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

Dossier related to: **FAD-2010-0374
CRL/100191**

Name of Feed Additive: **Safizym X**

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

Rapporteur Laboratory: **European Union Reference Laboratory
for Feed Additives (CRL-FA)
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Date: **13/01/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Safizym X*, 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. According to the Applicant, the *feed additive* contains *endo-1,4-β-xylanase* (EC 3.2.1.8) as the active agent, produced by the strain *Trichoderma reesei* (formerly known as *longibrachiatum*) CNCM MA 6-10W (CL 847). The additive is intended to be marketed in two different formulations: as solid *Safizym XP20* and liquid *Safizym XL200*. *Safizym XP20* has a guaranteed minimum *endo-1,4-β-xylanase* activity of 70000 IFP/g, while *Safizym XL200* has a guaranteed minimum *endo-1,4-β-xylanase* activity of 7000 IFP/ml.

The activity of *endo-1,4-β-xylanase* is expressed in Institut Français du Pétrole units (IFP). According to the Applicant, one IFP unit is the quantity of enzyme which liberates one μmole of reducing sugars (xylose equivalents) per minute from oat xylan at pH 4.8 and 50 °C.

Specifically, authorisation is sought for the use of *Safizym X* for chickens for fattening, turkeys for fattening and laying hens. The solid formulation of the *feed additive* (*Safizym XP20*) is intended to be incorporated into *premixtures* and/or complete *feedingstuffs*, while the liquid formulation (*Safizym XL200*) is sprayed into *feedingstuffs*. The *feed additive* is intended to be used in compound feed rich in non-starch polysaccharides (mainly arabinoxylans). The Applicant proposed the following minimum activities for *endo-1,4-β-xylanase* in complete *feedingstuffs*: 840 IFP/kg for laying hens, and 1400 IFP/kg for chickens and turkeys for fattening.

For the quantification of *endo-1,4-β-xylanase* in the *feed additive*, the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on reaction between released sugar and 3,5-dinitrosalicylic acid (DNS). The assay is based on the enzymatic hydrolysis of oat spelt xylan at pH 4.8 and 50 °C. The following performance characteristics were reported: - a relative standard deviation for *repeatability* (RSD_r) of 7.2% and - a relative standard deviation for *reproducibility* (RSD_R) of 17%.

For the quantification of *endo-1,4-β-xylanase* in the *premixtures* and *feedingstuffs* the Applicant proposed a single-laboratory validated and further verified colorimetric method based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on oat azo-xylan at pH 4.8 and 50 °C. The following performance characteristics were reported:

- for *premixtures*: - RSD_r ranging from 4.1 to 8.7 %; - RSD_R ranging from 14 to 22 %; and - a *recovery* rate (R_{Rec}) ranging from 89 to 107 %;

- for *feedingstuffs*: - RSD_r ranging from 4.6 to 12 %; - RSD_R ranging from 10 to 34 %; - R_{Rec} ranging from 97 to 113 %; and limits of detection (LOD) and quantification (LOQ) of 140 and 350 IFP/kg *feedingstuffs*, respectively.

Based on the satisfactory performance characteristics mentioned above, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods submitted by the Applicant, to determine *endo-1,4-β-xylanase* in *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

Safizym X, *endo-1,4-β-xylanase*, 4a1613, *Trichoderma reesei*, zootechnical additive, digestibility enhancers, chickens for fattening, turkeys for fattening, laying hens.

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use) and 10(2) (re-evaluation of additives already authorised) for *Safizym X*, under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers" [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003. The *feed additive* is already authorised under the following Commission Regulations: - (EC) No 1453/2004 for chickens for fattening, - (EC) No 943/2005 for turkeys for fattening, - (EC) No 1810/2005 for laying hens, - (EC) No 497/2007 for weaned piglets and - (EC) No 1501/2007 for ducks. According to the Applicant, the *feed additive* contains *endo-1,4-β-xylanase* (EC 3.2.1.8) as the active agent [2], produced by the strain *Trichoderma reesei* (formerly known as *longibrachiatum*) CNCM MA 6-10W (CL 847). The strain has been deposited at the Collection Nationale de Cultures de Microorganismes (CNCM) at the Institut Pasteur in Paris, France [3, 4]. The additive is intended to be marketed in two different formulations [3]: as solid *Safizym XP20* and liquid *Safizym XL200*. Maltodextrin and wheat flour are used as carriers of the solid formulation, while water, sorbitol, NaCl and calcium propionate are used for the liquid formulation. *Safizym XP20* has a guaranteed minimum *endo-1,4-β-xylanase* activity of 70000 IFP/g [3],

while *Safizym XL200* has a guaranteed minimum *endo-1,4-β-xylanase* activity of 7000 IFP/ml [3].

The activity of *endo-1,4-β-xylanase* is expressed in Institut Français du Pétrole units (IFP). According to the Applicant, one IFP unit is the quantity of enzyme which liberates one μmole of reducing sugars (xylose equivalents) per minute from oat xylan at pH 4.8 and 50 °C [5].

Specifically, authorisation is sought for the use of *Safizym X* for chickens for fattening, turkeys for fattening and laying hens [1]. The solid formulation of the *feed additive* (*Safizym XP20*) is intended to be incorporated into *premixtures* and/or complete *feedingstuffs*, while the liquid formulation (*Safizym XL200*) is sprayed into *feedingstuffs*. The *feed additive* is intended to be used in compound feed rich in non-starch polysaccharides (mainly arabinoxylans) [2]. The Applicant proposed the following minimum activities for *endo-1,4-β-xylanase* in complete *feedingstuffs*: 840 IFP/kg for laying hens; and 1400 IFP/kg for chickens and turkeys for fattening.

