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Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2007-185
	FAD-2007-0036
	CRL/070021
Product name:	Roxazyme G2 G& L
Active Substance(s):	Endo-1,4-beta-xylanase (EC 3.2.1.8)
	Endo-1,3(4)-beta-glucanase (EC 3.2.1.6)
	Endo-1,4-beta-glucanase (EC 3.2.1.4)
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
	Geel, Belgium
Report prepared by:	Sulhattin Yasar (CRL-FA)
Report checked by:	Piotr Robouch (CRL-FA)
Date:	26/03/2009
Report approved by:	Christoph von Holst
Date:	27/03/2009

EXECUTIVE SUMMARY

The current application authorisation is sought for *Roxazyme G2 G and L* under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Roxazyme G2 G and L* as a digestibility enhancer for chicken and turkey for fattening, laying hens, ducks and piglets (weaned). The product is intended to be marketed as solid (*Roxazyme G2 G*) and liquid (*Roxazyme G2 L*) formulations.

The active agents of *Roxazyme G2 G and L* are (1) endo-1,4-β-*xylanase* (2) endo-1,3(4)-β-*glucanase* and (3) endo-1,4-β-*glucanase* produced by *Trichoderma reesei*. The enzymatic activities are expressed in U-unit. According to the applicant one U-unit is the amount of the enzyme (endo-1,4-β-*xylanase* or endo-1,3(4)-β-*glucanase* or endo-1,4-β-*glucanase*) which releases 1 μmol of reducing sugar (glucose or xylose equivalent) per minute at 40°C, pH = 5.0 from the wheat arabinoxylan or barley β-glucan or carboxymethylcellulose, respectively.

The solid and liquid formulations (*Roxazyme G2 G and L*) have an endo-1,4-β-*xylanase* activity of 2700 U/g of *product*, an endo-1,3(4)-β-*glucanase* activity of 700 U/g of *product* and an endo-1,4-β-*glucanase* activity of 800 U/g of *product*. Both formulations are intended to be mixed into *premixtures* and/or *feedingstuffs* to provide in *feedingstuffs* a minimum endo-1,4-β-*xylanase* activity of 135 U/kg, endo-1,3(4)-β-*glucanase* activity of 35 U/kg and endo-1,4-β-*glucanase* activity of 40 U/kg.

For the determination of the activity of endo-1,4-β-*xylanase*, endo-1,3(4)-β-*glucanase* and endo-1,4-β-*glucanase* in the *feed additives* the applicant proposes a colorimetric method based on the formation of reducing sugar moieties released by the enzymes reacting with dinitrosalysilic acid (DNS). The method was ring-trial validated by four external laboratories for the *feed additives*.

- For the determination of endo-1,4-*betaxylanase* activity the reported performance characteristics were: (1) a relative standard deviation for repeatability (RSD_r) ranging from 4.0 to 5.0% and (2) a relative standard deviation (RSD) for between-laboratory reproducibility ranging from 5.0 to 10.0%.
- For the determination of endo-1,3(4)-*betaglucanase* activity the reported performance characteristics were: (1) a RSD_r ranging from 3.0 to 7.0% and (2) a RSD of reproducibility ranging from 4.0 to 9.0%.
- For the determination of endo-1,4-*betaglucanase* activity the reported performance characteristics were: (1) a RSD_r ranging from 4.0 to 8.0% and (2)

a RSD of reproducibility ranging from 2.0 to 9.0% for endo-1,4-*betaglucanase*.

Based on these acceptable performance characteristics, the applicant method is found suitable for official controls of the activities of the above mentioned three active substances in the *feed additives*.

For the determination of the activity of endo-1,4-β-*xylanase*, endo-1,3(4)-β-*glucanase* and endo-1,4-β-*glucanase* in the *premixtures* and *feedingstuffs* the applicant proposed three in-house validated colorimetric methods, based on the same analytical principle, but using different substrates compared to the assay on the feed additive: the measurement of the rate of release of water soluble dyed fragments by the enzymes from the dye cross-linked substrates.

