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Institute for Reference Materials and Measurements
**Community Reference Laboratory for Feed Additives**

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# CRL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

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| Dossier related to: | **FAD-2010-0006**<br>**CRL/ 100016** |
| Name of feed additive: | **Econase XT** |
| Active Agent (s): | **Endo-1,4-β-xylanase (E.C. 3.2.1.8)** |
| Rapporteur Laboratory: | **Community Reference Laboratory for Feed Additives (CRL-FA)**<br>**Geel, Belgium** |
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## EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Econase XT* under the category 'zootechnical additives', functional group 4(a) 'digestibility enhancers', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Econase XT* for laying hens, pigs for fattening and minor poultry species including ducks, geese, quails, pheasants and pigeons. The active agent of *Econase XT* is *endo-1,4-β-xylanase* (EC 3.2.1.8), produced by the strain *Trichoderma reesei* RF5427 (CBS 114044).

The activity of *endo-1,4-β-xylanase* is expressed as *xylanase* units (BXU). According to the Applicant, one BXU is the amount of enzyme that produces 1 nmol of reducing sugars from birch xylan as xylose in 1 second, under standard conditions (pH 5.3 and 50°C).

The product is intended to be marketed as liquid (*Econase XT L*) and powder (*Econase XT P*) formulations to be used in non-starch polysaccharides rich compound feed (mainly arabinoxylans). The main carriers of the powder and liquid formulations are wheat flour and water + sorbitol, respectively. The guaranteed minimum *endo-1,4-β-xylanase* activities are 400 000 BXU/g and 4 000 000 BXU/g in the liquid and powder formulation, respectively.

The minimum target activities of *endo-1,4-β-xylanase* in complete *feedingstuffs* are:

- 6000 BXU/kg for laying hens and minor poultry species; and
- 16000 BXU/kg for pigs for fattening.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive* and *premixtures*, the Applicant proposes 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. The following method performance characteristics were derived from the validation and verification studies:

* for the *feed additive*:
  - a relative standard deviation for *repeatability* ($RSD_r$) ranging from 2.1 to 3.7%;
  - a relative standard deviation for *intermediate precision* ($RSD_{int}$) ranging from 4.1 to 7.2 %; and
  - a *recovery rate* ($R_{Rec}$) ranging from 100 to 107 %

* for *premixtures*:
  - $RSD_r$ ranging from 3.9 to 8.9 %;
  - $RSD_{int}$ ranging from 4.6 to 23 %, and
  - $R_{Rec}$ ranging from 81 to 91 %.

For the determination of the activity of *endo-1,4-β-xylanase* in *feedingstuffs*, the Applicant proposes a single-laboratory validated and further verified method based on measurement of the rate of release of water soluble dyed fragments by *endo-1,4-β-xylanase* from the dye cross-linked wheat arabinoxylan in a form of "Xylazyme AX tablet". The following method performance characteristics were derived from the validation and verification studies for *feedingstuffs*:

- $RSD_r$ ranging from 4.4 to 7.6 %;
- $RSD_{int}$ ranging from 4.5 to 6.9 %;
- $R_{Rec}$ ranging from 101 to 121 %; and
- a limit of detection (LOD) and quantification (LOQ) of 750 and 2500 BXU/kg, respectively.

Based on the satisfactory performance characteristics reported, the CRL recommends for official control the single-laboratory validated and further verified colorimetric methods submitted by the Applicant for the determination of the activity of *endo-1,4-β-xylanase* 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**

*Econase XT*, *endo-1,4-β-xylanase*, *Trichoderma reesei,* digestibility enhancers, laying hens, pigs for fattening and minor poultry species including ducks, geese, quails, pheasants, pigeons

## 1. BACKGROUND

In the current application authorisation is sought for *Econase XT* under the category 'zootechnical additives', functional group 4(a) 'digestibility enhancers', according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation under article 4(1) (new use of a feed additive) is requested as the product has already been authorised by Commission Regulation (EC) 902/2009. Specifically, authorisation is sought for the use of *Econase XT* for laying hens, pigs for fattening and minor poultry species including ducks, geese, quails, pheasants and pigeons.

