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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

Zearalenone hydrolase

(FAD-2019-0088; CRL/190043)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2019-0088 - CRL/190043**
Name of Product:	***Zearalenone hydrolase***
Active Agent (s):	**Zearalenone hydrolase EC 3.1.1.-**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Zigmas Ezerskis** **12/06/2020**
Report approved by: Date:	**Christoph von Holst** **12/06/2020**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *zearalenone hydrolase* under the category/functional group 1(m) "technological additives" / "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action", 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 all terrestrial animal species. The product is intended to be marketed by the Applicant as preparation, namely ZENzyme®. The *active substance* in ZENzyme® is the purified enzyme *zearalenone hydrolase* (EC 3.1.1.-). According to the Applicant the preparation has a minimum guaranteed enzyme activity of 7 Unit/g, where:

> *"one unit corresponds to the enzymatic activity that hydrolyses 1 μmol zearalenone per minute in a 5 mg/L (15.71 μM) zearalenone solution in Teorell Stenhagen buffer, pH 7.5, with 0.1 mg/L BSA at 37 °C".*

ZENzyme® is intended to be used in *premixtures* and *feedingstuffs*, at a minimum recommended enzyme activity of 10 U/kg *feedingstuffs*.

For the quantification of the *zearalenone hydrolase* activity in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed an activity assay, where the decay of zearalenone triggered by the enzyme at specific experimental conditions is monitored with a single-laboratory validated and further verified method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). In these experiments the enzymatic activity is determined as zearalenone degradation over an enzymatic reaction time, where the corresponding concentration of zearalenone is determined at various points in time by means of the HPLC-MS/MS method.

Based on the experimental evidence available the EURL recommends for official control the enzymatic activity assay based on the HPLC-MS/MS method submitted by the Applicant for the quantification of the *zearalenone hydrolase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

zearalenone hydrolase, ZENzyme®, technological additives, substances for reduction of the contamination of feed by mycotoxins, all terrestrial animal species

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (authorisation of a feed additive) for *zearalenone hydrolase* under the category/functional group 1(m) "technological additives" / "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action", according to the classification system of Annex I of Regulation (EC) No 1831/2003 as amended by Commission Regulation (EC) No 386/2009 [1]. The authorisation is sought for the use of the *feed additive* for all terrestrial animal species [2].

The product is intended to be marketed by the Applicant as preparation, namely ZENzyme® [3]. The *active substance* in ZENzyme® is the purified enzyme *zearalenone hydrolase* (EC 3.1.1.-) produced by genetically modified Escherichia coli DSM 32731 (deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen - Braunschweig, Germany) [4]. ZENzyme® is produced by addition of the *active substance* to a maltodextrin carrier resulting in a minimum guaranteed enzyme activity of 7 Unit/g [5], where:

> *"one unit corresponds to the enzymatic activity that hydrolyses 1 µmol zearalenone per minute in a 5 mg/L (15.71 µM) zearalenone solution in Teorell Stenhagen buffer, pH 7.5, with 0.1 mg/L BSA at 37 °C".*

According to the Applicant *Zearalenone hydrolase* (*ZenA*) is used to degrade zearalenone (ZEN). The mycotoxin is detoxified by enzymatic hydrolysis via the opening of the lactone ring [6].

ZENzyme® is intended to be used in *premixtures* and *feedingstuffs*, at a minimum recommended enzyme activity of 10 U/kg *feedingstuffs* [7].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *zearalenone hydrolase* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of the *ZenA* activity in the *feed additive* (preparation i.e. ZENzyme®), *premixtures* and *feedingstuffs* the Applicant proposed an activity assay, where the decay of ZEN, triggered by the enzyme at specific experimental conditions, is monitored with a single-laboratory validated and further verified method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) [8-11].

The procedure comprises the following steps: (i) sample preparation and enzymatic reaction; (ii) HPLC-MS/MS analysis; and (iii) data evaluation.

i. Sample preparation and enzymatic reaction

As mentioned above, *ZenA* degrades ZEN. The enzyme hydrolyses the mycotoxin by opening the toxin's lactone ring and thus forming hydrolysed zearalenone (HZEN). HZEN may further undergo spontaneous decarboxylation resulting in decarboxylated hydrolysed zearalenone (DHZEN). The decarboxylation is accelerated by high temperatures and low pH [6]. However, the procedure presented by the applicant foresees exclusively the measurement of the decay of ZEN over time.

Feed additive: 150 to 250 mg of dried enzyme preparation (e.g. ZENzyme®) is dissolved in the sample buffer solution (Teorell Stenhagen buffer, pH 7.5 with 0.1 mg/L BSA) to a final concentration of 50 mg/mL. The solutions of the enzyme samples with known enzyme activity (e.g. from an indication on the label) are further diluted in sample buffer to a final enzyme concentration of 2.5 U/L. When the concentration of the dried enzyme sample is unknown, 14 standard dilutions have to be prepared and tested to obtain results in the detection range (from 1:1000 to 1:14000 dried enzyme preparation in sample buffer).

Premixtures or *feedingstuffs:* 10 g of sample is dissolved in the sample buffer solution. The mixture is kept under constant shaking for 60 min at room temperature. Afterwards, the solid particles are left to deposit. 1 mL of the supernatant is transferred into a reaction tube and centrifuged. The final supernatant is ready to be used for the activity assay. For feed samples with known enzyme activity (e.g. from an indication on the label) the clear extract is added directly into the reaction tube or diluted to a final enzyme concentration of 2 U/L. For feed samples with unknown concentration, at least three standard solutions of clear feed extract in sample buffer have to be prepared and tested to obtain results in the detection range (undiluted, diluted 1:5 and 1:10, v/v). According to the Applicant, the assay is able to detect a range of enzymatic activities between 0.02 and 0.15 U/L. For unknown samples further appropriate dilutions may be necessary.

All samples: Sample buffer and a solution containing ZEN is added into the reaction tube and vortexed (for the different type of samples, different volumes have to be transferred). The diluted extracts are added and the reaction starts by placing the tube in incubation in a pre-warmed water bath at 37 °C. A total of 7 subsamples are collected every 30 min starting from time zero. The enzymatic reaction for each subsample is stopped by applying heat (99 °C for 10 min). After inactivation, the subsamples are centrifuged and the supernatant is used for HPLC-MS/MS analysis [10].

ii. HPLC-MS/MS

The clear subsample is transferred into HPLC vials and diluted 1:20 in acetonitrile [10]. 1 µL is injected into a reversed phase HPLC. ZEN is detected by tandem mass spectrometry (m/z 317.1 > 131.0) and quantified using an external calibration [11,12].

iii. Data evaluation

To determine the ZEN-Units (U), the results obtained from the HPLC-MS/MS analysis expressed in µg/L are converted into µM and plotted against the sampling time. Within the linear range of the curve a linear regression line is calculated starting from the initial sampling (time zero). Only a coefficient of determination (R^2) larger than 0.985 is considered to be acceptable. The resulting slope of the curve is corresponding to the degradation of ZEN in µM per hour (rate of the enzymatic reaction). The absolute value of the slope is the enzymatic activity of *ZenA* expressed in µM per hour. The enzymatic activity is further calculated by a specific equation in order to express the results in U/g and U/kg, respectively, for the enzyme preparation and feed samples [10].

For the quantification of the enzyme activities in *premixtures*, the EURL suggests to dilute *premixture* samples with animal feed (according to the recommended inclusion rate) and to analyse them applying the assay for *feedingstuffs* as mentioned above.