- For the determination of endo-1,4-*betaxylanase* in the *premixtures and feedingstuffs* the reported performance characteristics were: (1) a RSD_r ranging from 10 to 15% and (2) a relative standard deviation for intermediate precision (RSD_R) ranging from 12 to 15%. The applicant determined only for the feedingstuffs a recovery rate ranging from 97 to 105% and the limit of detection (LOD) and limit of quantification (LOQ) of 15-30 and 70 U/kg *feedingstuffs*, respectively.
- For the determination of endo-1,3(4)-*betaglucanase* in the *premixtures and feedingstuffs* the reported performance characteristics were: (1) a RSD_r ranging from 3 to 5% and (2) a RSD_R ranging from 1 to 4%. The applicant determined only for the feedingstuffs a recovery rate ranging from 96 to 106% and the LOD and LOQ of 4-7 and 18 U/kg *feedingstuffs*, respectively.
- For the determination of endo-1,4-*betaglucanase* in the *premixtures and feedingstuffs* the reported performance characteristics were: (1) a RSD_r ranging from 3 to 16% and (3) a RSD_R ranging from 10 to 16%. The applicant determined only for the feedingstuffs a recovery rate ranging from 98 to 104% and the LOD and LOQ of 4-8 and 20 U/kg *feedingstuffs*, respectively.

The reported LOD and LOQ values were found to be below the minimum recommended activity levels in *feedingstuffs* of 135, 35 and 40 U/kg for endo-1,4-*betaxylanase*, endo-1,3(4)-*betaglucanase* and endo-1,4-*betaglucanase*, respectively. The premixture samples were validated at a target activity level of endo-1,4-*betaxylanase* of around 18000 U/kg, endo-1,3(4)-*betaglucanase* of around 5500 U/kg *premixture* and endo-1,4-*betaglucanase* of around 5000 U/kg *premixture*.

Based on these acceptable performance characteristics the CRL recommends three in-house validated methods for official controls for the determination of endo-1,4-*betaxylanase*,

endo-1,3(4)-*betaglucanase* and endo-1,4-*betaglucanase* activities in the *feedingstuffs* and *premixtures* in the frame of Authorisation.

Further testing or validation is not considered necessary.

KEYWORDS

Roxazyme G2 G and L; endo-1,4 β-*xylanase*; endo-1,3(4*)*-β-*glucanase*; endo-1,4 β-*glucanase*; *Trichoderma reesei*; digestibility enhancer; poultry and piglets.

1. BACKGROUND

Roxazyme G2 G and L is a product for which authorisation as feed additive is sought according to Article 4(1) and Article 10(2) of Regulation (EC) No 1831/2003, under the category 'zootechnical additives', functional groups 'digestibility enhancers' according to Annex I [1]. The product contains three active agents [2]: - endo-1,4-β *xylanase* (EC 3.2.1.8), - endo-1,3(4*)*-β-*glucanase* (EC 3.2.1.6) and - endo-1,4-β *glucanase* (EC 3.2.1.4) produced by the strains (M2C38) of *Trichoderma reesei*. The enzymatic activities of endo-1,4-β *xylanase*, endo-1,3(4*)*-β-*glucanase* and endo-1,4-β *glucanase* are expressed as U-unit (U), where one U-unit is the amount of enzyme which releases 1 μmol of reducing sugar (glucose or xylose equivalent) per minute at 40°C, pH = 5.0 from the wheat arabinoxylan or barley β-glucan or carboxymethylcellulose, respectively.

The product is intended to be marketed as a solid (*Roxazyme G2 G*) *and* a liquid (*Roxazyme G2 L*) formulations. Both formulations have an endo-1,4-β-*xylanase* activity of 2700 U/g of *product*, an endo-1,3(4*)*-β-*glucanase* activity of 700 U/g of *product* and an endo-1,4-β-*glucanase* activity of 800 U/g of *product* [3]. The minimum target activities in *feedingstuffs* for chicken and turkey for fattening, laying hens, ducks and piglet (weaned) are: 135 U/kg for endo-1,4-β-*xylanase,* 35 U/kg for endo-1,3(4*)*-β-*glucanase* and 40 U/kg for endo-1,4-β-*glucanase* [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis, submitted in connection with *Roxazyme G2 G and L*, (EFSA-Q-2007-185), and their suitability to be used for official controls in the frame of authorisation, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and qualitative 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 and heavy metals - cadmium, mercury and lead) are available at the respective Community Reference Laboratories [4].

