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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**EFSA-Q-2007-109** **FAD-2007-0018**
Product name:	**Danisco Xylanase L and G**
Active Substance(s):	**Endo-1,4-β-xylanase (EC 3.2.1.8)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA)** **Geel, Belgium**
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Date:	**09/10/2007**
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Date:	**10/10/2007**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Danisco Xylanase* under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Danisco Xylanase* as a digestibility enhancer for turkeys for fattening. The product is intended to be marketed as a powder (*Danisco Xylanase G*) and as liquid formulation (*Danisco Xylanase L*).

The active agent of *Danisco Xylanase* is endo-1,4-β-xylanase produced by a strain of *Trichoderma reesei* (ATCC PTA 5588). The enzymatic activity is expressed in units (U). One U is the amount of endo-1,4-β-xylanase that liberates 0.5 μmol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50^{o}C. The product has a target activity of 40000 U/g. *Danisco Xylanase G* is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas *Danisco Xylanase L* is sprayed directly onto feed to obtain an enzyme activity level of 1250 to 2500 U/kg in *feedingstuffs*.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a colorimetric method based on the quantification of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on commercially available cross-linked xylan substrates. Enzymatic activity of the sample is calculated using a reference enzyme standard. The applicant introduced some adaptations to the protocol. The modified methods have been single laboratory validated.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive,* the applicant proposes a method which measures the enzyme-catalysed formation of water soluble dyed fragments released from cross-linked wheat arabinoxylan. The rate of release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard, available from the applicant upon request. The analysis – however – is carried out at *different* conditions (pH 4.0 and 40^{o}C on a cross-linked wheat arabinoxylan) compared to those given in the proposed register entry (pH 5.3 and 50^{o}C on a cross-linked oat spelt xylan) and the enzymatic activity is calibrated against a reference enzyme of which the activity is obtained applying the conditions of the proposed register entry. Method performance characteristics include a limit of detection (LOD) of 1.2 U/g, limit of quantification (LOQ) of 1.5 U/g products and a relative standard deviation for repeatability (RSD_r) of 4.4%.

For the determination of the activity of endo-1,4-β-xylanase in *premixtures,* the applicant proposes a method based on the same principle as described above, but employing a different extraction procedure. The measurements are carried out at pH 5.3 and 40^{o}C on a cross-linked wheat arabinoxylan. Method performance characteristics include a LOD of 13.0 U/g, LOQ of 19.3 U/g, an RSD_r of 3.5 % and recovery rates of 96.4 %.

For the quantification of the activity of endo-1,4-β-xylanase in *feedingstuffs,* the applicant proposes a method, based on the same principle as described above, measuring enzymatic activity on a cross-linked wheat arabinoxylan at pH 4.2 and 50°C. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzyme, available from the applicant. Method performance characteristics include a LOD of 285 U/kg, a LOQ of 530 U/kg, a RSD_r of 7.5% and a recovery rate of 97%. In the case that identical blank feed samples are *not* available, a standard addition technique is employed.

Though the methods proposed by the applicant are based on well known principles and show acceptable performance characteristics, the CRL is concerned that the suggested approach of measuring the enzymatic activity at *different* conditions compared to the conditions of the proposed register entry and to the conditions of the determination of the activity of a reference enzyme, introduces additional uncertainty into the measurements. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions specified in the register entry.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as proposed in the Register entry, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

KEYWORDS

Danisco Xylanase, endo-1,4 β-xylanase, *Trichoderma reesei,* digestibility enhancer, turkeys for fattening

1. BACKGROUND

Danisco Xylanase is a product for which authorisation as feed additive is sought under the category 'zootechnical additives', functional groups 'digestibility enhancers', according to Annex I of Regulation (EC) No 1831/2003 [1]. It contains endo-1,4-β-xylanase as the active agent [2], produced by a strain *Trichoderma reesei* (ATCC PTA 5588), which has been deposited at the American Type Culture Collection (ATCC) in Manassas, VA, USA [3]. The activity of endo-1,4-β-xylanase is expressed as units (U). According to the applicant, one U is the amount of enzyme which liberates 0.5 μmol of reducing sugar (expressed as xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50^{o}C [4]. The product is intended to be marketed in two forms:

- *Danisco Xylanase G*, that is a solid formulation with a target endo-1,4-β-xylanase activity of 40000 U/g;
- *Danisco Xylanase L*, that is a liquid formulation with a target endo-1,4-β-xylanase activity of 40000 U/g.

Danisco Xylanase is intended to be mixed into *premixtures* and/or complete *feedingstuffs* to obtain enzyme activity levels of 1250 to 2500 U/kg in complete *feedingstuffs* for turkeys for fattening [4].

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 *Danisco Xylanase*, *cf*. EFSA-Q-2007-109, 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 quantitative composition of impurities in the additive

For the determination of arsenic and heavy metals (cadmium, mercury and lead), microbiological agents and mycotoxins, the applicant proposes well known methods published and approved by national standard offices [5] and therefore considered suitable for intended purposes. For official controls, various internationally accepted standard methods based on the same analytical techniques and routinely applied by official control authorities are available and recommended by the CRL.

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

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a colorimetric method based on the quantification of water soluble dyed fragments produced by the action of endo-1,4 β-xylanase on commercially available cross-linked xylan substrates. Enzymatic activity of the sample is calculated using a reference enzyme standard. The applicant introduced some adaptations to the protocol. The modified methods have been single laboratory validated [6,7,8].

When analysing the *feed additive*, three replicates of 1.0 g of either dry or liquid additive are extracted in 100 ml of extraction buffer and incubated with azurine cross-linked wheat arabinoxylan. The rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard, available from the applicant upon request. The analysis – however – is carried out at *different* conditions (pH 4.0 and 40°C on a cross-linked wheat arabinoxylan) compared to those given in the proposed Register entry (pH 5.3 and 50°C on a cross-linked oat spelt xylan) [4] and the enzymatic activity is calibrated against a reference enzyme of which the activity is obtained applying the conditions of the proposed Register entry. Method performance characteristics include a limit of detection (LOD) of 1.2 U/g, limit of quantification (LOQ) of 1.5 U/g products and a relative standard deviation for repeatability (RSD_r) of 4.4% [6].

When analysing *premixtures,* 1.0 g of sample is first suspended in 50 ml solution of ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), further diluted in sodium acetate buffer and incubated with cross-linked wheat arabinoxylan for 10 min. at 40°C and pH 5.3. Method performance characteristics include a LOD of 13.0 U/g, LOQ of 19.3 U/g, an RSD_r of 3.5 % and recovery rates of 96.4 % [7].

For the quantification of the activity of endo-1,4-β-xylanase in *feedingstuffs,* the applicant proposes a method, based on the same principle as described above, measuring enzymatic activity on cross-linked wheat arabinoxylan at pH 4.2 and 50°C for 60 min. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzyme, available from the applicant. Method performance characteristics include a LOD of 285 U/kg, a LOQ of 530 U/kg, an RSD_r of 7.5% and the recovery rates of 97% [8]. In the case that identical blank feed samples are *not* available, a standard addition technique is employed.

There are several published and ring-trial validated methods for the determination of the activity of endo-1,4-β-xylanase in different matrices. When measuring the activity of xylanase in a *product*, Bailey *et al*. (1992) reported RSD_R values of about 17% to 30%, depending on the substrate used [9]. Cosson *et al.* (1999) obtained RSD_r of 4.6 to 11.9% for the assay of xylanase in *feedingstuffs* using a dyed xylan substrate [10]. König *et al*. (2002) tested xylanase *products* in four laboratories using a wheat arabinoxylan and measuring the amount of released reducing sugars. The obtained within-laboratory RSD_R varied from 4.4 to 5.3% [11]. However, none of these studies were performed on *Danisco Xylanase*. For this reason, their applicability to the analysis of *Danisco Xylanase* cannot be evaluated. Nevertheless, the obtained precision data reported in this dossier could be compared with corresponding data from the below mentioned studies and are considered acceptable.

4. CONCLUSIONS AND RECOMMENDATIONS

The proposed methods for the determination of the enzyme activity in the various matrices show an acceptable performance profile. However, the different methods apply different analytical conditions, whereas a harmonised system would be required. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions specified in the Register entry.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as proposed in the Register entry, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Danisco Xylanase 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. 1831/14-2007.

[2] Main dossier, Section II, Subject 2, Item 1.3.

[3] Main dossier, Section II, Reference B 9.

[4] Annex III, Proposal of Register entry.

[5] Main dossier, Section II, Subject 2, Item 1.4.

[6] Main dossier, Section II, Reference B 21.

[7] Main dossier, Section II, Reference B 23.

[8] Main dossier, Section II, Reference B 22.

[9] Bailey, M.J. *et al.* Journal of Biotechnology, 23 (1992) 257-270.

[10] Cosson, T. *et al*. Animal Feed Science and Technology, 77 (1999) 345-353.

[11] König, J. *et al*. Anal. Bioanal. Chem., 374 (2002) 80-87.

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium.

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