EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

European Union Reference Laboratory
Feed Additives

JRC F.5/CvH/MGH/AS/Ares

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)

(FEED-2021-2299; CRL/210057)

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:	**FEED-2021-2299 - CRL/210057**
Name of Product:	***Preparation of 6-phytase (EC 3.1.3.26)***
Active Agent (s):	**6-phytase (EC 3.1.3.26)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Zigmas Ezerskis** **09/11/2022**
Report approved by: Date:	**Christoph von Holst** **10/11/2022**

EXECUTIVE SUMMARY

In the current application, an authorisation of a *preparation of 6-phytase (EC 3.1.3.26)* is sought under Article 4(1) for all poultry, all pig and all fish 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 (*HiPhorius™)* is a *6-phytase*, produced by *Aspergillus oryzae* DSM33737. Another *preparation of 6-phytase* from a different *Aspergillus oryzae* strain is currently authorised as *feed additive*.

The product is intended to be marketed in several solid and liquid formulations with a guaranteed minimum *6-phytase* activities ranging from of 10000 to 50000 FYT / g depending on the formulation. According to the Applicant, the solid formulations are intended to be used for poultry and swine while the liquid formulations are intended to be used also for aquaculture. *HiPhorius™* is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 200 FYT / kg *feedingstuffs* for pigs and poultry and a minimum phytase activity of 1000 FYT / kg *feedingstuffs* for fish.

The activity of *6-phytase* is expressed in phytase units (FYT). One FYT unit is defined as "the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM at pH 5.5 and 37 °C". This definition is in agreement with the *phytase* activity unit as stated in the EN ISO 30024.

For the quantification of the *phytase* activity in *feedingstuffs* the Applicant submitted the ring-trial validated colorimetric standard method EN ISO 30024 and also applied it, with minor experimental modifications, for the quantification of the *phytase* activity in the *feed additive* (*HiPhorius™*) and *premixtures.* The Applicant confirmed the equivalence of these modified methods to the ring-trial validated VDLUFA 27.1.3 and VDLUFA 27.1.4 methods.

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 *feed additive* (*HiPhorius™*), *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), *HiPhorius*^{TM}, zootechnical additives, digestibility enhancers, all pig, all poultry and all fish species.

1. BACKGROUND

In the current application, an 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, all pig and all fish species under the category/functional group 4 (a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1, 2].

According to the Applicant, the active agent of the product (*HiPhorius*^{TM}*)* is a *6-phytase*, produced by *Aspergillus oryzae* DSM33737 [3]. Another *preparation of 6-phytase* from a different *Aspergillus oryzae* strain is currently authorised as *feed additive* [4].

The product is marketed in solid (*HiPhorius*^{TM} *10* and *HiPhorius*^{TM} *40)* and liquid (*HiPhorius*^{TM} *20 L* and *HiPhorius*^{TM} *50 L)* formulations with a guaranteed minimum *6-phytase* (active agent) activities of 10000 and 40000 FYT / g for the solid formulations and of 20000 and 50000 FYT / g for the liquid formulations, respectively [3]. According to the Applicant, the solid formulations are intended to be used for poultry and swine while the liquid formulations are intended to be used for poultry, swine and aquaculture. *HiPhorius*^{TM} is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 200 FYT / kg *feedingstuffs* for pigs and poultry and a minimum phytase activity of 1000 FYT / kg *feedingstuffs* for fish [3].

The activity of *6-phytase* is expressed in phytase units (FYT). One FYT unit is defined as "the amount of enzyme that releases 1 μmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM at pH 5.5 and 37 °C" [3,5]. This definition is in agreement with the *phytase* activity unit as stated in the EN ISO 30024 [6].

Note: The analytical methods for the determination of other *6-phytase preparations* from different strains were already evaluated by the EURL in the frame of previous dossiers [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 *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)

For the quantification of the *phytase* activity in *feedingstuffs* the Applicant submitted the ring-trial validated colorimetric standard method EN ISO 30024 [6] based on the enzymatic reaction of *phytase* on phytate.

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 FYT / kg [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 FYT / kg *feedingstuffs*.

Similarly, for the quantification of the *phytase* activity in the *feed additive* and *premixtures* the Applicant applied EN ISO 30024, adapting the sample extraction and dilution of the *feed additive* (*HiPhorius*^{TM}) and the *premixtures* [8, 9].

The EURL is aware of the ring-trial validated VDLUFA 27.1.3 and VDLUFA 27.1.4 methods [10, 11] describing the preparation of *feed additives* and *premixtures* for the quantification of the *phytase* activity according to EN ISO 30024.

