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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® WX
(FAD-2013-0047; CRL/130025)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2013-0047 - CRL/130025**
Name of Feed Additive:	***Ronozyme® WX***
Active Agent (s):	**Endo 1,4-β-xylanase**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **27/05/2015**
Report approved by: Date:	**Christoph von Holst** **01/06/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 13(3) for *Ronozyme® WX* under the category/functional "zootechnical additives"/"digestibility enhancers". Specifically, authorisation is sought for the use of a new *Ronozyme® WX* produced from an optimized *Aspergillus oryzae* strain. The authorisation is sought for the use of the *feed additive* for poultry for fattening, piglets (weaned) and pigs for fattening.

According to the Applicant, *endo-1,4-β-xylanase* is the active agent of *Ronozyme® WX*. The Applicant expresses the *xylanase* enzymatic activity in xylanase units (FXU), defined as "the amount of enzyme which liberates 7.8 micromoles of reducing sugars (xylose equivalents) from azo-wheat arabinoxylan per minute at pH 6.0 and 50°C".

The product is intended to be marketed as solid and liquid formulations having a guaranteed minimum *xylanase* activity of 1000 FXU/g and 650 FXU/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 100 or 200 FXU/kg *feedingstuffs*, depending on the target species.

For the quantification of the *xylanase* activity in the *feed additive* the Applicant proposed a single-laboratory validated and further verified colorimetric method that requires the use of an automatic device (Konelab Analyzer). Upon request of the EURL of a manual method, the Applicant proposed to apply a ring-trial validated colorimetric method based on the quantification of the coloured compounds produced by the dinitro salicylic acid (DNSA) and the xylosylic moieties released by the action of *endo-1,4-β-xylanase* on arabinoxylan. Furthermore, for the quantification of the *xylanase* 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-1,4-β-xylanase* on oat spelt azo-xylan. In both methods the calibration is conducted using a reference standard with a known enzyme activity expressed in FXU.

Based on the satisfactory performance characteristics, recalculated from the experimental data available, the EURL recommends for official control the colorimetric methods mentioned above for the quantification of the *xylanase* 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-1,4-β-xylanase, *Ronozyme® WX*, "zootechnical additives"/"digestibility enhancers", poultry for fattening, pigs for fattening and piglets

1. BACKGROUND

Ronozyme® WX is a *feed additive* authorised by Commission Regulation (EC) No 1332/2004 for chickens and turkeys for fattening, and for piglets [1] and further extended to ducks and pigs for fattening by Commission Regulation (EC) No 2036/2005 [2] belonging to the "Enzymes" group listed in Directive 70/524/EEC. These regulations were further amended by Commission Implementing Regulation (EC) No 1206/2012 [3] according to article 10(2) of Regulation (EC) No 1831/2003. In the current application authorisation is sought under article article 13(3) (modification of the existing authorisation) of the Regulation (EC) No 1831/2003 [4] for *Ronozyme® WX* under the category/functional group "zootechnical additives"/"digestibility enhancers" [4]. Specifically, authorisation is sought for the use of a new *Ronozyme® WX* produced by an optimized *Aspergillus oryzae* production strain belonging to the same safe lineage as the one used to make the product currently on the EU market. The enzyme, the activity, the formulation, the production process, the target species and the conditions of use of the *feed additive* remain unchanged [5].

According to the Applicant, *endo-1,4-β-xylanase* is the active agent of *Ronozyme® WX* [6]. The Applicant expresses the xylanase enzymatic activity in xylanase units (FXU), defined as "the amount of enzyme which liberates 7.8 micromoles of reducing sugars (xylose equivalents) from azo-wheat arabinoxylan per minute at pH 6.0 and 50°C".

The product is intended to be marketed as solid (*Ronozyme® WX (CT)*) and liquid (*Ronozyme® WX (L)*) formulations having a guaranteed minimum *xylanase* activity of 1000 FXU/g and 650 FXU/ml respectively [6]. *Ronozyme® WX (CT)* is intended to be included through *premixtures* or directly in *feedingstuffs*, while *Ronozyme® WX (L)* is intended to be included directly in *feedingstuffs* to obtain a minimum activity of 100 or 200 FXU/kg *feedingstuffs*, depending on the target species [7].

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 *Ronozyme® WX* 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 [8]

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

For the quantification of the *xylanase* 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 wheat arabinoxylan by *endo-1,4-β-xylanase*. The coloured complexes formed by the reducing sugars and the stop solution (alkaline 4-Hydroxybenzhydrazide (PAHBAH)/bismut solution) are detected at 405 nm, being the measured absorbance proportional to the *xylanase* activity [10].

The validation and verification reports provided by the Applicant lead to acceptable method performance characteristics. However, this method requires the use of an "automatic device" (Konelab Analyzer), which is hardly available for official feed laboratories. Therefore the EURL does not consider this method suitable for official control.

Upon request of the EURL, the Applicant proposed to apply for the "new" *Ronozyme WX* a ring-trial validated method colorimetric method [11]. *Xylanase* cleaves non-starch polysaccharides (NSP) releasing xylosylic moieties with reducing ends. These reducing moieties are oxidised in an alkaline media by forming orange-yellow compounds with dinitro salicylic acid (DNSA) that are measured at a wavelength of 530 nm and quantified against a reference enzyme *"Ronoxyme WX"* available from the Applicant upon request [12].

The *feed additive* samples (1 g), are dissolved by stirring them for 45 min in 100 ml of 0.1 M acetate buffer at pH 5.0 with 20 mM calcium chloride and 0.04 % Tween 20. 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 40 ^{o}C. 0.3 ml of pre-incubated 1.5% arabinoxylan substrate is added. Sample extracts are mixed and incubated at 40^{o}C for 20 minutes. After the incubation time the reaction is stopped by adding 0.15 ml DNSA reagent, mixed and centrifuged for 20 s. Then, the mixture is boiled for 10 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 at 530 nm.

