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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**

**Ronozyme<sup>®</sup> HiPhos**  
*(FAD-2017-0021; CRL/160038)*



**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-2017-0021 - CRL/160038**

Name of Feed Additive: ***Ronozyme<sup>®</sup> HiPhos***

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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Date: **18/12/2017**

Report approved by: **Christoph von Holst**  
Date: **23/01/2018**

## EXECUTIVE SUMMARY

In the current application authorisation is sought under article 13(3) for *Ronozyme*<sup>®</sup> *HiPhos* under the category/functional "zootechnical additives"/"digestibility enhancers". Specifically, authorisation is sought for the reduction of the minimum dose in *feedingstuffs* for pigs for fattening.

According to the Applicant, the active agent of *Ronozyme*<sup>®</sup> *HiPhos* is *6-phytase*. 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".

The product is marketed in solid and liquid form with a guaranteed minimum *6-phytase* activity of 10000 FYT/g and 20000 FYT/g, respectively. It is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 250 FYT/kg *feedingstuffs*.

For the quantification of the *phytase* activity in *feedingstuffs* the Applicant submitted the ring-trial validated colorimetric standard method EN ISO 30024 and demonstrated the suitability of the method at 200 FYT/kg *feedingstuffs*. In addition, the Applicant applied this method with minor experimental modifications to analyse the *feed additive* (*Ronozyme*<sup>®</sup> *HiPhos*) and *premixtures* and obtained similar method performance characteristics.

Based on the performance characteristics available the EURL recommends for official control the colorimetric 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) is not considered necessary.

## KEYWORDS

*6-phytase*, *Ronozyme*<sup>®</sup> *HiPhos*, "zootechnical additives"/"digestibility enhancers", pigs for fattening

## 1. BACKGROUND

*Ronozyme*<sup>®</sup> *HiPhos* is a *feed additive* authorised by Commission Implementing Regulation (EU) No 837/2012 for poultry, weaned piglets, pigs for fattening and sows [1] under the category/functional group 4 (a) "zootechnical additives"/"digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003. This regulation was further amended by Commission Implementing Regulations (EU) No 1265/2012 and 2016/1881 [2][3].

In the current application, authorisation is sought under article 13(3) (modification of the existing authorisation) of the Regulation (EU) No 837/2012 for *Ronozyme*<sup>®</sup> *HiPhos* [4][5]. Specifically, authorisation is sought for the reduction of the minimum dose from 500 to 250 FYT/kg *feedingstuffs* for pigs for fattening [4][5][6].

According to the Applicant, the active agent of *Ronozyme*<sup>®</sup> *HiPhos* is *6-phytase* (EC 3.1.3.26), produced by the strain *Aspergillus oryzae* (DSM 22594) [7]. The strain was deposited at the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ) in Germany [8].

The product is marketed in solid and liquid form with a guaranteed minimum *6-phytase* (active agent) activity of 10000 FYT/g and 20000 FYT/g, respectively [2][3][5]. It is intended to be included through *premixtures* or directly in *feedingstuffs* to obtain a minimum activity of 250 FYT/kg *feedingstuffs* [5][6].

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" [1].

## 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 *Ronozyme*<sup>®</sup> *HiPhos* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

### 3. EVALUATION

#### ***Identification /Characterisation of the feed additive***

##### *Qualitative and quantitative composition of impurities in the additive*

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury and mycotoxins) are available from the respective European Union Reference Laboratories [10].

##### ***Description of the analytical methods for the determination of the active substance in the feed additive, premixtures and feedingstuffs***

For the quantification of the *phytase* activity in *feedingstuffs* the Applicant submitted the ring-trial validated colorimetric standard method EN ISO 30024 [11] 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 [11]:

- 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*.

Furthermore, the Applicant applied this method to analyse *feedingstuffs* samples containing lower levels of *phytase* (200 FTY/kg) and obtained similar performance characteristics ( $RSD_r = 4.2-13\%$  and  $RSD_{ip} = 6.3-13\%$ ) [12]. This confirms the applicability (extension of scope) of the standard method to the lower *feedingstuffs* dosage (200 FYT/kg).

