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European Union Reference Laboratory for Feed Additives



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**EURL Evaluation Report on the Analytical Methods  
submitted in connection with the Application for the  
Authorisation of a Feed Additive  
according to Regulation (EC) No 1831/2003**

Dossier related to: FAD-2011-0015 - CRL/100375

Name of Additive: *PHYZYME XP 5000 L and 10000 L  
PHYZYME XP 5000 G and 10000 TPT*

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

Rapporteur Laboratory: The Danish Plant Directorate (PL)  
Lyngby, Denmark

Report prepared by: Annette Plöger (PL)

Report revised by: Piotr Robouch (EURL-FA)  
Date: 30/01/2011

Report approved by: Christoph von Holst  
Date: 30/01/2011

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## EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *PHYZYME*, under the category/functional group 4(a) "zootechnical additive"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003. According to the Applicant, the *feed additive* contains *6-phytase* (EC 3.1.3.26) as active substance produced by the strain *Schizosaccharomyces pombe* (ATCC SD 5233). The product is intended to be marketed as different formulations: (i) liquid *PHYZYME XP 5000 L* and *PHYZYME XP 10000 L*; or (ii) solid *PHYZYME XP 5000 G* and *PHYZYME XP 10000 TPT*, with a guaranteed minimum activity of 5000 and 10000 U *6-phytase*/g, respectively. The activity of *6-phytase* is expressed in phytase units (U), where 1 U is defined as the amount of enzyme which hydrolyzes the phytate substrate producing 1  $\mu$ mole of phosphate per minute at pH 5.5 and 37°C.

Specifically, authorisation is sought for the use of *PHYZYME* for all minor poultry species. The *feed additive* is intended to be incorporated into *premixtures* and/or complete *feedingstuffs* with an activity ranging from 150 to 1000 U *6-phytase*/kg.

For the quantification of *6-phytase* in the *feed additive*, the Applicant submitted a single-laboratory validated and further verified method - similar to the ring-trial validated EN ISO 30024 method for complete *feedingstuffs* - based on colour formation of phosphorous produced at pH 5.5 and 37 °C with a molybdate-vanadate reagent. The following performance characteristics were derived from the validation and the verification studies:

- a relative standard deviation for *repeatability* ( $RSD_r$ ) of 3.7 %;
- a relative standard deviation for *intermediate precision* ( $RSD_{ip}$ ) ranging from 4.2 to 5.2 %; and
- a recovery rate ( $R_{Rec}$ ) ranging from 101 to 102 %.

Based on the satisfactory performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method submitted by the Applicant for the determination of *6-phytase* in the *feed additive*.

For the quantification of *6-phytase* in the *premixtures* and *feedingstuffs*, the Applicant proposed a single-laboratory validated and further verified method - similar to the above mentioned EN ISO 30024 method for *feedingstuffs*. For the *premixtures*, the sample is diluted with heat treated whole grain wheat flour. The performance characteristics derived from the validation and the verification studies are consistent with those published for the ring-trial validated EN ISO 30024 method, using the same analytical principle.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated colorimetric method (EN ISO 30024) for the determination of *6-phytase* in *premixtures* (after dilution with heat-treated whole grain flour) and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

## KEYWORDS

*PHYZYME XP 5000 L and 10000 L, PHYZYME XP 5000 G and 10000 TPT*, 6-phytase, zootechnical, digestibility enhancers, all poultry minor species

## 1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use) for *PHYZYME*, under the category/functional group 4(a) "zootechnical additive"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003. The *feed additive* was already authorised under the Commission Regulations (EC) No 785/2007 and (EC) No 379/2009 for chickens for fattening, turkeys for fattening, laying hens, ducks for fattening, piglets (weaned), pigs for fattening and sows. According to the Applicant the *feed additive* contains *6-phytase* as active substance. It is produced by the organism *Schizosaccharomyces pombe* (ATCC SD 5233). The product is intended to be marketed in different formulations as:

- a liquid (*PHYZYME XP 5000 L* and *PHYZYME XP 10000 L*); and
- a solid (*PHYZYME XP 5000 G* and *PHYZYME XP 10000 TPT*),

with a guaranteed minimum activity of 5000 or 10000 U *6-phytase* /g of product [1].

