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Community Reference Laboratory for Feed Additives



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CRL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorised as Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	FAD-2010-0019 CRL/090038
Name of Product:	Ronozyme HiPhos (M) (L)
Active Agent (s):	6-phytase (E.C. 3.1.3.26)
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA) Geel, Belgium
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Date:	11/10/2010
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Date:	12/10/2010

EXECUTIVE SUMMARY

Ronozyme HiPhos (M) (L) is a product for which authorisation as *feed additive* is sought under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003. The active agent of *Ronozyme HiPhos (M) (L)* is *6-phytase* (E.C. 3.1.3.26), produced by the strain *Aspergillus oryzae* (DSM 22594). The authorisation for *all pigs and poultry* is requested.

According to the Applicant, the enzymatic activity of the active agent is expressed in phytase units (FYT), where one FYT 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 temperature 37°C.

The product is intended to be marketed in two formulations: (i) as Granulate (M) with a minimum activity of 50000 FYT/g, and (ii) as Aqueous Liquid (L) with a minimum activity of 20000 FYT/g. The Applicant recommended a *6-phytase* activity in complete *feedingstuffs* ranging from 500 to 4000 FYT/kg.

For the determination of the activity of *6-phytase* in *feedingstuffs*, the Applicant proposed a colorimetric method based on the ring trial validated ISO 30024:2009 method. The performance characteristics of the ISO 30024:2009 method, reported for *feedingstuffs* containing 500 to 1500 FYT/kg, are:

- a relative standard deviation for *repeatability* (RSD_r) of 10%;
- a relative standard deviation for *reproducibility* (RSD_R) of 12%;
- a limit of detection (LOD) and quantification (LOQ) of 20 and 60 FYT/kg, respectively.

Furthermore, the Applicant estimated, for *feedingstuffs* containing 500 to 2170 FYT/kg, a *recovery rate* (R_{Rec}) ranging from 98 to 109%.

For *premixtures* samples, the Applicant suggested a method protocol which differs from the ISO 30024:2009 method only regarding slight modifications of the extraction procedure. The performance characteristics of this method are: RSD_r of 5% and a relative standard deviation for *intermediate precision* (RSD_{ip}) of 5%; a limit of detection (LOD) and quantification (LOQ) of 20 and 60 FYT/kg, respectively.

For the determination of the activity of *6-phytase* in *feed additive*, the Applicant provided, upon request of CRL, experimental data applying the ISO 30024:2009 method to the *feed additive*. The following performance characteristics derived from experimental data, were

recalculated by the CRL: - $RSD_r = 1.35\%$, and - $RSD_{ip} = 2.12\%$, thus confirming the applicability of the ISO method to the product.

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

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 (M) (L), *6-phytase*, *Aspergillus oryzae*, digestibility enhancer, all pigs and poultry

1. BACKGROUND

Ronozyme HiPhos (M) (L) is a product for which authorisation as *feed additive* is sought under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1]. The active agent of *Ronozyme HiPhos (M) (L)* is *6-phytase* (E.C. 3.1.3.26), produced by a strain of *Aspergillus oryzae* (DSM 22594) [2]. The strain has been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), Germany [3].

In the current application submitted according to Article 4(1) of Regulation (EC) No 1831/2003, the authorisation for *all pigs and poultry* is requested [1].

According to the Applicant, the enzymatic activity of the active agent is expressed in phytase units (FYT), where one FYT 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 temperature 37°C [4].

The product is intended to be marketed in two formulations: (i) as Granulate (M) with a minimum activity of 50000 FYT/g, and (ii) as Aqueous Liquid (L) with a minimum activity of 20000 FYT/g [2].

The Applicant proposed a minimum content of *6-phytase* activity in complete *feedingstuffs* of 500 FYT/kg. Furthermore the applicant recommended an activity range from 500 to 4000 500 FYT/kg in complete *feedingstuffs* [2].

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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL 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 (M) (L)*, 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

Quantitative and qualitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective Community Reference Laboratories [5].

