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**CRL Evaluation Report on the Analytical Methods submitted in
connection with Section II, 2.6 (Control Methods) of the Application
for Authorisation as a Feed Additive
according to Regulation (EC) No 1831/2003**

Dossier related to: **FAD-2008-0013**
CRL / 070030

Product name: **endo-1,4- β -xylanase (Ronozyme WX)**

Active Substance(s): **endo-1,4- β -xylanase (EC 3.2.1.8)**

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Date: **12/08/2009**

EXECUTIVE SUMMARY

The current application authorisation is sought for *endo-1,4- β -xylanase* (*Ronozyme WX*) under the category 'zootechnical additives', group 4(a), 'digestibility enhancer' for poultry, piglets (weaned) and pigs for fattening, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for *endo-1,4- β -xylanase* (*Ronozyme WX*) according to Article 4(1) and Article 10(2) of Regulation (EC) No 1831/2003.

The active agent of *Ronozyme WX* is *endo-1,4- β -xylanase* produced by submerged fermentation of *Aspergillus oryzae* (DSM 10287). The enzymatic activity of *endo-1,4- β -xylanase* is expressed in xylanase unit (FXU). One FXU-unit is the amount of *endo-1,4- β -xylanase* which liberates 7.8 micromoles of reducing sugars (xylose equivalents) per minute from azo-wheat arabinoxylan at pH 6.0 and 50 °C. The product is intended to be marketed in two forms, as liquid formulation (*Ronozyme WX L*) with an enzymatic activity of 650 FXU/ml and as granulate formulation (*Ronozyme WX CT*) with an enzymatic activity of 1000 FXU/g. Both *products* are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain a minimum enzyme activity level of 100 FXU/kg of *feedingstuffs* for poultry and pigs for fattening, and a minimum enzyme activity level of 200 FXU/kg of *feedingstuffs* for piglets (weaned).

For the determination of *endo-1,4- β -xylanase* activity in *feed additives*, *premixtures* and *feedingstuffs* the applicant proposes in-house validated colorimetric methods based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4- β -xylanase* on azo-wheat arabinoxylan substrate for *feed additives* and azurine-crosslinked wheat arabinoxylan substrate for *premixtures* and *feedingstuffs*. The intensity of the colour is proportional to the *endo-1,4- β -xylanase* activity, which is quantified based on a reference enzyme *Ronozyme*, available from the applicant upon request. For the quantification of *endo-1,4- β -xylanase* activity in *premixtures* and *feedingstuffs* the analytical method foresees using the standard addition technique.

The results of in-house validation of the analytical method determining *endo-1,4- β -xylanase* activity in *feed additives* are: - a relative standard deviation for repeatability (RSD_r)

ranging from 1.3 to 7.0 %; - a relative standard deviation for intermediate precision (RSD_R) ranging from 7.0 to 12.3 %; - a limit of quantification (LOQ) of 0.7 FXU/g of *product* and - a recovery rate (RR) of 97.8 %. The reported method performance characteristics are acceptable. Therefore, the proposed method is considered suitable for determination of *endo-1,4- β -xylanase* in *feed additives* for official control purpose in the frame of authorisation.

For the determination of *endo-1,4- β -xylanase* activity in *premixtures* the following performance characteristics of the analytical method based on a standard addition technique, were provided, upon request by the CRL: - $RSD_I = 14.2$ %, - $RSD_R = 38.5$ %, - LOD = 75 FXU/kg of *premixture*, and - a RR ranging from 85 to 90 %. The performance characteristics were determined for premixtures samples with an enzyme activity range of 15000 to 30000 FXU/kg of *premixture*. Better method precision values may be obtained by diluting the premixture sample into blank feed matrix and applying the corresponding method for the determination of *endo-1,4- β -xylanase* activity in *feedingstuffs*. However, this alternative approach was not applied in the present dossier and the corresponding validation data is missing; hence the suitability of such method for official controls could not be evaluated. Therefore, the method proposed by the applicant is considered suitable for determination of *endo-1,4- β -xylanase* in *premixtures* for official control purpose within the range of the activity levels included in the validation which is 15000 to 30000 FXU/kg.

For the determination of *endo-1,4- β -xylanase* activity in *feedingstuffs* the following performance characteristics for the method based on a standard addition technique were reported: - RSD_I ranging from 6.6 to 16.8 %, - RSD_R ranging from 16.5 to 28.1 %, - LOD = 50 FXU/kg of *feedingstuffs*, and - RR ranging from 96 to 112 %. The performance characteristics were determined for the feed samples with an enzyme activity range of 160 to 2000 FXU/kg of *feedingstuffs*. The reported method performance characteristics are acceptable. Therefore, the proposed method is considered suitable for determination of *endo-1,4- β -xylanase* in *feedingstuffs* for official control purpose in the frame of authorisation.

