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Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II – 5.2 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**EFSA-Q-2006-119** **FAD-2006-0019**
Name of Additive:	**Natugrain Wheat® TS and TS L**
Active Substance(s):	**Endo-1,4-β-xylanase (E.C. 3.2.1.8)**
Rapporteur Laboratory:	**Plantedirektoratet, Lyngby, Denmark**
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EXECUTIVE SUMMARY

In the current application authorisation is sought for *Natugrain Wheat® TS* and *TS L*, under the category 'zootechnical additives' and the functional group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, *cf*. EFSA-Q-2006-119, authorisation is sought to use *Natugrain Wheat® TS* and *TS L* as a digestibility enhancer for turkeys for fattening.

The active agent of *Natugrain Wheat® TS* and *TS L* is a thermostable endo-1,4-β-xylanase, produced by a strain of *Aspergillus niger (*CBS 109.713*).* Enzymatic activity is expressed in thermostable xylanase units (TXU). One TXU is defined as the amount of enzyme that liberates 5 μmol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH 3.5 and 55^{o} C. The additive is intended to be marketed as a powder (*Natugrain Wheat® TS*) and as liquid formulation (*Natugrain Wheat® TS L*). *Natugrain Wheat® TS* and *Natugrain Wheat® TS L* contain 5600 TXU/g of product and are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of 400 to 800 TXU/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 an *in-house* developed and validated viscosimetric method based on the fact that endo-1,4-β-xylanase catalyses the hydrolysis of glycosidic bonds in the substrate wheat arabinoxylan. The decrease in sample viscosity, expressed in terms of a drop time, is a measure for the endo-1,4-β-xylanase's activity and is determined using a falling ball viscosimeter at pH 3.5 and 55^{o}C. The quantification is performed via an endo-1,4-β-xylanase's standard curve based on reference enzyme obtainable from the applicant. Method performance characteristics, when measured on various matrices (*feed additive, premixtures* and *feedingstuffs*), include relative standard deviation for repeatability (RSD_r) of 2.4 to 5.7%, within-laboratory relative standard deviation for reproducibility (RSD_R) of 2.4 to 11.1% and recovery rates ranging from 85 to 115%. The limit of detection (LOD) is 11 TXU/kg and the limit of quantification (LOQ) is 36 TXU/kg *feedingstuffs*. Based on acceptable performance characteristics, the method is considered to be suitable for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

KEYWORDS

Natugrain Wheat® TS, Natugrain Wheat® TS L, endo-1,4-β-xylanase, digestibility enhancer, *Aspergillus niger*

BACKGROUND

Natugrain Wheat® TS is a feed additive for which authorisation is sought under the category 'zootechnical additives' and the functional group 'digestibility enhancers', according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. It contains endo-1,4-β-xylanase as the active agent [2], produced by a strain of *Aspergillus niger* [3], which has been deposited at Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands, under the register number CBS 109.713 [4]. Enzymatic activity is expressed in thermostable xylanase units (TXU). According to the applicant, one TXU is defined as the amount of enzyme that liberates 5 μmol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH 3.5 and 55^{o} C [1].

The additive is intended to be marketed as a powder (*Natugrain Wheat® TS*) and as liquid formulation (*Natugrain Wheat® TS L*). Both formulations have a target enzymatic activity of 5600 TXU/g of product [3].

According to EFSA-Q-2006-119, *Natugrain Wheat® TS* is intended to be mixed into premixtures and/or feedingstuffs, whereas *Natugrain Wheat® TS L* is sprayed directly onto feedingstuffs. Both formulations are used to obtain enzyme activity levels of 400 to 800 TXU/kg in complete feedingstuffs for turkeys for fattening [5].

The product has been already authorised to be used for chickens for fattening under the register number 62 [6].

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 *Natugrain Wheat® TS* and *TS L*, *cf*. EFSA-Q-2006-119, and their suitability to be used for official controls in the frame of authorisation, were evaluated.

EVALUATION

Identification/Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

For the determination of arsenic and selenium, heavy metals (lead, mercury and cadmium), microbiological agents and mycotoxins, the applicant proposes methods based on well known techniques [7] and therefore considered suitable for intended purposes with an exception of an old-fashioned semi-quantitative determination of heavy metal ions, using visual inspection of colours [8]. For official controls, various 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 *in-house* developed and validated viscosimetric method based on the fact that thermostable endo-1,4-β-xylanase catalyses the hydrolysis of glycosidic bonds in the substrate wheat arabinoxylan to yield xylose and consequently reduces the viscosity of sample solution. This decrease of sample viscosity is determined electronically using a falling ball viscosimeter at defined conditions. In falling ball tests, the viscosity is proportional to the time required for a ball to fall through the test solution contained in a precise and temperature controlled glass tube or syringe. The drop time is registered at regular time intervals and is a measure for the endo-1,4-β-xylanase's activity, subtracting the obtained value from reagent blanks. The quantification is performed via a polynomial calibration curve of a reference endo-1,4-β-xylanase, that can be available from the applicant under request. The measurements are carried out at pH 3.5 and 55^{o}C and therefore the activity is expressed in terms of TXU [9]. Method's validation has been performed according to the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

For the determination of the activity of endo-1,4-β-xylanase in *feed additive*, 2.0 g of the dry product or 1.0 g of the liquid additive are suspended in 200 or 100 ml of citric acid buffer (pH 3.3), stirred and centrifuged (only if a dry product is analysed). Mixing 5 ml of the substrate solution (dissolved in water) and 1 ml of diluted enzyme extract (dissolved in citric acid buffer) yields a pH of 3.5 of the final solution to be incubated at 55°C. General assay conditions are then followed [9,10]. The relative standard deviation for repeatability (RSD_r)

ranges from 2.4 to 5.1% and the within-laboratory relative standard deviation for reproducibility (RSD_R) varies from 3.3 to 5.9% [10,13].