The enzymatic activity assay using high performance liquid chromatography coupled to HPLC-MS/MS for the quantification of the *zearalenone hydrolase* activity in the *feed additive* and *feedingstuffs* has been single-laboratory validated and further verified [13-15]. The Applicant presented the following performance characteristics for the determination of the enzymatic activity in the *feed additive* and *feedingstuffs* containing 150 to 789 U/g and 6 to 120 U/kg, respectively: a relative standard deviation for *repeatability* (RSD_r) ranging from 5.9 to 16.3 %, a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 8.5 to 16.3 %, and a *recovery* rate (R_{Rec}) ranging from 80 to 97 %.

Based on the experimental evidence available the EURL recommends for official control the enzymatic activity assay based on the HPLC-MS/MS method submitted by the Applicant for the quantification of the *ZenA* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Note: Several standard methods for mycotoxins determination in the *feedingstuff* field are available and possibly fit for purpose for ZEN determination (i.e. EN ISO 17194:2019, EN ISO 15792:2009, ISO 17372:2008) [16-18]. In particular the EN ISO 17194:2019 standard on the determination of various mycotoxins (including ZEN) also applies HPLC-MS/MS [16]. The standard may therefore be applicable to the quantification of ZEN as alternative to the HPLC-MS/MS method presented by the Applicant. However, prior to the use of the EN ISO 17194:2019 or the other relevant standards, these methods need to be checked for compatibility with the protocol of the sample preparation and enzymatic reaction as specified in the previous chapter and which cannot be modified.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not considered necessary by the EURL.

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, as last amended by Regulation (EU) 2015/1761) 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 an enzymatic activity assay with an HPLC-MS/MS method submitted by the Applicant for the quantification of the *ZenA* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *zearalenone hydrolase* activity in the *feed additive, premixtures* and *feedingstuffs*:

- an enzymatic activity assay based on the determination of the *zearalenone* degradation from the action of *zearalenone hydrolase* at pH 7.5 and 37 ºC measured with high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS).

One unit (U) corresponds to the enzymatic activity that hydrolyses 1 µmol zearalenone per minute in a 5 mg/L (15.71 µM) zearalenone solution in Teorell Stenhagen buffer, pH 7.5, with 0.1 mg/L BSA at 37 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *zearalenone hydrolase* 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 SANTE_E5_FWD. APPL. 1831-0082-2019 & Annex I – submission number 1575965647414-2494

[2] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[3] *Technical dossier, Section II: 2.1 Identity of the additive

[4] *Technical dossier, Section II: 2.2 Characterisation of the active substance/agent

[5] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[6] *Technical dossier, Section II: 2.2.1.1 Enzyme

[7] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[8] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance

[9] *Technical dossier, Section II – Annex II_01 Annex entry proposal.pdf

[10] *Technical dossier, Section II – Annex II_53 SOP ZENzyme activity assay.pdf

[11] *Technical dossier, Section II – Annex II_52 SOP Determination of ZEN using LCMSMS.pdf

[12] *Technical dossier, Section II – Annex II_54 Validation report_PartA_Determination of the enzymatic activity_LC-MSMS.pdf

[13] *Technical dossier, Section II – Annex II_55 Validation report_PartB_ZENzyme activity assay.pdf

[14] *Technical dossier, Section II - Annex II_56 Verification Additive.pdf

[15] *Technical dossier, Section II - Annex II_57 Verification Feed.pdf

[16] EN ISO 17194:2019 - Animal feeding stuffs: Methods of sampling and analysis - Determination of Deoxynivalenol, Aflatoxin B1, Fumonisin B1 & B2, T-2 & HT-2 toxins, Zearalenone and Ochratoxin A in feed materials and compound feed by LC-MS/MS

[17] EN ISO 15792:2009 - Animal feeding stuffs - Determination of zearalenone in animal feed - High performance liquid chromatographic method with fluorescence detection and immunoaffinity column clean-up

[18] ISO 17372:2008 Animal feeding stuffs -- Determination of zearalenone by immunoaffinity column chromatography and high performance liquid chromatography

*Refers to Dossier no: FAD-2019-0088

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Wageningen Food Safety Research[1] (WFSR), Wageningen (NL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Istituto Superiore di Sanità. Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Roma (IT)

[1] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.