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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-2007-132
	FAD-2007-0019
Product name:	RONOZYME® P, BIO-FEED PHYTASE, ZY PHYTASE
Active Substance(s):	6-phytase (EC 3.1.3.26)
Rapporteur Laboratory:	Danish Plant Directorate
	Lyngby, Denmark
Report prepared by:	Annette Plöger (PD)
Report checked by:	Sulhattin Yasar, Piotr Robouch (CRL-FA)
Date:	16/05/2008
Report approved by:	Christoph von Holst (CRL-FA)
Date:	02/06/2008

EXECUTIVE SUMMARY

In the current application authorisation is sought for Ronozyme® P[1] under the category "zootechnical additives", the functional groups 4(a) and 4(c), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use this product as a digestibility enhancer for *pigs* and *poultry*, and as a substance which favourably affects the environment.

The active agent of Ronozyme® P is 6-phytase, produced by a microorganism *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 under specific conditions (pH 5.5 and 37°C). The product is intended to be placed on the market as solid formulation Ronozyme® P5000 (CT) containing at least 5000 FYT/g and as liquid form Ronozyme® P20000 (L) containing at least 20000 FYT/g of the product. The product is intended to be incorporated into premixtures and/or complete feedingstuffs to obtain enzyme activity levels of minimum 250 FYT/kg (poultry and pigs except sows) and 750 FYT/kg (sows) of complete feedingstuffs.

For the determination of the activity of Ronozyme® P in the *feed additive* and *premixtures*, the applicant proposes single-laboratory validated colorimetric methods, based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37°C by the 6-phytase. 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 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 analysis of the enzyme activity 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 similar performance characteristics, the CRL recommends for official control the use of the method requiring conventional instruments, which are easily available in official feed laboratories.

[1] Ronozyme® P refers to the trade names, Ronozyme® P, Bio-Feed Phytase, ZY Phytase.

The method for the determination of the enzyme activity in *premixtures* is similar to the corresponding method for the analysis of feedingstuffs, and shows values for relative standard deviation for repeatability (RSD_r) between 1.2 to 5.1% and for intermediate precision (within-laboratory RSD_R) between 2.4 to 4.1%. The values for the percentage recovery rate were between 95 and 99%. Based on the obtained method performance characteristics the method is considered suitable for official control purposes.

For the quantification of 6-phytase activity in *feedingstuffs* the applicant proposes a method which is identical to the harmonised method developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA). This method is currently under evaluation to become standards of the European Committee for Standardisation (CEN) and International Organisation for standardisation (ISO). It is based on the same principle as the method for the determination of the phytase activity in the feed additive. The method has been fully ring trial validated on various feed samples that contained different phytase products including Ronozyme® P, covering a phytase activity from 750 to 1500 FYT/kg. The obtained precision of the method was 10 % for the RSD_r and 12% for RSD_R. These precision data have been calculated from the pooled results of all enzyme products included in the study and therefore apply irrespective of the specific phytase to be analysed. Therefore, 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® P, 6-phytase, *Aspergillus oryzae*, digestibility enhancer, substance which favourably affects the environment, pigs, poultry, sows.

1. BACKGROUND

Ronozyme® P5000 (CT) and Ronozyme® P20000 (L) are products for which authorisation is sought under the category ‘zootechnical additives’, functional group ‘digestibility enhancer’ and ‘substance which favourably affects the environment’, according to Annex I of Regulation (EC) No 1831/2003 [1]. Ronozyme® P5000 (CT) and Ronozyme® P20000 (L)

contain 6-phytase (EC 3.1.3.26) as the active agent, produced by a microorganism *Aspergillus oryzae* (DSM 14223) [1], which is deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen in Braunschweig, Germany.

One FYT unit is the quantity of enzyme which liberates 1 micromole of inorganic phosphate per minute from sodium phytate under specific conditions (pH 5.5 and 37^{o}C). According to the applicant, the product is intended to be marketed as solid formulation Ronozyme® P5000 (CT), containing 5000 FYT/g and as liquid form Ronozyme® P20000 (L) containing 20 000 FYT/g. The 6-phytase is intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain enzyme activity levels in complete *feedingstuffs* of minimum 250 FYT/kg (poultry, pigs except sows) and 750 FYT/kg (sows) [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 Bio-Feed® Phytase, Ronozyme® and ZY® Phytase (cf. EFSA-Q-2007-132), 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 contaminants 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 proposes different colorimetric methods, based on the same principle, which is the release of inorganic phosphate during the hydrolysis of sodium phytate

at pH 5.5 and 37°C by the enzyme. 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 which is present in the samples and which is *not* related to the phytase activity (endogenous phosphate) is measured in a separate analysis and subtracted from the response of the enzymatic activity measurement [3-5].

