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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-2008-0418
FAD-2008-0023
CRL/080021

Name of Additive: Natugrain Wheat TS
Natugrain Wheat TS L

Active Substance(s): Endo-1,4- β -xylanase (E.C. 3.2.1.8)

Rapporteur Laboratory: The Community Reference Laboratory for
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Date: 06/01/2009

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Natugrain Wheat TS* and *Natugrain Wheat TS L*, in accordance with article 4(1) and 10(2) of Regulation (EC) No 1831/2003. Authorisation is sought to use *Natugrain Wheat TS* and *Natugrain Wheat TS L* as a digestibility enhancer for chicken for fattening and ducks 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.

The active agent of *Natugrain Wheat TS* and *Natugrain Wheat TS L* is thermostable endo-1,4- β -xylanase, produced by a strain of *Aspergillus niger*-CBS 109.713. The additive is intended to be marketed as powder (*Natugrain Wheat TS*) and as liquid formulation (*Natugrain Wheat TS L*). Both formulations contain an endo-1,4- β -xylanase activity of 5600 TXU/g product. They are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a recommended endo-1,4- β -xylanase activity level ranging from 280 to 800 TXU per kg *feedingstuffs*. Enzymatic activity of endo-1,4- β -xylanase is expressed in thermostable xylanase units (TXU). One TXU is defined as the amount of enzyme that liberates 5 μ mol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH = 3.3 and 55°C.

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* validated viscosimetric method. Endo-1,4- β -xylanase catalyses the hydrolysis of glycosidic bonds in the substrate wheat arabinoxylan to yield xylose and reduces consequently the viscosity of sample solution. The decrease in viscosity of sample solution, expressed in terms of a drop time, is a measure for the endo-1,4- β -xylanase activity and is determined using a falling ball viscosimeter at pH = 3.3 and 55°C. The quantification is performed using an endo-1,4- β -xylanase standard curve based on reference enzyme with known activity provided by the applicant. The method performance characteristics, determined for the *feed additive*, *premixtures* and *feedingstuffs* matrices are: - a relative standard deviation for repeatability (RSD_r) ranging from 2.4 to 5.7%; - a relative intermediate precision (RSD_R) ranging from 3.4 to 11.8%; - a recovery rate ranging from 82 to 115%; - a limit of detection (LOD) of 11 TXU/kg *feedingstuffs* and - a limit of quantification (LOQ) of 36 TXU/kg *feedingstuffs*.

Based on acceptable performance characteristics, the applicant 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 product 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]. The product contains an active agent of endo-1,4- β -xylanase produced by a strain of *Aspergillus niger* (CBS 109.713) [2], which is deposited at Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands [3].

The additive is intended to be marketed as a powder (*Natugrain Wheat TS*) and as liquid formulation (*Natugrain Wheat TS L*). Both formulations have an activity of thermostable endo-1,4- β -xylanase of 5600 TXU/g product. One TXU is defined as the amount of enzyme that liberates 5 μ mol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH = 3.3 and 55°C [2].

Natugrain Wheat TS is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas *Natugrain Wheat TS L* is sprayed directly onto feedingstuffs for ducks and chicken for fattening. Both formulations are used to obtain a recommended endo-1,4- β -xylanase activity ranging from 280 to 800 TXU per kg *feedingstuffs* [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 (EFSA) for each application. For this particular dossier, the methods of analysis submitted in connection with *Natugrain Wheat TS* and *Natugrain Wheat TS L*, cf. EFSA-Q-2008-0418, 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

When required by EU legislation, analytical methods for official control of impurities in the *additive* (e.g. arsenic and heavy metals - cadmium, mercury and lead) are available from the respective Community Reference Laboratories [4].

Description of the analytical methods for the determination of the active agent(s) 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 an *in-house* validated viscosimetric method. Thermostable endo-1,4- β -xylanase catalyses the hydrolysis of glycosidic bonds in the wheat arabinoxylan substrate to yield xylose and consequently reduces the viscosity of sample solution. The decrease in viscosity of sample solution is determined electronically using a falling ball viscosimeter at defined conditions. Viscosity is proportional to the time required for a ball to fall through the test solution contained in a temperature controlled glass tube or syringe. The drop time, registered at regular time intervals, is a measure for the endo-1,4- β -xylanase activity. The quantification is performed via a third degree polynomial calibration curve of a reference endo-1,4- β -xylanase standard with known activity (available from the applicant upon request). Measurements are carried out at pH = 3.3 and 55°C and the activity is expressed in TXU. The viscosimetric measurement is described in [5] and is referenced in all the method protocols for the determination of enzyme activity in the feed additive, premixtures and feedingstuffs.

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) and stirred. In the case of the *dry* product, the solution needs to be centrifuged. 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 3.3 of the final solution to be incubated at 55°C [6]. The viscosity measurements are then carried out according to general assay conditions [5]. The following performance characteristics are reported: a relative standard deviation for repeatability (RSD_r) ranging from 2.4 to 5.1%; a relative standard deviation for intermediate precision (RSD_R) ranging from 3.7 to 6.9% and a recovery rate ranging from 94 to 113% [8].

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) and 0.5 g of ground premixture sample is added. The mixture is stirred for 50 min and centrifuged. The supernatant is further diluted using citric acid buffer [9]. The viscosity measurements are then carried out according to general assay conditions [5]. The reported method performance characteristics are: a RSD_r of 6 %; a RSD_R of 12% and a recovery rate of 82% [8]. The validation experiment was conducted on *premixture* samples covering an activity range of 120 TXU/g. The method is

considered suitable for the intended purpose within the activity range covered by the validation study.

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 [10] and analysed according to general assay conditions [5]. The reported method performance characteristics are: a RSD_T of 3.4%; a RSD_R of 3.4%; a recovery rate of 102% [8]. Furthermore, the method validation includes a limit of detection (LOD) of 11 TXU/kg *feedingstuffs* and a limit of quantification (LOQ) of 36 TXU/kg *feedingstuffs* [11].

There are other analytical methods for the determination of the endo-1,4- β -xylanase enzyme activity [12]. However, there are no data demonstrating that these methods work for the *Natugrain Wheat* products. Therefore, the suitability of these methods for official controls could not be demonstrated for these products.

Taking into account the acceptable method performance characteristics provided by the applicant the in-house validated method submitted is considered suitable, and therefore recommended by the CRL for official control purposes.

CONCLUSIONS AND RECOMMENDATIONS

For the determination of the activity of endo-1,4- β -xylanase in the *feed additive, premixture* and *feedingstuffs*, an in-house validated viscosimetric method is considered to be suitable for official control purposes within the authorisation frame of *Natugrain Wheat TS and Natugrain Wheat TS L*.

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 = 3.3 and 55°C.

One thermostable xylanase activity unit (TXU) is defined as the amount of enzyme that liberates 5 μ mol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH = 3.3 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 on 10 October 2008.

REFERENCES

- [1] *Reference SANCO/D/2 Forw. Appl. 1831/29-2007.
- [2] *Annex III, Proposal of Register entry.
- [3] Section II "REG2.2.4.a2".
- [4] 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 Community Reference Laboratories. O.J., L 136, 24.5.2006.
- [5] * Register 1 "REG01a_PM01002_01e_Xylanase activity, General Method".
- [6] * Register 2 "REG02a_PM01003-01e_Xylanase-activity"_solid product.
- [7] * Register 3 "REG03a_PM01004-01e_xylanase-activity "_liquid product.
- [8] * Register 1 "REG01c Validation Report No. 61862.02.UK1 Volume I".
- [9] * Register 5 "REG05a_PM01006-01e_Xylanase activity Mineral Premix".
- [10] * Register 4 "REG04a_PM01005_01e_Xylanase activity Feed".
- [11] * Register 4 "REG04b_PM01005_01e Validation Report".
- [12] Cosson, T. *et al.* Animal Feed Science and Technology, 77 (1999) 345-353.
- [13] König, J. *et al.* Anal. Bioanal. Chem., 374 (2002) 80-87.

*Refers to Dossier number FAD-2008-0023-*Natugrain Wheat TS* – *Natugrain Wheat TSL*

RAPPORTEUR LABORATORY

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