Further testing or validation is not considered necessary.

KEYWORDS

Endo-1,4- β -xylanase, *Ronozyme WX (CT and L)*, *Aspergillus oryzae*, digestibility enhancer, poultry, piglets (weaned) and pigs for fattening

1. BACKGROUND

Endo-1,4- β -xylanase (*Ronozyme WX*) is a product for which authorisation as feed additive is sought under the category ‘zootechnical additives’, functional groups ‘digestibility enhancers’ for poultry, piglets (weaned) and pigs for fattening, according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorisation is sought for *endo-1,4- β -xylanase* according to Article 4 (1) and Article 10 (2) of Regulation (EC) No 1831/2003 [1]. The additive has already been authorised for chickens for fattening, turkey for fattening and piglets [2] and for pigs for fattening and ducks [3].

The active agent of *Ronozyme WX* is *endo-1,4- β -xylanase* (EC 3.2.1.8) produced by submerged fermentation of *Aspergillus oryzae* (DSM 10287) deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) under the number DSM 10287 in Germany [4]. The activity of *endo-1,4- β -xylanase* is expressed in FXU unit, which corresponds to its equivalents units as "FXU(W)", "M:FXU(W)" and "FXUW(P)" at various parts of the dossier documentation. According to the applicant one FXU-unit is the amount of *endo-1,4- β -xylanase* which liberates 7.8 micromoles of reducing sugars (xylose equivalents) per minute from azo-wheat arabinoxylan at pH 6.0 and 50 °C.

The *product* is intended to be marketed in two forms [4]:

- as liquid formulation (*Ronozyme WX L*) with a target *endo-1,4- β -xylanase* activity of 650 FXU/ml;
- as granulate formulation (*Ronozyme WX CT*) with a target *endo-1,4- β -xylanase* activity of 1000 FXU/g.

These products are intended to be incorporated into premixtures and/or complete *feedingstuffs* to obtain a minimum enzyme activity level of 100 FXU/kg in *feedingstuffs* for

poultry and pigs for fattening, and a minimum enzyme activity level of 200 FXU/kg in *feedingstuffs* for piglets (weaned) [5].

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 *Ronozyme WX*, 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 [6].

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

For the determination of *endo-1,4- β -xylanase* activity in *feed additives, premixtures and feedingstuffs* the applicant proposes in-house validated colorimetric methods based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4- β -xylanase* on azo-wheat arabinoxylan substrate for *feed additives* and azurine-cross linked wheat arabinoxylan substrate for *premixtures* and *feedingstuffs*. The intensity of the colour is proportional to the *endo-1,4- β -xylanase* activity, which is quantified based on a reference enzyme "*Ronozyme*", available from the applicant upon request.

For the determination of *endo-1,4- β -xylanase* activity in *feed additives* either liquid or granulate products are suspended in phosphate buffer (pH = 6.0). The suspension is diluted to a final concentration between 0.3 FXU/ml and 0.7 FXU/ml. The substrate (azo-wheat

arabinoxylan dyed with remazol brilliant blue) is added and incubated at 50 °C for 30 minutes. The reaction is stopped adding ethanol with hydrochlorid acid and the unconverted substrate is precipitated. The blue colouring of the supernatant due to unprecipitated remazol-stained substrate degrading products is proportional to the *endo-1,4- β -xylanase* activity and is measured on a spectrophotometer at 585 nm. The activity is quantified relative to a reference enzyme standard, available from the applicant upon request [7]. The method has two protocols, one for a robotic and another for manual method. Upon request by the CRL, the applicant clarified that the validation study applies to the manual method, and later confirmed the comparability of both methods [9]. The method is in-house validated and the reported method performance characteristics were [7]: - RSD_r ranging from 1.3 to 7.0 % (as recalculated by the CRL), - RSD_R ranging from 7.0 to 12.3 % (as recalculated by the CRL), - LOQ = 0.7 FXU/g of *product* and - RR= 97.8 %. The reported method performance characteristics are acceptable. Therefore, the proposed method is considered suitable for determination of *endo-1,4- β -xylanase* in *feed additives* for official control purpose in the frame of authorisation.

