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

Dossier related to:	FAD-2010-0034 EURL/100132
Product Name:	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:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
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Report approved by: Date:	Christoph von Holst 18/04/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) (new use) for *Natugrain®TS* and *Natugrain®TS L*, under the category/functional group 4(a) 'zootechnical additives' / 'digestibility enhancers', 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 chickens reared for laying, turkeys for breeding purposes, turkeys reared for breeding, minor avian species, other then ducks, and ornamental birds. The products was previously authorised for weaned piglets, chickens for fatting, laying hens, turkeys for fattening and ducks for fattening.

The products contain two active agents : *endo-1,4-β-xylanase* and *endo-1,4-β-glucanase*. The product is intended to be marketed as a powder (*Natugrain®TS*) and a 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 an *endo-1,4-β-xylanase* activity ranging from 280 to 840 TXU/kg *feedingstuffs* and an *endo-1,4-β-glucanase* activity ranging from 125 to 375 TGU/kg *feedingstuffs*. The enzymatic activity of *endo-1,4-β-xylanase* is expressed in thermostable xylanase units (TXU); where 1 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. The enzymatic activity of *endo-1,4-β-glucanase* is expressed in thermostable glucanase units (TGU), where 1 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 *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposes a *single-laboratory* validated viscosimetric method, further verified by an independent laboratory. *Endo-1,4-β-xylanase* catalyses the hydrolysis of glycosidic bonds in the substrate standard wheat arabinoxylan to yield xylose and reduces consequently the viscosity. The decrease in viscosity of the substrate enzyme 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 following performance characteristics, determined for the *feed additive, premixtures* and *feedingstuffs* were reported:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.5 to 9.9 %;
- a relative *intermediate precision* (RSD_{ip}) ranging from 4.2 to 9.9 %;
- a *recovery* rate (R_{rec}) ranging from 82 to 115 %; and

- a limit of detection (LOD) and quantification (LOQ) of 11 and 36 TXU/kg *feedingstuffs,* respectively.

For the determination of *endo-1,4-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposes an single laboratory validated viscosimetric method, further verified by an independent laboratory. *Endo-1,4-β-glucanase* catalyses the hydrolysis of glycosidic bonds in the substrate standard barley betaglucan to yield glucose and reduces consequently the viscosity. The decrease in viscosity of the substrate enzyme 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 following performance characteristics, determined for the *feed additive, premixtures* and *feedingstuffs*, were reported:

- RSD_r ranging from 4.1 to 10.4 %;
- RSD_{ip} ranging from 7.5 to 12.3 %;
- R_{rec} from 85 to 115%; and
- LOD and LOQ of 16 and 49 TGU/kg *feedingstuffs*, respectively.

Based on the performance characteristics presented, the EURL recommends for official control the two viscosimetric methods submitted by the Applicant for the determination of *endo-1,4-β-xylanase* and *endo-1,4-β-glucanase* in the *feed additive, premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

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

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

1. BACKGROUND

Natugrain®TS is a product for which authorisation is sought under articles 4(1) (new use for a variety of species) under the category / the functional group 'zootechnical additives' / '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, while the strain of *Aspergillus niger* (CBS 109.713) is deposited at Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands [3].

The 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 55°C [2].

The 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 [19].

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 minimum activity of thermostable *endo-1,4-β-xylanase* of 5600 TXU/g, and a minimum activity of thermostable *endo-1,4-β-glucanase* of 2500 TGU/g [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 activity of *endo-1,4-β-xylanase* of 280 TXU/kg *feedingstuffs* and a minimum activity of *endo-1,4-β-glucanase* of 125 TGU/kg *feedingstuffs* [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 European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Natugrain®TS* and *Natugrain®TS L*, and their suitability to be used for official controls in the frame of 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, and dioxins) are available from the respective European Union 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 Conditions [5]

For the determination of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposes a single laboratory validated viscosimetric method. 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 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 *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 the abovementioned general assay conditions [5]. The performance characteristics determined during method validation and verification by an independent laboratory are summarised in Table 1.

For the determination 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 containing the enzymes further

diluted using citric acid buffer [12]. The viscosity measurements are then carried out according to general assay conditions [5]. The performance characteristics determined during method validation and verification by an independent laboratory are summarised in Table 1.

For the determination 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 [14] and analysed according to general assay conditions [5]. The performance characteristics determined during method validation and verification by an independent laboratory are summarised in Table 1. Furthermore, the Applicant reported LOD and LOQ of 11 and 36 TXU/kg *feedingstuffs* [15].

Other analytical methods for the determination of *endo-1,4-β-xylanase* enzyme activity [17, 18] were tested in the frame of an interlaboratory comparison including four laboratories. However, there are no experimental data demonstrating that these 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.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified viscosimetry method submitted by the Applicant for the determination of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

Table 1: Summary of method performance for the determination of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs* containing 7900-9000, 480 and 0.96 TXU/g, respectively

	RSD_r		RSD_{ip}		Recovery	
	Validation	Verification	Validation	Verification	Validation	Verification
Feed Additive	2.5 %- 3 % [9]	2.4 % - 3.7 % [10,11]*	4.2 % - 7.5 %[9]	3.7 % [10,11]*	85 % - 115 % [8]	97 % - 102 % [10,11]
Premixtures	9.9 % [9]	5.4 % [13]*	9.9 % [9]	5.4 % [13]*	82 % - 115 % [8]	98 % [13]
Feedingstuffs	6.5 % [9]	6.5 % [16]*	8.2 % [9]	6.5 % [16]*	85 % - 115 % [8]	97 % [16]

* values recalculated by the EURL

Endo-1,4-β-glucanase

General Assay Conditions [19]

For the determination of *endo-1,4-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposes an single-laboratory 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 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 determination of *endo-1,4-β-glucanase* in the *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 [20, 21]. The viscosity measurements are then carried out according to the abovementioned general assay conditions [19]. The performance characteristics determined during method validation and verification by an independent laboratory are summarised in Table 2.

For the determination of *endo-1,4-β-glucanase* 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 [25]. The viscosity measurements are then carried out according to general assay conditions [19]. The performance characteristics determined during method validation and verification by an independent laboratory are summarised in Table 2.

For the determination of *endo-1,4-β-glucanase* 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 [27]. The viscosity measurements are carried out according to general assay conditions [19]. The performance characteristics determined during method validation and verification by an independent laboratory are summarised in Table 2. Furthermore, the Applicant reported LOD and LOQ of of 16 and 49 TGU/kg *feedingstuffs*, respectively [22].

Other analytical methods for the determination of the *endo-1,4-β-glucanase* enzyme activity have been reported in the literature [17, 18]. However, there are no experimental data demonstrating that these 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.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified viscosimetry method submitted by the Applicant for the determination of *endo-1,4-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs*, within the concentration range covered by the experimental data.

Table 2: Summary of method performance for the determination of *endo-1,4-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs* containing 3600-4550, 170 and 0.38 TGU/g, respectively

	RSD_r		RSD_{ip}		R_{rec}	
	Validation	Verification	Validation	Verification	Validation	Verification
Feed Additive	4.1 % - 5.0 % [9]	1.7 % - 2.3 % [23, 24]*	7.5 % - 8.0 % [9]	1.9 % - 2.3 % [23, 24]*	85 % - 115 % [22]	93 % - 98 % [23, 24]
Pre-mixtures	10.4 % [9]	5.6 % [26]*	12.3 % [9]	5.6 % [26]*	85 % - 115 % [22]	83 % [26]
Feeding-stuffs	7.4 % [9]	6.3 % [28]*day1	12.3 % [9]	# [28]*	85 % - 115 % [22]	104 % [28]

* values recalculated by the EURL
(#) unreliable results were obtained on the second day

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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- the single laboratory validated and further verified 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 for the determination of *endo-1,4-β-xylanase* in the *feed additive*, *premixture* and *feedingstuffs*

- the single laboratory method and further verified 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 for the determination of *endo-1,4-β-glucanase* in the *feed additive*, *premixture* and *feedingstuffs*

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

For quantification of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs:*

- 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.

For quantification of *endo-1,4-β-glucanase* in *feed additive*, *premixtures* and *feedingstuffs:*:

- 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.

5. 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 European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Reference SANCO/D/2 Forw. Appl. 1831/0026-2010
[2] * Application, Proposal for Register Entry – Annex A
[3] * Technical dossier, Section II – Identity, Characterization and Conditions of Use of the Additive, Methods of Control
[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
[5] * Technical dossier, Section II – Annex, Vol 3 Reg 1a
[6] * Technical dossier, Section II – Annex, Vol 3 Reg 2a
[7] * Technical dossier, Section II – Annex, Vol 3 Reg 3a
[8] * Technical dossier, Section II – Annex, Vol 3 Reg 1b
[9] * Technical dossier, Section II – Annex, Vol 3 Reg 7b
[10] * Technical dossier, Section II – Annex, Vol 3 Reg 2c

[11] * Technical dossier, Section II – Annex, Vol 3 Reg 3c
[12] * Technical dossier, Section II – Annex, Vol 3 Reg 5a
[13] * Technical dossier, Section II – Annex, Vol 3 Reg 5c
[14] * Technical dossier, Section II – Annex, Vol 3 Reg 4a
[15] * Technical dossier, Section II – Annex, Vol 3 Reg 4b
[16] * Technical dossier, Section II – Annex, Vol 3 Reg 4c
[17] Cosson, T. et al. Animal Feed Science and Technology, 77 (1999) 345-353.
[18] König, J. et al. Anal. Bioanal. Chem., 374 (2002) 80-87.
[19] * Technical dossier, Section II – Annex, Vol 3 Reg 6a
[20] * Technical dossier, Section II – Annex, Vol 3 Reg 7a
[21] * Technical dossier, Section II – Annex, Vol 3 Reg 8a
[22] * Technical dossier, Section II – Annex, Vol 3 Reg 6b
[23] * Technical dossier, Section II – Annex, Vol 3 Reg 7c
[24] * Technical dossier, Section II – Annex, Vol 3 Reg 8c
[25] * Technical dossier, Section II – Annex, Vol 3 Reg 10a
[26] * Technical dossier, Section II – Annex, Vol 3 Reg 10c
[27] * Technical dossier, Section II – Annex, Vol 3 Reg 9a
[28] * Technical dossier, Section II – Annex, Vol 3 Reg 9c
*Refers to FAD-2010-0034

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was European Union 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

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (POL)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien