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Community Reference Laboratory for Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorised as Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2010-0009** **CRL/ 0090037**
Name of Product:	**Ronozyme RumiStar (CT) (L)**
Active Agent (s):	**α-amylase (E.C. 3.2.1.1)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA)** **Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (CRL-FA)** **24/08/2010**
Report approved by: Date:	**Christoph von Holst (CRL-FA)** **27/08/2010**

EXECUTIVE SUMMARY

Ronozyme RumiStar (CT) (L) is a product for which authorisation as *feed additive* is sought under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003. The active agent of *Ronozyme RumiStar (CT) (L)* is *α-amylase* (E.C. 3.2.1.1), produced by a strain of *Bacillus Licheniformis* (DSM 21564). The authorisation for *dairy cows* in the lactation period is requested.

The enzymatic activity of the *α-amylase* is expressed in "KNU" units. According to the Applicant, one KNU is defined as the amount of enzyme that releases 6 µmol p-nitrophenol per minute from 1.86 mM ethylidene-G_7-p-nitrophenyl-maltoheptaoside at pH 7.0 and 37 °C.

The product is intended to be marketed in two formulations: (i) as Coated Thermo tolerant granulate (CT) with a minimum activity of 160 KNU/g, and (ii) as Aqueous Liquid (L) with a minimum activity of 240 KNU/g. The Applicant recommended *α-amylase* activity in complete *feedingstuffs* ranging from 300 to 600 KNU/kg.

For the determination of the activity of *α-amylase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposed two single-laboratory validated and further verified colorimetric methods. In all methods the activity of the samples is calibrated against reference enzyme standards with known activity determined at the definition conditions of the activity unit.

The following performance characteristics for the determination of *α-amylase* in *feed additives*, *premixtures* and *feedingstuffs*, were recalculated by the CRL:

- for *feed additives*, a relative standard deviation for *repeatability* (RSD_r) ranging from 1.6 to 3.3 %, a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 3.5 to 9.1 %;
- for *premixtures*, RSD_r ranging from 3.9 to 6.5 %, RSD_{ip} ranging from 3.6 to 6.5 %;
- for *feedingstuffs*, RSD_r ranging from 4.1 to 7.2 %, RSD_{ip} ranging from 5.1 to 13.6 %, a recovery rate (R_{Rec}) ranging from 92.6 to 114.4 %, a limit of detection (LOD) and quantification (LOQ) of 2 and 7 KNU/kg *feedingstuffs*, respectively.

Based on the satisfactory performance characteristics mentioned above, the CRL recommends for official control the methods submitted by the applicant for the determination of *α-amylase* 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

Ronozyme RumiStar (CT) (L), α-amylase, *Bacillus Licheniformis*, digestibility enhancer, Dairy cows

1. BACKGROUND

Ronozyme RumiStar (CT) (L) is a product for which authorisation as *feed additive* is sought under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1]. The active agent of *Ronozyme RumiStar (CT) (L)* is *α-amylase* (E.C. 3.2.1.1), produced by a strain of *Bacillus Licheniformis* (DSM 21564) [2]. The strain has been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany [3].

In the current application submitted according to Article 4(1) of Regulation (EC) No 1831/2003, the authorisation for *dairy cows* in the lactation period is requested [1,4].

According to the Applicant, enzymatic activity of the active agents is expressed in "KNU" units [5], where one *α-amylase* unit (KNU) is defined as the amount of enzyme that releases 6 µmol p-nitrophenol per minute from 1.86 mM ethylidene-G_7-p-nitrophenyl-maltoheptaoside at pH 7.0 and 37 °C.

The product is intended to be marketed in two formulations: (i) as Coated Thermo tolerant granulate (CT) with a minimum activity of 160 KNU/g, and (ii) as Aqueous Liquid (L) with a minimum activity of 240 KNU/g [2].

The Applicant recommended *α-amylase* activity in complete *feedingstuffs* ranging from 300 to 600 KNU/kg [2].

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 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 or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Ronozyme RumiStar (CT) (L)*, 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

Quantitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective Community Reference Laboratories [6].

