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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**

**Preparation of endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase and  
endo-1,4-beta-glucanase  
(FAD-2021-0052; CRL/200087)**





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Dossier related to: **FAD-2021-0052 - CRL/200087**

Name of Feed Additive: ***Preparation of endo-1,4-beta-xylanase,  
endo-1,3(4)-beta-glucanase and  
endo-1,4-beta-glucanase***

Active Agent (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: **European Union Reference Laboratory for  
Feed Additives (EURL-FA)  
JRC Geel, Belgium**

Report prepared by: **María José González de la Huebra**

Report checked by: **Zigmas Ezerskis**  
Date: **10/03/2023**

Report approved by: **Christoph von Holst**  
Date: **13/03/2023**

## EXECUTIVE SUMMARY

In the current application an authorisation of a *preparation of endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase and endo-1,4-beta-glucanase* is sought for the category / functional group 4(a) "zootechnical additives" / "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for poultry for fattening, poultry for laying, turkeys for fattening, piglets (weaned) and pigs for fattening.

According to the Applicant, the *feed additive* contains three enzymes as active substances namely: *endo-1,4-beta-xylanase* (EC 3.2.1.8), *endo-1,3(4)-beta-glucanase* (EC 3.2.1.6) and *endo-1,4-beta-glucanase* (EC 3.2.1.4) produced by the *Trichoderma reesei* ATCC 74444 strain.

The enzymatic activity for each of the three enzymes is expressed in international units (U), where one international unit (U) is the amount of enzyme (*endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase or endo-1,4-beta-glucanase*) that releases 1 micromole of reducing sugar (glucose or xylose) equivalents from wheat arabinoxylan, barley beta-glucan and carboxy-methyl-cellulose, respectively, per minute at pH 5.0 and 40 °C.

The *feed additive* is intended to be marketed as solid (*RONOZYME<sup>®</sup> MultiGrain* (GT)) and as liquid (*RONOZYME<sup>®</sup> MultiGrain* (L)) formulations, having minimum activities of 2700 U / g (or U / ml) for *endo-1,4-beta-xylanase*, 700 U / g (or U / ml) for *endo-1,3(4)-beta-glucanase*, and 800 U / g (or U / ml) for *endo-1,4-beta-glucanase*.

The *feed additive* is intended to be incorporated through *premixtures* or directly into *compound feed* (for the solid formulation) or directly sprayed onto the *compound feed* (for the liquid formulation). For pigs for fattening (new animal category) the Applicant recommends including the *feed additive* in *compound feed* in order to obtain minimum activities of 270, 70 and 80 U / kg *compound feed* for *endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase and endo-1,4-beta-glucanase* respectively, while for the other animal categories the recommended activities are the same as specified in the Commission Implementing Regulation (EU) No 403/2013.

For the determination of the *endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase and endo-1,4-beta-glucanase* activities in the *feed additive* the Applicant submitted a ring-trial validated colorimetric method based on the enzymatic hydrolysis of the respective substrates and the colour formation of the released reducing sugars with 3,5-dinitrosalicylic acid (DNS) at pH 5.0 and 40 °C.

For the determination of the *endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase and endo-1,4-beta-glucanase* activities in *premixtures* and *compound feed* the Applicant submitted

single-laboratory validated and further verified colorimetric methods based on the measurement of the soluble oligomeric cleavage products from the respective dye-labelled substrates.

Based on the acceptable performance characteristics presented the EURL recommends for official control the ring-trial validated and the single-laboratory validated and further verified colorimetric methods submitted by the Applicant for the determination of the activities of *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* in the *feed additive, premixtures* and *compound feed*.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

## KEYWORDS

*RONOZYME<sup>®</sup> MultiGrain*; preparation of *endo-1,4-beta-xylanase* (EC 3.2.1.8), *endo-1,3(4)-beta-glucanase* (EC 3.2.1.6) and *endo-1,4-beta-glucanase* (EC 3.2.1.4); *Trichoderma reesei* ATCC 74444; zootechnical additives; digestibility enhancer; poultry for fattening, poultry for laying, turkeys for fattening, piglets (weaned) and pigs for fattening.

## 1. BACKGROUND

In the current application an authorisation of a *preparation of endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* is sought for poultry for fattening, poultry for laying, turkeys for fattening, piglets (weaned) and pigs for fattening under the category / functional group 4(a) "zootechnical additives" / "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1, 2]. According to the Applicant the *preparation of endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* subject of this application is a variant of the *feed additive* with identification number 4a1602i [3], which is currently authorised by Commission Implementing Regulation (EU) No 403/2013 [4].