Feed additive

For the analysis of the enzyme activity in the *feed additive,* the applicant submitted two protocols, which differ in terms of the equipment used – robot [3] versus conventional instruments [5]. When analysing the feed additive, the activity of the product is calibrated against a phosphate standard curve. The methods have been single-laboratory validated obtaining the values in the Table below [6,7]:

	Robot [3,6]	**Conventional** [5,6]
Standardization	enzyme	Inorganic phosphate
Wavelength	405 nm	415 nm
Relative Intermediate-precision	3.2 %	1.4 -2.6 %
Relative repeatability	1.2 %	0.5 – 1.4 %
Recovery	101%	101 %
LOD	0.08 FYT/ml ≈ 8 FYT/g*	0.02 -0.05 FYT/ml extract ≈ 2 - 5 FYT/g*
LOQ	1.7 FYT/g	0.1 FYT/ml ≈ 10 FYT/g*

**: Calculated by the rapporteur laboratory of this report.*

Since both methods display similar performance characteristics, the CRL recommends for official control the use of the method requiring conventional instruments, which are easily available in official feed laboratories. Samples are extracted with acetate buffer supplemented by Tween 20 and incubated with substrate under defined conditions. After incubation at the defined conditions, the phosphate concentration is determined as described above.

Premixtures

For the determination of the 6-phytase activity in *premixtures,* the applicant proposes the colorimetric method mentioned above [4], which was validated by a single-laboratory study [8]. According to the protocol, 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 appropriately diluted and an aliquot of the dilution is subjected to incubation. The method was validated on two different premixtures obtaining 1.2 and 5.1% for the RSD_r and 2.4 to 4.1 % for the within-laboratory RSD_R. The values for the percentage recovery rate were between 95 and 99%. This method is considered suitable for official control purposes.

Feedingstuffs

For the quantification of the 6-phytase activity in *feedingstuffs,* the applicant proposes the method [5] which is identical to the harmonised method developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA). This method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN) and for ISO [9]. According to the protocol, 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 the defined conditions, the phosphate concentration is determined as described above - based on spectrophotometry and an inorganic phosphate standard curve. The method has been fully ring trial validated on various feed samples that contained different phytase products including Ronozyme® P, covering a phytase activity from 750 to 1500 FYT/kg. The obtained precision of the method was 10 % for the RSD_r and 12% for RSD_R. These precision data have been calculated from the pooled results of all enzyme products included in the study and therefore apply irrespective of the specific phytase to be analysed [11]. Samples with phytase activity values below 750 FYT/kg have not been included in the study.

Additional method performance characteristics have been determined by the applicant in the single-laboratory validation study of this method [7], namely the (1) LOQ which was 50 FYT/kg, (2) the percentage rate of recovery which was close to 100 % and the (3) the precision on samples containing a *low* phytase activity of about 200 FYT/kg. This level is close to the minimum enzyme activity level of 250 FYT/kg of the present application. The results of these experiments delivered sufficient precision data for laboratory RSD_R, ranging between 2.6 % and 4.0%.

Therefore, the CRL recommends this harmonised method developed by FEFANA [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® P:

- Two single-laboratory validated methods provided by the applicant for the *feed additive* and *premixture*;
- The harmonised method developed by FEFANA for the *feedingstuffs*.

Further testing or validation is not considered necessary.

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

Quantification in feed:

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.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Ronozyme® P5000 (CT) and Ronozyme® P20000 (L) have been sent to the Community Reference Laboratory for Feed Additives Authorisation.

The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Application FAD-2007-0019_Annex_III: Application for Ronozyme P5000(CT) and Ronozyme P20000 (L).

[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 1.01.a EB-SM-0511.02-D Phytase activity, colorimetric on Konelab, FYT(V)

[4] * Appendix 1.12 Method PHY-102/04E Determination of Phytase Activity in Premix Sample

[5] * Appendix 1.13 Method PHY-101/04E Determination of Phytase Activity in Tel Quel (per se) and Feed Samples

[6] * Appendix 1.01.b Validation of the FYT(V) method PSL-SM-0511.01/EB-SM-0511.02

[7] * Appendix 1.14 REPORT No. 1011669 Validation of the Method PHY-101/04E Determination of Phytase Activity in Tel Quel (per se) and Feed Samples.

[8] * Supplementary information obtained on request from CRL (April, 25th, 2008): Report No. 2000569 VALIDATION of the Method PHY-102/04E Determination of Ronozyme R (CT) in Premix Sample

[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] Gisele 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 International (2008) Vol. 91, No. 2 259-267

*Refers to dossier application number: FAD-2007-0019

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Danish Plant Directorate, Lyngby, Denmark. 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

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