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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 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- 2007-133 FAD-2007- 0027
Product name:	RONOZYME®NP
Active Substance(s):	6-phytase (EC 3.1.3.26)
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA) Geel, Belgium
Report prepared by:	Christoph von Holst (CRL-FA)
Report checked by: Date:	Giuseppe Simone (CRL-FA) 27 02 2008
Report approved by: Date:	Christoph von Holst (CRL-FA) 27 02 2008

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Ronozyme®NP* under the category 'zootechnical additives' and 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 *Ronozyme®NP* as a digestibility enhancer for *chickens for fattening* and as a substance which favourably affects the environment.

The active agent of *Ronozyme®NP* is 6-phytase, produced by a strain of *Aspergillus oryzae* (DSM 17594). Enzymatic activity is expressed in FYT (phytase) units. One FYT unit is defined as the amount of enzyme that liberates one μmol of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37°C. The additive is intended to be marketed as a solid formulation *RONOZYME NP (CT)* containing 10,000 FYT/g and as liquid formulation *RONOZYME NP (L)* containing 20,000 FYT/g. The products are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a recommended enzyme activity level rainging from 1500 to 3000 FYT/kg in *feedingstuffs*.

For the determination of the activity of 6-phytase in *feed additive*, *premixtures* and *feedingstuffs,* the applicant proposes colorimetric methods, based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37°C by the enzyme phytase. The released phosphate forms with molybdate and vanadate ions a coloured complex that is measured on a spectrophotometer at 415 nm and quantified against the phosphate standard curve. The phosphate content which is present in the samples and which is *not* related to the phytase activity (endogenous phosphat) is measured in a separate analysis and subtracted from the response of the enzymatic activity measurement.

For the determination of the enzyme activity in the *feed additive* the applicant submitted two very similar protocols, which mainly differ in terms of the equipment utilised (robot versus conventional instruments). Since both methods deliver very similar results, the CRL recommends the use of the method requiring conventional instruments, which are easier available in official feed laboratories. The method has been single-laboratory validated obtaining values for the relative standard deviation for repeatability (RSD_r) ranging from 0.5 to 1.4%. The relative standard deviation for intermediate precision (within-laboratory RSD_R) varied from 1.4 to 2.6%.

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 RSD_r between 1.2 to 5.1% and for the within-laboratory RSD_R between 2.4 to 4.1 %. The values for the percentage recovery rate were between 95 and 99%.

For the determination of the enzyme activity in *feedingstuffs* the applicant proposed a method which is identical with the harmonised method developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA). This method is applicable to the

analysis of different phytase products and has been validated through an interlaboratory study. The limit of quantification is 50 FYT/kg feedingstuffs and the obtained values for the recovery rate are close to 100 %. Precision data for the method were taken from the interlaboratory study, obtaining 10 % for the RSD_r and 12% for the relative standard deviation for *reproducibility*. 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. This method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN).

Based on acceptable performance characteristics, the proposed methods are considered suitable for determination of phytase's activity in *feed additive*, *premixtures* and *feedingstuffs* for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

KEYWORDS

Ronozyme®NP, 6-phytase, *Aspergillus oryzae*, digestibility enhancer, substance, which favourably affects the environment, chicken for fattening

1. BACKGROUND

Ronozyme®NP is a product for which authorisation as feed additive is sought under the category 'zootechnical additives', functional groups 'digestibility enhancers' and 'substances, which favourably affect the environment', according to Annex I of Regulation (EC) No 1831/2003 [1]. *Ronozyme®NP* contains 6-phytase (E.C. 3.1.3.26) as the active agent [2], produced by a strain of *Aspergillus oryzae*, which is deposited at the Deutsche Sammlung von Mikroorganismen und Zell-kulturen (D.S.M.Z.) under the number DSM 17594 in Braunschweig, Germany [3].

The activity of 6-phytase is expressed as FYT (phytase) units. 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 a temperature of 37°C during 30 minutes incubation [4]. The additive is marketed in two forms [5]:

- *Ronozyme NP –(CT)*, which is a solid formulation with activity of 10,000 FYT/g;
- *Ronozyme NP (L)*, which is a liquid formulation with activity of 20,000 FYT/g.

The dry product is mixed into feedingstuffs with the other feed materials after incorporation in a premixture and the liquid product form is designed to be sprayed directly onto the compound feedingstuffs [6]. The minimum inclusion level in feedingstuffs is 1500 FYT/kg and the recommended level ranges from 1500 to 3000 FYT/kg *feedingstuffs* [5].

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 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. For this particular dossier, the methods of analysis submitted in connection with *Ronozyme®NP* (EFSA-Q-2007-133), and their suitability to be used for official controls in the frame of authorisation, 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 official control are available from the respective Community Reference Laboratories [7].

