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

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2008-430
	FAD-2008-0008
	CRL/070029
Product name:	Ronozyme NP (CT, L, M)
Active Substance(s):	6-phytase (EC 3.1.3.26)
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Date:	02/03/2009

EXECUTIVE SUMMARY

In the current application authorisation is sought for Ronozyme NP (CT, L, M) 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 for *poultry, piglets (weaned) and pigs for fattening*, and as a substance which favourably affects the environment.

The active agent of Ronozyme NP (CT, L, M) is 6-phytase (EC 3.1.3.26), produced by *Aspergillus oryzae* (DSM 17594). 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 coated thermo tolerant granulate formulation (Ronozyme NP CT) containing 10000 FYT/g of *product*, as a granulate formulation (Ronozyme NP M) containing 50000 FYT/g of *product* and as a liquid formulation (Ronozyme NP 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 600 FYT/kg of *feedingstuffs* for laying hens, 1000 FYT/kg of *feedingstuffs* for piglets (weaned) and pigs for fattening and 1500 FYT/kg of *feedingstuffs* for poultry excluding laying hens.

For the determination of the activity of 6-phytase in the *feed additive* and *premixtures*, the applicant submitted an in-house validated colorimetric method, 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 phytase activity - is measured in a separate analysis and subtracted from the response of the enzymatic activity measurement.

For the determination of the enzyme activity of 6-phytase in the *feed additive*, the applicant submitted two protocols, which differ in terms of the equipment used - robot versus conventional instruments. Since both methods show comparable performance characteristics, the CRL recommends for official control the use of the method requiring conventional instruments, easily available in official feed laboratories.

The method for the determination of the enzyme activity in *premixtures* is similar to the corresponding method for the analysis of *feedingstuffs*. The method was validated on two different premixtures at the activity range of 80000 to 1700000 FTY/kg of *premixture*. 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_R) ranging from 2.4 to 4.2% and (3) a recovery rate ranging from 95 to 99%. Based on these acceptable performance characteristics the method is considered to be suitable for official control at the target activity ranges.

For the determination of the 6-phytase activity in *feedingstuffs*, the applicant submitted the harmonised method developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA). This method is currently under evaluation to become a CEN (European Committee for Standardisation) and ISO (International Organisation Standardization) standard. This method is similar to the one for the determination of the phytase activity in the *feed additive*. The method was ring trial validated covering a phytase activity from 500 to 1500 FYT/kg of *feedingstuffs* on various feed samples including different phytase products such as Ronozyme P. The performance characteristics obtained were: (1) a RSD_r of 10%, (2) a relative standard deviation for between-laboratory reproducibility of 12% and (3) a limit of detection (LOD) and limit of quantification (LOQ) of 20 and 60 FTY/kg of *feedingstuffs*, respectively. Both limits are well below of the minimum enzyme activity level of 600 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 the acceptable method performance characteristics the CRL recommends this method for official controls to determine the activity of 6-phytase in *feedingstuffs* at the target activity levels.

Further testing or validation is not considered necessary.

KEYWORDS

Ronozyme NP (CT, L, M), 6-phytase, enzyme activity, *Aspergillus oryzae* (DSM 17594)

1. BACKGROUND

Ronozyme NP (CT, L, M) 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 *poultry, piglets (weaned) and pigs for fattening*, and as a substance which favourably affects the environment. Ronozyme NP (CT, L, M) contains 6-phytase (EC 3.1.3.26) as the active agent, produced by a microorganism *Aspergillus oryzae* (DSM 17594) [1].

The activity of 6-phytase is expressed as FTY (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:

- coated thermo tolerant granulate formulation (Ronozyme NP CT) containing 10000 FYT/g of *product*,
- granulate formulation (Ronozyme NP M) containing 50000 FYT/g of *product* and
- liquid formulation (Ronozyme NP L) containing 20000 FYT/g of *product*.

The 6-phytase is intended to be incorporated into premixtures and/or complete *feedingstuffs* to obtain a minimum enzyme activity level of 600 FYT/kg of *feedingstuffs* for laying hens, 1000 FYT/kg of *feedingstuffs* for piglets (weaned) and pigs for fattening and 1500 FYT/kg of *feedingstuffs* poultry excluding laying hens [1].

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 NP (CT, L, M) (cf. EFSA-Q-2008-430), 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 [2].

