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EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for the Authorisation of Feed Additives according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2011-0042**
	CRL/110015
Name of Product:	**Ronozyme HiPhos (GT)**
Active Substance(s):	**6-phytase (E.C. 3.1.3.26)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (CRL-FA) Geel, Belgium**
Report prepared by:	**Dijana Mitić (EURL-FA)**
Report revised by: Date:	**Piotr Robouch(EURL-FA) 02/04/2012**
Report approved by: Date:	**Christoph von Holst 03/04/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Ronozyme HiPhos (GT),* under the category "zootechnical additives" functional groups 4(a) "digestibility enhancers" and 4(c) "substances which favourably affect the environment", according to the classification system of Annex I of Regulation (EC) No 1831/2003. According to the Applicant, the active agent of *Ronozyme HiPhos (GT)* is *6-phytase* (EC 3.1.3.26), produced by the strain *Aspergillus oryzae* (DSM 22594). The product is intended to be marketed as a white granulate with a guaranteed minimum *6-phytase* activity of 10000 FYT/g.

The activity of *6-phytase* is expressed in phytase units (FYT). According to the Applicant, 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.

Specifically, authorisation is sought for the use of *Ronozyme HiPhos (GT)* for poultry and pigs. The *feed additive* is intended to be used in feed rich in phytin-bound phosphorus. It is intended to be used in *premixtures* and/or complete *feedingstuffs,* with a minimum *6-phytase* activity in complete *feedingstuffs* of 1000 FYT/kg for sows and 500 FYT/kg for other pigs and poultry.

For the determination of the activity of *6-phytase* in the *feed additive, premixtures* and *feedingstuffs*, the Applicant proposed the ring trial validated EN ISO 30024:2009 method, based on colorimetry. The following performance characteristics were reported for *feedingstuffs* samples containing 500 to 1500 FYT/kg:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.2 to 11 %;
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 3.3 to 13 %;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 5.4 to 15 %;
- a *recovery* rate (R_{Rec}) ranging from 80 to 108 %; and
- a limit of quantification (LOQ) of 60 FYT/kg *feedingstuffs*.

Furthermore, the Applicant applied the EN ISO 30024:2009 method to the product and premixtures and reported similar performance characteristics, thus extending the original scope of the international standard method to the *feed additive* and *premixtures.*

Based on the experimental evidence and the performance characteristics presented, the EURL recommends for official control the EN ISO 30024:2009 method, for the determination of the activity of the *6-phytase* 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

Ronozyme HiPhos (GT), *6-phytase*, *Aspergillus oryzae*, zootechnical additive, digestibility enhancers, substances which favourably affect the environment, poultry and pigs.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) for *Ronozyme HiPhos (GT),* under the category "zootechnical additives" functional groups 4(a) "digestibility enhancers" and 4(c) "substances which favourably affect the environment" [1,2], according to the classification system of Annex I of Regulation (EC) No 1831/2003. According to the Applicant, the active agent of *Ronozyme HiPhos (GT)* is *6-phytase* (EC 3.1.3.26), produced by the strain *Aspergillus oryzae* (DSM 22594) [1,2]. The strain was deposited at the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ) in Germany [3]. The product is intended to be marketed as a white granulate with a guaranteed minimum *6-phytase* activity of 10000 FYT/g [2,4]. Cellulose, dextrin and sodium sulphate are used as carriers.

The activity of *6-phytase* is expressed in phytase units (FYT). According to the Applicant, 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 [4].

Specifically, authorisation is sought for the use of *Ronozyme HiPhos (GT)* for poultry and pigs [1]. The *feed additive* is intended to be used in feed rich in phytin-bound phosphorus. It is intended to be used in *premixtures* and/or complete *feedingstuffs,* with a minimum *6-phytase* activity in complete *feedingstuffs* of 1000 FYT/kg for sows and 500 FYT/kg for other pigs and poultry [2]. Furthermore, the Applicant recommends a maximum phytase activity of 4000 FYT/kg *feedingstuffs* for the species mentioned above [2,4].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 HiPhos (GT)*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

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, mycotoxins and dioxins) are available from the respective European Union Reference Laboratories [5].

