of *endo-1,3(4)-β-glucanase* is expressed in beta-glucanase units (BU). Upon request by the EURL, the Applicant defined the BU unit as the amount of enzyme producing 1 nmol of reducing sugar (expressed as glucose equivalents) from a barley beta-glucan substrate per second at pH 4.8 and 50 °C.

The *feed additive* is intended to be used in *premixtures* or complete *feedingstuffs* or in compound feed rich in non-starch polysaccharides (mainly glucans), with a minimum *endo-1,3(4)-β-glucanase* activity of 10000 BU/kg in complete *feedingstuffs.*

For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feed additive, premixtures* and *feedingstuffs,* the Applicant submitted a single-laboratory validated and further verified spectrophotometric method, based on colour formation of released sugars with 3,5-dinitrosalicylic acid (DNS). The assay is based on the enzymatic hydrolysis of the barley beta-glucan MEGAZYME substrate at pH 4.8 and 50 °C. The reaction products are determined by spectrophotometry at 540 nm using a standard calibration curve prepared with glucose which was subjected to reaction with DNS prior to spectrophotometric measurement. The following performance characteristics were reported:

- for the *feed additive*: - a relative standard deviation for *repeatability* (RSD_r) ranging from 5.6 to 7.1 %; - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 6.3 to 12.1 % and – a *recovery rate* (R_{Rec}) of 107 %;
- for *premixtures*: - RSD_r ranging from 10 to 10.7 %; - RSD_{ip} ranging from 7.4 to 10.8 %; and - R_{Rec} ranging from 90 to 128 %;
- for *feedingstuffs*: - RSD_r ranging from 5.8 to 11.5 %; - RSD_{ip} ranging from 10.1 to 12.1 %; - R_{Rec} ranging from 104 to 107 %; while limit of quantification (LOQ) was set to the lowest content experimentally analysed of 570 BU/kg.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified spectrophotometric method, based on the quantification of released sugars produced by the action of *endo-1,3(4)-β-glucanase* on barley beta-glucan at pH 4.8 and 50 °C, to determine the activity of *endo-1,3(4)-β-glucanase* in *feed additive*, *premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

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

Econase GT, *endo-1,3(4)-β-glucanase*, *Trichoderma reesei*, zootechnical additive, digestibility enhancers, chickens for fattening, weaned piglets.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new authorisation) for *Econase GT,* under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers" [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Econase GT* for chickens for fattening and weaned piglets.

According to the Applicant, the *feed additive* contains *endo-1,3(4)-β-glucanase* (EC 3.2.1.6) as the active agent [1,2], produced by the strain *Trichoderma reesei* (CBS 126896). The strain was deposited at the "Centraalbureau voor Schimmelcultures" (CBS) in the Nederlands [3].

The additive is intended to be marketed as different formulations [2,3]:

- solid *Econase GT 200 P* and *Econase GT P*, with a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 200 and 2000 BU/mg, respectively; and
- liquid *Econase GT 400 L,* with a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 400 BU/mL.

Water and sorbitol are the main carriers of the liquid formulation, while wheat flour is used for the solid formulations.

The activity of *endo-1,3(4)-β-glucanase* is expressed in beta-glucanase units (BU). Upon request by the EURL, the Applicant defined the BU unit as the amount of enzyme producing

1 nmol of reducing sugar (expressed as glucose equivalents) from a barley beta-glucan substrate per second at pH 4.8 and 50 °C [4].

The *feed additive* is intended to be used in *premixtures* or complete *feedingstuffs* or in compound feed rich in non-starch polysaccharides (mainly glucans), with a minimum *endo-1,3(4)-β-glucanase* activity of 10000 BU/kg in complete *feedingstuffs* [2,3].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 *Econase GT*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

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, mycotoxins and dioxins) are available from the respective European Union Reference Laboratories [5].

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

For the determination of the activity of *endo-1,3(4)-β-glucanase* in the *feed additive, premixtures* and *feedingstuffs,* the Applicant submitted a single-laboratory validated and further verified spectrophotometric method [6], based on colour formation of released sugars with 3,5-dinitrosalicylic acid (DNS). The assay is based on the enzymatic hydrolysis of the barley beta-glucan at pH 4.8 and 50 °C.

