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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

Preparation of 6-phytase (EC 3.1.3.26)

(FAD-2020-0083; CRL/200044)

European Union Reference Laboratory
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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

Dossier related to:	**FAD-2020-0083 - CRL/200044**
Name of Product:	***Preparation of 6-phytase***
Active Agent (s):	**6-phytase (EC 3.1.3.26)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **03/09/2021**
Report approved by: Date:	**Christoph von Holst** **06/09/2021**

EXECUTIVE SUMMARY

In the current application, authorisation of a *preparation of 6-phytase (EC 3.1.3.26)* is sought under Article 4 for all poultry and all pig species under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the active agent of the product is *6-phytase*, produced by fermentation of the genetically modified strain *Trichoderma reesei* CBS 146250. Other *preparation of 6-phytase* from a different *Trichoderma reesei* strain is currently authorised as *feed additive*.

The activity of *6-phytase* is expressed in phytase units (FTU) where "one FTU is the amount of enzyme which releases one micromole of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C". This definition is in agreement with the phytase activity unit as described in EN ISO 30024.

The product is intended to be marketed in three different formulations namely *Axtra® PHY GOLD 30 L* (liquid), *Axtra® PHY GOLD 30 T* (granular and thermostable) and *Axtra® PHY GOLD 65 G* (granular) with a guaranteed minimum *6-phytase* activity of 30000 FTU / g for the *Axtra® PHY GOLD 30 L* and the *Axtra® PHY GOLD 30 T* formulations and 65000 FTU / g for the *Axtra® PHY GOLD 65 G* formulation. The product is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum recommended activity of 300 FTU / kg *feedingstuffs* for laying hens and other birds for laying and 500 FTU / kg *feedingstuffs* for the other target species.

The Applicant submitted single-laboratory validated and further verified methods for the quantification of the *phytase* activity in the *product (Axtra® PHY GOLD), premixtures* and *feedingstuffs*. The submitted methods are very similar to the ring-trial validated EN ISO 30024 method.

Upon request of the EURL, the Applicant compared both protocols confirming that equivalent results are obtained when applying the slightly different methods to *feedingstuffs* containing the product *(Axtra® PHY GOLD).*

Additionally the EURL is aware of other ring-trial validated VDLUFA methods specifically describing the preparation of *premixtures* (VDLUFA 27.1.3) and *feed additives* (VDLUFA 27.1.4) for the quantification of their *phytase* activity according to EN ISO 30024.

Based on the performance characteristics available the EURL recommends for official control the ring-trial validated EN ISO and VDLUFA colorimetric methods mentioned above for the quantification of the phytase activity in the *product*, *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

Preparation of 6-phytase (EC 3.1.3.26), *Axtra PHY GOLD*, zootechnical additives, digestibility enhancers, all pig and all poultry species.

1. BACKGROUND

In the current application, authorisation of a *preparation of 6-phytase (EC 3.1.3.26)* is sought under Article 4(1) (authorisation of a new feed additive) for all poultry and all pig species [1, 2] under the category/functional group 4 (a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003.

According to the Applicant, the *active agent* of the product is *6-phytase*, produced by fermentation of the genetically modified strain *Trichoderma reesei* CBS 146250 [3]. Other *preparation of 6-phytase* from a different *Trichoderma reesei* strain is currently authorised as *feed additive* [4, 5].

The activity of *6-phytase* is expressed in phytase units (FTU) where "one FTU is the amount of enzyme which releases one micromole of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37 °C" [3]. This definition is in agreement with the *phytase* activity unit as described in EN ISO 30024 [6].

The product is marketed as three different formulations so called *Axtra® PHY GOLD 30 L* (liquid), *Axtra® PHY GOLD 30 T* (granular and thermostable) and *Axtra® PHY GOLD 65 G* (granular) with a guaranteed minimum *6-phytase* (active agent) activity of 30000 FTU / g and 65000 FTU / g for the liquid and granular formulations, respectively [7]. *Axtra® PHY GOLD* is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum recommended activity of 300 FTU / kg *feedingstuffs* for laying hens and other birds for laying and 500 FTU / kg *feedingstuffs* for the other target species [2, 7].

Note: The analytical methods for the determination of another *Axtra® PHY* from a different strain of *Trichoderma reesei* were already evaluated by the EURL in the frame of previous dossiers [8].

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 the *preparation of 6-phytase (EC 3.1.3.26)* 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)

The Applicant submitted single-laboratory validated and further verified methods for the quantification of the *phytase* activity in the *product (Axtra® PHY GOLD)* [9], *premixtures* [10-12] and *feedingstuffs* [11-13]. According to the Applicant the submitted methods are very similar to the ring-trial validated ISO method (EN ISO 30024) [6] thus the standard EN ISO 30024 should be applicable for *Axtra® PHY GOLD* [14].

In the EN ISO 30024, samples containing phytase are incubated with sodium phytate, triggering the release of inorganic phosphate and forming a yellow complex with an acidic molybdate / vanadate reagent. The optical density of the yellow complex is measured at 415 nm and the inorganic phosphate released is quantified against a phosphate standard calibration curve. The following performance characteristics were reported for *feedingstuffs* at nominal phytase activities ranging from 500 to 1500 FTU / kg *feedingstuffs* [6]:

- a relative standard deviation for repeatability (RSD_r) ranging from 2.2 to 11 %;
- a relative standard deviation for reproducibility (RSD_R) ranging from 5.4 to 15 %; and
- a limit of quantification (LOQ) of 60 FTU / kg *feedingstuffs*.

Additionally the method VDLUFA 27.1.4 describes the preparation of phytases as *feed additives* for the determination of their phytase activity according to EN ISO 30024. This combination of methods has been ring-trial validated for phytase samples with activities between 6000 and 58000 FTU / g leading to the following performance characteristics [15]:

- a RSD_r ranging from 2.3 to 4.6 %; and
- a RSD_R ranging from 6.1 to 21 %.

Furthermore, the VDLUFA 27.1.3 method [16], based on a solid dilution using maize meal, describes specifically the preparation of *premixtures* for quantification of the phytase activity according to EN ISO 30024. This combination of methods has been ring-trial validated for

premixtures with phytase activities between 13000 to 228000 FTU / kg leading to the following performance characteristics [16]:

- a RSD_r ranging from 3.3 to 7.6 %; and
- a RSD_R ranging from 8.3 to 23 %.

Upon EURL request, the Applicant compared the analytical protocol submitted [13] with the one described in the EN ISO 30024 [17]. After their evaluation, the EURL identified some modifications done by the Applicant. However, the Applicant confirmed that despite the differences identified by the EURL both protocols will lead to equivalent results [18]. This conclusion was supported by experimental data [19].

Furthermore, as the VDLUFA methods specifically describe the preparation of *feed additives* (VDLUFA 27.1.4) and *premixtures* (VDLUFA 27.1.3) for the quantification of their *phytase* activity according to EN ISO 30024, the experiments provided by the Applicant proved also the suitability of the mentioned methods for these two other matrices.

Based on the performance characteristics available the EURL recommends for official control the ring-trial validated EN ISO and VDLUFA colorimetric methods mentioned above for the quantification of the phytase activity in the *product*, *premixtures* and *feedingstuffs*.

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.

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 the ring-trial validated colorimetric methods based on the enzymatic reaction of *phytase* on phytate for the quantification of the *phytase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of phytase activity in the *feed additive*:

- colorimetric method based on the enzymatic reaction of *phytase* on phytate - VDLUFA 27.1.4

For the quantification of phytase activity in *premixtures*:

- colorimetric method based on the enzymatic reaction of *phytase* on phytate - VDLUFA 27.1.3

For the quantification of phytase activity in *feedingstuffs*:

- colorimetric method based on the enzymatic reaction of *phytase* on phytate - EN ISO 30024

One *phytase* unit (FTU) is the amount of enzyme which releases one micromole of inorganic orthophosphate from a sodium phytate substrate per minute at pH 5.5 and 37°C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the *preparation of 6-phytase (EC 3.1.3.26)* 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: FORW. APPL. 1831-0073-2020

[2] *Annex I Submission number 1602852355009-2704

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

[4] Commission Implementing Regulation (EU) 2016/899 of 8 June 2016 concerning the authorisation of a 6-phytase produced by Trichoderma reesei (ATCC SD-6528) as a feed additive for all poultry species and all porcine species (other than suckling piglets) (holder of authorisation Danisco (UK) Ltd) O.J. L 152, 9.6.2016

[5] Commission Implementing Regulation (EU) 2017/896 of 24 May 2017 concerning the authorisation of a preparation of 6-phytase, produced by Trichoderma reesei (ATCC SD-6528) as a feed additive in solid form for all poultry species and all porcine species (other than suckling piglets) (holder of the authorisation Danisco (UK) Ltd) O.J. L 138, 25.5.2017

[6] EN ISO 30024:2009 - Animal feeding stuffs - Determination of phytase activity

[7] *Technical dossier, Section II: 2 Introduction

[8] EURL Evaluation Report:
https://ec.europa.eu/jrc/sites/jrcsh/files/finirep-fad-2015-0048-axtra-phy-20000tpt2.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2013-0049-axtraphy15000l.pdf

[9] *Technical dossier, Annexes_II_42-44

[10] *Technical dossier, Annexes_II_45

[11] *Technical dossier, Annex_II_46

[12] *Technical dossier, Annex_II_47

[13] *Technical dossier, Annex_II_48

[14] *Technical dossier, Section II: 2.6 Methods of analysis and reference samples

[15] Association of German Agricultural Analytic and Research Institute (VDLUFA): Method 27.1.3 Preparation of mineral feeds and mineral premixtures for the determination of the phytase activity

[16] Association of German Agricultural Analytic and Research Institutes (VDLUFA): Method 27.1.4 Preparation of feed additives for the determination of the phytase activity

[17] *Supplementary information, Method comp_phytase activity in feed_ISO 30024 vs Danisco F_031_08_EURL_TRC.pdf

[18] *Supplementary information, Annex_1_ Reply_Conf.pdf

[19] Supplementary information, Annex_II_2.1_Conf.pdf

*Refers to Dossier no: FAD-2020-0083

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

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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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