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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-2006-060** **FAD-2006-0005**
Product name:	**Ronozyme® P/Bio-Feed® Phytase**
Active Substance(s):	**6-phytase (EC 3.1.3.26)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA)** **Geel, Belgium**
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EXECUTIVE SUMMARY

In the current application authorisation is sought for *Ronozyme® P/Bio-Feed® Phytase* 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® P/Bio-Feed® Phytase* as a digestibility enhancer for ducks and as a substance, which favourably affects the environment.

The active agent of *Ronozyme® P/Bio-Feed® Phytase* is 6-phytase, produced by a strain of *Aspergillus oryzae* (DSM 14223). 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^{o}C. The additive is intended to be marketed as a solid formulation (*Ronozyme® P5000 (CT)/Bio-Feed® Phytase CT 2X)* containing 5000 FYT/g and as liquid formulation (*Ronozyme® P20000 (L)/Bio-Feed® Phytase L 4X*) containing 20000 FYT/g. The products are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a recommended enzyme activity level of 500 to 1000 FYT/kg in *feedingstuffs*.

For the determination of the activity of 6-phytase in *feed additive*, *premixtures* and *feedingstuffs,* the applicant proposes a colorimetric method, based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37^{o}C by the enzyme phytase. 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 applicant submitted method's validation data on *feed additive* and *premixtures*, obtained in a single laboratory. For the *feed additive*, method performance characteristics include a limit of quantification (LOQ) of 0.1 FYT/ml, a relative standard deviation for repeatability (RSD_r) of 0.5 to 1.4% and a relative within-laboratory standard deviation for reproducibility (RSD_R) of 1.4 to 2.6%. For the *premixtures*, an RSD_r of 1.2 to 5.1%, a within-laboratory RSD_R of 2.4 to 4.1 % and recovery rates of 95% and 99% were obtained.

This method has been developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) for the measurement of phytase activity in *feedingstuffs* and validated in an inter-laboratory study on various phytase products including *Ronozyme® P/Bio-Feed® Phytase*. The obtained values for the RSD_R, ranging from 5 to 14%, are considered acceptable and the method is therefore suitable for official control purposes. 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® P/Bio-Feed® Phytase, 6-phytase, *Aspergillus oryzae*, digestibility enhancer, substance, which favourably affects the environment, ducks

1. BACKGROUND

Ronozyme® P/Bio-Feed® Phytase 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® P/Bio-Feed® Phytase* contains 6-phytase (E.C. 3.1.3.26) as the active agent [2], produced by a microorganism *Aspergillus oryzae*, which is deposited at the Deutsche Sammlung von Mikroorganismen und Zell-kulturen (D.S.M.Z.) under the number DSM 14223 in Braunschweig, Germany.

The activity of 6-phytase is expressed as FYT (phytase) units. According to the applicant, one FYT unit is the amount of enzyme which liberates one μmol of inorganic phosphate per minute from sodium phytate at pH 5.5 and 37°C. The additive is marketed in two forms [3]:

Ronozyme® P5000 (CT)/Bio-Feed® Phytase CT 2X, which is a solid formulation with a minimum guaranteed activity of 5000 FYT/g;

Ronozyme® P20000 (L)/Bio-Feed® Phytase L 4X, which is a liquid formulation with a minimum guaranteed activity of 20000 FYT/g.

The additive is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of minimum 250 to recommended 500-1000 FYT/kg *feedingstuffs* [4].

6-phytase, produced by *Aspergillus oryzae* (DSM 14223), has been already authorised under number EC N° 1614 (i) as a feed additive for chickens for fattening, laying hens, turkeys for fattening, piglets, pigs for fattening and sows (Commission Regulation (EC) N° 255/2005) [5] and provisionally authorised under N° 50 as a feed additive for salmonids (Commission Regulation (EC) N° 521/2005) [6].

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® P/Bio-Feed® Phytase*, *cf.* EFSA-Q-2006-060, 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 arsenic and heavy metals, the applicant proposes inductively coupled plasma mass spectrometry (ICP-MS) method, which is considered suitable for the intended purposes. For the analysis of microbiological agents, methods described by the Joint FAO/WHO Expert Committee on Food Additives, European Pharmacopoeia and slightly modified standard ISO methods (ISO 4832, ISO 6887-1, ISO 6759, ISO 16654) [8] are proposed and considered suitable for intended purposes. For official controls various standard methods based on the same analytical techniques and routinely applied by official control authorities are available and recommended by the CRL.