The proposed methods [8-9] have been already proposed and evaluated by the EURL in the frame of a different *preparation of 6-phytase* previously submitted by the Applicant [7]. Taking into account that at that time, the Applicant confirmed that the modifications of the EN ISO 30024 intended to extend its scope to the *feed additive* and *premixtures* [8, 9] were fully equivalent to those described in the ring-trial validated VDLUFA 27.1.3 and VDLUFA 27.1.4 methods [12], the EURL considers that this statement is still valid and fully applicable to the current application.

Furthermore, in the frame of the homogeneity and stability studies the Applicant applied the proposed methods to each of the *feed additive* formulations i.e. *HiPhorius* 10; *HiPhorius* 40, *HiPhorius* 20 L and *HiPhorius* 50 L [13], to two different *premixtures* (for poultry and pigs) and to pelleted and mash *feedingstuffs* for poultry, pig and fish [14-15]. The evaluation of these experimental data by the EURL led to acceptable performance characteristics [16] demonstrating thus the applicability of the proposed methods to all the products and matrices, which are subject of this dossier.

Based on the performance characteristics available the EURL recommends for official control the ring-trial validated colorimetric methods (VDLUFA 27.1.4, VDLUFA 27.1.3 and EN ISO 30024) for the quantification of the *phytase* activity in the *feed additive*, *premixtures* and *feedingstuffs*, respectively.

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, 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 ring-trial validated colorimetric methods (VDLUFA 27.1.4, VDLUFA 27.1.3 and EN ISO 30024) based on the enzymatic reaction of *phytase* on phytate for the quantification of the *phytase* activity in the *feed additive*, *premixtures* and *feedingstuffs*, respectively.

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 the phytate - VDLUFA 27.1.4

For the quantification of phytase activity in *premixtures*:

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

For the quantification of phytase activity in *feedingstuffs*:

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

One phytase unit (FYT) is the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM 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] Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform
https://webgate.ec.europa.eu/esfc/#/applications/4309
https://open.efsa.europa.eu/questions/EFSA-Q-2022-00082

[2] *Application, Annex I

[3] *Technical dossier, Section II: 2.1 & 2.2 Identity & Characterisation of the active substance

[4] Commission implementing Regulation (EU) (EU) 2016/1881 of 24 October 2016 amending Implementing Regulation (EU) No 837/2012 as regards the minimum activity of 6-phytase produced by Aspergillus oryzae (DSM 22594) as feed additive for sows (holder of authorisation DSM Nutritional Products Ltd)

[5] * Technical dossier, Section II, Section II: 2.6 Methods of analysis

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

[7] EURL evaluation Reports:
https://joint-research-centre.ec.europa.eu/document/download/00758e91-5f7d-4a7b-9ae8-0d4989c11b48_en?filename=finrep_fad-2021-0066_0067_vtr-phytase.pdf
https://joint-research-centre.ec.europa.eu/document/download/e1f4e27a-94ff-41bb-a726-087db2267a59_en?filename=finrep_fad-2020-0083_axtra-phy-gold.pdf
https://joint-research-centre.ec.europa.eu/document/download/a88c64f9-9665-4337-9db2-53a9a87ac543_en?filename=finrep-fad-2019-0005-nutrasep.pdf
https://joint-research-centre.ec.europa.eu/document/download/fe95dc43-222e-4953-8be9-4f42e06520c3_en?filename=finrep-fad-2017-0021-ronozyme_hiphos.pdf

[8] *Technical dossier, Section II, Annex 2.6.3

[9] *Technical dossier, Section II, Annex 2.6.4

[10] Asssociation of German Agricultural Analytic and Research Institute (VDLUFA) (Ed.) 2012: Method 27.1.3 Preparation of mineral feeds and mineral premixtures for the determination of the phytase activity. Methods Book III. The Chemical analysis of feedingstuffs, 3nd Edition, 8th Supplementary volume, VDLUFA-Publishing house, Darmstadt

[11] Asssociation of German Agricultural Analytic and Research Institute (VDLUFA): Method 27.1.4 Preparation of feed additives for the determination of the phytase activity.

[12] *Supplementary Information – Applicant statement methods equivalence with VDLUFA 27.1.4 and VDLUFA 27.1.3 cf. FAD-2017-0021, Ares (2018) 361178

[13] *Technical dossier, Section II, Annexes 2.4.1, 2.4.2, 2.4.3 and 2.4.4

[14] *Technical dossier, Section II, Annexes 2.4.5 and 2.4.6

[15] *Supplementary Information, "eurl_anova_hiphorius.pdf "

*Refers to Dossier no: FEED-2021-2299

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:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)