Description of the analytical methods for the determination of the active agents in the feed additive, premixtures and feedingstuffs

For the determination of the activity of *6-phytase* in *premixtures* [6] and *feedingstuffs* [7], the Applicant proposed a colorimetric method based on the ring trial validated ISO 30024:2009 method [8], measuring the inorganic phosphate released by the enzyme from the sodium phytate (phytic acid dodecasodium salt, $C_6H_6O_{24}P_6Na_{12}$). The extraction procedure for *premixtures* [6] and *feedingstuffs* [7] are similar. Acetate buffer containing EDTA and Tween[®]20 is used in *premixtures* samples, while distilled water containing Tween[®]20 is used in *feedingstuffs* samples. After extraction, the solution is diluted and an aliquot of the dilution is submitted to incubation at pH = 5.5 and 37°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. The performance characteristics of the ISO 30024:2009 method, reported for *feedingstuffs* containing 500 to 1500 FYT/kg [8] are:

- a relative standard deviation for *repeatability* (RSD_r) of 10%;
- a relative standard deviation for *reproducibility* (RSD_R) of 12%;
- a limit of detection (LOD) and quantification (LOQ) of 20 and 60 FYT/kg, respectively.

Furthermore, the Applicant conducted experiments on feedingstuffs samples containing 6-phytase of this application at activities ranging from 500 to 2170 FYT/kg. Based on the obtained results, a *recovery* rate (R_{Rec}) ranging from 98 to 109% [12] was estimated.

For *premixtures* samples, the Applicant suggested a method protocol which differs from the ISO 30024:2009 method only regarding slight modifications of the extraction procedure. The modified regards the used extraction solution: acetate buffer containing EDTA and Tween[®]20 instead of distilled water containing Tween[®]20. The performance characteristics of the Applicant's method are [6]: RSD_r of 5% and a relative standard deviation for *intermediate precision* (RSD_{ip}) of 5%; a limit of detection (LOD) and quantification (LOQ) of 20 and 60 FYT/kg, respectively.

For the determination of the activity of 6-phytase in *feed additive*, the Applicant proposed two colorimetric method based on the same principle, but different in terms of the equipment used – robot [9] versus conventional method [7]. In both methods, 6-phytase reacts with sodium phytate (phytic acid dodecasodium salt, $C_6H_6O_{24}P_6Na_{12}$) and releases inorganic phosphate, forming a yellow complex determined spectrophotometrically. The following performance characteristics were reported [7,10-11]: - RSD_r ranging from 2 to 3.4%; and, - a relative standard deviation for *intermediate precision* (R_{ip}) ranging from 2 to 6.3% .

Additionally, the Applicant provided experimental data, upon request from the CRL, applying the ISO 30024:2009 method to the *feed additive* [12]. The following performance characteristics derived from experimental data, were recalculated by the CRL [13]: - $RSD_r = 1.35\%$, and - $RSD_{ip} = 2.12\%$, thus confirming the applicability of the ISO method to the product.

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

Based on the satisfactory performance characteristics presented, the CRL recommends for official control the ISO 30024:2009 method, for the determination of the activity of the 6-*phytase* in *feed additive*, and *feedingstuffs*. For *premixtures* the CRL recommends the method suggested by the Applicant which differs only slightly from the ISO 30024:2009 by applying an other extraction reagent.

Recommended text for the register entry (analytical method)

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

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ronozyme HiPhos (M) (L)* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

- [1] Reference SANCO/D/2 Forw. Appl. 1831/0014-2010
- [2] *Application, Proposal for Register Entry
- [3] *Technical dossier, Section II – Appendix II_14_DSMZ 2009
- [4] *Technical dossier, Section II – 2.6. Method of analysis and reference samples
- [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 – Appendix II_45_PHY-102_05E_ISO 78-2
- [7] *Technical dossier, Section II – Appendix II_44_PHY-101_05E_ISO 78-2
- [8] ISO 30024:2009 “FYT Animal feeding stuffs - Determination of phytase activity”
- [9] *Technical dossier, Section II – Appendix II_40_0744.02-ISO78-2
- [10] *Supplementary Information – 3_Report_CRL Verification FYT(B)
- [11] *Supplementary Information – 3_Amendment to CRL Verification (FYT(B))
- [12] * Supplementary Information – Ronozyme HiPhos Info for CRL_KV20100907
- [13] * Additional Information – Precision data as recalculated by the CRL

* Refers to Dossier No. FAD-2010-0019

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community 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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- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)