EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

D08/FSQ/CVH/SY/D(2008)19607

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-2008-0013 FAD-2007-0044
Name of Additive:	Natugrain®TS and Natugrain®TS L
Active Substance(s):	Endo-1,4-β-xylanase (E.C. 3.2.1.8) Endo-1,4-β-glucanase (E.C. 3.2.1.4)
Rapporteur Laboratory:	The Community Reference Laboratory for Feed Additives (CRL-FA)
Report prepared by:	Sulhattin Yasar (CRL-FA)
Report checked by:	Piotr Robouch (CRL-FA) Christoph von Holst (CRL-FA)
Date:	31/07/2008
Report approved by:	Christoph von Holst (CRL-FA)
Date:	01/08/2008

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Natugrain®TS* and *Natugrain®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. Authorisation is sought to use *Natugrain®TS* and *Natugrain®TS L* as a digestibility enhancer for piglets (weaned), laying hens, chicken and turkeys for fattening and ducks.

The two active agents of *Natugrain®TS* and *Natugrain®TS L* are (1) thermostable endo-1,4-β-xylanase, produced by a strain of *Aspergillus niger*-CBS 109.713, and (2) thermostable endo-1,4-β-glucanase, produced by a strain of *Aspergillus niger*-DSM 18404. The additive is intended to be marketed as powder (*Natugrain®TS*) and as liquid formulation (*Natugrain®TS L*). Both formulations contain an endo-1,4-β-xylanase activity of 5600 TXU/g product and an endo-1,4-β-glucanase activity of 2500 TGU/g product. They are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a minimum endo-1,4-β-xylanase activity level of 280 up to a maximum recommended of 840 TXU per kg *feedingstuffs* and a minimum endo-1,4-β-glucanase activity level of 125 to a maximum recommended of 375 TGU 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 µmol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH = 3.5 and 40°C. Enzymatic activity of endo-1,4-β-glucanase is expressed in thermostable glucanase units (TGU). One TGU is defined as the amount of enzyme that liberates 1 µmol of reducing sugars (glucose equivalents) from barley betaglucan per minute at pH = 3.5 and 40°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 = 3.5 and 55°C. The quantification is performed using an endo-1,4-β-xylanase standard curve based on reference enzyme 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*; - a limit of quantification (LOQ) of 36 TXU/kg *feedingstuffs*.

For the determination of the activity of endo-1,4-β-glucanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes an *in-house* validated viscosimetric method. Endo-1,4-β-glucanase catalyses the hydrolysis of glycosidic bonds in the substrate barley betaglucan to yield glucose 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-glucanase activity and is determined using a falling ball viscosimeter at pH = 3.5 and 40°C. The quantification is performed using an endo-1,4-β-glucanase standard curve based on reference enzyme provided by the applicant. The method performance characteristics, determined for the *feed additive, premixtures* and *feedingstuffs* matrices, are: - a RSD_r ranging from 4.1 to 10.4%; - a RSD_R ranging from 7.5 to 12.3%; - the recovery rate ranging from 88 to 117%; - a LOD of 16 TGU/kg *feedingstuffs*; - a LOQ of 49 TGU/kg *feedingstuffs*.

Based on acceptable performance characteristics, both methods are considered to be suitable for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

KEYWORDS

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

BACKGROUND

Natugrain®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 two active agents: –endo-1,4-β-xylanase produced by a strain of *Aspergillus niger* (CBS 109.713) and –endo-1,4-β-glucanase produced by a strain of *Aspergillus niger* (DSM 18404) [2]. The strain of *Aspergillus niger* (DSM 18404) is deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany, and the strain of *Aspergillus niger* (CBS 109.713) 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, 5600 TXU/g of product, and an activity of thermostable endo-1,4-β-glucanase of 2500 TGU/g of product [2]. 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 40^{o}C [2]. Enzymatic activity of endo-1,4-β-glucanase is expressed in thermostable glucanase units (TGU). One TGU is defined as the amount of enzyme that liberates 1 μmol of reducing sugars (glucose equivalents) from barley betaglucan per minute at pH = 3.5 and 40^{o}C [2].

Natugrain®TS is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas *Natugrain®TS L* is sprayed directly onto feedingstuffs for poultry (laying hens, chicken and turkey for fattening, ducks) and piglets (weaned). Both formulations are used to obtain a minimum endo-1,4-β-xylanase activity level of 280 up to a maximum recommended of 840 TXU per kg *feedingstuffs* and a minimum endo-1,4-β-glucanase activity level of 125 to a maximum recommended of 375 TGU 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®TS* and *Natugrain®TS L*, *cf.* EFSA-Q-2008-0013, 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 various contaminants including heavy metals and mycotoxins suitable methods to be used in the frame of official control 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

Endo-1,4-β-xylanase

General Assay Condition [5]

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes *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 with known activity available from the applicant upon request. Measurements are carried out at pH = 3.5 and 55^{o}C and the activity is expressed in TXU.

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 3.5 of the final solution to be incubated at 55°C [6][7]. The viscosity measurements are then carried out according to general assay conditions [5]. The following performance characteristics are reported: - a RSD_r ranging from 2.4 to 5.1%; - a RSD_R ranging from 3.7 to 6.9%; - 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%; - a recovery of 82% [8].

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_r of 3.4%; - a RSD_R of 3.4%; and - a recovery rate of 102% [8]. Furthermore, the method validation includes a LOD of 11 TXU/kg *feedingstuffs* and a 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],[13] that have even been tested between four laboratories. However, there are no data demonstrating that the methods work for the products related to this dossier. 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-FA for official control purposes.

Endo-1,4-β-glucanase

General Assay Condition [14]

For the determination of the activity of endo-1,4-β-glucanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes an in-house validated viscosimetric method. Thermostable endo-1,4-beta-glucanase catalyses the hydrolysis of glycosidic bonds in the substrate (barley betaglucan) to yield glucose and reduces consequently 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-glucanase activity. The quantification is performed via a third degree polynomial calibration curve of a reference endo-1,4-β-glucanase available from the applicant upon request. The measurement is carried out at pH = 3.5 and 40°C. The activity is expressed in TGU.

For the endo-1,4-β-glucanase determination in *feed additive*, 2.0 g of the dry product or 1.0 g of the liquid product are suspended in 200 or 100 ml of citric acid buffer (pH = 3.5) and

stirred for 30 minutes. In the case of the *dry* product, the solution needs to be centrifuged. Mixing 5 ml of the substrate solution (suspended in water) and 1 ml of diluted enzyme extract (dissolved in citric acid) yields a pH of 3.5 of the final solution to be incubated at 40°C [15],[16]. The viscosity measurements are then carried out according to general assay conditions [14]. The RSD_r ranges from 4.1 to 5.0% and the RSD_R varies from 7.5 to 8.0% [17].

For the endo-1,4-β- glucanase determination in *premixtures*, 50 g of corn meal are suspended in 500 ml of citric acid buffer (pH = 3.5) and 0.5 g of ground premixture sample is added. The mixture is stirred for 60 min and centrifuged. The supernatant is diluted using citric acid buffer [18]. The viscosity measurements are then carried out according to general assay conditions [14]. The RSD_r 10% and RSD_R 12% are reported [17].

For the endo-1,4-β- glucanase determination in *feedingstuffs*, 50 g of the ground feed sample are suspended in 500 ml of citric acid buffer (pH = 3.5). After stirring and centrifugation, the supernatant is further diluted in citric acid buffer [19]. The viscosity measurements are carried out according to general assay conditions [14]. The reported method performance characteristics are: - a RSD_r 7%; - a RSD_R 12%; - a recovery rate 88-117%; - a LOD 16 TGU/kg *feedingstuffs*; - a LOQ 49 TGU/kg *feedingstuffs* [17].

There are analytical methods for the determination of the endo-1,4-β-glucanase enzyme activity [12],[13]. However, there are no data demonstrating that the methods work for the products related to this dossier. 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-FA for official control purposes.

CONCLUSIONS AND RECOMMENDATIONS

For the determination of the activity of endo-1,4-β-xylanase and endo-1,4-β-glucanase in the *feed additive*, *premixture* and *feedingstuffs*, two in-house validated viscosimetric methods, based on the same principle, are considered to be suitable for official control purposes in the authorisation frame of *Natugrain®TS and Natugrain®TS L*.

Further testing or validation is not considered necessary.

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

- For quantification of endo-1,4-β-xylanase activity in feed:

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 40° C.

- For quantification of endo-1,4-β-glucanase activity in feed:

Viscosimetric method based on decrease of viscosity produced by action of endo-1,4-β-glucanase on the glucan-containing substrate (barley betaglucan) at pH = 3.5 and 40°C.

One thermostable glucanase activity unit (TGU) is defined as the amount of enzyme that liberates 1 µmol of reducing sugars (glucose equivalents) from barley betaglucan per minute at pH = 3.5 and 40° C.

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Natugrain®TS* and *Natugrain®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 16 May 2008.

REFERENCES

[1] *Reference SANCO/D/2 Forw. Appl. 1831/29-2007.

[2] *Annex III, Proposal of Register entry.

[3] * Section II, Annex "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] * Section II, Register 1 "REG01a_PM01002_01e_Xylanase activity, General Method".

[6] * Section II, Register 2 "REG02a_PM01003-01e_Xylanase-activity"_solid product.

[7] * Section II, Register 3 "REG03a_PM01004-01e_xylanase-activity "_liquid product.

[8] * Section II, Register 1 "REG01c_Validation Report No. 61862.02.UK1 Volume I".

[9] * Section II, Register 5 "REG05a_PM01006-01e_Xylanase activity Mineral Premix".

[10] * Section II, Register 4 "REG04a_PM01005_01e_Xylanase activity Feed".

[11] * Section II, 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.

[14] * Section II, Register 6 "REG06a_PM01230_01e_Glucanase activity, General method".

[15] * Section II, Register 6_" REG07_PM123101e_Glucanase activity"_solid product

[16] * Section II, Register 6_"REG08a_PM123201e_Glucanase activity"_liquid product.

[17] * Section II, Register 6 _"REG06b_PM01230_01e_Validation Report".

[18] * Section II, Register 10 "REG10a_PM01234_01e_Glucanase acitivity, Mineral Premix".

[19] * Section II, Annex REG09a_PM01233_01e_Glucanase activity, Feed.

*Refers to Dossier number FAD-2007-0044-*Natugrain®TS – Natugrain®TSL*

RAPPORTEUR LABORATORY

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

ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Plantedirektoratets Laboratorium, Lyngby, DK.
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana, SL.
- Põllumajandusuuringute Keskus, Saku, Harjumaa, EE.
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig, DE.
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, CZ.