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

Feed additive

For the determinations of endo-1,4-*betaxylanase*, endo-1,3(4)-*betaglucanase* and endo-1,4-*betaglucanase* in the *feed additive* the applicant proposes an absolute colorimetric method based on the formation of reducing sugar moieties released by the enzymes reacted with dinitrosalysilic acid (DNS), where the colour change is proportional to the reducing sugar (glucose or xylose equivalents) from the respective substrates measured at 530 nm [5]. 0.2 to 5.0 g of samples are added with a 100 ml of acetate buffer (pH = 5.0) and stirred at room temperature for 30 minutes. The samples and known enzyme standard solutions used for internal control of the assay are further diluted with the buffer to a final concentration ranging from 0.2 to 0.7 U/ml for endo-1,4-*betaxylanase* and endo-1,3(4)-*betaglucanase* activities, and from 0.15 to 0.25 U/ml for endo-1,4-*betaglucanase* activity, respectively. The tubes containing 0.03 ml of diluted sample or of the level control are pre-incubated for 5 min at 40 ^{o}C. The tubes are further incubated at 40 ^{o}C for 20 min after the addition of 0.3 ml of the substrate solution (1.5% w/v wheat arobinoxylan for endo-1,4-*betaxylanase*; 1.5% w/v barley

betaglucan for endo-1,3(4)-*betaglucanase*; 4.0% w/v carboxymethylcellulose for endo-1,4-*betaglucanase*). The reaction is stopped adding 0.15 ml DNS solution. The blank samples are assayed without incubation at 40 ^{o}C and added with DNS solution just before the addition of the above mentioned substrate solutions. The final solutions are centrifuged at 14000 rpm for a few seconds and incubated in a boiling bath for 10 min. After cooling in an ice bath and mixing with 1.5 ml water, the absorbance is measured at 530 nm by the use of a double-beam spectrophotometer or a microplate reader. A glucose standard curve is used to calculate the endo-1,3(4)-*betaglucanase* and the endo-1,4-*betaglucanase* activities, whereas a xylose standard curve is used to calculate the endo-1, 4-*betaxylanase* activity.

The method was ring-trial validated by four external laboratories for the *feed additives* [5] and the reported performance characteristics were listed in Table 1 including relative standard deviation for repeatability (RSD_r) and Relative standard deviation (RSD) for reproducibility.

Table 1: Performance profile of the methods for the enzyme determination in the feed additive

	endo-1,4-*betaxylanase*	endo-1,3(4)-*betaglucanase*	endo-1,4-*betaglucanase*
RSD_r	4-5%	3-7%	4-8%
RSD of reproducibility	5-10%	4-9%	2-9%

Based on the acceptable performance characteristics presented in Table 1, the applicant method is found suitable for official controls of endo-1,4-*betaxylanase*, endo-1,3(4)-*betaglucanase* and endo-1,4-*betaglucanase* activities in the feed additive "*Roxazyme G2 G and L*".

Feedingstuffs and premixtures

For the *feedingstuffs and premixtures* the applicant submitted, upon request from the CRL, three modified method protocols determining the activity of endo-1,4-*betaxylanase* [6], endo-1,3(4)-*betaglucanase* [8] and endo-1,4-*betaglucanase* [10], respectively. These methods are the same analytical principle based on a standard addition technique. 50 g of feed (mash or pellets) or premix samples (solid dilution 1/100 in corn meal) are extracted in 500 ml buffer containing sodium acetate trihydrate and sodium dihydrogen phosphate (pH = 4.2) for 45 min.

50 ml or 2 ml extract [6, 10 or 8] is transferred into a tube and centrifuged. The premix extracts are further diluted with buffer to obtain an activity level of 270, 70 and 80 U/kg for endo-1,4-*betaxylanase*, endo-1,3(4)-*betaglucanase* and endo-1,4-*betaglucanase*, respectively, which is equivalent to 100 mg/kg *Roxazyme G2* in *feedingstuffs*. 10 ml sample extracts are spiked with the different concentration of *Roxazyme G2* standard solution [6, 10]. Finally, 0.2 ml sample extract (spiked/non-spiked) is added with:

- 0.25 ml azoxylan from Megazyme for the determination of endo-1,4-*betaxylanase* activity and incubated for 150 min at 50 ^{o}C [6];
- 0.2 ml azobarley glucan substrate from Megazyme for the determination endo-1,3(4)-*betaglucanase* activity and incubated for 60 min at 50 ^{o}C [8];
- 0.2 ml azocarboxymethylcellulose substrate from Megazyme for the determination of endo-1,4-*betaglucanase* activity and incubated for 90 min at 50 ^{o}C [10].

