EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

JRC.DDG.D.6/CvH/SY/mds/Ares (2010) 114543

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

Dossier related to:	FAD-2009-0012 CRL/090001
Product name:	6-phytase (Ronozyme P)
Active Substance(s):	*6-phytase* (EC 3.1.3.26)
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives, Belgium (CRL-FA).
Report prepared by:	Sulhattin Yasar (CRL-FA)
Report checked by:	Piotr Robouch (CRL-FA)
Date:	16/02/2010
Report approved by:	Christoph von Holst (CRL-FA)
Date:	26/02/2010

EXECUTIVE SUMMARY

In the current application authorisation is sought for Ronozyme P under the category "zootechnical additives", functional groups 4(a) and 4(c), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Authorisation is sought to use this product as a digestibility enhancer and as a substance which favourably affects the environment.

The active agent of Ronozyme P is *6-phytase* (EC 3.1.3.26), produced by *Aspergillus oryzae* (DSM 14223). The activity of *6-phytase* is expressed in FYT (phytase) units. According to the applicant, one FYT unit is the quantity of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate at pH = 5.5 and 37^{o}C. The product is intended to be placed on the market as a liquid formulation Ronozyme P (L) containing 20000 FYT/g of *product*.

The product is intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain a minimum enzyme activity level of 500 FYT/kg of *feedingstuffs* for salmonids.

For the determination of the activity of *6-phytase* in the *feed additive*, *premixtures* and *feedingstuffs* the applicant submitted three colorimetric methods, based on the release by the *6-phytase* of inorganic phosphate during the hydrolysis of sodium phytate at pH = 5.5 and 37^{o}C. The released phosphate forms with molybdate and vanadate ions a coloured complex that is measured at 405 or 415 nm and quantified against a phosphate curve. The content of endogenous phosphate - present in the samples and not related to the added phytase activity - is measured in a separate analysis and subtracted from the response of the enzymatic activity measurement.

For the determination of the activity of *6-phytase* in the *feed additive*, the applicant submitted two single-laboratory validated protocols, which differ in terms of the equipment used - robot versus conventional instruments. The method was further verified by a second laboratory for the protocol using robot instrument to prove the successful transferability of the method. Therefore, the CRL recommends for official control the use of the method requiring conventional instruments, easily available in official control laboratories.

The method for the determination of the *6-phytase* activity in *premixtures* was single-laboratory validated on two different *premixtures* at the activity range of 80000 to 1700000

FYT/kg of *premixtures*. The following performance characteristics were reported: (1) a relative standard deviation for repeatability (RSD_r) ranging from 1.2 to 5.1%, (2) a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 2.4 to 4.2% and (3) a recovery rate ranging from 95 to 99%. These results are acceptable. Alternatively, the CRL recommends to make a solid dilution of *premixture* samples containing *6-phytase* with ground cereal feedingstuffs and to apply the method of analysis for *feedingstuffs* for the determination of *6-phytase* activity, as described hereafter.

For the determination of the *6-phytase* activity in *feedingstuffs*, the applicant submitted the standard method ISO 30024:2009. This method is similar to the one for the determination of the *phytase* activity in the *feed additive*. The method is fully ring trial validated covering a phytase activity from 500 to 1500 FYT/kg of *feedingstuffs* on various feed samples containing different phytase products such as Ronozyme P. The performance characteristics were reported: (1) a RSD_r of 10%, (2) a relative standard deviation for reproducibility (RSD_R) of 12% and (3) a limit of detection (LOD) and limit of quantification (LOQ) of 20 and 60 FYT/kg of *feedingstuffs*, respectively. Both limits are well below of the minimum enzyme activity level of 500 FYT/kg, proposed by the applicant. These precision data have been calculated from pooled results of all enzyme products including a feed additive that contained the specific enzyme of the present application.

Based on acceptable method performance characteristics the CRL recommends the ISO 30024:2009 for official controls to determine the activity of *6-phytase* in *feedingstuffs* and *premixtures*.