According to the Applicant, the *feed additive* contains as active substances three enzymes namely: (i) *endo-1,4-beta-xylanase* (EC 3.2.1.8) (*xylanase*), (ii) *endo-1,3(4)-beta-glucanase* (EC 3.2.1.6) (*beta-glucanase*) and (iii) *endo-1,4-beta-glucanase* (EC 3.2.1.4) (*cellulase*) produced by the *Trichoderma reesei* ATCC 74444 strain [5].

The enzymatic activities of each of the three enzymes are expressed in international units (U), where one international unit (U) is the amount of enzyme (*endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase* or *endo-1,4-beta-glucanase*) that releases 1 micromole of reducing

sugar (xylose or glucose) equivalents from the respective substrate, i.e. wheat arabinoxylan for *endo-1,4-beta-xylanase*, barley beta-glucan for *endo-1,3(4)-beta-glucanase* and carboxymethyl-cellulose for *endo-1,4-beta-glucanase* per minute at pH 5.0 and 40 °C [6].

The *feed additive* is intended to be marketed as an off-white granular powder (*RONOZYME<sup>®</sup> MultiGrain (GT)*) and as liquid (*RONOZYME<sup>®</sup> MultiGrain (L)*) formulations, having the following guaranteed minimum activities: 2700 U / g (or U / ml for the liquid formulation) *endo-1,4-beta-xylanase*; 700 U / g (or U / ml for the liquid formulation) *endo-1,3(4)-beta-glucanase* and 800 U / g (or U / ml for the liquid formulation) *endo-1,4-beta-glucanase* [3].

*RONOZYME<sup>®</sup> MultiGrain (GT)* (solid formulation) is intended to be incorporated through *premixtures* or directly into *compound feed* while *RONOZYME<sup>®</sup> MultiGrain (L)* (liquid formulation) is designed to be sprayed directly onto the *compound feed* [7].

For the additional animal category (pigs for fattening) the Applicant recommends including the *feed additive* in *compound feed* in order to obtain a minimum activity for *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* respectively of 270, 70 and 80 U / kg *compound feed* [7] while for the other animal categories the recommended activities are the same as specified in the corresponding Commission Implementing Regulation [4].

Note: The EURL has evaluated analytical methods for the determination of *endo-1,4-beta-xylanase* (EC 3.2.1.8); *endo-1,3(4)-beta-glucanase* (EC 3.2.1.6) and *endo-1,4-beta-glucanase* (EC 3.2.1.4) in the frame of a previous dossier [8].

## 2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *preparation of endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase and endo-1,4-beta-glucanase* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

## 3. EVALUATION

***Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)***

For the quantification of the *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities in the *feed additive* the Applicant submitted a ring-trial validated colorimetric method based on the enzymatic hydrolysis of the respective substrates and the colour formation of the released reducing sugars with 3,5-dinitrosalicylic acid (DNS) at pH 5.0 and 40 °C [6].

The *feed additive* is extracted with acetate buffer and further diluted. Aliquots of the diluted extracts are then mixed with the appropriate substrate (wheat arabinoxylan for *endo-1,4-beta-xylanase*, barley betaglucan for *endo-1,3(4)-beta-glucanase* and carboxy-methyl-cellulose for *endo-1,4-beta-glucanase*) and submitted to an incubation process at pH 5.0 and 40 °C. After the incubation, the reactions are stopped by adding a DNS solution. The reaction products are determined colorimetrically at 530 nm. The enzymatic activities, are calculated by using external glucose (for *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase*) and xylose (for *endo-1,4-beta-xylanase*) calibration curves. This method was ring-trial validated by four external laboratories and the performance characteristics as reported by the Applicant are depicted in Table 1 [6].

Furthermore, in the frame of the stability studies [9-10] the Applicant applied the method described above to the products subject of this dossier, thus demonstrating its applicability.

Based on the satisfactory performance characteristics presented, the EURL recommends for official control the ring-trial validated colorimetric method submitted by the Applicant for the determination of the *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities in the *feed additive*.

For the determination of the *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities in *premixtures* and *compound feed* the Applicant submitted single-laboratory validated and further verified colorimetric methods based on the measurement of the soluble oligomeric cleavage products from the respective dye-labelled substrates (azo-xylan (birchwood) for *endo-1,4-beta-xylanase*, azo-barley glucan for *endo-1,3(4)-beta-glucanase* and azo-carboxy-methyl-cellulose for *endo-1,4-beta-glucanase*) [11, 16-18].

