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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-2008-378
	FAD-2008-0010
	CRL/080005
Product name:	FINASE L & P
Active Substance(s):	3-phytase (EC 3.1.3.8)
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Date:	17/03/2009
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Date:	17/03/2009

EXECUTIVE SUMMARY

In the current application authorisation is sought for FINASE L & P under the category zootechnical additives, group 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 FINASE L & P as a digestibility enhancer for laying hens, turkeys for fattening, sows, ducks for fattening, pheasants and other game birds and as a substance which favourably affects the environment. The additive is intended to be marketed in two forms, as a powder (FINASE P) and as liquid (FINASE L) formulations.

The active agent of FINASE L & P is 3-phytase (EC 3.1.3.8), produced by a microorganism *Trichoderma reesei*. The enzyme activity is expressed in PPU units. According to the applicant, one PPU unit is the quantity of enzyme which liberates one µmole of inorganic phosphate from sodium phytate per minute at pH = 5.0 and 37^{o}C. According to the applicant, Finase L & P have a guaranteed minimum activity of 5000 PPU/g of *product*. Finase L & P are intended to be incorporated into premixtures and/or complete *feedingstuffs* to obtain an enzyme activity level ranging from 250 to 1000 PPU/kg of *feedingstuffs* for laying hens, turkeys for fattening, sows, ducks for fattening, pheasants and other game birds.

For the determination of the 3-phytase activity in the *feed additive*, the applicant proposes a spectrophotometric method. The method is based on the release by the 3-phytase of inorganic phosphate during the hydrolysis of sodium phytate. The released phosphate forms a colour with phosphomolybdate complex that is measured at 820 nm and quantified against a phosphate standard curve. The method is in-house validated and the following performance characteristics are reported: (1) a relative standard deviation for repeatability (RSD_r) ranging from 3.0 to 6.1 %, (2) a relative standard deviation for intermediate precision (RSD_R) ranging from 4.9 to 10.8 % and (3) a recovery rate of 100%. Therefore, the method is considered suitable for the intended purposes.

For the determination of the 3-phytase activity in *premixtures*, the applicant proposes a method, based on the same method principle as for the additive. The assay requires an extraction of the enzyme in a buffer containing EDTA, albumin and Tween followed by centrifugation. The method is in-house validated and the following performance characteristics are reported: (1) a RSR_r ranging from 2.3 to 3.5%, (2) a RSD_R ranging from

3.9 to 6.0 % and (3) a recovery rate ranging from 99 to 114%. The results of a ruggedness test indicated that the addition of 20 mM copper reduces the enzyme activity by 17 to 25 %. Therefore the applicant submitted method is not considered suitable for the intended purposes due to the interference of copper – a substance which is often present in *premixtures* at high levels. An alternative approach, considered valid by the CRL for the determination of the phytase activity in *premixtures*, is based on the dilution of the premixture sample into blank feed matrix and applying the corresponding method for the determination of the phytase activity in *feedingstuffs*. However, this method is not applied in the present dossier and the corresponding validation data is missing; hence the suitability of such method for official controls could not be evaluated.

For the determination of the 3-phytase activity in *feedingstuffs*, the applicant proposes a method, based on the same principles as for the *feed additive*. The method is in-house validated and the following performance characteristics are reported: (1) a RSD_r ranging from 3.9 to 8.9%, (2) a RSD_R ranging from 5.1 to 7.4 % and (3) a limit of detection (LOD) and limit of quantification (LOQ) of 23 and 36 PPU/ kg of *feedingstuffs*, respectively. The recovery rate ranges from 82 to 94% within the target activity range of 250 and 500 PPU/kg of *feedingstuffs*.

A harmonised method is available for the determination of the phytase in *feedingstuffs*, and is currently evaluated to become a standard of the European Committee for Standardisation (CEN). However, this harmonised method requires a pH = 5.5 which is different from the one used by the applicant (pH = 5.0) at which the enzymatic activity unit of the applicant product is defined. Hence, the CRL could not evaluate the harmonised method for official control for the determination of 3-phytase activity at the defined condition within the authorisation frame of FINASE L & P.

Based on the acceptable performance characteristics the method submitted by the applicant is considered suitable for official controls to determine the activity of 3-phytase in *feedingstuffs* at the target activity level.

KEYWORDS

FINASE L & P, 3-phytase, *Trichoderma reesei*, zootechnical additives, laying hens, turkeys for fattening, sows, ducks for fattening, pheasants and other game birds.

1. BACKGROUND

FINASE L & P is a feed additive containing 3-phytase (EC 3.1.3.8) produced by a microorganism *Trichoderma reesei* for which authorisation is sought under the category "zootechnical additives", 'functional group 4(a)' as a digestibility enhancer for laying hens, turkeys for fattening, sows, ducks for fattening, pheasants and other game birds, and 'functional group 4(c)' as a substance which favourably affects the environment, according to Annex I of Regulation (EC) No 1831/2003. Specifically authorisation is sought according to the article 4(1) and 10(2) of Regulation (EC) No 1831/2003 [1].

The enzyme activity is expressed in PPU units. According to the applicant, one PPU unit is the quantity of enzyme which liberates one µmole of inorganic phosphate from sodium phytate per minute at pH = 5.0 and 37^{o}C. The additive is intended to be marketed in a powder (FINASE P) and a liquid (FINASE L) formulation, having a minimum guaranteed activity of 5000 PPU/g of product.

FINASE L & P are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain an enzyme activity level ranging from 250 to 1000 PPU/kg of *feedingstuffs*.