Further testing or validation is not considered necessary.

KEYWORDS

Ronozyme P, *6-phytase*, enzyme activity, *Aspergillus oryzae* (DSM 14223)

1. BACKGROUND

Ronozyme P is a feed additive for which authorisation is sought under the category 'Zootechnical additives', functional group 4(a) and 4(c), according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought to use this product as a digestibility enhancer for *salmonids* and as a substance which favourably affects the environment. Ronozyme P contains *6-phytase* (EC 3.1.3.26) as an active agent, produced by a microorganism *Aspergillus oryzae* (DSM 14223) [1].

The activity of *6-phytase* is expressed as FYT (phytase) units. According to the applicant, one FYT unit is the quantity of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate at pH = 5.5 and 37^{o}C.

The additive is intended to be marketed as liquid formulation Ronozyme P (L) containing 20000 FYT/g of *product* [2]. The *6-phytase* is intended to be incorporated into premixtures and/or complete *feedingstuffs* to obtain a minimum enzyme activity level of 500 FYT/kg of *feedingstuffs* for salmonids [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis submitted in connection with Ronozyme P (L), and their suitability to be used for official controls, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

For the determination of various undesirable substances including heavy metals and mycotoxins suitable methods to be used in the frame of official control are available from the respective Community Reference Laboratories [3].

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

For the determination of the activity of *6-phytase* in the *feed additive, premixtures and feedingstuffs,* the applicant submitted three colorimetric methods, based on the same principle: the release by the enzyme of inorganic phosphate during the hydrolysis of sodium phytate at pH = 5.5 and 37°C. The released phosphate forms with molybdate and vanadate ions a coloured complex that is measured at 405 or 415 nm and quantified against a phosphate standard curve. The phosphate content present in the samples and not related to the added phytase activity (endogenous phosphate) is measured in a separate analysis and subtracted from the response of the enzymatic activity measurement [4, 5, 6]. The applicant confirmed that the provided validation data referring to the present product (Ronozyme P) applies to the other products of Ronozyme regardless whether the product is liquid or solid.

Feed additive

For the determination of the *6-phytase* activity in the *feed additive,* the applicant submitted two protocols based on the same principle, but differ in terms of the equipment used – robot [4] versus conventional instruments [6]. The method requires a sample extraction in acetate buffer supplemented by Tween 20 and incubation with substrate at pH = 5.5 and 37°C. The phosphate concentration is determined using a spectrophotometry and calculated against an inorganic phosphate standard curve [6]. Both protocols were single-laboratory validated [7, 8]. Furthermore, a verification study was performed [11]. Comparable performance characteristics were reported as presented the Table below:

Therefore, the CRL recommends for official control the use of the conventional method requiring instruments, easily available at official feed laboratories, to determine the activity of *6-phytase* in *feed additives*.

	Robot [11] Verification Study		**Conventional** [8] Single-Lab Study
	Lab1(Applicant)	Lab2 (Verifying)	Applicant
Relative standard deviation for repeatability (RSD_r), %	3.9	1.6	0.5 -1.4 %
Relative standard deviation for intermediate precision (RSD_{ip}), %	4.4	3.7	1.4 - 2.6 %
Limit of Detection (LOD) – FYT/ml	0.032	0.083	0.02-0.05
Limit of Quantification (LOQ) – FYT/ml extract	0.105	0.277	0.1-0.8

Premixtures

For the determination of the *6-phytase activity* in *premixtures*, the applicant submitted the single-laboratory validated colorimetric method mentioned above [5]. Two portions of premix sample, of about 5.0 g each, are weighed into 100 ml Erlenmeyer flasks and extracted with 100 ml acetate buffer containing EDTA and 0.5% Tween 20. After the 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 method was validated on the samples of *premixtures* at a *6-phytase* activity ranging from 80000 to 1700000 FYT/kg of *premixture* [9]. The following performance characteristics were reported: (1) a RSD_r ranging from 1.2 to 5.1%, (2) a RSD_{ip} ranging from 2.4 to 4.2% and (3) a recovery rate ranging from 95 to 99%. These results are acceptable. However, the CRL recommends an alternative approach, based on the dilution of *premixture* samples containing *6-phytase* with ground cereal feedingstuffs and the application of the method of analysis for *feedingstuffs* for the determination of *6-phytase* activity, as described hereafter.