For the determination of the activity of endo-1,4-β-xylanase in *premixtures*, 50 g of corn meal are suspended in 500 ml of citric acid buffer (pH 3.3). Afterwards, 0.5 g of ground premixtures sample is added and the mixture is stirred for 50 min followed by centrifugation and further dilution of supernatant using citric acid buffer. General assay conditions are followed [9,10]. The RSD_r is 5.7 % and the within-laboratory RSD_R is 11.1% [11,13].

For the determination of the activity of endo-1,4-β-xylanase in *feedingstuffs*, 50 g of the ground feed sample are suspended in 500 ml of citric acid buffer (pH 3.3). After stirring and centrifugation, the supernatant is further diluted in citric acid buffer and analysed according to general assay conditions [9,12]. Method performance characteristics include RSD_r of 3.4%, a within-laboratory RSD_R of 2.4%, a limit of detection (LOD) of 11 TXU/kg and a limit of quantification (LOQ) of 36 TXU/kg *feedingstuffs* [12,13]. Based on these performance characteristics, the method is considered suitable for the intended purpose.

Regarding the application of the protocol in the frame of official control the CRL favours the use of inter-laboratory validated methods. There are several published and ring-trial validated methods for the determination of the activity of endo-1,4-β-xylanase in various matrices. It must be, however, noted that these are *colorimetric* methods, based on the detection and quantification of the amount of reducing sugars or a specific dye released by xylanase using a xylan-containing substrate. However, none of these studies were performed on *Natugrain Wheat® TS* and *TS L*. For this reason and due to the fact that the methods are based on a *different* principle compared to the method of this dossier, their applicability to the analysis of *Natugrain Wheat® TS* and *TS L* cannot be evaluated. Nevertheless, the obtained precision data reported in this dossier could be compared with corresponding data from the above mentioned studies on colorimetric methods. When measuring the activity of xylanase in a *product*, Cosson *et al* (1999) reported RSD_r of 7.2% and showed RSD_r varying from 4.6 to 11.9% for the assay of xylanase in *feed* using a dyed xylan substrate [14]. König *et al*. (2002) tested xylanase *products* in four laboratories using a substrate wheat arabinoxylan and measuring the amount of released reducing sugars. The obtained within-laboratory RSD_R varied form 4.4 to 5.3% [15]. Based on this comparison and due to above mentioned reasons, the CRL recommends to use the applicant's method for official control purposes in the frame of authorisation.

CONCLUSIONS AND RECOMMENDATIONS

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes an *in-house* developed and validated viscosimetric method based on the fact that xylanase catalyses the hydrolysis of glycosidic bonds in substrate wheat arabinoxylan. The decrease in sample viscosity, expressed in the terms as a drop time, is a measure for the endo-1,4-β-xylanase's activity and is determined using a falling ball viscosimeter at pH 3.5 and 55°C. The quantification is performed via an endo-1,4-β-xylanase's standard curve. Based on acceptable performance characteristics, the method is considered to be suitable for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Viscosimetric method based on decrease of viscosity produced by action of endo-1,4-β-xylanase on the xylan-containing substrate (wheat arabinoxylan) at pH 3.5 and 55°C.

One thermostable xylanase activity unit (TXU) is defined as the amount of enzyme that liberates 5 µmol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH 3.5 and 55° C.

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Natugrain Wheat® TS* and *Natugrain Wheat® TS L* have been sent to the Community Reference Laboratory for Feed Additives.

The dossier has been made available to the CRL by EFSA.

REFERENCES

[1] Main dossier, Section II, Subject 2, Item 1.2.

[2] Reference SANCO/D/2 Forw. Appl. 1831/011-2006.

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

[4] Main dossier, Section II, Annex 2.2.4.a.

[5] Annex III, Proposal of Register entry.

[6] Commission Regulation (EC) No 1458/2005 of 8 September 2005, concerning the permanent and provisional authorisations of certain additives in feedingstuffs and the provisional authorisation of new uses of certain additives already authorised in feedingstuffs. O.J., L 233, 9.9.2005, p.3.

[7] Main dossier, Section II, Annexes 2.5.1.a.15-2.5.1.a.24.

[8] Main dossier, Section II, Annex 2.5.1.a.22.

[9] Main dossier, Section II, Annex 2.5.1.a.1.

[10] Main dossier, Section II, Annexes 2.5.1.a.2 and 2.5.1.a.3.

[11] Main dossier, Section II, Annex 2.5.1.a.5.

[12] Main dossier, Section II, Annex 2.5.1.a.4.

[13] Validation report No 61862.02.UK1, supplementary information.

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

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

RAPPORTEUR LABORATORY

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