The incubation is stopped adding 1.3 ml ethanol (95% v/v). The samples extracts are then centrifuged at 11000 to 20000 *g* for 3 to 10 min, and the absorbance readings of supernatants are made at 590 nm.

The methods were in-house validated for the determination of endo-1,4-*betaxylanase*, endo-1,3(4)-*betaglucanase and* endo-1,4-*betaglucanase in the feedingstuffs and premixtures* [7, 9, 11]. The reported performance characteristics are listed in Table 2, including relative standard deviation for repeatability (RSD_r), relative standard deviation for intermediate precision (RSD_R), recovery rate (RR), Limit of detection (LOD) and Limit of quantification (LOQ).

Table 2: Performance profile of the methods for the enzyme determination in the premixture and in feed

		endo-1,4-*betaxylanase* [7]		endo-1,3(4)-*betaglucanase* [9]		endo-1,4-*betaglucanase* [11]	
		Feed	Premix	Feed	Premix	Feed	Premix
*RR		97-105%	-	96-106%	-	98-104%	-
RSD_r		10-11%	15%	3-5%	4%	3-10%	16%
RSD_R		12%	15%	1-3%	4%	10-14%	16%
LOD, feed	U/kg	15-30	-	4-7	-	4-8	-
LOQ, feed	U/kg	70	-	18	-	20	-

*Calculated by the Rapporteur from the applicant data, cf. linearity in [7, 9, 11].

The LOD and LOQ values presented in Table 2 are found to be below the minimum recommended activity levels in *feedingstuffs* of 135, 35 and 40 U/kg for endo-1,4-*betaxylanase*, endo-1,3(4)-*betaglucanase* and endo-1,4-*betaglucanase*, respectively [2]. The premixture samples were validated at a target activity level of endo-1,4-*betaxylanase* of around 18000 U/kg, endo-1,3(4)-*betaglucanase* of around 5500 U/kg *premixture* and endo-1,4-*betaglucanase* of around 5000 U/kg *premixture*.

Based on the acceptable performance characteristics the proposed methods are considered suitable for the determination of endo-1,4-*betaxylanase*, endo-1,3(4)-*betaglucanase* and endo-1,4-*betaglucanase* activities in the *feedingstuffs* and *premixtures* for official control purposes in the frame of authorisation.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of *Roxazyme G2 G and L* authorisation, the CRL recommends the methods proposed by the applicant for determination of endo-1,4-β-*xylanase*, endo-1,3(4)-β-*glucanase* and endo-1,4-β-*glucanase* in *feed additives* and *feedingstuffs* and *premixtures* for chicken and turkey for fattening, laying hens, ducks and piglets (weaned) for official control purposes.

Further testing or validation is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Characterisation of the active substances in the *feedingstuffs*:

- Colorimetric method measuring water soluble dye released by action of endo-1,4-β-*xylanase* from dye cross-linked birchwood azoxylan substrate;
- Colorimetric method measuring water soluble dye released by action of endo-1,3(4)-β-*glucanase* from dye cross-linked azobarley glucan substrate;
- Colorimetric method measuring water soluble dye released by action of endo-1,4-β-*glucanase* from dye cross-linked azocarboxymethylcellulose substrate.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Roxazyme G2 G and L* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Application/Reference SANCO/D/2 Forw. Appl. 1831/025-2007.

[2] * Application/Annex III.pdf. Proposal of Register entry.

[3] * Technical dossier/Section II_Identity.pdf: Item 2.1.3.

[4] 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 Community reference laboratories, Official Journal of the European Union L 136.

[5] *Technical dossier/Appendix 2-4. [Konig et al. (2002). Determination of xylanase, β-glucanase and cellulase activity. *Anal Bional Chem*, 374:80-87].

[6] *Supplementary information (27/02/09) Method XYL-103_03E.

[7] *Supplementary information (27/02/09) XYL-103_03E Validation.

[8] *Supplementary information (27/02/09) Method GLU-103_03E.

[9] *Supplementary information (27/02/09) GLU-103_03E Validation.

[10] *Supplementary information (27/02/09) Method CEL-103_03E.

[11] *Supplementary information (27/02/09) CEL-103_03E Validation.

*Refers to Dossier number: FAD-2007-0036

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

– Federaal Voedingslabo Tervuren (AFSCA-FAVV), Tervuren, Belgium

– Plantedirektoratets Laboratorium, Lyngby Denmark.

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