For the quantification of *premixtures* and *feedingstuffs* the applicant proposes two analytical assays of the method: - an assay based on the use of a control feed and - an assay based on a standard addition technique. The CRL recommends the latter for official control since a blank feed is usually not available at the official control laboratories. The standard addition assay is described as follows: the samples are extracted in phosphate buffer (pH = 6.0). For premixture samples the buffer contains additional ethylenediaminetetraacetic acid (EDTA) and bovine serum albumin (BSA). Five sample extracts are spiked with five different activities of an enzyme standard solution. The enzyme standard is available from the applicant upon request. Three sample extracts remain without spiking. After adding the substrate (azurine-cross linked wheat arabinoxylan) the solution is incubated at 50 °C for 60 minutes. The reaction is stopped adding Trizma base and the unconverted substrate is precipitated. The converted substrate is soluble and will dye the supernatant blue. The intensity of the colour is proportional to the *endo-1,4- β -xylanase* activity and is measured on a spectrophotometer at 600 nm [8]. For the quantification the response corresponding to the analysis of the samples without added enzymes (target sample) and of the spiked samples are plotted against the added activities. From this calibration curve the activity of the target sample is calculated.

For the determination of *endo-1,4- β -xylanase* activity in *premixtures* the following performance characteristics of the in-house validated analytical method, based on a standard addition technique, were provided, upon request by the CRL 0: - $RSD_T = 14.2\%$, - $RSD_R = 38.5\%$, - $LOD = 75$ FXU/kg of *premixture*, and - RR ranging from 85 to 90 %. The performance characteristics were determined for the premixture samples at *endo-1,4- β -xylanase* activity range of 15000 to 30000 FXU/kg of *premixture*. Better method precision values can be obtained by diluting the premixture sample into blank feed matrix and applying the corresponding method for the determination of *endo-1,4- β -xylanase* activity in *feedingstuffs*. However, this alternative approach was not applied in the present dossier and the corresponding validation data is missing; hence the suitability of such method for official controls could not be evaluated. Therefore, the method proposed by the applicant is considered suitable for determination of *endo-1,4- β -xylanase* in *premixtures* for official control purpose in the frame of authorisation.

For the determination of *endo-1,4- β -xylanase* activity in *feedingstuffs* the following performance characteristics of the in-house validated method, based on a standard addition technique were reported: - RSD_T ranging from 6.6 to 16.8 %, - RSD_R ranging from 16.5 to 28.1 %, - $LOD = 50$ FXU/kg of *feedingstuffs*, and - RR ranging from 96 to 112 %. The performance characteristics were determined for the feed samples with an enzyme activity range of 160 to 2000 FXU/kg of *feedingstuffs*. The reported method performance characteristics are acceptable. Therefore, the proposed method is considered suitable for determination of *endo-1,4- β -xylanase* in *feedingstuffs* for official control purpose in the frame of authorisation.

Based on acceptable performance characteristics the proposed methods are recommended for official control purpose for the determination of *endo-1,4- β -xylanase* activity in *feed additives*, *premixtures* and *feedingstuffs* in the frame of authorisation.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of *Ronozyme WX* authorisation, the CRL recommends the colorimetric methods proposed by the applicant for determination of *endo-1,4- β -xylanase* activity - in *feed additives*, *premixtures* and *feedingstuffs* for poultry, pigs for fattening 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)

- For *feed additive*: Colorimetric method measuring water soluble dyed fragments released by the enzyme from azo-wheat arabinoxylan substrate dyed with remazol brilliant blue.
- For *premixture* and *feedingstuffs*: Colorimetric method measuring water soluble dyed fragments released by the enzyme from azurine-cross linked wheat arabinoxylan substrate.
- One FXU-unit is 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 CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ronozyme WX* 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. 1831/019-2008.
- [2] Commission Regulation (EC) No 1332/2004 of 20 July 2004 concerning the permanent authorisation of certain additives in feedingstuffs, Official Journal of the European Union L 247, 21.7.2004
- [3] Commission Regulation (EC) No 2036/2005 of 14 December 2005 concerning the permanent authorisations of certain additives in feedingstuffs and the provisional authorisation of a new use of certain additives already authorised in feedingstuffs, Official Journal of the European Union L 328, 15.12.2005
- [4] * Section II: Identity, characterization and condition of use of the additive; methods of control
- [5] * Annex III, Proposal of Register Entry.
- [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 Community reference laboratories, Official Journal of the European Union L 136.
- [7] * Appendix 2-51.
- [8] * Appendix 2-52.
- [9] * Supplementary information _03/03/2009.
- [10] * Supplementary information_validation of analysis of premix samples
 - * Refers to Dossier number: FAD-2008-0013

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Austrian Agency for Health and Food Safety, Vienna, Austria

8. ACKNOWLEDGEMENTS

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- Danish Plant Directorate, Lyngby, Denmark
- National Veterinary Research Institute, Pulawy, Poland