Feedingstuffs

For the determination of the *6-phytase* activity in *feedingstuffs,* the applicant submitted the standard method ISO 30024:2009 [10]. Two portions of pellet or mash, of about 50 g each are weighed into 500 ml Erlenmeyer flasks and extracted with a mixture of 500 ml distilled water and 0.5 ml 10% Tween 20. After incubation at pH = 5.5 and 37 ^{o}C for 30 min, the phosphate concentration is determined using a spectrophotometer and calculated against an inorganic phosphate standard curve. The method was fully ring trial validated on various feed samples that contained different phytase products including equivalent to Ronozyme P, covering a phytase activity from 500 to 1500 FYT/kg of *feedingstuffs*. The following performance characteristics were obtained: (1) a RSD_r of 10%, (2) a relative standard deviation for reproducibility (RSD_R) of 12% and (3) an LOD and LOQ of 20 and 60 FYT/kg of *feedingstuffs*, respectively. Both limits are well below the minimum enzyme activity level of 500 FYT/kg proposed by the applicant. These precision data have been calculated from pooled results of all enzyme products including a feed additive that contained the specific enzyme of the present application [10].

Based on acceptable performance characteristics the CRL recommends the ISO 30024:2009 [10] for official controls to determine the activity of *6-phytase* in *feedingstuffs* and *premixtures.*

4. CONCLUSIONS AND RECOMMENDATIONS

Two colorimetric methods based on the same principle are recommended by the CRL for official control when determining *6-phytase* in various matrices containing Ronozyme P:

– For the *feed additive* a single-laboratory validated and verified colorimetric method;

– For the *feedingstuffs* and *premixtures* the ISO 30024:2009 "Animal feeding stuffs – Determination of phytase activity".

Further testing or validation is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

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

Colorimetric method based on reaction of vanadomolybdate on inorganic phosphate produced by action of *6-phytase* on a phytate-containing substrate (sodium phytate) at pH 5.5 and 37°C, quantified against a standard curve from inorganic phosphate.

One FYT unit is defined as the quantity of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate at pH = 5.5 and 37°C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Ronozyme P 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] * Application for Authorisation - Ref:SANCO/D/2:FORW.APPL. 1831/010-2009.

[2] * FAD-2009-0012_Annex A: Proposal for register entry for Ronozyme P.

[3] 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 Community reference laboratories.

[4] * Appendix 2-16 EB-SM-0614.02 : Absolute Phytase activity, colorimetrically on Konelab, AFYT(V).

[5] * Appendix 2-21 Method PHY-102/04E Determination of Phytase Activity in Premix Samples.

[6] * Appendix 2-18 Method PHY-101/04E Determination of Phytase Activity in Tel Quel (per se) and Feed Samples.

[7] * Appendix 2-17 Validation of the AFYT(V) method PSL-SM-0614.01.

[8] * Appendix 2-19 Report No. 1011669 Validation of the Method PHY-101/04E Determination of Phytase Activity in Tel Quel (per se) and Feed Samples.

[9] * Appendix 2.41 Report No. 2000569 VALIDATION of the Method PHY-102/04E Determination of Ronozyme P (CT) in Premix Samples.

[10] ISO standard 30024:2009 (E): Animal feedingstuffs – Determination of phytase activity.

[11] * Appendix 2.59 CRL verification report form for applicant.

*Refers to Dossier number: FAD-2009-0012

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.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

-Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien.

-Plantedirektoratets Laboratorium, Lyngby.

-Service Commun Des Laboratories Laboratoire Rennes (SCL), Rennes.

- Ústredný Kontrolný A Skúšobný Ústav Poľnohospodársky, V Bratislave.