



EUROPEAN COMMISSION  
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)  
European Union Reference Laboratory for Feed Additives

 Ref. Ares(2019)3101222 - 10/05/2019

JRC F.5/CvH/MGH/AS/Ares

**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**

**Endo-1,4-beta-xylanase (4a8)**  
*(FAD-2018-0071; CRL/180062)*



**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**

Dossier related to: **FAD-2018-0071 - CRL/180062**

Name of Feed Additive: ***Endo-1,4-beta-xylanase produced by  
Trichoderma reesei (CBS 114044) (4a8)***

Active Agent (s): **Endo-1,4-beta-xylanase (E.C. 3.2.1.8)**

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: **Stefano Bellorini**  
Date: **07/05/2019**

Report approved by: **Christoph von Holst**  
Date: **10/05/2019**

## EXECUTIVE SUMMARY

*Econase*<sup>®</sup> *XT* is the trade name of a *feed additive* containing as active substance *endo-1,4-beta-xylanase* (EC 3.2.1.8) produced from *Trichoderma reesei* (CBS 114044). This *feed additive* is currently authorised by different Commission Regulations under the category/functional 4(a) "zootechnical additives"/"digestibility enhancers" according to the classification system of Annex I of Regulation (EC) No 1831/2003 (*feed additive* identification number 4a8). In the current application a renewal of the *feed additive* authorisation under Article 14 of the Regulation (EC) No 1831/2003 is requested for different avian and porcine species.

The *endo-1,4-beta-xylanase* activity is expressed in BXU units, where "one BXU is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as xylose equivalents, from birch xylan at pH 5.3 and 50 °C". The *feed additive* is intended to be marketed as light-brown powder formulations (*Econase XT 25*; *Econase XT 5 P* and *Econase XT P*) or as brown liquids (*Econase XT 25L* and *Econase XT L*) with minimum activities ranging from 160000 BXU/g to 4000000 BXU/g. *Endo-1,4-beta-xylanase (4a8)* is intended to be incorporated directly or through *premixtures* at a minimum *endo-1,4-beta-xylanase* activity in *feedingstuffs* of 8000, 16000 or 24000 BXU/kg depending on the target species.

For the quantification of the activity of *endo-1,4-beta-xylanase* in the *feed additive* and *premixtures* the Applicant submitted a single-laboratory validated and further verified spectrophotometric method, based on the formation of reducing sugars reacting with 3,5-dinitrosalicylic acid (DNS) at pH 5.3 and 50 °C while for the *feedingstuffs* the Applicant submitted a different single-laboratory validated and further verified spectrophotometric method based on the quantification of water soluble dye fragments produced at pH 5.0 and 50 °C by the action of *endo-1,4-β-xylanase* on commercially available azurine cross-linked wheat arabinoxylan substrates from Megazyme. For the *feed additive* and *premixtures* external calibration is performed using a commercially available xylose standard, while for *feedingstuffs* external calibration is carried out using a xylanase standard with known enzyme activity and subjected to the same experimental conditions than the *feedingstuffs* samples. For all matrices the measurements are performed by spectrophotometry at 540 nm.

According to the results provided by the Applicant in the frame of the respective validation and verification studies, relative standard deviations for repeatability (RSD<sub>r</sub>) and for intermediate precision (RSD<sub>ip</sub>) ranging from 2.1 to 8.9 % and from 4.1 to 7.2 %, respectively, were obtained for the quantification of the activity of *endo-1,4-beta-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*.

Based on the performance characteristics available the EURL recommends for official control these methods for the quantification of the total *endo-1,4-beta-xylanase* activity in these three matrices.

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

*Endo-1,4-beta-xylanase (4a8)*, *Econase*<sup>®</sup> *XT*, zootechnical additive, digestibility enhancers, turkeys and chickens for fattening, turkeys reared for breeding, chickens reared for laying and piglets (weaned)

## 1. BACKGROUND

*Econase*<sup>®</sup> *XT* is the trade name of a *feed additive* containing as active substance *endo-1,4-beta-xylanase* (EC 3.2.1.8) produced from *Trichoderma reesei* (CBS 114044) [1]. This feed additive is currently authorized for weaned piglets, chickens for fattening, chickens reared for laying, turkeys for fattening and turkeys reared for breeding [2] and for laying hens, minor poultry species and pigs for fattening [3] [4], under the category/functional 4(a) "zootechnical additives"/"digestibility enhancers" according to the classification system of Annex I of Regulation (EC) No 1831/2003 (*feed additive* identification number 4a8) [1][5][6]. In the current application a renewal of the *feed additive* authorisation under Article 14 of the Regulation (EC) No 1831/2003 is requested for weaned piglets, chickens for fattening, chickens reared for laying, turkeys for fattening and turkeys reared for breeding [1][5].