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 FINASE L & P, (cf. EFSA-Q-2008-378) were evaluated for their suitability for official controls.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and qualitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the *additive*: arsenic, heavy metals and mycotoxins are available at 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

Feed additive

For the determination of 3-phytase activity in *feed additive*, a spectrophotometric method is proposed, which is based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH = 5.0 and 37^{o}C by the enzyme phytase. 0.5 g sample of liquid or solid product is extracted with citrate buffer (pH = 5.0) and subjected to the enzyme reaction at 37^{o}C after addition of phytate. The reaction is stopped by addition of trichloroacetic acid. Blank samples are also prepared. Ammonium molybdate is added. The released orthophosphate forms a coloured phosphomolybdate complex that is measured at 820 nm and quantified against a phosphate standard curve [3]. The method is in-house validated and the following performance characteristics are reported: (1) a relative standard deviation for repeatability (RSD_r) ranging from 3.0 to 6.1 %, (2) a relative standard deviation for intermediate precision (RSD_R) ranging from 4.9 to 10.8 %, (3) a recovery rate of 100.5%, (4) a limit of detection (LOD) determined to be 0.97 PPU/g of *product* and (5) a limit of quantification (LOQ) determined to be 1.75 PPU/g of *product* [4]. These performance characteristics are acceptable and the applicant method is considered suitable for the intended purposes.

Premixtures

For the determination of 3-phytase activity in *premixtures*, the applicant proposes a similar method to the method for *feed additive*. 5 g sample is extracted with citrate buffer containing EDTA, albumin and Tween. The extract is centrifuged, and phytase activity in the extract is analysed according to the above mentioned assay [3]. The method is in-house

validated and the following performance characteristics are reported: (1) a recovery rate ranging from 99 to 114%, (2) a RSD_r ranging from 2.3 to 3.5%, (3) a RSD_R ranging from 3.9 to 6.0 %, and (4) an LOD and LOQ determined to be 0.31 and 0.46 PPU/g of *premixtures*, respectively. The method ruggedness studied the effect of cations on the phytase activity [3]. The results revealed that the addition of 20 mM Copper reduced the enzyme activity of Finase L by 17 to 25%. Therefore, the method submitted by the applicant is considered not suitable for the intended purposes due to the interference of Cu, a substance often present in *premixtures*.

An alternative approach, considered valid by the CRL for the determination of the phytase activity in *premixtures*, is based on the dilution of the premixture sample into blank feed matrix and applying the corresponding method for the determination of the phytase activity in *feedingstuffs*. However, this method is not applied in the present dossier and the corresponding validation data is missing; hence the suitability of such method for official controls could not be evaluated.

Feedingstuffs

For the determination of 3-phytase activity in *feedingstuffs*, the applicant proposes a method similar to the method for *feed additive*. 2.5 g feed sample is extracted with 20 ml buffer (pH = 5.0) and centrifuged. The enzyme purification is carried out by gel filtration on a Sephadex G-25 column. The enzyme activity is determined according to the above mentioned assay [3]. The method was in-house validated and the following performance characteristics are reported: (1) a RSD_r ranging from 3.9 to 8.9 %, (2) a RSD_R ranging from 5.1 to 7.4% and (3) a recovery rate ranging from 82 to 94% at the target levels of 250 and 500 PPU/kg of spiked into broiler feeds, respectively [5]. The applicant provided additional information upon request from the CRL: - an LOD and LOQ of 23 and 36 PPU/kg of *feedingstuffs*, respectively [6]. A harmonised method is available for the determination of the phytase in *feedingstuffs*, and is currently evaluated to become a standard of the European Committee for Standardisation (CEN) [7]. However, this harmonised method requires a pH = 5.5 which is different from the one used by the applicant (pH = 5.0) at which the enzymatic activity unit of the applicant product is defined. Hence, the CRL could not evaluate the harmonised method for official control for the determination of 3-phytase activity at the defined condition within the authorisation frame of FINASE L & P.

Based on the above acceptable performance characteristics the method submitted by applicant is considered suitable for official controls to determine the activity of 3-phytase in *feedingstuffs* at the target activity levels.

4. CONCLUSIONS AND RECOMMENDATIONS

The reported method performance characteristics are acceptable and the proposed analytical methods are therefore considered suitable for determination of 3-phytase in *feed additives* and *feedingstuffs* (not in *premixtures*) for laying hens, turkeys for fattening, sows, ducks for fattening, pheasants and other game birds.

Further testing or validation is not considered necessary.

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

Characterisation of the active substances in the *feedingstuffs*:

Colorimetric method based on reaction of ammoniummolybdate on inorganic phosphate produced by action of 3-phytase on a phytate-containing substrate (sodium phytate) at pH 5.0 and 37^{o}C, quantified against a standard curve from inorganic phosphate.

One PPU unit is the quantity of enzyme which liberates one µmole of inorganic phosphate from sodium phytate per minute at pH = 5.0 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 FINASE L & P 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/SANCO/D/2 Forw. Appl. 1831/013-2008

[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, Official Journal of the European Union L 136. 24.5.2006.

[3] *Annexes/Enclosure 37. Analytical method

[4] *Annexes/Enclosure 42a. In-house method validation for phytase activity.

[5] *Supplementary Information/Validation report for Finase.

[6] * Supplementary Information /LDLQ for Finase L P: Research report no. 208355.

[7] CEN-method draft: Animal feeding stuffs – Determination of phytase activity. CEN TC 327 WG 3 (version 19 June 2008).

*Refers to Dossier number: FAD-2008-0010.

7. RAPPORTEUR LABORATORY

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

8. ACKNOWLEDGEMENTS

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- Sächsische Landesanstalt für Landwirtschaft, Leipzig, Germany
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