At first samples were prepared as follows:

- The *feed additive* (FA) sample (0.5 g for solid and 1 g for liquid formulations) is extracted in 0.1 M acetate buffer (pH = 4.8);

- The *premixtures* (PM) sample (5 g) is extracted in 0.1 M acetate buffer (pH = 4.8) containing 2% EDTA, 1.25% albumin and 0.01% Tween, stirred for 30 minutes at room temperature and then centrifuged for 10 minutes;
- The *feedingstuffs* (FS) sample (5 g) is extracted in 0.1 M acetate buffer (pH = 4.8) after stirring on magnetic stirrer for 30 minutes. The extract is centrifuged and diluted.

A beta-glucan MEGAZYME substrate solution is then equilibrated at 50 °C for 5 minutes. For each matrix, an aliquot of the extracted sample is added to the substrate and incubated at 50 °C for 5 or 60 minutes (for FA/PM or FS). After 5 minutes, the reaction is stopped in each solution by adding 2 mL of DNS solution and boiling for 5 minutes. 10 mL of water are added to each solution cooled to room temperature. The reaction products are determined by spectrophotometry at 540 nm using a standard glucose (subjected to reaction with DNS) calibration curve.

The potential spectral interferences due to the various matrices are quantified - and corrected for – by measuring the absorbance at 540 nm of the blank solutions, containing all the reagents mentioned above to which the same amounts of extracted samples (FA, PM or FS) are added after incubation.

The performance characteristics derived from validation [7,8] and verification [9-14] studies are presented in Table 1. Furthermore, the limit of quantification (LOQ) was set to the lowest content experimentally analysed of 570 BU/kg *feedingstuffs* [7,8]

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified spectrophotometric method, based on the quantification of released sugars produced by the action of *endo-1,3(4)-β-glucanase* on barley beta-glucan at pH 4.8 and 50 °C, to determine the activity of *endo-1,3(4)-β-glucanase* in *feed additive*, *premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

Table 1: Performance characteristics for the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive (FA), premixtures (PM)* and *feedingstuffs (FS)*

	Concentration (BU/kg)	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
		Validation	Verification	Validation	Verification	Validation	Verification
FA	$19x10^7$-$25x10^8$	5.9-7.1 [7]	5.6 [9]	8.2-12.1 [7]	6.3 [9]	-	107 [9]
PM	$3x10^6$-$3x10^7$	10.7 [7]	10.0* [10]	7.4-10.8 [8]	-	90 - 128 [7]	114* [10]
FS	8000-85000	5.8-9.2 [7]	11.5 [13]	10.1-12.1 [7]	11.5* [13]	104 - 107 [7]	107 [13]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*, respectively.
R_{Rec}: a recovery rate
* - recalculated by the EURL

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 spectrophotometric method, based on the quantification of released sugars produced by the action of *endo-1,3(4)-β-glucanase* on barley beta-glucan at pH 4.8 and 50 °C, to determine the activity of *endo-1,3(4)-β-glucanase* in *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:*

- spectrophotometric (DNS) method, based on the quantification of released sugars produced by the action of *endo-1,3(4)-β-glucanase* on barley beta-glucan at pH 4.8 and 50 °C.

One beta-glucanase unit (BU) is the amount of enzyme producing 1 nmol of reducing sugar (expressed as glucose equivalents) from a barley beta-glucan substrate per second at pH 4.8 and 50 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Econase GT* 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 SANCO/G1: Forw. Appl. 1831/0120-2011

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

[3] *Technical dossier, Section II, Identity, characterisation and conditions of use of the additive; methods of analysis

[4] *Supplementary Info, E-mail from Roal Oy 08_05_12

[5] 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

[6] *Technical dossier, Section II, Annex_II_82_beta-glucanase assay method

[7] *Technical dossier, Section II, Annex_II_79a_Validation report on beta-glucanase_report

[8] *Technical dossier, Section II, Annex_II_79b_Validation report on beta-glucanase_enclosures

[9] *Technical dossier, Section II, Annex_II_80a_verification report_feed additive

[10] *Technical dossier, Section II, Annex_II_80b_verification report_premixtures

[11] *Technical dossier, Section II, Annex_II_81a_verification report_Annex IV_feed additive

[12] *Technical dossier, Section II, Annex_II_81b_verification report_Annex IV_premixtures

[13] *Technical dossier, Section II, Annex_II_80c_verification report_feedingstuffs

[14] *Technical dossier, Section II, Annex_II_81c_verification report_Annex IV_feedingstuffs

*Refers to Dossier No. FAD-2011-0033

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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Fødevarestyrelsen, Ringsted (DK)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)