Description of the analytical methods for the determination of the active agent in 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, which however are based on the same principle: Phytase releases phosphate from the substrate myo-inositol-hexaphosphate (phytate). This reaction takes place under defined conditions [4]. The released inorganic phosphate is determined by forming a yellow complex with an acidic molybdate/vanadate reagent, which is then measured at a wavelength of 415 nm. Finally the released inorganic phosphate is quantified with a phosphate standard curve. Since endogenous phosphate present in the samples can interfere with the unbiased determination of the enzyme activity, the response corresponding to the phosphate content in the samples is measured in a separate analysis *without* incubation, thus determining the blank phosphate value. Prior to the quantification of the enzyme activity of the sample the response of the blank phosphate value is *subtracted* from the response of the enzymatic activity measurement.

For the determination of the enzyme activity in the *feed additive* the applicant submitted two very similar protocols, which mainly differ in terms of the equipment utilised. One method utilises a robot for conducting the analysis [8], whereas the alternative methods use conventional instrumentation [9]. Since both methods deliver very similar results [10], the CRL recommends the use of the method requiring conventional instruments, which are easier available in official feed laboratories. Samples are extracted with acetate buffer supplemented by Tween 20 and incubated with substrate under defined conditions. When analysing solid formulation of the additive, the extraction buffer is additionally supplemented by 0.06% albumin from bovine serum (BSA). After incubation at the defined conditions the phosphate concentration is determined as described above. The method has been single-laboratory validated obtaining values for the relative standard deviation for repeatability ranging from 0.5 to 1.4%. The relative within-laboratory standard deviation for reproducibility varied from 1.4 to 2.6%.

The method for the determination of the enzyme activity in *premixtures* [11] is similar to the corresponding method for the analysis of feedingstuffs, and shows values for the RSD_r between 1.2 to 5.1% and for the within-laboratory RSD_R between 2.4 to 4.1 %. The values for the percentage recovery rate were between 95 and 99% [12].

For the determination of the enzyme activity in *feedingstuffs* the applicant proposed a method [9], which is identical to the harmonised method developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) [13]. This method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN). According to the protocol two portions of pellets 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 of 10 % Tween 20. After incubation at the defined conditions the phosphate concentration is determined as described above. This method is applicable to the analysis of different phytase products and has been validated through an interlaboratory study. The limit of quantification is 50 FYT/kg feedingstuffs and the obtained values for the recovery rate are close to 100 %. Precision data for the method were taken from the interlaboratory study, obtaining 10 % for the RSD_r and 12% for the relative standard deviation for *reproducibility*. 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 [14].

Based on acceptable performance characteristics, the proposed methods are considered suitable for determination of phytase's activity in *feed additive*, *premixtures* and *feedingstuffs* for official control purposes in the frame of the authorisation.

4. CONCLUSIONS AND RECOMMENDATIONS

For the quantification of the 6-phytase activity in *feedingstuffs*, the applicant proposes a colorimetric method that has been validated in an inter-laboratory study, showing acceptable performance characteristics. The applicant also provided validation data of analytical methods for the analysis of the *feed additive* and of *premixtures*, obtaining acceptable performance characteristics. Therefore, the proposed methods are considered suitable for determination of phytase's activity in the *feed additive, premixtures* and *feedingstuffs* for official control purposes in the frame of authorisation.

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°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 NP (CT) nd Ronozyme NP (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. 1831/008-2006.

[2] Section II, chapter 2.2.1

[3] Appendix 2-8_JP6500_Strain deposition certificate.pdf

[4] Section II, chapter 2.1.3 Table 2-1

[5] Annex III. Proposal of Register entry

[6] Section II, chapter 2.4.2

[7] 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,

[8] Appendix 2-16_JP6500_AFYT-V.pdf

[9] Appendix 2-20_JP6500_Phytase per se and in feed_Phy-1-1-04E.pdf

[10] Section II, chapter 2.5.1.1

[11] Appendix 2-22_ActivityPremix-PHY-10-04_E.pdf

[12] Supplementary information obtained on request from CRL (11 Jan 2008): Report_2000569 (Validation PHY-102-04E)_06-June-2007.pdf

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

[14] Gizzi G. et al. (2007): Determination of phytase activity in feed: Results of a collaborative study. Journal of AOAC International, accepted for publication.

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

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) and Annex II of Commission Regulation (EC) No 378/2005, as amended by Commission Regulation (EC) No 850/2007.

8. ACKNOWLEDGEMENTS

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- Plantedirektoratets Laboratorium, Lyngby, Denmark.
- Unit for Pathology of Animal Nutrition and Environmental Hygiene of the National Veterinary Institute, Ljubljana, Slovenia.
- National Veterinary Research Institute, Puławy, Poland
- Central Institute for Supervising and Testing in Agriculture, Praha, Czech Republic