The Applicant defines one phytase unit (U) as "the amount of enzyme which hydrolyzes the phytate substrate producing 1 µmole of phosphate per minute at pH 5.5 and 37 °C" [2]. Furthermore, the Applicant confirmed upon request from EFSA and the EURL that the FTU units used throughout the dossier are identical [12] to the international unit U defined in the EN ISO 30024 standard method [9] (1FTU = 1U).

Specifically, authorisation is sought for the use of *PHYZYME* for all minor poultry species [1]. The *feed additive* is intended to be incorporated into *premixtures* and/or complete *feedingstuffs* with an activity ranging from 150 to 1000 U *6-phytase*/kg [2,3].

## 2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *PHYZYME XP 5000 L and 10000 L & PHYZYME XP 5000 G and 10000 TPT*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

## 3. EVALUATION

### *Qualitative and quantitative composition of impurities in the feed additive*

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective European Union Reference Laboratories [4].

### *Description of the analytical methods for the determination of the active agents in the feed additive, premixtures and feedingstuffs*

For the determination of *6-phytase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted a single-laboratory validated method [5-8], based on colour formation of phosphorous with a molybdate-vanadate reagent. The standard operating procedure is similar to the one described in the ring trial validated spectrophotometric method EN ISO 30024 [9] for complete *feedingstuffs*. Dedicated sample treatments are prescribed for each matrix investigated:

- \* About 1 gram of *feed additive* is dissolved in acetate buffer (pH 5.5) containing 1 mM calcium chloride (100 ml for dry enzyme samples and 10 ml for liquid enzyme samples). The solutions are further diluted and incubated with sodium phytate for 60 min. at 37 °C, to release inorganic phosphate [5].
- \* For the *premixtures*, the sample is diluted with heat-treated wholegrain wheat flour. This mixture is milled. Five to 10 grams of whole grain wheat flour are added to 0.5 – 5.0 g of the milled *premixture* sample. Then, 100 ml of acetate buffer pH 5.5 containing 1 mM calcium chloride are added. Further dilution may be required after stirring and filtration.

The phytase activity is determined in the filtrate. For calculation, the endogenous phytase activity present in the heat treated wholegrain wheat flour must be subtracted from the premix result, and the dilution in wheat flour must be taken into account [6].

- \* For the *feedingstuffs*, 10 g of milled feed is stirred for 10 min in acetate buffer pH 5.5 containing 1 mM calcium chloride. This extract is diluted four times before determination of phytase activity as above [6].

The inorganic phosphate creates a yellow coloured complex when reacting with molybdate-vanadate reagent. The yellow colour is measured at a wavelength of 415 nm. The extent of colour formation is related to the enzyme activity using a phosphate standard calibration curve [5,6].

The Applicant submitted the validation reports of his method for the *feed additive* [7], *premixtures* and *feedingstuffs* [8]. Additionally, he provided verification data obtained by a second independent laboratory for the *feed additive* [10]. Furthermore, the Applicant provided experimental data obtained by another expert laboratory applying the EN ISO 30024 standard method [11], and proving that both methods (Applicant and EN ISO 30024) provide similar results for *feedingstuffs* samples [12]. All the performance characteristics reported are summarized in Table 1. No specific experimental data for *premixtures* are provided since *premixture* samples are first diluted with heat-treated wholegrain wheat flour and then analysed as *feedingstuff* samples.

Based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified colorimetric method submitted by the Applicant for the analysis of 6-phytase in the *feed additive*. Furthermore, the EURL recommends for official control the ring-trial validated EN ISO 30024 colorimetric method for the determination of 6-phytase in the *premixtures* (after dilution with heat-treated whole grain flour) and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

**Table 1** Performance characteristics of analytical methods for the determination of the activity of *6-phytase* in the *feed additive (FA)*, *premixtures (PM)* and *feedingstuffs (FS)* derived from the validation and verification studies of the Applicant method, compared with the results for FS (\*) obtained applying the EN ISO 30024 method.