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 *feed additive*, *premixtures* and *feedingstuffs,* the applicant proposes a colorimetric method, based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37^{o}C by the enzyme phytase [9,10,11]. Samples are extracted with acetate buffer supplemented by Tween 20 and incubated with substrate under defined conditions. When analysing solid formulation of the additive (*Ronozyme® P5000 (CT)/Bio-Feed® Phytase CT 2X),* the extraction buffer is additionally supplemented by 0.06% albumin from bovine serum (BSA). For the extraction of *premixtures*, a supplementary ethylenediaminetetraacetic acid (EDTA) is used. The reaction is stopped by adding of molybdate/vanadate reagent, afterwards forming a coloured complex with released inorganic phosphate. This complex is then measured on a spectrophotometer at 415 nm and enzymatic activity is quantified against a phosphate standard curve.

The applicant submitted method's validation data on *feed additive* and *premixtures*, obtained in a single laboratory. For the *feed additive*, a limit of quantification (LOQ) was 0.1 FYT/ml. Two enzymatic samples (solid (5284 FYT/g) and liquid (3940 FYT/g)) were analysed, obtaining a relative standard deviation for repeatability (RSD_r) of 0.5 to 1.4%. Intermediate precision was estimated on six enzymatic samples, containing 2888 to 5782 FYT/g, within 5 days, obtaining a relative within-laboratory standard deviation for reproducibility (RSD_R) of 1.4 to 2.6% [13].

Two samples of *premixtures*, containing on average 82.5 and 1717 FYT/g, were tested, obtaining an RSD_r of 1.2 to 5.1%. Intermediate precision was also estimated on two samples of *premixtures*, containing on average 78.5 and 1642 FYT/g, within 5 days, obtaining a relative within-laboratory RSD_R of 2.4 to 4.1 %. Recovery rates were 94.8% and 98.7% [14].

This method has been developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) for the measurement of phytase activity in *feedingstuffs* [11] and validated in an inter-laboratory study on various phytase products including *Ronozyme® P/Bio-Feed® Phytase*. The obtained values for the RSD_R, ranging from 5 to 14% [12], are considered acceptable and the method is therefore suitable for official control purposes. 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.

4. CONCLUSIONS AND RECOMMENDATIONS

For the quantification of the 6-phytase activity in various matrices, the applicant proposes a colorimetric method that has been developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) for the measurement of phytase activity in *feedingstuffs* and validated in an inter-laboratory study on various phytase products (including *Ronozyme® P/Bio-Feed® Phytase*) and showing acceptable performance characteristics. The applicant provided method's validation data on *feed additive* and *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.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Ronozyme® P5000 (CT)/Bio-Feed® Phytase CT 2X* and *Ronozyme® P20000 (L)/Bio-Feed® Phytase L 4X* 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, Subject 2, Item 1.2.

[3] Section II, Subject 2, Item 1.3.

[4] Annex III. Proposal of Register entry.

[5] Commission Regulation (EC) No 255/2005 concerning the permanent authorisation of certain additives in feedingstuffs, OJ L 45, 16.2.2005, p. 3.

[6] Commission Regulation (EC) No 521/2005 concerning the permanent authorisation of an additive and the provisional authorisation of new uses of certain additives already authorised in feedingstuffs, OJ L 84, 4.2.2005, p. 3.

[7] Section II, Appendix 1.10.

[8] Section II, Appendices 1.02-1.09.

[9] Section II, Appendix 1.12.

[10] Section II, Appendix 1.13.

[11] CEN-method draft: Animal feedingstuffs – Determination of phytase activity; Working document N 410 of CEN TC 327.

[12] Gisele Gizzi and Christoph von Holst (2005). Validation study on a new method for a determination of phytase activity in feed: Results from an interlaboratory study conducted according to the IUPAC harmonised protocol. European Commission, DG JRC, IRMM, Geel.

[13] Section II, Appendix 1.14.

[14] Validation report, submitted upon request of the CRL-FA (received 8 June 2007).

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium.

8. ACKNOWLEDGEMENTS

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