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

CRL Feed Additives Authorisation

D08/FSQ/CVH/GS/(2006) D/16033

CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**EFSA-Q-2005-276**
Name of Additive:	**Safizym X®**
Active Substance(s):	**Endo-1,4-β-xylanase (EC 3.2.1.8)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives Authorisation (CRL-FAA), Geel, Belgium**
Report prepared by:	**Dalia Garalevičienė (CRL-FAA)**
Report checked by:	**Giuseppe Simone (CRL-FAA)**
Date:	**06/06/2006**
Report approved by:	**Christoph von Holst (CRL-FAA)**
Date:	**29/06/2006**

EXECUTIVE SUMMARY

In the current application authorisation is sought for Safizym X® under the category zootechnical additives, group 4(d), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Safizym X® as a zootechnical feed additive for piglets, according to EFSA-Q-2005-276. The additive is intended to be marketed in two forms, as a powder (Safizym XP20®) and as liquid formulation (Safizym XL200®).

The active agent of Safizym X® is endo-1,4-β-xylanase, produced by a microorganism *Trichoderma longibranchiatum* (CNCM MA 6-10W). According to the nomenclature of the International Union of Biochemistry and Molecular Biology (IUBMB), endo-1,4-β-xylanase has the number EC 3.2.1.8.

The activity of endo-1,4-β-xylanase is expressed as IFP (Institut Français du Pétrole) units. According to the applicant, one IFP unit is the quantity of enzyme which liberates one μmole of reducing sugars in equivalent xylose per minute from oat xylan under specific conditions (pH 4.8 and 50°C). According to the applicant, Safizym XP20® and Safizym XL200® have a guaranteed minimum activity of 70000 IFP/g and 7000 IFP/ml of product, respectively. The additive also contains a residual activity of endo-1,3-(4)- β–glucanase. Safizym XP20® is intended to be incorporated into premixtures or complete feedingstuffs, whereas the liquid formulation Safizym XL200® is sprayed onto the feedingstuffs to obtain enzyme activity levels of minimum 840 to recommended 1680 IFP/kg in complete feedingstuffs.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, the applicant proposes an absolute colorimetric method based on reducing sugar properties. Xylose is released from the substrate oat spelt xylan when incubated with endo-1,4-β-xylanase. Method's transferability on Safizym XP20® has been checked among three laboratories [*Anim. Feed Sci. Techn.* 1999, 77:345-353] and similar results were obtained. Therefore, the method is considered suitable for the intended purpose.

For the determination of the endo-1,4-β-xylanase activity in *premixtures*, the applicant proposes a relative colorimetric method, based on the principle that xylanase releases water soluble dyed fragments, when incubated with oat azo-xylan. Method's transferability on Safizym XP20® has been checked among two laboratories, obtaining similar results. Based on the method performance characteristics, that include a limit of detection (LOD) of 0.14 IFP/g, limit of quantification (LOQ) of 0.35 IFP/g and the within-laboratory relative standard deviation for repeatability (RSD_r) of 6.5 %, the method is considered suitable for the intended purpose.

For the quantification of the endo-1,4-β-xylanase activity in *feedingstuffs*, the applicant proposes the same method as for *premixtures*, just the sample extraction is modified and the

incubation time is prolonged. The enzyme activity in *feedingstuffs* is quantified against matrix matched standards (blank feed samples supplemented by a known dose of Safizym XP20® with declared activity). The transferability of the method has been checked among two laboratories [*Anim. Feed Sci. Techn*. 1999, 77:345-353] obtaining similar results. A separate check among two laboratories has been performed on the liquid formulation Safizym XL200®. The LOD and LOQ correspond to 140 IFP/kg and 350 IFP/kg of feedingstuffs, respectively, and the average within-laboratory RSD_r is 8.3 % for the powder and 9.2% for the liquid formulation. Taking into account the target enzyme activity level of 840 to 1680 IFP/kg of complete feedingstuffs and the acceptable values of method performance characteristics, the proposed method is considered fit for official controls to determine the activity of the endo-1,4-β-xylanase in target feedingstuffs at the target activity level, when the standard feed (blank feed supplemented by a known dose of Safizym XP20® with declared activity) is available.

It is recommended by the CRL that for the preparation of matrix matched standards for the quantification of the enzyme activity in feedingstuffs, the declared activity of endo-1,4-β-xylanase in Safizym XP20® is confirmed by applying the method proposed for the pure additive and the actual measured activity is taken into account for the calculation of the final enzyme activity in feedingstuffs.

In the case, that the standard feed (blank feed supplemented by a known dose of Safizym XP20® with declared activity) is not available, a standard addition method is recommended.

Further testing or validation is not considered necessary.

KEYWORDS

Safizym X®, endo-1,4-β-xylanase, *Trichoderma longibranchiatum*, other zootechnical additives, piglets

TABLE OF CONTENTS

1. BACKGROUND

Safizym X® is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003 [1]. Safizym X® contains endo-1,4-β-xylanase as the active agent [2], produced by a microorganism *Trichoderma longibranchiatum* (CNCM MA 6-10W) [3], which is deposited at the Collection Nationale de Cultures de Microorganismes (C.N.C.M.) at the Institute Pasteur, Paris, France.

The activity of endo-1,4-β-xylanase is expressed as IFP (Institut Français du Pétrole) units. According to the applicant, one IFP unit is the quantity of enzyme which liberates one μmole of reducing sugars in equivalent xylose per minute from oat xylan under specific conditions (pH 4.8 and 50°C). The additive is presented in two forms [4]:

- Safizym XP20®, which is a solid formulation with a minimum guaranteed activity of 70000 IFP/g
- Safizym XL200®, which is a liquid formulation with a minimum guaranteed activity of 7000 IFP/ml

Endo-1,4-β-xylanase, produced by a microorganism *Trichoderma longibranchiatum* (CNCM MA 6-10W), is already authorised (EC N° 1613) as a feed additive for chickens for fattening (Commission Regulation (EC) N° 1453/2004), turkeys for fattening (Commission Regulation (EC) N° 943/2005) and laying hens (Commission Regulation (EC) N° 1810/2005).

Safizym XP20® is intended to be incorporated into premixtures or complete feedingstuffs, whereas the liquid formulation Safizym XL200® is sprayed onto the feedingstuffs [5] to

obtain enzyme activity levels of minimum 840 to recommended 1680 IFP/kg in complete feedingstuffs [6].

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, 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 Safizym X®, *cf.* EFSA-Q-2005-276, and their suitability to be used for official controls, were evaluated.

3. EVALUATION

The numbering system under this point refers to that of Section II of the "Guidelines for the assessment of additives in feedingstuffs, PART II: Enzymes and Micro-organisms (2.5 Control methods)", in the following referred as "the Guidelines".

Description of the some analytical methods listed under 2.5.1 of the Guidelines

Quantitative analysis of the active agent in the additive Safizym X®

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, the applicant proposes an *absolute* colorimetric method [7]. The method is based on the fact that endo-1,4-β-xylanase catalyses the hydrolysis of oat xylan to yield xylose. Under alkaline conditions, xylose reduces the added 3,5-dinitro-salicylic acid to a coloured compound 3-amino-5-nitrosalicylic acid, which has a strong absorbance in the range of 550 to 600 nm. The optical density of the solution is measured on a spectrophotometer. The calibration is done against the xylose standard and the activity of endo-1,4-β-xylanase in the additive is calculated [8]. The relative within-laboratory standard deviation for repeatability (RSD_r) is 7.2 %.

Method's transferability on Safizym XP20® has been checked among three laboratories [7] and similar results were obtained. Therefore, the method is considered suitable for its purpose.

For the determination of the residual activity of β-glucanase present in the feed additive, a colorimetric method based on reducing sugars is proposed [7], which is considered suitable for the intended purpose.

Quantitative analysis of impurities

Analyses of heavy metals (arsenic, cadmium, lead and mercury), mycotoxins (aflatoxin B_1, ochratoxin A, T-2 toxin, zearalenone and sterigmatocystine) and microbiological agents (aerobic mesophillic bacteria, pathogenic staphylococci, sulphite reducing bacteria, total coliforms and *Escherichia coli*, *Salmonella* species, yeasts and moulds) are performed by an external laboratory. The methods have been internally developed and validated [9]. Only protocols of analytical results and short method descriptions, but neither full method protocols (and/or references) nor validation data have been provided. Therefore, the CRL cannot evaluate the suitability of the internal methods for official controls. Corresponding ISO/CEN standard methods are recommended by the CRL for official control purposes. ISO 14718 method is proposed for the analysis of aflatoxin B_1 [10] and is considered fit for official controls.

Description of the qualitative and quantitative analytical methods for routine control of the active agent in premixtures and feedingstuffs (cf. pt. 2.5.2 of the Guidelines)

For the determination of the activity of endo-1,4-β-xylanase in *premixtures,* the applicant proposes a *relative* colorimetric method, based on the principle that endo-1,4-β-xylanase releases water soluble dyed fragments from a substrate oat azo-xylan [11]. The formed dyed fragments are then measured on a spectrophotometer. The enzyme activity is quantified against a standard line realised with the Safizym XP20® with declared activity [11].

The transferability of the method has been performed among two laboratories using three inclusion levels of Safizym XP20® [12]. Based on the obtained method performance characteristics, that include a limit of detection (LOD) of 0.14 IFP/g, limit of quantification (LOQ) of 0.35 IFP/g and within-laboratory RSD_r of 6.5 % [9], [12], the method is considered suitable for the intended purpose.

For the quantification of the endo-1,4-β-xylanase activity in *feedingstuffs,* the applicant proposes the same method as for *premixtures,* just the extraction is modified and the incubation time is prolonged to allow for detection of lower activity levels in the feedingstuffs [11]. The enzyme activity in feedingstuffs is quantified against matrix matched standards (blank feed samples supplemented by known dosages of Safizym XP20® with declared activity).

Method's transferability on Safizym XP20® has been checked among two laboratories, using mashed and pelleted broiler feed, and the similar results were obtained [7]. A separate check among two laboratories, using starter and finisher broiler feed, has been performed on Safizym XL200® [9]. The LOD and LOQ correspond to 140 IFP/kg and 350 IFP/kg of

feedingstuffs [9], respectively, and the average within-laboratory RSD_r is 8.3 % for the powder [7] and 9.2% for the liquid formulation [9].

Taking into account the target enzyme activity level of 840 to 1680 IFP/kg of complete feedingstuffs and the acceptable values of method performance characteristics, the proposed method is considered fit for official controls to determine the activity of the endo-1,4-β-xylanase in target feedingstuffs at the target activity level, when the standard feed (blank feed supplemented by a known dose of Safizym XP20® with declared activity) is available.

4. CONCLUSIONS AND RECOMMENDATIONS

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, the applicant proposes an *absolute* colorimetric method based on reducing sugar properties and calibrated against the xylose standard [7]. The method has shown acceptable transferability results on Safizym XP20®among three laboratories [7] and is therefore considered suitable for the intended purpose.

For the determination of the activity of endo-1,4-β-xylanase in *premixtures*, a *relative* colorimetric method is proposed [11]. Based on the acceptable performance characteristics, the method is considered suitable for the intended purpose.

For the quantification of the endo-1,4-β-xylanase activity in *feedingstuffs*, the applicant proposes the same method as for *premixtures*, just the extraction is modified and the incubation time is prolonged [11]. Taking into account the target enzyme activity level and the acceptable values of method performance characteristics, the proposed method is considered fit for official controls to determine the activity of the endo-1,4-β-xylanase in target feedingstuffs at the target activity level, when the standard feed (blank feed supplemented by a known dose of Safizym XP20® with declared activity) is available.

For the determination of any impurities in the additive, methods internally developed and validated by an external laboratory are proposed [9]. However, neither full method protocols nor validation data have been provided. Therefore, the CRL cannot evaluate the suitability of the internal methods for official controls. Corresponding ISO/CEN standard methods are recommended by the CRL for official control purposes. ISO 14718 method is proposed for the analysis of aflatoxin B_1 [10] and is considered fit for official controls.

Recommendations

For the preparation of matrix matched standards for the quantification of the enzyme activity in feedingstuffs, the declared activity of endo-1,4-β-xylanase in Safizym XP20® must be confirmed by applying the method proposed for the pure additive and the actual measured

activity must be taken into account for the calculation of the final enzyme activity in feedingstuffs.

In the case, that the standard feed (blank feed supplemented by a known dose of Safizym XP20® with declared activity) is not available, a standard addition method is recommended.

Further testing or validation is not considered necessary.

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

Analytical method evaluated by the CRL: Reducing sugar assay for endo-1,4-β-xylanase by colorimetric reaction of dinitrosalicylic acid reagent on reducing sugar yield, or any other method with comparable performance characteristics.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Safizym XP20® and Safizym XL200® have been sent to the Community Reference Laboratory for Feed Additives Authorisation.

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

6. REFERENCES

[1] Reference SANCO/D/1 Forw. Appl. 1831/023-2005.

[2] Section II, Subject 2, Item 2.1.

[3] Section II, Subject 2, Item 2.2.

[4] Section II, Subject 2, Items 2.1-2.3.

[5] Section II, Subject 2. Item 5.1.

[6] Annex III. Proposal of Register entry.

[7] Cosson T., Perez Vendrell A.M., Gonzalez Teresa B., Rene D., Taillade P. and Brufau J. 1999. Enzymatic assays for xylanase and β-glucanase feed enzymes. *Anim. Feed Sci. Techn*. 77:345-353.

[8] Section II, Appendix 2.22.

[9] Additional information submitted by the applicant (received by e-mail on 8 March 2006).

[10] Additional information submitted by the applicant (received by e-mail on 18 April 2006).

[11] Section II, Appendix 2.23.

[12] Additional information submitted by the applicant (received by e-mail on 23 February 2006).

7. RAPPORTEUR LABORATORY

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