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 *Safizym X*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

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, mycotoxins and dioxins) are available from the respective European Union Reference Laboratories [6].

Description of the analytical methods for the quantification of the active substance in feed additive, premixtures and feedingstuffs

For the quantification of *endo-1,4-β-xylanase* in the *feed additive*, the Applicant submitted a single-laboratory validated and further verified method, based on colour formation of released sugar (xylose) with 3,5-dinitrosalicylic acid (DNS) [7]. The assay is based on the enzymatic hydrolysis of oat spelt xylan at pH 4.8 and 50 °C. The enzyme is diluted in 100 mM sodium acetate buffer (pH 4.8). One mL of xylan solution is then added to various volumes of the enzyme dilution (from 0.2 to 1.0 mL completed to 1.0 mL with sodium acetate buffer) in tests tubes and incubated at 50 °C for 30 minutes. The reaction is stopped by placing the tubes in a boiling bath for 10 minutes. Then 3 mL of DNS is added and the tubes are incubated in a boiling bath for 15 minutes to obtain good reveal of colour. After the tubes have been cooled in a water bath, 16 mL of water is added. The absorbances of the sample solutions are measured spectrophotometrically at 550 nm and quantified against the xylose standard curve. The calculated enzymatic activity is then expressed as xylose equivalents. The performance characteristics determined in the frame of intercomparison study between three laboratories [8], are presented in Table 1.

For the determination of the *endo-1,4-β-xylanase* activity in *premixtures* and *feedingstuffs*, the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of water soluble dyed fragments from the substrate oat azo-xylan [9, 10]. For *premixtures* [10], the samples (1 g) are suspended in 50 mL of citrate phosphate buffer (pH 4.8), stirred for 10 minutes, centrifuged at 1500 g for 5 minutes and further diluted in buffer. For *feedingstuffs* [9], the samples (20 g) are suspended in 100 mL of citrate phosphate buffer. After 30 minutes the aliquots are centrifuged at 1500 g for 10 minutes and the supernatant is collected. The substrate solution (250 µL of azo-xylan) is added to the samples and incubated at 50 °C for 60 minutes for *premixtures* (4 hours for *feedingstuffs*). The reaction is stopped by adding 1 mL of 95% ethanol and stirring vigorously. After 30 minutes the tubes are centrifuged for 10 minutes at 1500 g and the absorbances of the sample solutions are measured spectrophotometrically at 585 nm and compared to the standard line. The standard line is based on the extraction of known dose of reference enzyme (*Safizym XP20*, available from the applicant upon request). The method does not differentiate between endogenous and added enzyme and measures the total enzyme present in the feed. The performance characteristics determined in the frame of intercomparison study between three laboratories [8], as well as from the additional inter-laboratory study done by the Applicant [10], are presented in Table 1. Furthermore, the Applicant reported limits of detection (LOD) and quantification (LOQ) of 140 and 350 IFP/kg *feedingstuffs*, respectively.

Table 1: Performance characteristics for the quantification of *endo-1,4-β-xylanase* in the *feed additive (FA)*, *premixtures (PM)* and *feedingstuffs (FS)*

	Concentration range (IFP/g)	RSD _r (%)	RSD _R (%)	Recovery (%)
FA	62000-70000	7.2 [8]	16.6 [8]	na
PM	140-700	4.1 – 8.7 [10]	14 – 22 [10]	89 - 107 [10]
FS	0.35-2.8	4.6 – 12 [8,10]	10 – 34 [8,10]	97 - 113 [8,10]

RSD_r and RSD_R: relative standard deviation for *repeatability* and *reproducibility*, respectively.

na - not available

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory-validated and further verified colorimetric methods submitted by the Applicant, to determine the *endo-1,4-β-xylanase* in *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.

4. CONCLUSIONS AND RECOMMENDATIONS

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

- a single-laboratory validated and further verified colorimetric (DNS) method, submitted by the Applicant, based on the enzymatic hydrolysis of oat spelt xylan at pH 4.8 and 50 °C for the quantification of *endo-1,4-β-xylanase* in the *feed additive*; and
- a single-laboratory validated and further verified colorimetric method, submitted by the Applicant, based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on oat azo-xylan at pH 4.8 and 50 °C for *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- colorimetric (DNS) method based on the *endo-1,4-β-xylanase* hydrolysis of oat spelt xylan at pH 4.8 and 50 °C.

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

- colorimetric method based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4-β-xylanase* on oat azo-xylan at pH 4.8 and 50 °C.

1 IFP unit is the quantity of enzyme which liberates one μmole of reducing sugars (xylose equivalents) per minute from oat xylan at pH 4.8 and 50 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Safizym X* 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] *Application, Reference SANCO/D/2 Forw. Appl. 1831/00170 (10431)-2010
 - [2] *Application, Proposal for Register Entry – Annex A
 - [3] *Technical dossier, Section II, Identity, characterisation and conditions of use of the additive; methods of analysis
 - [4] *Technical dossier, Section II – Annex_II_2_1 Certificate of deposition
 - [5] *Supplementary Information, E-mail from Lesaffre 09_12_2011
 - [6] 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 to Community Reference Laboratories
 - [7] *Technical dossier, Section II-Annex_II_6_1
 - [8] *Technical dossier, Section II-Annex_II_6_3
 - [9] #Additional Information, Xylanase-feed without control feed
 - [10] *Technical dossier, Section II-Annex_II_6_4
- *Refers to Dossier No. FAD-2010-0374
#Refers to Dossier No. FAD-2006-0037

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

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:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)