The *endo-1,4-beta-xylanase* activity is expressed in BXU units, where "one BXU is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as xylose equivalents, from birch xylan at pH 5.3 and 50 °C" [1].

*Endo-1,4-beta-xylanase (4a8)* is intended to be marketed as light-brown powder formulations (*Econase XT 25*; *Econase XT 5 P* and *Econase XT P*) or as brown liquids (*Econase XT 25L* and *Econase XT L*) with a minimum activities ranging from 160000 to 4000000 BXU/g [7].

The *feed additive* is intended to be incorporated directly or through *premixtures* at a minimum *endo-1,4-beta-xylanase* activity in *feedingstuffs* of 8000, 16000 or 24000 BXU/kg depending on the target species [1][8].

Note: The analytical methods for the quantification of *endo-1,4-beta-xylanase (4a8)* in the relevant matrices were already evaluated and recommended by the EURL in the frame of previous dossiers [9].

## 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 *endo-1,4-beta-xylanase (4a8)* 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* activity in the *feed additive* and *premixtures* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released xylose with dinitrosalicylic acid (DNS) [10]. The assay is based on the enzymatic hydrolysis of xylanase on the beech xylan substrate at pH 5.3 and 50 °C.

According to the Applicant, the change of substrate, from birch to beech xylan for the quantification of the *endo-1,4-beta-xylanase* activity was triggered by the lack of a commercially available birch xylan substrate. Consequently, upon request of the EURL the Applicant provided experimental evidence proving that the change of the substrate did not have a significant impact on the determination of the *endo-1,4-beta-xylanase* activity in the product subject of this Application [11].

The *feed additive* sample is extracted with 0.05 M citrate buffer (pH 5.3), and for solid *feed additive* formulations the solution is stirred for 30 min at room temperature, let stand for few minutes and diluted appropriately. For *premixture* an aliquot is extracted with 40 ml of 0.05 M citrate buffer (pH 5.3) containing 2 % (w/v) ethylenediaminetetraacetic acid (EDTA), stirred for 30 min at room temperature and centrifuged 10 min followed by appropriate dilution.

The obtained supernatants (0.2 ml) are placed into a test tube together with 1.8 ml of the beechwood xylan substrate (1.0 % and pH 5.3) and shaken on a vortex mixer before incubation at 50 °C for 5 min. After incubation 3.0 ml of a DNS solution are added to the tubes. The activity of *endo-1,4-beta-xylanase* is then determined by colorimetry at 540 nm using a xylose standard (external) calibration curve [10].

Additionally, for the quantification of *endo-1,4-beta-xylanase* in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of the water soluble dye fragments produced by the action of xylanase on a commercially available azurine cross-linked wheat arabinoxylan substrate (Megazyme) at pH 5.0 and 50 °C [12].

Two aliquots of 2.5 g ground feed are mixed with 20 ml of 0.05 M acetate buffer (pH 5.0) and stirred for 30 min. An aliquot of the mixture (10 ml) is then centrifuged for another 10 min. An aliquot (1.0 ml) of the obtained supernatant is equilibrated at 50 °C for 5 min, a Xylazyme tablet containing the azurine cross-linked wheat arabinoxylan substrate is then added, and incubated at 50 °C during 30 min.

The reaction is stopped by adding 5.0 ml of a stop solution (Trizma base solution 1 %). Samples are vigorously mixed, let cool down for 5 min and mixed again. Finally, the solutions are filtered through paper filter and the absorbance of the filtrate is measured against a blank at 590 nm. External calibration is performed with xylanase with known enzyme activity which was subjected to the same experiments and measured by spectrophotometry at 590 nm. Tables 1 and 2 present the performance characteristics reported by the Applicant based on experimental data obtained in the frame of the validation [13] and verification [14,15,16] studies.

