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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

Econase® XT,
(FAD-2019-0029; CRL/190003)

European Union Reference Laboratory
Feed Additives

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-2019-0029 - CRL/190003**
Name of Product:	***Econase® XT***
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**
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Report approved by: Date:	**Christoph von Holst** **22/11/2019**

EXECUTIVE SUMMARY

Econase® XT is the trade name of a *feed additive* preparation containing as active substance *endo-1,4-beta-xylanase* (EC 3.2.1.8) produced by *Trichoderma reesei* (CBS 140027). In the current application authorisation is sought under Article 4 (1) for *Econase® XT* under the category / functional group 4 (a) "zootechnical additives"/"digestibility enhancers" according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* 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 nanomol 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 5P* and *Econase XT P*) or as brown liquids (*Econase XT 25L* and *Econase XT L*) with minimum *endo-1,4-beta-xylanase* activities ranging from 160 000 to 4 000 000 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 8 000, 16 000 or 24 000 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), 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, by the action of *endo-1,4-β-xylanase* on commercially available azurine cross-linked wheat arabinoxylan substrates. 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 validation and verification studies, relative standard deviations for *repeatability* (RSD_r) and for *intermediate precision* (RSD_{ip}) ranging from 2.1 to 8.9 % and from 4.1 to 7.2 %, respectively, were obtained for the quantification of *endo-1,4-beta-xylanase* in the *feed additive, premixtures* and *feedingstuffs.* Additionally the Applicant estimated a limit of quantification (LOQ) of 4491 BXU/kg *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,, *Econase® XT*, zootechnical additive, digestibility enhancers, turkeys and chickens for fattening, turkeys reared for breading, chickens reared for laying laying hens, laying birds of minor poultry species, minor poultry species other than laying birds, piglets (weaned) and pigs for fattening

1. BACKGROUND

Econase® XT is the trade name of a *feed additive* preparation containing as active substance *endo-1,4-beta-xylanase* (EC 3.2.1.8) produced by *Trichoderma reesei* (CBS 140027) [1]. In the current application authorisation is sought under Article 4(1) (authorisation of a feed additive) for *Econase® XT* under the category / functional group 4(a) "zootechnical additives"/"digestibility enhancers" according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for the use of the *feed additive* for turkeys and chickens for fattening, turkeys reared for breading, chickens reared for laying, laying hens, laying birds of minor poultry species, minor poultry species other than laying birds, piglets (weaned) and pigs for fattening [1-2].

The *endo-1,4-beta-xylanase* activity is expressed in BXU units, where "one BXU is the amount of enzyme, which liberates one nanomol per second of reducing sugars, expressed as xylose equivalents, from birch xylan at pH 5.3 and 50 °C" [3].

Endo-1,4-beta-xylanase 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 *endo-1,4-beta-xylanase* activities ranging from 160 000 to 4 000 000 BXU/g [4-5].

The *feed additive* is intended to be incorporated directly or through *premixtures* at a minimum *endo-1,4-beta-xylanase* activity in *feedingstuffs* of 8 000, 12 000, 16 000, 20 000 or 24 000 BXU/kg depending on the target species[1, 6].

Note: The analytical methods for the quantification of *endo-1,4-beta-xylanase* in the relevant matrices were already evaluated and recommended by the EURL in the frame of previous dossiers [7].

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 *Econase® XT* 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 colourimetric method, based on the colour formation of released xylose with dinitrosalycilic acid (DNS) [8]. The method is based on the enzymatic hydrolysis of xylanase on the beech xylan substrate at pH 5.3 and 50 °C.

In the frame of a previous *endo-1,4-beta-xylanase* dossier [5] the Applicant justified the change of substrate, from birch to beech xylan for the quantification of the *endo-1,4-beta-xylanase* activity for the lack of a commercially available birch xylan substrate. Additionally, 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 [9]. Consequently, the EURL considered these evidences also applicable for the new product subject of this Application.

A sample of the solid *feed additive* is extracted with 0.05 M citrate buffer (pH 5.3), stirred for 30 min at room temperature, let stand for few minutes and diluted appropriately. For the liquid formulations of the *feed additive* the sample is directly diluted with the citrate buffer. For *premixtures* an aliquot of the sample 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, centrifuged for 10 min and the supernatants are appropriately diluted.

An aliquot (0.2 ml) of the diluted supernatants is placed into a test tube together with 1.8 ml of the beechwood xylan substrate (1.0 % (w/v) 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 colourimetry at 540 nm using a xylose standard (external) calibration curve [8].

For the quantification of *endo-1,4-beta-xylanase* in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colourimetric 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 [10].

Two aliquots of 2.5 g ground *feedingstuffs* 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 ^{o}C for 5 min, the azurine cross-linked wheat arabinoxylan substrate is then added, and incubated at 50 ^{o}C for 30 min.

The reaction is stopped by adding 5.0 ml of a stop solution (Trizma base solution 1 % (w/v)). Samples are vigorously mixed, let to 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 using xylanase with known enzyme activity expressed in BXU and available from the Applicant upon request. These calibration solutions were subjected to the same experiments as the unknown samples and measured by spectrophotometry at 590 nm. Table 1 presents acceptable performance characteristics reported by the Applicant based on experimental data obtained in the frame of the validation [11] and verification [12-14] studies. Additionally the Applicant estimated a limit of quantification (LOQ) of 4491 BXU/kg *feedingstuffs* [11].

Table 1: Performance characteristics for the colourimetric 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_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	2.1-3.7 [11]	3.7 [12]	4.1-7.2 [11]	4.5 [12]	100-101 [11]	107 [12]
PM	3.9 [11]	8.9 [13]	4.6-23 [11]	7.2 [13]	81-91 [11]	86 [13]
FS	4.4-6.7 [11]	7.6 [14]	4.5-6.9 [11]	6.7 [14]	101-110 [11]	121 [14]

RSD_r; RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision;* R_{Rec}*: Recovery rate*

Table 2: Precision performance characteristics as recalculated by the EURL for the colourimetric determination of *endo-1,4-beta-xylanase* activity in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS) obtained in the frame of the stability studies [15-17].

Matrices	RSD_r (%)	RSD_{ip} (%)
	Validation	Verification
FA	2.8-6.6 [18]	2.8-7.5 [18]
PM	5.8-7.2 [19]	6.2-11.2 [19]
FS (mash)	6.0-9.9 [20]	6.5-9.9 [20]
FS (pellets)	7.4-8.4 [21]	8.2-9.6 [21]

RSD_r; RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*

These studies, however, have been carried out with an *endo-1,4-beta-xylanase* [7] currently authorised and produced by *Trichoderma reesei* (CBS 114044) which is a different production strain than the one from the current application. To check, whether the performance of the method assessed using the data from the previous studies also applies to the activity measurement of the current application, the results of the enzyme stability studies as presented in the current application [15-17] were further investigated.

In these stability studies [15-17] the Applicant applied the methods described above to the products subject of this authorisation request, i.e. *endo-1,4-beta-xylanase* (EC 3.2.1.8) produced by *Trichoderma reesei* (CBS 140027) leading to similar precision values. Table 2 presents the performance characteristics recalculated by the EURL [18-21] and based on experimental data obtained by the Applicant in the frame of the stability studies [15-17]. Consequently, the EURL considers the proposed method suitable for the quantification of the total activity of *endo-1,4-beta-xylanase* (EC 3.2.1.8) produced by *Trichoderma reesei* (CBS 140027) in these three matrices.

For the quantification of the *endo-1,4-beta-xylanase* in *feedingstuffs* the Applicant presented an additional single-laboratory validated [22] and further verified [23] Enzyme Linked Immunosorbent Assay (ELISA) method [24], showing acceptable method performance characteristics. However, according to the Applicant this method is specific for feeds containing *Econase XT* [22], whereas the measurement principle of the previously described method is applied in the majority of methods used for the activity measurements of the other xylanase *feed additives* currently authorised. Given the generally accepted requirement of official control laboratories to use the same measurement principle for as many as possible analytes, the EURL does not recommend the ELISA method for official control purposes.

Based on the performance characteristics available the EURL recommends for official control the single-laboratory validated and further verified colourimetric methods submitted 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 colourimetric methods submitted by the Applicant 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*:

- colourimetric method based the enzymatic reaction of endo-1,4-beta-xylanase on the beech xylan substrate

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

- colourimetric method based the enzymatic reaction of *endo-1,4-beta-xylanase* on the azurine cross-linked wheat arabinoxylan substrate

One *endo-1,4-beta-xylanase* unit (BXU) is the amount of enzyme, which liberates one nanomol 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* 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. 1831-0033-2019

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

[3] Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.6 Methods of analysis and reference samples

[4] *Application, Annex 1 (Submission No:1555592750133-2402)

[5] *Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.1.5 Physical state of each form of the product

[6] *Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.5 Conditions of use

[7] 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
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0071-econasext.pdf

[8] *Technical dossier, Section II, Annexes II.11

[9] Supplementary Information FAD 2018-0071– Research_report_212058.pdf

[10] *Technical dossier, Section II, Annexes II.63

[11] *Technical dossier, Section II, Annexes II.58

[12] *Technical dossier, Section II, Annexes II.59

[13] *Technical dossier, Section II, Annexes II.60

[14] *Technical dossier, Section II, Annexes II.62

[15] *Technical dossier, Section II, Annexes II.29; II.30; II.31; II.32; II.33

[16] *Technical dossier, Section II, Annexes II.37a; II.37b and II.37c

[17] *Technical dossier, Section II, Annexes II.40a; II.40b and II.40c

[18] Supplementary Information, ANOVA_EURL_FA.pdf

[19] Supplementary Information, ANOVA_EURL_PM.pdf

[20] Supplementary Information, ANOVA_EURL_FS_P.pdf

[21] Supplementary Information, ANOVA_EURL_FS_M.pdf

[22] *Technical dossier, Section II, Annexes II.64

[23] *Technical dossier, Section II, Annexes II.65

[24] *Technical dossier, Section II, Annexes II.66

*Refers to Dossier no: FAD-2019-0029

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

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