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* (*Ronozyme<sup>®</sup> HiPhos*) and the *premixtures* [9].

The EURL identified instead the ring-trial validated VDLUFA 27.1.3 and VDLUFA 27.1.4 methods [14][15] describing the preparation of *feed additives* and *premixtures* for quantification of the *phytase* activity according to EN ISO 30024.

Upon the EURL request, the Applicant confirmed that the modifications of the EN ISO 30024 intended to extend its scope to the *feed additive* and *premixtures* are fully equivalent to those described in the ring-trial validated VDLUFA 27.1.3 and VDLUFA 27.1.4 methods. Furthermore the Applicant confirmed that they have been involved in the development and validation of both VDLUFA methods and that these methods can be applied for official

control of the phytase activity in the *feed additive* and *premixtures* [16]. The EURL re-evaluated the experimental data reported by the Applicant in the frame of the stability studies for the *feed additive* and the *premixtures* [13] and calculated  $RSD_r = 0.6 - 15 \%$  and  $RSD_{ip} = 2.2 - 15 \%$  [17][18]. These performance characteristics are in good agreement with those reported in the EN ISO 30024 thus confirming the applicability (extension of scope) to the analysis of the *feed additive* (*Ronozyme<sup>®</sup> HiPhos*) and the *premixtures*.

Based on the performance characteristics available the EURL recommends for official control the colorimetric 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) is not considered necessary.

#### 4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for official control the colorimetric method 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 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  $\mu\text{mol}$  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 *Ronozyme*<sup>®</sup> *HiPhos* 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] Commission Implementing Regulation (EU) No 837/2012 of 18 September 2012 concerning the authorisation of 6-phytase (EC 3.1.3.26) produced by *Aspergillus oryzae* (DSM 22594) as feed additive for poultry, weaned piglets, pigs for fattening and sows (holder of authorisation DSM Nutritional Products)
- [2] Commission Implementing Regulation (EU) No 1265/2012 of 17 December 2012 amending Implementing Regulation (EU) No 837/2012 as regards the minimum activity of a preparation of 6-phytase (EC 3.1.3.26) produced by *Aspergillus oryzae* (DSM 22594) as feed additive for poultry, weaned piglets, pigs for fattening and sows (holder of authorisation DSM Nutritional Products)
- [3] Commission Implementing Regulation (EU) No 2016/1881 of 24 October 2016 amending Implementing Regulation (EU) No 837/2012 as regards the minimum activity of 6-phytase (EC 3.1.3.26) produced by *Aspergillus oryzae* (DSM 22594) as feed additive for sows (holder of authorisation DSM Nutritional Products Ltd)
- [4] \*Application, Reference SANTE/E5: FORW. APPL. 1831-0014-2017
- [5] \*Application, Proposal for register entry, Annex A
- [6] \*Technical dossier, Section II: 2.5 Conditions of use
- [7] \*Technical dossier, Section II: 2.2 Characterisation of the active substance
- [8] \*Technical dossier, Section II, Annex II\_5\_DSMZ 2009
- [9] \*Technical dossier, Section II, Annex II\_17
- [10] 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
- [11] EN ISO 30024:2009 - Animal feeding stuffs - Determination of phytase activity
- [12] \*Technical dossier, Section II, Annex II\_19
- [13] \*Technical dossier, Section II, Annex II\_13-15
- [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, 3<sup>rd</sup> Edition, 8<sup>th</sup> Supplementary volume, VDLUFA-Publishing house, Darmstadt
- [15] Association of German Agricultural Analytic and Research Institute (VDLUFA): Method 27.1.4 Preparation of feed additives for the determination of the phytase activity.



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

[17] \*Supplementary information, ANOVA\_FAs.pdf

[18] \*Supplementary information, ANOVA\_PM.pdf

\*Refers to Dossier no: FAD-2017-0021

## **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)