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 different 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^{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 standard curve. The phosphate content present in the samples and not related to the phytase activity (endogenous phosphate) is measured in a separate analysis and subtracted from the response of the enzymatic activity measurement [3, 4, 5]. The applicant confirmed, upon request from the CRL, that the submitted validation data referring to another product (Ronozyme P) applies to the present product (Ronozyme NP) 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, which differ in terms of the equipment used – robot [3] versus conventional instruments [5]. The phosphate concentration is determined using a spectrophotometry and calculated against an inorganic phosphate standard curve. The performance characteristics of the in-house validated methods are listed in the table below:

	Robot [3,6]	**Conventional** [5,7]
Target analyte	Inorganic phosphate	Inorganic phosphate
Wavelength	405 nm	415 nm
Relative standard deviation for repeatability (RSD_r)	0.3 - 0.7 %	0.5 -1.4 %
Relative standard deviation for intermediate precision (RSD_R)	0.5 %	1.4 - 2.6 %
LOD	≈ 6 FYT/g*	≈ 4 FYT/g*

**: Approximate mean value calculated by the rapporteur.*

Both methods display comparable performance characteristics. However, the CRL recommends for official control the use of the conventional method requiring instruments, easily available in official feed laboratories. Samples are extracted with acetate buffer supplemented by Tween 20 and incubated with substrate at pH = 5.5 and 37°C. The phosphate concentration is determined as described above [4].

Premixtures

For the determination of the 6-phytase activity in *premixtures*, the applicant submitted the in-house validated colorimetric method mentioned above [4]. 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°C for 30 min. The method was validated on two different premixtures at a 6-phytase activity ranging from 80000 to 1700000 FTY/kg of *premixture* [8]. The following performance characteristics were obtained: (1) a RSD_r ranging from 1.2 to 5.1%, (2) a RSD_R ranging from 2.4 to 4.2% and (3) a recovery rate ranging from 95 to 99%. Based on these acceptable performance characteristics the method is considered to be suitable for official controls at the target activity ranges.

Feedingstuffs

For the determination of the 6-phytase activity in *feedingstuffs,* the applicant submitted the harmonised method developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) [5]. This method is currently under evaluation to become a CEN (European Committee for Standardisation) and for ISO (International Organisation Standardization) standards [9, 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 spectrophotometry and calculated against an inorganic phosphate standard curve. The method is fully ring trial validated on various feed samples that contained different phytase products including equivalent to Ronozyme NP (CT, L, M), 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 between-laboratory reproducibility 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 600 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 [11].

Furthermore, the applicant provided the following additional performance characteristics determined during his in-house validation study [7]: (1) an LOQ of 50 FYT/kg of *feedingstuffs*, (2) a recovery rate of 100 % and (3) a RSD_R ranging from 1.3 to 5.0 %, obtained with samples containing a 6-phytase activity ranging from 200 to 800 FYT/kg of *feedingstuffs*.

Based on the acceptable performance characteristics the CRL recommends the harmonised draft CEN method [9, 10] for official controls to determine the activity of 6-phytase in feedingstuffs at the target activity levels.

4. CONCLUSIONS AND RECOMMENDATIONS

Three colorimetric methods based on the same principle are recommended by the CRL for official control when determining 6-phytase in various matrices containing Ronozyme NP (CT, L, M):

• Two in-house validated methods provided by the applicant for the *feed additive* and *premixture*;

• The harmonised draft CEN method "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)

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^{o}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^{o}C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Ronozyme NP (CT) , (M) and (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] * FAD-2008-0008_Annex III: Proposal of register entry (February 2008) for Ronozyme NP

[2] 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.

[3] * Appendix 2-16 (page 88-103) EB-SM-0614.02 : Absolute Phytase activity, colorimetrically on Konelab, AFYT(V)

[4] * Appendix 2-22 (page 169-175) Method PHY-102/04E Determination of Phytase Activity in Premix Samples

[5] * Appendix 2-20 (page 151-159) Method PHY-101/04E Determination of Phytase Activity in Tel Quel (per se) and Feed Samples

[6] * Appendix 2-17 (page 104-136) Validation of the AFYT(V) method PSL-SM-0614.01

[7] * Appendix 2-21 (page 160-166) Report No. 1011669 Validation of the Method PHY-101/04E Determination of Phytase Activity in Tel Quel (per se) and Feed Samples.

[8] * Appendix 2.42 (page 369-373) Report No. 2000569 VALIDATION of the Method PHY-102/04E Determination of Ronozyme P (CT) in Premix Samples

[9] CEN-method draft: Animal feedingstuffs – Determination of phytase activity; working document of CEN CEN/TC 327/WG 3N 113.

[10] Draft international standard ISO/DIS 30024: Animal feedingstuffs – Determination of phytase activity. ISO/TC 34/SC 10.

[11] Gizzi G. Thyregod P., von Holst C., Bertin G., Vogel K., Faurschou-Isaksen M.,Betz R., Murphy R., Brandt Andersen B.:" Determination of Phytase Activity in Feed: Interlaboratory Study" J. AOAC Int. (2008) Vol. 91, 259-267

*Refers to Dossier number: FAD-2008-0008

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Laboratoire de Rennes - Service Commun des Laboratoires, France.

8. ACKNOWLEDGEMENTS

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- Austrian Agency for Health and Food Safety, Wien, Austria.
- Danish Plant Directorate, Lyngby, Denmark.
- National Research Institute of Animal Production in Krakow, Lublin, Poland.
- Natonal Veterinary Laboratory, Pulawy, Poland.