Description of the analytical methods for the quantification of the active substance in feed additive, premixtures and feedingstuffs

For the determination of the activity of *6-phytase* in *feed additive* [6, 7], *premixtures* [8] and *feedingstuffs* [6], the Applicant proposed a colorimetric method based on the ring trial validated EN ISO 30024:2009 method [9], measuring the inorganic phosphate released by the enzyme from the sodium phytate (phytic acid dodecasodium salt, $C_6H_6O_{24}P_6Na_{12}$).

Acetate buffer containing EDTA and Tween®20 is used for extraction from *feed additive* and *premixtures* samples, while distilled water containing Tween®20 is used for extraction from *feedingstuffs* samples. After extraction, the solution is diluted and an aliquot of the dilution is submitted to incubation at pH = 5.5 and 37 ^{o}C for 30 min. The released inorganic phosphate is determined measuring at 415 nm the yellow complex formed after addition of the acidic molybdate/vanadate reagent. The released inorganic phosphate is quantified with a phosphate standard curve.

Upon the request of the EURL the Applicant applied the EN ISO 30024:2009 method to the product and premixtures. The experimental data provided [10] confirmed the applicability of the ISO method to the product. The performance characteristics reported are presented in Table 1. Furthermore, the Applicant reported a precision of 5% for *premixtures* [8], validated at two concentrations (80000 and $1.8x10^6$ FYT/kg) [11], and a limit of quantification (LOQ) of 60 FYT/kg *feedingstuffs* [9].

Table 1: Performance characteristics for the quantification of *6-phytase* in the *feed additive (FA)* and *feedingstuffs (FS)*

	Concentration (FYT/kg)	RSD_r (%)	RSD_{ip} (%)	RSD_R (%)	R_{Rec} (%)
FA [10]	$1.0x10^7 – 1.2x10^7$	1.2 – 3.2	0.7 – 1.9	-	-
FS [9]	500 - 1500	2.2 - 11	3.3 - 13	5.4 – 15	80 – 108[#]

RSD_r, RSD_{ip}, RSD_R: relative standard deviation for *repeatability, intermediate precision* and *reproducibility*, respectively.

R_{Rec}: a recovery rate; [#] recalculated by the EURL

Based on the satisfactory performance characteristics presented, the EURL recommends for official control the EN ISO 30024:2009 method, for the determination of the activity of the *6-phytase* 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 EN ISO 30024:2009 method, for the determination of the activity of the *6-phytase* in the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *6-phytase* in the *feed additive, premixtures* and *feedingstuffs:*

- colorimetric method measuring the inorganic phosphate released by the 6-phytase from the phytate (EN ISO 30024:2009).

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 *Ronozyme HiPhos (GT)* 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 SANCO/G1: Forw. Appl. 1831/0109-2011

[2] *Application, Proposal for Register Entry – Annex A

[3] *Technical dossier, Section II, Annex II_13_DSMZ 2009

[4] *Technical dossier, Section II, Identity, characterisation and conditions of use of the additive; methods of analysis

[5] 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

[6] *Technical dossier, Section II, Annex II_2_PHY 101_06E_ISO 78-2

[7] *Technical dossier, Section II, Annex II_1_0744.02-ISO78-2

[8] *Technical dossier, Section II, Annex II_3_PHY 102_06E_ISO 78-2

[9] ISO 30024:2009 "FYT Animal feeding stuffs - Determination of phytase activity"

[10] *Supplementary Information, Ronozyme HiPhos (G)_Info for EURL-FA_KV20120216

[11] *Supplementary Information, Answer to EURL Additional Information on Ronozyme HiPhos (G), 03_04_12

*Refers to Dossier No. FAD-2011-0042

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Państwowy Instytut Weterynaryjny, Puławy (PL)