Calibration is performed against external calibration curve of a *Ronozyme WX* standard with a known certified activity expressed in FXU [12]. This method was ring-trial validated for *xylanase* products commercially available. The following performance characteristics were reported [11]:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 4.4 to 5.3 % and
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.9 to 10.1 %

From the experimental data available, the EURL recalculated for the *feed additive* precision values (repeatability and intermediate precision) of 6.4 and 11 % [13][14]. These performance characteristics are in good agreement with those reported in the ring-trial validation and demonstrate the applicability the method to the *feed additive* (*Ronozyme WX).*

For the quantification of *xylanase* activity in *premixtures* and *feedingstuffs* the Applicant proposes a single-laboratory validated and further verified [15] colorimetric method based on the quantification of the water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on oat spelt azo-xylan. The quantification of the *xylanase* activity is based on a reference enzyme *"Ronoxyme WX"* available from the Applicant upon request [16]. The *premixtures* (1 g and 49 g of wheat), *feedingstuffs* samples (50 g) and wheat (50 g) are extracted in 500 ml of 0.04 M phosphate/acetate buffer with 0.02 % Tween 20 (pH 6.0) for 60 minutes and a 2 ml aliquot is further centrifuged during 3 minutes and appropriately spiked (wheat extract) and diluted. An aliquot (0.2 ml) of the diluted final extract is pre-incubated for 5 min at 65 ^{o}C and 0.2 ml of 1% azo-xylan (oat spelt), preheated at 40 ^{o}C, is added. Sample extracts are mixed and then incubated at 65^{o}C for 90 minutes (or 180 min for samples with activity ≤ 100 FXU/kg). After the incubation time the reaction is stopped by adding 1 ml of a stop and precipitant solution (water:ethanol (5:95 v:v)). Samples are then mixed, let at room temperature for 15 min and centrifuged for 3 min. Finally, the absorbance of the clear supernatant solution is measured at 590 nm. Calibration is performed against external calibration curve of feed blank (wheat) extract spiked with a *Ronozyme WX* standard with a known certified activity expressed in FXU [16]. The following performance characteristics were recalculated by the EURL from the experimental data available [17]. For *premixtures*: a relative standard deviation for *repeatability* (RSD_r) ranging from 4.6 to 6.9 % and a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 5.8 to 6.1 % and for the *feedingstuffs:* RSD_r ranging from 7.1 to 21.4 %;and RSD_{ip} ranging from 8.6 to 24.4 %. Additionally the Applicant reported a limit of quantification (LOQ) in feedingstuffs of 53 FXU/kg.

Based on the performance characteristics available, the EURL recommends for official control the colorimetric methods mentioned above for the quantification of the *xylanase* 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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the proposed colorimetric methods based on the enzymatic reaction of xylanase on for the quantification of *xylanase* activity in the *feed additive* and the single-laboratory validated and further verified colorimetric method for the quantification of *xylanase* activity in *premixtures* and *feedingstuffs*

.***Recommended text for the register entry (analytical method)***

For the quantification of *xylanase* activity in *the feed additive*,

- colorimetric method measuring coloured compound produced by the dinitro salicylic acid (DNSA) and the xylosylic moieties released by the action of *xylanase* on arabinoxylan.

For the quantification of *xylanase* activity in *premixtures* and *feedingstuffs*,

- colorimetric method measuring water soluble dye released by action of *xylanase* from dye-labelled oat spelt azo-xylan.

One *xylanase* units (FXU), corresponds to the amount of enzyme which liberates 7.8 micromoles of reducing sugars (xylose equivalents) from azo-wheat arabinoxylan per minute at pH 6.0 and 50°C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ronozyme®WX* 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 1332/2004 of 20 July 2004, concerning the permanent authorisation of certain additives in feedingstuffs

[2] Commission Regulation (EC) No 2036/2005 of 14 December 2005, concerning the permanent authorisation of certain additives in feedingstuffs and the provisional authorisation of a new use of certain additives already authorised in feedingstuffs

[3] Commission Implementing Regulation (EC) No 1206/2012 of 14 December 2012, concerning authorisation of a preparation of endo-1,4-beta xylanase produced by *Aspergillus oryzae* (DSM 10287) as a feed additive for poultry for fattening, weaned piglets and pigs for fattening and amending Regulations (EC) No 1332/2004 and (EC) No 2036/2005 (holder of the authorisation DSM Nutritional Products)

[4] *Application, Reference SANCO/G1: Forw. Appl. 1831/0007-2014

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

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

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

[8] 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

[9] *Technical dossier, Annexes, Part II Appendix 2-43 & 2-44

[10] *Technical dossier, Annexes, Part I Appendix 2-1

[11] König J., Grasser R., Pikor H. and Vogel K. Anal. Bioanal. Chem. 374 (2002) 80-87

[12] Supplementary Information, Method Ronozyme WX_01E

[13] Supplementary Information, ANNEX IV_Verification Report_Ronozyme WX-01E_20111025.pdf & ANNEX III_Ronozyme WX_01E_20110617.pdf

[14] Supplementary Information, Ronozyme_ver_FA_ANOVA.pdf

[15] *Technical dossier, Annexes, Part II Appendix 2-45 & 2-46

[16] *Technical dossier, Annexes, Part I Appendix 2-2

[17] *Supplementary Information, Ronozyme_ver_PM_FS_ANOVA.pdf

*Refers to Dossier no: FAD-2013-0047

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

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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid[1] (ES)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)

[1] Name and address according to Regulation (EC) No 885/2009: Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid