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CRL Evaluation Report on the Analytical Methods submitted in  
connection with Section II, 2.6 (Control Methods) of the Application  
for Authorisation as a Feed Additive  
according to Regulation (EC) No 1831/2003

Dossier related to: FAD-2008-0040  
CRL/ 080016

Product name: 6-Phytase (Finase EC)

Active Substance(s): 6-phytase (EC 3.1.3.26)

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## EXECUTIVE SUMMARY

In the current application authorisation is sought for *6-Phytase (Finase EC)* under the category zootechnical additives, functional group 4(a) as a digestibility enhancer for chickens for fattening, chickens for laying, laying hens, turkeys for fattening, turkeys for breeding, piglets, pigs for fattening, sows, ducks, and other minor poultry species (i.e. geese, quail, pigeons, pheasants and 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. The *additive* is intended to be marketed in two forms, as a liquid (*Finase EC 10 L*) and as a solid (*Finase EC 40 P*) formulation.

The active agent of *Finase EC* is *6-phytase* (EC 3.1.3.26), produced by a fungus *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  $\mu$ mole of inorganic phosphate from sodium phytate per minute at pH = 5.0 and 37°C. *Finase EC L* and *Finase EC P* have a minimum *6-phytase* activity of 10000 and 40000 PPU/g, respectively.

*Both formulations* are intended to be incorporated into premixtures and/or complete *feedingstuffs* to obtain a minimum recommended enzyme activity level of (a) 125 PPU/kg of *feedingstuffs* for chickens for fattening, chickens for laying, laying hens, turkeys for fattening, turkeys for breeding, ducks and minor species (i.e. geese, quail, pigeons, pheasants and game birds) and (b) 250 PPU/kg of *feedingstuffs* for piglets, pigs for fattening and sows.

For the determination of *6-phytase* activity in the *feed additive* the applicant proposes a spectrophotometric method. The method is based on the release of inorganic phosphate from *6-phytase* during the hydrolysis of sodium phytate and the quantification is done against a phosphate standard curve. The method was in-house validated by the applicant and later was verified by a second independent laboratory. The reported performance characteristics were: - a relative standard deviation for repeatability (RSD<sub>r</sub>) ranging from 3.5 to 6.1%, - a relative standard deviation for intermediate precision (RSD<sub>R</sub>) ranging from 3.5 to 5.3%, - a recovery rate (RR) ranging from 100 to 101%, - a limit of detection (LOD) ranging from 0.1 to 1.0 PPU/g of *product* and - a limit of quantification (LOQ) of *product* ranging from 1.0 to 1.8 PPU/g

For the determination of *6-phytase* activity in *premixtures* the applicant proposes a method based on the same method principle as for the *feed additive*. The method was in-house

validated by the applicant and later was verified by a second independent laboratory. The reported performance characteristics were: - a  $RSD_r$  ranging from 3.5 to 5.5%, - a  $RSD_R$  of 8.5%, - LOD ranging from 0.3 to 10 PPU/g of *premixture*, - a LOQ ranging from 0.5 to 50 PPU/g of *premixture* and a RR of 102%. Due to reported matrix effects for some *premixtures*, the method is not applicable to *premixtures* with elevated levels of zinc and copper.

For the determination of *6-phytase* activity in *feedingstuffs* the applicant proposes a method, based on the same principles as for the *feed additive*. The method was in-house validated by the applicant and later was verified by a second independent laboratory. The reported performance characteristics were: - a  $RSD_r$  ranging from 3.9 to 8.9%, - a  $RSD_R$  ranging from 5.1 to 7.4%, - a LOD ranging from 23 to 35 PPU/kg of *feedingstuffs*, - LOQ ranging from 36 to 50 PPU/kg of *feedingstuffs* and - RR ranging from 64 to 101%. A very low RR of 64% was obtained at an enzymatic activity level of 125 PPU/kg of *feedingstuffs*.

Based on these acceptable performance characteristics the applicant's in-house validated and verified methods are recommended for official control purposes within the frame of authorisation for the determination of *6-phytase* activity in

- *feed additives*
- *premixtures* with the exception of samples with high amounts of zinc and copper
- *feedingstuffs* at the activity level of 250 PPU/kg, but not at the activity level of 125 PPU/kg due to the low recovery rate obtained at this level.

Further testing or validation is not considered necessary.

## KEYWORDS

*Finase EC, 6-phytase, Trichoderma reesei, Zootechnical Additives*

## 1. BACKGROUND

*Finase EC* is a feed additive containing *6-phytase* (EC 3.1.3.26) 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 chickens for fattening, chickens for laying, laying hens, turkeys for fattening, turkeys for breeding, piglets,

pigs for fattening, sows, ducks, and minor species (i.e. geese, quail, pigeons, pheasants and 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 [1]. The strain RF7265 of *Trichoderma reesei* is deposited in The Centraalbureau voor Schimmelcultures (CBS) in the Netherlands with the deposit number CBS 122001.

The activity of 6-*phytase* is expressed in PPU units. According to the applicant, one-PPU unit is the quantity of enzyme which liberates one  $\mu$ mole of inorganic phosphate from sodium phytate per minute at pH = 5.0 and 37°C. The additive is intended to be marketed in a liquid (*Finase EC L*) and a solid (*Finase EC S*) formulation, having a minimum guaranteed activity of 10000 and 40000 PPU/g, respectively. *Both formulations* are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain a minimum recommended enzyme activity level of 125 PPU/kg of *feedingstuffs* for chickens for fattening, chickens for laying, laying hens, turkeys for fattening, turkeys for breeding, ducks and minor species (i.e. geese, quail, pigeons, pheasants and game birds) and of 250 PPU/kg of *feedingstuffs* for piglets, pigs for fattening and sows [2].

## 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 (FAD-2008-0040), the methods of analysis submitted in connection with 6-*phytase* (*Finase EC*) 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* (i.e. arsenic, heavy metals and mycotoxins) are available at the respective Community Reference Laboratories [3].

### *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 *6-phytase* activity in *feed additive* a spectrophotometric method is proposed [4], which is based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH = 5.0 and 37°C by the enzyme *6-phytase*. A 0.5 g sample of liquid or solid product is extracted in citrate buffer (pH = 5.0) and incubated at 37°C after addition of phytate substrate. The reaction is stopped by addition of trichloroacetic acid and followed by the addition of ammonium molybdate and ascorbic acid. The released orthophosphate forms a coloured phosphomolybdate complex, which is measured at 820 nm and quantified against a phosphate standard curve [4]. The method is in-house validated [5] and verified by a second independent laboratory [6, 7] for the *feed additive* samples at an enzymatic activity range of 1200 to 50000 PPU/g or ml of *product*. The reported method performance characteristics for *feed additive* were presented in Table 1:

**Table 1.** The results of validation and verification studies for the analytical method determining *6-phytase* activity in *feed additives*. LAB 1 is "validating laboratory (applicant)"; LAB 2 is "verifying laboratory (second independent)". \* the value was recalculated by the CRL.

	<i>Feed Additive</i>	
	LAB 1 [5]	LAB 2 [7]
LOD = limit of detection	1.0 PPU/g	0.1 PPU/g
LOQ = Limit of quantification	1.8 PPU/g	1.0 PPU/g
RSD <sub>r</sub> , % = Relative standard deviation for repeatability	6.1	*3.5
RSD <sub>R</sub> , % = Relative standard deviation for intermediate precision	4.9-5.3	*3.5
RR = Recovery rate	100	101
Enzymatic activity range	1200-50000 PPU/g	9000-43817 PPU/g

Based on these acceptable performance characteristics, the applicant in-house validated method, which was verified by a second independent laboratory, is recommended for official control purposes for the determination of *6-phytase* activity in *feed additives* in the frame of authorisation.

### Premixtures

For the determination of *6-phytase* activity in *premixtures* the applicant proposes a method, based on the same method principle as for the *feed additive* [4]. A 5 g sample is extracted in citrate buffer containing EDTA, albumin and Tween. The extract is centrifuged, and its phytase activity level is determined according to the above mentioned assay [4]. The

method is in-house validated [5] and verified by a second independent laboratory [6, 7] for the premixture samples at an enzymatic activity range of 250 to 540 PPU/g. The reported method performance characteristics for *premixture* were presented in the Table 2:

**Table 2.** The results of validation and verification studies for the analytical method determining *6-phytase* activity in *premixtures*. LAB 1 is "validating laboratory (applicant)"; LAB 2 is "verifying laboratory (second independent)". (-) Not investigated. \* Recalculated by the CRL

	<i>Premixture</i>	
	LAB 1 [5]	LAB 2 [7]
LOD	0.3 PPU/g	10 PPU/g
LOQ	0.5 PPU/g	50 PPU/g
RSD <sub>r</sub> , %	3.5	*5.5
RSD <sub>R</sub> , %	(-)	*8.5
RR	(-)	102
Enzymatic activity range	450 PPU/g	240-540 PPU/g

The results of a ruggedness test indicated that the addition of 20 mM copper reduces the enzyme activity by 17 % (-), and 1 mM ZnCl<sub>2</sub> reduces the activity by 27 % [5]. Therefore the applicant's method is considered suitable for official control purposes but not for those samples that contain elevated levels of zinc and copper. 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 are not available; hence the suitability of such method for official controls could not be evaluated.

### Feedingstuffs

For the determination of *6-phytase* activity in *feedingstuffs* the applicant proposes a method similar to the method for *feed additive* [4]. A 2.5 g of feed sample is extracted in 20 ml buffer (pH = 5.0) and centrifuged. The enzyme purification is carried out by gel filtration on a Sephadex G-25M column. The enzyme activity is determined according to the above mentioned assay [4]. The method was in-house validated [5] and verified by a second independent laboratory [6, 7] for the *feedingstuffs* at an enzymatic activity range of 125 to 750 PPU/kg. The reported method performance characteristics were presented in the Table 3.

**Table 3.** The results of validation and verification studies for the analytical method determining *6-phytase* activity in *feedingstuffs*. LAB 1 is "validating laboratory (applicant)"; LAB 2 is "verifying laboratory (second independent)". \* Recalculated by the CRL. \*\* A low recovery rate of 64% obtained at a minimum recommended enzyme activity level of 125 PPU/kg of *feedingstuffs*.

	<i>Feedingstuffs</i>	
	LAB 1 [5]	LAB 2 [7]
LOD	23 PPU/kg	35 PPU/kg
LOQ	36 PPU/kg	50 PPU/kg
RSD <sub>r</sub> , %	3.9-8.9	*5.0
RSD <sub>R</sub> , %	5.1-7.4	*7.0
RR	**64-94	101
Enzymatic activity range	125-750 PPU/kg	439-698 PPU/kg

A harmonised method is available for the determination of phytase activity 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 *6-phytase* activity at the defined condition within the authorisation frame of *6-phytase (Finase EC)*.

Based on these acceptable performance characteristics the applicant in-house validated method, which is verified by a second independent laboratory, is considered suitable for official controls to determine the activity of *6-phytase* in *feedingstuffs* at the target activity level of 250 PPU/kg, but not at the activity level of 125 PPU/kg due to low recovery rate.

Further testing or validation is not considered necessary.

#### 4. CONCLUSIONS AND RECOMMENDATIONS

Based on these acceptable performance characteristics the applicant's in-house validated and verified methods are recommended for official control purposes within the frame of authorisation for the determination of *6-phytase* activity in

- *feed additives*
- *premixtures* with the exception of samples with high amounts of zinc and copper
- *feedingstuffs* at the activity level of 250 PPU/kg, but not at the activity level of 125 PPU/kg due to the low recovery rate obtained at this level

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 *feed additives, premixtures* and *feedingstuffs*:

Colorimetric method quantifying the activity of *6-phytase* by measuring released inorganic phosphate from sodium phytate by analysing the colour formed by the reduction of a phosphomolybdate complex.

One-PPU unit is the quantity of enzyme which liberates one  $\mu$ mole of inorganic phosphate from sodium phytate per minute at pH = 5.0 and 37°C

## **5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL**

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of 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] \* Reference SANCO/D/2 Forw. Appl. 1831/019-2008.
  - [2] \* Annex III, Proposal of Register Entry.
  - [3] 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.
  - [4] \* Section II. ENCL\_II\_62-Assay of phytase activity.
  - [5] \*Section II. ENCL\_II\_59\_Part1\_validation data for phytase activity revised
  - [6] \* Section II. ENCL\_II\_61\_Verification report-Annex IV.
  - [7] \* Section II. ENCL\_II\_60\_b\_Verification report-Annex III.
- \*Refers to Dossier No: FAD-2008-0040.

## **7. RAPPORTEUR LABORATORY**

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



## **8. ACKNOWLEDGEMENTS**

The following National Reference Laboratories contributed to this report:

- Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Institut für Futtermittel, Wien, Austria.
- Thüringer Landesanstalt für Landwirtschaft, Jena, Deutschland
- National Veterinary Research Institute, Pulawy, Poland
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig