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

D08/FSQ/CVH/DG/D(2007) 11850

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-2006-320** **FAD-2006-0037**
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 (CRL-FA)** **Geel, Belgium**
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EXECUTIVE SUMMARY

In the current application authorisation is sought for *Safizym X* 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. Specifically, *cf.* EFSA-Q-2006-320, authorisation is sought to use *Safizym X* as a digestibility enhancer for ducks.

The active agent of *Safizym X* is endo-1,4-β-xylanase, produced by a strain of *Trichoderma longibranchiatum* CNCM MA 6-10W. Enzymatic activity is expressed in IFP (Institut Français du Pétrole) units. One IFP unit is defined as the amount of enzyme that liberates one μmol of reducing sugars (xylose equivalents) from oat xylan per minute at pH 4.8 and 50^{o}C. The additive is intended to be marketed in two forms, namely as a powder (*Safizym XP20*) containing 70000 IFP/g and as liquid formulation (*Safizym XL200*) containing 7000 IFP/ml of the product. *Safizym X* is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of minimum 700 IFP/kg in *feedingstuffs.* The recommended enzyme activity in feed is 2800 IFP/kg. *Safizym X* also contains a residual activity of endo-1,3-(4)-β–glucanase.

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 the fact that endo-1,4-β-xylanase releases xylose from the substrate oat spelt xylan. Released sugar reduces the added 3,5-dinitro-salicylic acid to a coloured compound that is measured spectrophotometrically and quantified against the xylose standard curve. Transferability of the method on *Safizym XP20* has been checked among three laboratories [*Anim. Feed Sci. Techn.* 1999, 77:345-353] obtaining similar results.

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 from the substrate oat azo-xylan. The formed dyed fragments are then measured with a spectrophotometer and quantification is performed via a standard line based on the reference enzyme *Safizym XP20,* available from the applicant upon request. Method performance characteristics, when checked between two laboratories, include a limit of detection (LOD) of 0.14 IFP/g, a limit of quantification (LOQ) of 0.35 IFP/g, a relative standard deviation for repeatability (RSD_r) of 6.5% and an intermediate relative standard deviation for reproducibility (RSD_R) of 16.6%.

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 which are blank feed samples fortified with a known dose of the reference enzyme *Safizym XP20*. In the case that a matrix matched blank feed is not available the

applicant proposes the use of the standard addition technique for quantifying the enzyme activity in feed.

Transferability of the method on *Safizym XP20*, using matrix matched standards, has been checked between two laboratories [*Anim. Feed Sci. Techn*. 1999, 77:345-353] obtaining similar results. The applicant also reported on another study with independent measurements between two laboratories. The LOD and LOQ correspond to 140 IFP/kg and 350 IFP/kg of *feedingstuffs*, respectively. The average RSD_r is 8.3% for powder form and 9.2% for liquid form. The average intermediate RSD_R is 15.8% for the liquid form and 24% for the powder form. Since the method shows acceptable performance characteristics, it is considered fit for official controls in the frame of the authorisation.

The CRL recommends that 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 or – in the case of major deviations - substituted by the actual measured activity of the enzyme.

Further testing or validation is not considered necessary.

KEYWORDS

Safizym X, endo-1,4-β-xylanase, *Trichoderma longibranchiatum*, digestibility enhancer, ducks

1. BACKGROUND

Safizym X is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'digestibility enhancers', 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* CL 847, which is deposited at the Collection Nationale de Cultures de Microorganismes (C.N.C.M.) under the reference CNCM MA 6-10W at the Institut Pasteur, Paris, France [3].

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 μmol of reducing sugars (xylose equivalents) per minute from oat xylan at pH 4.8 and 50^{o}C. The additive is marketed 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 No 1613) as a feed additive for chickens for fattening (Commission Regulation (EC) No 1453/2004), turkeys for fattening (Commission Regulation (EC) No 943/2005) and laying hens (Commission Regulation (EC) No 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 700 IFP/kg in complete *feedingstuffs* [6]. The recommended enzyme activity in feed is 2800 IFP/kg.

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 for each application. For this particular dossier, the methods of analysis submitted in connection with *Safizym X, cf.* EFSA-Q-2006-320, 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

For the determination of arsenic and heavy metals (cadmium, lead and mercury), mycotoxins and microbiological agents, the applicant proposes validated methods based on well known techniques [7]. The methods are therefore considered suitable for the intended purposes. However, based only on provided method summaries, the suitability of methods for official controls can not be evaluated, with an exception of an ISO 14718 method for the analysis of aflatoxin B_1, which is considered fit for official controls. Various standard methods based on the same analytical techniques and routinely applied by official control authorities are available and recommended by the CRL.

Description of the analytical methods for the determination of the active agent in the feed additive

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 the fact that endo-1,4-β-xylanase catalyses the hydrolysis of oat xylan to yield xylose. Released xylose reduces the added 3,5-dinitro-salicylic acid to a coloured compound 3-amino-5-nitrosalicylic acid, whose absorbance is measured on a spectrophotometer at 550 nm. The quantification is done against the xylose standard curve.

Transferability of the method on *Safizym XP20* has been checked among three laboratories obtaining average relative standard deviation for repeatability (RSD_r) of 7.2% and intermediate relative standard deviation for reproducibility (RSD_R) of 16.6% [8]. The precision data reported could be compared with corresponding data from other inter-laboratory studies on quantification of xylanase in the product. Bailey *et al*. (1992) reported values for the inter-laboratory RSD_R ranging from 17 to 30%, when analysing activity of endo-1,4-β-xylanase using birchwood glucuronoxylan [11]. König *et al*. (2002) tested endo-1,4-β-xylanase products using a substrate wheat arabinoxylan and obtained within-laboratory RSD_R of 4.4 to 5.3% [12]. Based on this comparison the performance characteristics of the

applicant's method are considered acceptable. The proposed method is therefore suitable for official control purposes.

For the determination of the residual activity of β-glucanase present in the *feed additive*, a colorimetric method based on reducing sugar properties is proposed [8], which is considered suitable for the intended purpose.

Description of the analytical methods for the determination of the active agent in premixtures and feedingstuffs

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 chromogenic substrate oat azo-xylan. The formed dyed fragments are then measured on a spectrophotometer. Enzymatic activity is quantified against a standard line of *Safizym XP20*, available from the applicant upon request [9]. Transferability of the method has been checked between two laboratories on three inclusion levels of *Safizym XP20* (140, 280 and 700 IFP/g *premixtures*). Method performance characteristics include average RSD_r of 6.5% (8.7, 6.6 and 4.1% for 140, 280 and 700 IFP/g *premixtures*, respectively) and average intermediate RSD_R of 16.6% (22.0, 13.7 and 14.0% for 140, 280 and 700 IFP/g *premixtures*) [10]. The limit of detection (LOD) is 0.14 IFP/g and the limit of quantification (LOQ) is 0.35 IFP/g,

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* [8,9]. The enzyme activity in *feedingstuffs* is quantified against matrix matched standards (blank feed samples supplemented by known dosages of *Safizym XP20* with declared activity). In the case that standard blank feed is not available, the applicant proposes the use of the standard addition technique for quantifying the enzyme activity in feed [13].

Transferability of the method on *Safizym XP20*, using matrix matched standards, has been checked between two laboratories at three inclusion levels (350, 1400 and 2800 IFP/kg) in mashed and pelleted broiler feed, and the similar results were obtained [8]. A separate check has been performed on *Safizym XL200* (at the same inclusion levels) between two laboratories, using broiler starter and finisher feed [10]. For *powder form*, average RSD_r is 8.3% (4.6 and 11.9% for 1400 IFP/kg of *pelleted* and *mashed* feed, respectively) and average intermediate RSD_R is 16.6% (33.9, 10.3 and 28.3% for 350, 1400 and 2800 IFP/kg *feedingstuffs*). For *liquid form*, average relative standard deviation for repeatability (RSD_r) is 9.2 % (4.6 and 11.9% for 1400 IFP/kg of *broiler* feed) and average intermediate relative standard deviation for reproducibility (RSD_R) is 15.8% (21.2, 10.8 and 15.5% for 350, 1400

and 2800 IFP/kg *feedingstuffs*). The LOD and LOQ correspond to 140 IFP/kg and 350 IFP/kg of *feedingstuffs* [10].

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 in the frame of authorisation. In the case that standard blank feed is not available, the same method, quantifying enzymatic activity by standard addition, is proposed.

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. The method has shown acceptable transferability results on *Safizym XP20* among three laboratories and is therefore considered suitable for the official controls.

For the determination of the activity of endo-1,4-β-xylanase in *premixtures* and *feedingstuffs*, a *relative* colorimetric method is proposed, just the extraction is modified and the incubation time is prolonged when analysing *feedingstuffs*. Based on acceptable performance characteristics, the method is considered fit for official controls in the frame of authorisation.

Recommendations

The CRL recommends that 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 or – in the case of major deviation - substituted by the actual measured activity of the enzyme.

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

Colorimetric method based on quantification of water soluble dyed fragments produced by reaction of endo-1,4-β-xylanase with oat azo-xylan substrate.

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

6. REFERENCES

[1] Reference SANCO/D/2 Forw. Appl. 1831/020-2006.

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

[3] Section II, Appendix 2.2a.

[4] Section II, Subject 1, Items 1.1-1.3.

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

[6] Annex III. Proposal of Register entry.

[7] Section II, Appendix 2.37.

[8] 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.

[9] Section II, Appendix 2.35.

[10] Section II, Appendix 2.36.

[11] Bailey M.J., Biely P. and Poutanen K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. of Biotechnology*, 23:257-270.

[12] König J., Grasser R., Pikor H. and Vogel K. 2002. Determination of xylanase, β-glucanase and cellulase activity. *Anal. Bioanal. Chem*., 374:80-87.

[13] Supplementary information obtained upon request of the CRL-FA (received 24/04/2007).

7. RAPPORTEUR LABORATORY

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

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Central Institute for Supervising and Testing in Agriculture, Praha, Czech Republic.
- Plantedirektoratets Laboratorium, Lyngby, Denmark.
- Sächsische Landesanstalt für Landwirtschaft, Leipzig, Germany.
- Service Commun des Laboratoires, Laboratoire de Rennes, France.
- Thüringer Landesanstalt für Landwirtschaft, Jena, Germany.