



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
European Union Reference Laboratory for 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)
(FAD-2019-0042; CRL/190023)



**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-2019-0042 - CRL/190023**

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: **Stefano Bellowini (EURL-FA)**
Date: **22/11/2019**

Report approved by: **Christoph von Holst**
Date: **22/11/2019**

EXECUTIVE SUMMARY

In the current application, authorisation of a *Preparation of 6-phytase (EC 3.1.3.26)* is sought under Article 4(1) for all porcine species under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers".

According to the Applicant, the active agent is *6-phytase*. The *phytase* activity is expressed in *phytase* units (FTU). One FTU unit as described in the EN ISO 30024 is defined as "the amount of enzyme that releases 1 µmol of inorganic phosphate from sodium phytate per minute under reaction conditions of pH 5.5 and 37 °C".

The product is intended to be marketed in solid and liquid formulations denoted as *Optiphos® Plus* 5000 G, 5000 CT and 5000 L with a guaranteed minimum *6-phytase* activity of 5000 FTU/g. The product is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 250 FTU/kg *feedingstuffs*.

For the quantification of the *phytase* activity the Applicant submitted the ring-trial validated colourimetric standard methods EN ISO 30024 (for *feedingstuffs*) and VDLUFA 27.1.4 (for the *feed additive*). In addition, the Applicant applied also the VDLUFA 27.1.4 with minor experimental modifications to analyse the *premixtures* and obtained similar method performance characteristics. However the EURL is aware of the ring-trial validated VDLUFA 27.1.3 method specifically describing the preparation of *premixtures* for quantification of the *phytase* activity according to EN ISO 30024.

Based on the performance characteristics available the EURL recommends for official control the colourimetric methods mentioned above for the quantification of the *phytase* 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

Preparation of 6-phytase (EC 3.1.3.26), *Optiphos® Plus*, "zootechnical additives"/"digestibility enhancers", weaned piglets, suckling piglets, pigs for fattening, sows, minor reproductive pig species and minor growing pigs 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 feed additive) for weaned piglets, suckling piglets, pigs for fattening, sows, minor reproductive pig species and minor growing 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 the genetically modified strain *Komagataella phaffii* BSY-0007 (DSM 32854) [1, 4].

The product is marketed as dry granulated (*Optiphos[®] Plus 5000 G*), coated (*Optiphos[®] Plus 5000 CT*) and as liquid (*Optiphos[®] Plus 5000 L*) formulations with a guaranteed minimum *6-phytase* (active agent) activity of 5000 FTU/g [1, 4]. It is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 250 FTU/kg *feedingstuffs* [1, 5].

The activity of *6-phytase* is expressed in *phytase* units (FTU). One FTU unit as described in the EN ISO 30024 is defined as "the amount of enzyme that releases 1 µmol of inorganic phosphate from sodium phytate per minute under reaction conditions of pH 5.5 and 37 °C" [6].

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)

For the quantification of the *phytase* activity in *feedingstuffs*, the Applicant submitted the ring-trial validated colourimetric 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 FTU/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 FTU/kg *feedingstuffs*.

Furthermore, the Applicant has verified the method in *feedingstuffs* containing *Optiphos[®] Plus* [7]. The re-evaluation of the data provided by the Applicant in the frame of the verification study, carried out by the EURL, led to similar performance characteristics. ($RSD_r = RSD_{ip} = 7.6\%$) [8], thus confirming the applicability of the standard method EN ISO 30024 to *feedingstuffs* supplemented with *Optiphos[®] Plus*.

For the quantification of the *phytase* activity in the product (*Optiphos[®] Plus* 5000 G, 5000 CT & 5000 L) and in *premixtures* the Applicant submitted the ring-trial validated colourimetric method VDLUFA 27.1.4 [9] based on the enzymatic reaction of *phytase* on phytate.

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 [9]:

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

The Applicant provided verification studies of this method combination to the three product formulations (*Optiphos[®] Plus* 5000 G, 5000 CT & 5000 L) [10] and to *premixtures* [11].

The EURL re-evaluated the experimental data reported by the Applicant in the frame of the verification studies for the *feed additive* [10] and *premixtures* [11] and calculated $RSD_r = 2.1 - 3.0 \%$ and $RSD_{ip} = 2.6 - 3.1 \%$ for the *feed additive* [12] and $RSD_r = 7.1 \%$ and $RSD_{ip} = 8.8 \%$ for *premixtures* [13]. These performance characteristics are in good agreement with those reported in the VDLUFA 27.1.4 /EN ISO 30024 combination thus confirming the applicability to the analysis of the products (*Optiphos[®] Plus 5000 G, 5000 CT & 5000 L*) as well as the extension of scope to *premixtures*.

The EURL was informed that the VDLUFA 27.1.4/EN ISO 30024 combination could lead to some issues when applied to mineral premixtures. Additionally the EURL is aware of the ring-trial validated VDLUFA 27.1.3 method [14], based on a solid dilution using maize meal, describing 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 [14]:

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

Based on the performance characteristics available the EURL recommends for official control the EN ISO 30024 and the combined VDLUFA/EN ISO 30024 colourimetric methods mentioned above for the quantification of the *phytase* activity in the *feed additive*, *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.

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 colourimetric 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*:

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

For the quantification of *phytase* activity in *premixtures*:

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

For the quantification of *phytase* activity in *feedingstuffs*:

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

One *phytase* unit (FTU) is the amount of enzyme that releases 1 μmol of inorganic phosphate from phytate 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, Proposal for registry entry, Annex A
- [2] *Application, Annex 1 (Submission No:1560928633369-2428)
- [3] *Application, Reference SANTE/E5: FORW. APPL. 1831-0050-2019
- [4] *Technical dossier, Section II: 2.1 Identity of the additive
- [5] *Technical dossier, Section II: 2.5 Conditions of use
- [6] EN ISO 30024:2009 - Animal feeding stuffs - Determination of phytase activity
- [7] *Technical dossier, Annex_II_64
- [8] *Supplementary information, EURL_ANOVA_FS.pdf
- [9] Association of German Agricultural Analytic and Research Institute (VDLUFA): Method 27.1.4 Preparation of feed additives for the determination of the phytase activity
- [10] *Technical dossier, Annex_II_62
- [11] *Technical dossier, Annex_II_65
- [12] *Supplementary information, EURL_ANOVA_FA.pdf
- [13] *Supplementary information, EURL_ANOVA_PM.pdf

- [14] Association 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, 3rd Edition, 8th Supplementary volume, VDLUFA-Publishing house, Darmstadt

*Refers to Dossier no: FAD-2019-0042

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:

- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- 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)