**Table 1:** Performance characteristics for the quantification of *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities in the *feed additive*

	<i>endo-1,4-beta-xylanase</i>	<i>endo-1,3(4)-beta-glucanase</i>	<i>endo-1,4-beta-glucanase</i>
<b>Activity (U/g)</b>	6450 – 52172	1588 – 17773	2367 – 17948
<b>RSD<sub>r</sub>, (%)</b>	4 – 5	3 – 7	4 – 8
<b>RSD<sub>R</sub>, (%)</b>	5 – 10	4 – 9	2 – 9

RSD<sub>r</sub>; RSD<sub>R</sub>: relative standard deviations for *repeatability* and *reproducibility*, respectively.

**Table 2:** Performance characteristics for the quantification of *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities in *premixtures*

	<i>endo-1,4-beta-xylanase</i>		<i>endo-1,3(4)-beta-glucanase</i>		<i>endo-1,4-beta-glucanase</i>	
	Val [13]	Ver [12]	Val [14]	Ver [12]	Val [15]	Ver [12]
<b>Activity (U/g)</b>	31.1–6.5	31.1–6.4	8.4–16.9	8.6–15.8	10.3–19.5	10.4–19.0
<b>RSD<sub>r</sub>, (%)</b>	8.5	5.5	8.3	6.9	4.7	2.8
<b>RSD<sub>ip</sub>, (%)</b>	8.9	6.2	12.6	7.9	4.7	2.9
<b>Recovery (%)</b>	-	99	-	93	-	98

RSD<sub>r</sub>; RSD<sub>ip</sub>: relative standard deviations for *repeatability* and *intermediate precision*, respectively.

The *premixture* (mixed with wheat middlings) and the *compound feed* (mash or pellets) samples are extracted with acetate buffer at pH 4.5. Aliquots of the obtained extracts are mixed with the appropriate substrate and incubated at 50 °C. After the incubation, the reaction is stopped by adding a “stop and precipitation” solution. The reaction products are determined colorimetrically at 590 nm. The *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities are calculated by using external standard calibration curves using a Roxazyme G2G standard [11, 16-18].

These methods were single-laboratory validated [13-15] and further verified [12, 19-21] for *premixtures* and for *compound feed* and the performance characteristics as reported by the Applicant, are depicted in Table 2 (for *premixtures*) and in Table 3 (for *compound feed*).

Furthermore, the Applicant estimated limits of quantification (LOQ) of 62 U / kg *compound feed* for *endo-1,4-beta-xylanase*; 19 U / kg *compound feed* for *endo-1,3(4)-beta-glucanase* and 32 U / kg *compound feed* for *endo-1,4-beta-glucanase* which are below the minimum activities recommended for each of the target enzymes in *compound feed* [7].

Based on the satisfactory performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods submitted by the Applicant for the determination of the *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities in *premixtures* and *compound feed*.

**Table 3:** Performance characteristics for the quantification of *endo-1,4-beta-xylanase*, *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* activities in *compound feed*

	<i>endo-1,4-beta-xylanase</i>		<i>endo-1,3(4)-beta-glucanase</i>		<i>endo-1,4-beta-glucanase</i>	
	Val [13]	Ver [19]	Val [14]	Ver [20]	Val [15]	Ver [21]
<b>Activity (U/kg)</b>	459–678	437–719	126–282	110–260	77–293	75–287
<b>RSD<sub>r</sub>, (%)</b>	9.5–18.1	1.6–18.7	5.5–18.1	3.6–15.7	3.0–17.0	1.6–17.0
<b>RSD<sub>ip</sub>, (%)</b>	11.7–16.9	12.8–19.9	11.0–17.2	9.5–14.4	3.7–16.9	4.9–16.4
<b>Recovery (%)</b>	-	95–106	-	90–104	-	96–105

RSD<sub>r</sub>; RSD<sub>ip</sub>: relative standard deviations for *repeatability* and *intermediate precision*, respectively.



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***Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)***

The evaluation of corresponding methods of analysis is not relevant for the present application.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

#### **4. CONCLUSIONS AND RECOMMENDATIONS**

In the frame of this authorisation the EURL recommends for official control: i) the ring-trial validated colorimetric (DNS) method for the determination of *endo-1,4-beta-xylanase*; *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* in the *feed additive* and ii) the single-laboratory validated and further verified colorimetric methods for the determination of *endo-1,4-beta-xylanase*; *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* in *premixtures* and *compound feed*.

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

For the determination of *endo-1,4-beta-xylanase* in the *feed additive*:

- colorimetric (DNS) method based on the enzymatic hydrolysis of the wheat arabinoxylan substrate
- For the determination of *endo-1,3(4)-beta-glucanase* in the *feed additive*:
  - colorimetric (DNS) method based on the enzymatic hydrolysis of the barley betaglucan substrate

For the determination of *endo-1,4-beta-glucanase* in the *feed additive*:

- colorimetric (DNS) method based on the enzymatic hydrolysis of the carboxy-methyl-cellulose substrate

For the determination of *endo-1,4-beta-xylanase* in *premixtures* and *compound feed*:

- colorimetric method based on the enzymatic reaction on the azo-xylan (birchwood) substrate

For the determination of *endo-1,3(4)-beta-glucanase* in *premixtures* and *compound feed*:

- colorimetric method based on the enzymatic reaction on the azo-barley-glucan substrate

For the determination of *endo-1,4-beta-glucanase* in *premixtures* and *compound feed*:

- colorimetric method based on the enzymatic reaction on the azo-carboxy-methyl-cellulose substrate

One *endo-1,4-beta-xylanase* unit (U) is the amount of enzyme which releases 1 micromole of reducing sugar (xylose equivalent) per minute from wheat arabinoxylan at 40 °C and pH 5.0

One *endo-1,3(4)-beta-glucanase* unit (U) is the amount of enzyme which releases 1 micromole of reducing sugar (glucose equivalent) per minute from barley beta-glucan at 40 °C and pH 5.0.

One *endo-1,4-beta-glucanase* unit (U) is the amount of enzyme which releases 1 micromole of reducing sugar (glucose equivalent) per minute from carboxy-methyl-cellulose at 40 °C and pH 5.0.

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *preparation of endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* 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 SANTE/E5: FORW. APPL. 1831-0042-2021
- [2] \*Application: Annex I – submission number 1616063688614-2904
- [3] \*Technical dossier, Section II: 2.1 Identity of the additive
- [4] Commission Implementing Regulation (EU) 403/2013 of 2 May 2013 concerning the authorisation of a preparation of *endo-1,4-beta-xylanase, endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-glucanase* produced by *Trichoderma reesei* (ATCC 74444) as a feed additive for poultry for fattening and for laying and for weaned piglets and amending Regulations (EC) No 1259/2004, (EC) No 1206/2005 and (EC) No 1876/2006 (holder of authorisation DSM Nutritional Products)
- [5] \*Technical dossier, Section II: 2.2 Characterisation of the active substance(s)/agent(s)
- [6] Suppl. information: Annex 0- König 2002\_Determination of xylanase glucanase cellulase activity.pdf
- [7] \*Technical dossier, Section II: 2.5 Conditions of use of the additive
- [8] EURL evaluation Report (FAD-2007-0036)  
[https://joint-research-centre.ec.europa.eu/publications/fad-2007-0036\\_en](https://joint-research-centre.ec.europa.eu/publications/fad-2007-0036_en)
- [9] \*Technical dossier, Section II: Annex 2-18\_Per se stability 24 months Multigrain GT.pdf

- [10] \*Technical dossier, Section II: Annex 2-19\_Per se stability 24 months Multigrain L
- [11] Suppl. information:: Annex IId Method Ronozyme MultiGrain\_PREMIX-102\_01E.pdf
- [12] Suppl. information: Annex IIIId Verification Study Report Premix-102\_01E.pdf
- [13] Suppl. information: Annex IVa Verification Report MultiGrain Xylanase.pdf
- [14] Suppl. information: Annex IVb Verification Report MultiGrain Glucanase.pdf
- [15] Suppl. information: Annex IVc Verification Report MultiGrain Cellulase.pdf
- [16] Suppl. information: Annex Va Method Ronozyme MultiGrain\_XYL-101\_02E\_new.pdf
- [17] Suppl. information: Annex Vb Method Ronozyme MultiGrain\_CEL-101\_01E\_new.pdf
- [18] Suppl. information: Annex IIb Method Ronozyme MultiGrain\_GLU-101\_02E.pdf
- [19] Suppl. information: Annex IIIa Verification Study Report XYL-101\_02E.pdf
- [20] Suppl. information: Annex IIIb Verification Study Report GLU-101\_02E.pdf
- [21] Suppl. information: Annex IIIc Verification Study Report CEL-101\_01E.pdf

\*Refers to Dossier no: FAD-2021-0052

## **7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES**

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

## **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)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)