The active agent of *Econase XT* is *endo-1,4-β-xylanase* (EC 3.2.1.8), produced by the strain *Trichoderma reesei* RF5427 (CBS 114044) [2]. The production strain has been deposited at the Centraalbureau voor Schimmelcultures (CBS) in Utrecht, Netherlands [3].

The activity of *endo-1,4-β-xylanase* is expressed as *xylanase* units (BXU). According to the Applicant, one BXU is the amount of enzyme that produces 1 nmol of reducing sugars from birch xylan as xylose in 1 second, under standard conditions (pH 5.3 and 50°C) [4].

The product is intended to be marketed as liquid (*Econase XT L*) and powder (*Econase XT P*) formulations to be used in non-starch polysaccharides rich compound feed (mainly arabinoxylans). The main carriers of the powder and liquid formulations are wheat flour and water + sorbitol, respectively. The guaranteed minimum *endo-1,4-β-xylanase* activities are 400 000 BXU/g and 4 000 000 BXU/g in the liquid and powder formulation respectively [2].

The minimum target *endo-1,4-β-xylanase* activities in complete *feedingstuffs* are:

- 6000 BXU/kg for laying hens and minor poultry species [2]; and
- 16000 BXU/kg for pigs for fattening [2].

## 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 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 (EFSA) for each application or group of applications. For this 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

### *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, aflatoxin B1 and dioxins) are available from the respective Community Reference Laboratories [5].

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

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive* and *premixtures*, the Applicant proposes a single-laboratory validated and further verified spectrophotometric method [6], based on the measurement of reducing sugars released by the action of *endo-1,4-β-xylanase* on 1% birch xylan substrate in the presence of 3.5-dinitrosalicylic acid (DNS) at pH 5.3 and 50°C. The *feed additive* samples are prepared by extracting 0.5 g of feed additive in 25 ml of 0.05 M citrate buffer (pH = 5.3). The *premixture* samples are prepared by extracting 5 g of premixture in 40 ml of citrate buffer containing 2% EDTA, stirred for 30 minutes at room temperature and then centrifuged for 10 minutes. The samples are suitably diluted and 1.8 ml substrate solution containing 1% birch xylan is added. The samples are then incubated at 50$^{o}$C for 5 minutes. After exactly 5 minutes, 3.0 ml DNS solution is added. The blank sample undergoes a similar procedure, but the enzyme solution is added after the reaction is stopped by DNS. All the samples are then boiled for 5 minutes and cooled down to room temperature. The absorbance is then measured against the enzyme blank at 540 nm. A four points calibration curve is constructed using xylose (Merck 8689) standard solution for the determination of unknown enzyme activity. The method was single-laboratory validated [7] and further verified [8-11] and the reported performance characteristics are presented in Table 1. The Applicant attributes the high *intermediate precision* for piglet premixture (23%) to a stronger inhibitory effect due to some cations present in piglet premixtures at high concentration [7].

For the determination of the activity of *endo-1,4-β-xylanase* in *feedingstuffs*, the Applicant proposes a single-laboratory validated and further verified method based on the Megazyme method [12]. The method is based on measurement of the rate of release of water soluble dyed fragments by *endo-1,4-β-xylanase* from the dye cross-linked wheat arabinoxylan in a form of "Xylazyme AX tablet". The feed sample (2.5 g) is extracted with 20 ml 0.05 M acetate buffer (pH = 5.0) for 30 minutes at room temperature. After centrifugation, the supernatants are equilibrated for 5 minutes at 50°C and Xylazame AX tablet is added. The reaction is stopped after 30 minutes by addition of 1% Trizma Base and filtered. Colour intensity is measured at 590 nm on a spectrophotometer. The activity of the sample is calibrated against known amount of xylanase activity determined at the definition conditions of the activity unit (pH 5.3 and 50$^{o}$C).

The method was single-laboratory validated [7] and further verified [13, 14] and the reported performance characteristics are presented in Table 1. Furthermore, the Applicant investigated a limit of detection (LOD) and a limit of quantification (LOQ) analysing two blank *feedingstuffs* containing respectively 750 and 2500 BXU/kg of naturally occurring

(endogenous) *xylanase*. A total of 36 replicate samples per blank were analysed to derive a standard deviation (Std) of 250 BXU/kg. Applying the following formulas: LOD = $Y_0$ + 3*Std and LOD = $Y_0$ + 10*Std, they derive two sets of LOD and LOQs – depending on the endogenous *xylanase* content.

The CRL considers that above mentioned method does not distinguish between "endogenous" and "added" *xylanase*, but allows the determination of total *xylanase*. It is therefore assumed that only one set of LOD/LOQ characterises the method. The CRL computed this unique set after "blank" correction, to obtain LOD = 3*Std = 750 BXU/kg and LOQ = 10*Std = 2500 BXU/kg[1] of *feedingstuffs* [7].

Based on the satisfactory performance characteristics presented in Table 1, the CRL recommends for official control the single-laboratory validated and further verified colorimetric methods submitted by the Applicant for the determination of *endo-1,4-β-xylanase* 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.

**Table 1:** Method performance characteristics for the determination of *endo-1,4-β-xylanase* in *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

| | $RSD_r$ (%) | | $RSD_{int}$ (%) | | $R_{Rec}$ (%) | |
|---|---|---|---|---|---|---|
| | Validation | Verification | Validation | Verification | Validation | Verification |
| FA | 2.1-3.7 [7] | 3.7 [8,9] | 4.1-7.2 [7] | 4.5 [8,9] | 100-101 [7] | 107 [8,9] |
| PM | 3.9 [7] | 8.9 [10,11] | 4.6-23 [7] | 7.2 [10,11] | 81-91 [7] | 86 [10,11] |
| FS | 4.4-6.7 [7] | 7.6 [13,14] | 4.5-6.9 [7] | 6.9 [13,14] | 101-110 [7] | 121 [13,14] |

$RSD_r$,$RSD_{int}$ - relative standard deviation for *repeatability* and *intermediate precision*,
$R_{Rec}$ – recovery rate

[1] Upon the request by the CRL, the Applicant confirmed that 1 BXU = 1 FAXU [15]: - BXU when using birch xylan as substrate (for *feed additive* and *premixtures*) and - FAXU when using arabinoxylan as substrate for *feedingstuffs.*

## 4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the CRL recommends for official control the colorimetric methods submitted by the Applicant to determine the activities of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*.

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

Characterisation of the active substances in the *feed additive* and *premixtures*:

- Colorimetric method measuring reducing sugars released by action of *endo-1,4-β-xylanase* on birch xylan substrate in the presence of 3,5-dinitrosalicilic acid (DNS).

Characterisation of the active substances in the *feedingstuffs*:

- Colorimetric method measuring water soluble dye released by action of *endo-1,4-β-xylanase* from azurine cross-linked wheat arabinoxylan substrates.

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Econase XT* 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] *Application/Ref:SANCO/D/2: Forw. Appl. 1831/008-2010
[2] *Application, Proposal for Register Entry, Annex A
[3] *Technical Dossier, Section II, Annex II_conf_11_certificate of deposition
[4] *Technical Dossier, Section II, 2.6.1. Methods of analysis for the active substance
[5] 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
[6] *Technical Dossier, Section II, Annex II_41_assay method
[7] *Technical Dossier, Section II, Annex II_40_validation of asay methods
[8] *Technical Dossier, Section II, Annex II_40a_Verification feed additive
[9] *Technical Dossier, Section II, Annex II_40a_Verification feed additive 1
[10] *Technical Dossier, Section II, Annex_II_40a Verification Premix

[11] *Technical Dossier, Section II, Annex_II_40a Verification Premix 1
[12] *Technical Dossier, Section II, Annex II_42_in feed assay method
[13] *Technical Dossier, Section II, Annex_II_40a Verification Feedingstuffs
[14] *Technical Dossier, Section II, Annex_II_40a Verification Feedingstuffs 1
[15] *Supplementary Information, E-mail from Applicant 17 September 2010
* Refers to Dossier No. FAD-2010-0006

## 7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was the Community 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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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)