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

FUM*zyme*®

(FAD-2013-0002; CRL/120037)

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

Dossier related to:	**FAD-2013-0002 - CRL/120037**
Name of Product:	**FUMzyme®**
Active Substance(s):	**Fumonisin esterase (EC 3.1.1.87)**
Rapporteur Laboratory:	**European Reference Laboratory for Feed Additives (EURL-FA)**
Report prepared by:	**Johanna Keltti**
Report revised by: Date:	**Piotr Robouch (EURL-FA)** **30/08/2013**
Report approved by: Date:	**Christoph von Holst** **02/09/2013**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *FUMzyme®* (EC 3.1.1.87) under the category/functional group 1(m) "technological additives"/"substances for reduction of the contamination of feed by mycotoxins", according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the feed additive for pigs. *FUMzyme®* is used as feed additive for its ability to degrade fumonisin B1. The enzyme detoxifies this mycotoxin by cleavage of the toxin's diester bonds and removal of the propane-1,2,3-tricarboxylic acid (trivial name: tricarballylic acid) side chains. According to the Applicant, the active substance in *FUMzyme®* preparation is *fumonisin esterase* (EC 3.1.1.87) which is added to maltodextrin carrier to result in a minimum guaranteed enzyme activity of 3000 U/g. The Applicant defined the enzyme activity unit (U) as follows:

> *One unit is the enzymatic activity that releases 1 µmol tricarballylic acid per minute from 100 µM fumonisin B1 in 20 mM Tris-Cl buffer pH 8.0 with 0.1 mg/ml bovine serum albumin at 30 °C.*

FUMzyme® is intended to be used in *premixtures* and *feedingstuffs*, with a proposed enzyme activity ranging from 15 to 300 U/kg *feedingstuffs*.

For the determination of the *fumonisin esterase* activity in the *feed additive* and *feedingstuffs* the Applicant proposed a single laboratory validated and further verified High Performance Liquid Chromatography coupled to a tandem mass spectrometry (HPLC-MS/MS) method. The method is based on the quantification of the tricarballylic acid (TCA) released from the action of the enzyme on fumonisin B_1. The following performance characteristics were recalculated by the EURL based on the experimental data provided in the frame of the validation and verification studies:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.3 to 7.8 %;
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 3.4 to 8.2 %;
- a recovery rate (R_{Rec}) ranging from 89 to 115 %;
- a limit of quantification (LOQ) of 2.1 U/kg *feedingstuffs* and
- a limit of detection (LOD) of 0.6 U/kg *feedingstuffs*..

For the determination of the enzyme activities in *premixtures*, the Applicant suggested to dilute *premixture* samples with blank feeds (mainly consisting of wheat and maize) and to analyse them applying the method for *feedingstuffs* presented above.

Based on the satisfactory experimental evidence available the EURL recommends for official control the HPLC-MS/MS method submitted by the Applicant for the determination of the *fumonisin esterase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

KEYWORDS

FUMzyme®, Fumonisin esterase, technological additives, substances for reduction of the contamination of feed by mycotoxins, pigs.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) for *FUMzyme®* (EC 3.1.1.87) under the category/functional group 1(m) "technological additives"/"substances for reduction of the contamination of feed by mycotoxins", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for the use of the feed additive for pigs [2].

FUMzyme® is used as feed additive for its ability to degrade fumonisin B1. The enzyme detoxifies this mycotoxin by cleavage of the toxin's diester bonds and removal of the propane-1,2,3-tricarboxylic acid side chains. In this report, the trivial name 'tricarballylic acid' (TCA) is used for propane-1,2,3-tricarboxylic acid.

According to the Applicant, the active substance in *FUMzyme®* is *fumonisin esterase* which is added to maltodextrin carrier to result in a minimum guaranteed enzyme activity of 3000 U/g [3].

The Applicant defined the enzyme activity unit (U) [3] as follows:

> *One unit is the enzymatic activity that releases 1 µmol tricarballylic acid per minute from 100 µM fumonisin B1 in 20 mM Tris-Cl buffer pH 8.0 with 0.1 mg/ml bovine serum albumin at 30 °C.*

FUMzyme® is intended to be used in *premixtures* and *feedingstuffs*, with a proposed enzyme activity ranging from 15 to 300 U/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 *FUMzyme®,* 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 feed additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective European Union Reference Laboratories [4].

Description of the analytical methods for the determination of the active agents in the feed additive, premixtures and feedingstuffs

For the determination of the *fumonisin esterase* activity in the *feed additive* and *feedingstuffs* the Applicant proposed a single laboratory validated and further verified High Performance Liquid Chromatography coupled to a tandem mass spectrometry (HPLC-MS/MS) method. The method is based on the quantification of the tricarballylic acid (TCA) released from the fumonisin B_1 (FB1) [3].

The *feed additive* (1 g) is dissolved in 10 ml of the buffer solution (pH 8.0) containing Tris base and bovine serum albumin. Similarly 10 g of *feedingstuffs* sample is extracted in 100 ml of the buffer solution. The dissolved *feed additive* and the extracted, *feedingstuffs* samples, are further diluted with the same buffer to reach enzymatic activities in the reaction mixture of 0.15 to 3.0 U/kg. Aliquots (100 µl) of each test samples are added to 0.9 ml of FB1 (100 µM) solution and incubated at 30 ºC. On five time intervals ranging from 15 minutes to 4 hours a sample of the reaction mixture is taken for the analysis and heated at 99 ºC for 5 minutes to stop the reaction [5]. TCA is then quantified in the five samples by HPLC-MS/MS (parent ion is 174.904 and fragment ion for quantification is 69.000) using external calibration. The measured TCA amounts are plotted versus time and the enzyme activity is then derived from the slope of the linear range of this curve. Detailed instrumental settings of the specific MS/MS system used were provided by the Applicant as supplementary information [8].

Furtheremore, the Applicant suggested to dilute *premixture* samples with blank feed (mainly consisting of wheat and maize) and to analyse them applying the above mentioned method for the determination of *fumonisin esterase* activity in *feedingstuffs* [8].

The performance characteristics recalculated by the EURL [9] - based on the experimental data reported in the frame of the validation [6] and verification [7] studies are presented in the Table 1. Furthermore, the Applicant determined a limit of detection (LOD) and limit of quantification (LOQ) of 0.6 and 2.1 U/kg *feedingstuffs* [6].

Table 1: Performance characteristics of analytical methods for the determination of *fumonisin esterase* in *feed additive* (FA) and *feedingstuffs* (FS), recalculated by the EURL [9] based on the experimental data provided in the frame of the validation [6] and verification [7] studies.

		RSD_r (%)		**RSD_{ip} (%)**		**R_{rec} (%)**	
	Activity [8]	Valid. [9]	Verif. [9]	Valid. [9]	Verif. [9]	Valid. [9]	Verif. [9]
FA	3000 U/g	2.3-6.0	2.7-3.6	3.4-6.5	3.7	113-115	99
FS	15 – 300 U/kg	3.4-7.8	2.6-6.8	5.2-7.3	8.2	89-98	101

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec} : recovery rate

Based on the satisfactory experimental evidence available the EURL recommends for official control the HPLC-MS/MS method submitted by the Applicant for the determination of the *fumonisin esterase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single laboratory validated and further verified HPLC-MS/MS method submitted by the Applicant for the determination of *fumonisin esterase* in the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

Determination of *fumonisin esterase* activity in the *feed additive, premixtures* and *feedingstuffs*:

- High Performance Liquid Chromatography coupled to a tandem mass spectrometry (HPLC-MS/MS) method based on the quantification of the tricarballylic acid released from the action of the enzyme on fumonisin B1 at pH 8.0 and 30 ºC

One unit (U) is the enzymatic activity that releases 1 µmol tricarballylic acid per minute from 100 µM fumonisin B1 in 20 mM Tris-Cl buffer pH 8.0 with 0.1 mg/ml bovine serum albumin at 30 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference sample of *FUMzyme®* 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. 1831/0001-2013
[2] Application, Proposal for Register Entry
[3] Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; methods of analysis
[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 to Community Reference Laboratories
[5] Technical dossier, Section II – Annex 71
[6] Technical dossier, Section II – Annex 72
[7] Technical dossier, Section II – Annex 73
[8] Supplementary information, SIN-20130627
[9] Supplementary information, EURL-recalculation

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the 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:

- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala
- 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)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)

- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SL)
- Państwowy Instytut Weterynaryjny, Puławy (PL)