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

For the determination of the activity of *α-amylase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposed two single-laboratory validated and further verified colorimetric methods. The methods are based on the same principle: incubation of the commercially availablesubstrate Red Starch with *Ronozyme® RumiStar α-amylase* depolymerizes the substrate by an endo-mechanism to produce low-molecular weight dyed fragments. The low-molecular weight dyed fragments remain in solution after addition of ethanol to the reaction mixture, whereas the high molecular weight material is precipitated. The high molecular weight material is removed by centrifugation and the clear colored supernatant is measured photometrically at 510 nm. The *α-amylase* in the assay solution is quantified against a certified *α-amylase* standard, available from the Applicant upon request. The activity of the reference enzyme standard was detected under the conditions defining the alpha-amylase unit (pH 7 and 37^{o}C) [5].

The analysis of *feed additive, premixtures* and *feedingstuffs* are carried out using the same experimental protocol, with slightly different extraction conditions:

- *feed additives* are extracted for 45-60 minute, at room temperature and pH 5 [7];
- *premixtures* are extracted for 60 minute, at room temperature and pH 8 [8];
- *feedingstuffs* are extracted for 90 minute, at 50^{o}C and pH 8 [7].

The Applicant provided an additional method for the determination of the activity of *α-amylase* in the *feed additive*, based on a similar colorimetric principle, but using an automatic device (robot) [5], and incubating for 5 minute, at 37^{o}C and pH 7. The *α-amylase* release yellow colored p-nitrophenol from ethylidene-G_7-p-nitrophenyl-maltoheptaoside, measured photometrically at 405 nm [13]. The CRL consider the use of the automatic device (robot), may not be easily available at Official Control laboratories.

Table 1: Method performance characteristics for the determination of *endo-1,4-β-xylanase* in *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

	RSD_r (%)		RSD_{int} (%)		R_{Rec} (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	1.6 [9]	3.3 [11]	3.5 [9]	9.1 [11]	95-103 [9]	98-114 [11]
PM	3.9 [10]	6.5 [12]	3.6 [10]	6.5 [12]	91-101[10]	98-112 [11]
FS	4.1 [9]	7.2 [11]	5.1 [9]	14 [11]	95-103 [9]	93-114 [11]

RSD_r,RSD_{int} - relative standard deviation for *repeatability* and *intermediate precision*,
R_{Rec} – recovery rate (calculated as activity measured/activity expected)

The performance characteristics derived from the validation [9,10] and verification [11] studies, were recalculated by the CRL [12] and are presented in Table 1. Furthermore, the Applicant reported for the determination of *α-amylase* in the *feedingstuffs,* a limit of detection (LOD) and quantification (LOQ) of 2 and 7 KNU/kg *feedingstuffs*, respectively.

Based on the above mentioned performance characteristics, the CRL recommends for official control the single-laboratory validated and further verified conventional colorimetric methods, submitted by the Applicant, for the determination of the activity of the *α-amylase* in *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 CRL recommends for official control the single-laboratory validated and further verified colorimetric methods, submitted by the Applicant, for the determination of the activity of the *α-amylase* in *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *α-amylase* in *feed additive*, *premixtures* and *feedingstuffs*:

- colorimetric method based on the quantification of the dyed fragments produced by the action of *α-amylase* on Red Starch substrates.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ronozyme RumiStar (CT) (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] Reference SANCO/D/2 Forw. Appl. 1831/0010-2010
[2] *Application, Proposal for Register Entry
[3] *Technical dossier, Section II – Annex – 2-25_Weihs 2008_DSMZ.pdf
[4] *Technical dossier, Section II – 2.5. Condition of Use of the Additives
[5] *Technical dossier, Section II – 2.6. Method of analysis and reference samples
[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, Section II – Annex – 2-51_Method AMY 101-02
[8] *Technical dossier, Section II – Annex – 2-52_Method AMY 102-02
[9] *Technical dossier, Section II – Annex – 2-53_Jung and Vogel 2008_2500707
[10] *Technical dossier, Section II – Annex – 2-54_Jung and Vogel 2008_2500724
[11] *Technical dossier, Section II – Annex – 2-55_Biopract study plan Ver AMY 101_102
[12] *Additional Information – Precision data as recalculated by the CRL.xls
[13] *Technical dossier, Section II – Annex – 2-57_AnRC 2010_SM-0221
[14] *Technical dossier, Section II – Annex – 2-58_Validation SM-0221
[15] *Technical dossier, Section II – Annex – 2-60_CRL verf form for appl SM 0221

* Refers to Dossier No. FAD-2010-0009

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community 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:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)