Matrix	Activity range	RSD <sub>r</sub>		RSD <sub>ip</sub>		R <sub>rec</sub>		LOQ U/kg
		Valid.	Verif.	Valid	Verif	Valid	Verif	
FA [7,10]	5 – 7.6 kU/g	3.7%	3.7%	4.2%	5.2%	101%	102%	
PM [8]	255 U/g	9.5%	--	14%	--		--	488
FS [8]	1040 U/kg	10%	--	11%	--	99%	--	61
FS [11] *	300 -800 U/kg	--	0.7-2.8%#	--	0.6-3.5%#	--	86-96%#	
FS [9] * EN ISO 30024	500-1700 U/kg	6 - 11%		RSD <sub>ip</sub> : 3 – 13% RSD <sub>R</sub> : 5 – 15%				60

RSD<sub>r</sub>, RSD<sub>ip</sub> and RSD<sub>R</sub>: relative standard deviation for *repeatability*, *intermediate precision* and *reproducibility*.

R<sub>rec</sub>: recovery rate; LOQ: limit of quantification

(#) values recalculated by the Rapporteur; Data obtained on the 15/11/2010 were used for RSD<sub>r</sub>

#### 4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single laboratory validated and further verified method based on the enzymatic hydrolysis of sodium phytate at pH = 5.5 and 37 °C for the quantification of *6-phytase* in the *feed additive*. No specific experimental data for *premixtures* are provided since *premixture* samples are first diluted with heat-treated wholegrain wheat flour and then analysed as *feedingstuff* samples. Hence, for the determination of *6-phytase* in *premixtures* (after dilution with heat-treated whole grain flour) and *feedingstuffs*, the EURL recommends for official control the ring-trial validated EN ISO 30024 method.

##### ***Recommended text for the register entry (analytical method)***

For the determination of *6-phytase* in *feed additive*:

- colorimetric method based on the quantification of the inorganic phosphate released by the enzyme from the sodium phytate

For the determination of *6-phytase* in *premixtures* and *feedingstuffs*:

- EN ISO 30024: colorimetric method based on the quantification of the inorganic phosphate released by the enzyme from the sodium phytate, (after dilution with heat-treated whole grain flour)

For the determination of *6-phytase* in *premixtures* and *feedingstuffs*:

- EN ISO 30024: colorimetric method based on the quantification of the inorganic phosphate released by the enzyme from the sodium phytate

One phytase unit (U) is the amount of enzyme, which hydrolyzes the phytate substrate producing 1  $\mu$ mole of phosphate per minute at pH 5.5 and 37 °C.

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *PHYZYME XP 5000 L and 10000 L and PHYZYME XP 5000 G and 10000 TPT* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

## 6. REFERENCES

- [1] \*Reference SANCO/D/2 Forw. Appl. 1831/0009-2010
- [2] \*Application, Proposal for Register Entry
- [3] \* Technical dossier, Section II – Identity, characterisation and conditions of use of the additive; Methods of analysis
- [4] 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 to Community Reference Laboratories
- [5] \*Technical dossier, Annexes Section II-Annex\_II\_B148\_MoA Method of Analysis (FA)
- [6] \*Technical dossier, Annexes Section II-Annex\_II\_B149\_MoA (FS&PM)
- [7] \*Technical dossier, Annexes Section II-Annex\_II\_B150\_ValidationReport (FA)
- [8] \*Technical dossier, Annexes Section II-Annex\_II\_B151\_ValidationReport (FS&PM)
- [9] EN ISO 30024, Animal feeding stuffs – Determination of phytase activity
- [10] \*Technical dossier, Annexes Section II-Annex\_II\_B152\_VerificationReport (FA)
- [11] \*Technical dossier, Annexes Section II-Annex\_II\_B153\_VerificationReport (FS&PM)
- [12] \*Supplementary information – Applicant e-mail dated 09/12/2011
  - Refers to Dossier No. FAD-2011-0015

## 7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was The 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, as last amended by Regulation (EC) No 885/2009.

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## 8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ) (CZ)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)