**Table 1:** Performance characteristics for the determination of *endo-1,4-beta-xylanase* activity in the *feed additive (FA) premixtures (PM)* and *feedingstuffs (FS)* obtained in the frame of the validation (Val) and verification (Ver) studies.

Matrices	RSD <sub>r</sub> (%)		RSD <sub>ip</sub> (%)		R <sub>Rec</sub> (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	2.1-3.7 [13]	3.7 [14]	4.1-7.2 [13]	4.5 [14]	100-101 [13]	107 [14]
PM	3.9 [13]	8.9 [15]	4.6-23 [13]	7.2 [15]	81-91 [13]	86 [15]
FS	4.4-6.7 [13]	7.6 [16]	4.5-6.9 [13]	6.69 [16]	101-110 [13]	121 [16]

RSD<sub>r</sub>; RSD<sub>ip</sub>: relative standard deviation for *repeatability* and *intermediate precision*; R<sub>Rec</sub>: *Recovery rate*

Based on the performance characteristics available the EURL recommends for official control the single-laboratory validated and further verified methods proposed by the Applicant for the quantification of the total *endo-1,4-beta-xylanase* activity in the *feed additive, premixtures* and *feedingstuffs*.

---

***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)***

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

***Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)***

The evaluation of corresponding methods of analysis is not considered necessary by the EURL.

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 the validated and further verified colorimetric methods for the quantification of *endo-1,4-beta-xylanase* in the *feed additive, premixtures* and *feedingstuffs*.

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

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

- colorimetric method based the enzymatic reaction of *endo-1,4-beta-xylanase* on the birch xylan substrate at pH 5.3 and 50 °C

for the quantification of *endo-1,4-beta-xylanase* in *feedingstuffs*:

- colorimetric method based the enzymatic reaction of *endo-1,4-beta-xylanase* on the azurine cross-linked wheat arabinoxylan substrate at pH 5.3 and 50 °C

One *endo-1,4-beta-xylanase* unit (BXU) is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as xylose equivalents, from birch xylan at pH 5.3 and 50 °C.

#### **5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL**

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *endo-1,4-beta-xylanase (4a8)* 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, Proposal for Register Entry – Annex A
  - [2] Commission Regulation (EC) No 902/2009 of 28 September 2009 concerning the authorisation of an enzyme preparation of endo-1,4-beta-xylanase produced by *Trichoderma reesei* (CBS 114044) as a feed additive for weaned piglets, chickens for fattening, chickens reared for laying, turkeys for fattening and turkeys reared for breeding (holder of authorisation Roal Oy)
  - [3] Commission Implementing Regulation (EU) No 1110/2011 of 3 November 2011 concerning the authorisation of an enzyme preparation of endo-1,4-beta-xylanase produced by *Trichoderma reesei* (CBS 114044) as a feed additive for laying hens, minor poultry species and pigs for fattening (holder of authorisation Roal Oy)
  - [4] Commission Implementing Regulation (EU) 2018/1569 of 18 October 2018 amending Implementing Regulation (EU) No 1110/2011 concerning the authorisation of an enzyme preparation of endo-1,4-beta-xylanase produced by *Trichoderma reesei* (CBS 114044) as a feed additive for laying hens, minor poultry species and pigs for fattening (holder of authorisation Roal Oy)
  - [5] \*Application, Reference SANTE/E5: FORW. APPL. 1831-0074-2018
  - [6] \*Application, Annex 1 (Submission No:1538377219331-2292)
  - [7] \*Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.1.3 Qualitative and quantitative composition
  - [8] \*Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.5 Conditions of use
  - [9] EURL Evaluation Reports:  
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2007-0020.pdf>  
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0006.pdf>
  - [10] \*Technical dossier, Section II, Annexes II.10
  - [11] Supplementary Information – Research\_report\_212058.pdf
  - [12] \*Technical dossier, Section II, Annexes II.56
  - [13] \*Technical dossier, Section II, Annexes II.51
  - [14] \*Technical dossier, Section II, Annexes II.52
  - [15] \*Technical dossier, Section II, Annexes II.53
  - [16] \*Technical dossier, Section II, Annexes II.55
- \*Refers to Dossier no: FAD-2018-0071

## 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)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)