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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-2010-0008 CRL/100004

Name of Additive: *Optiphos*

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

Rapporteur Laboratory: European Union Reference
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Date: 17/05/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) for *Optiphos* under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for chickens and turkeys for fattening, laying hens, chickens reared for laying, turkeys reared for breeding, other birds for fattening and laying, weaned piglets, pigs for fattening and sows. The active agent is *6-phytase* (E.C. 3.1.3.26), produced by the strain *Pichia pastoris*.

According to the Applicant, enzymatic activity of the active agents is expressed in "FTU" units, where: one FTU is defined as the amount of enzyme that catalyzes the release of 1.0 micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 buffer at 37°C.

The *feed additive* is intended to be marketed in two granulate formulations (*Optiphos G 4000* and *Optiphos CT 4000*) and one liquid formulation (*Optiphos L 8000*).

Optiphos G 4000 is composed by pregelatinised starch, wheat meal and *6-phytase*, with a minimum activity of 4000 FTU/g. *Optiphos CT 4000* is composed by pregelatinised starch, wheat meal, distilled monoglyceride, palm oil, corn grid and *6-phytase*, with a minimum activity of 4000 FTU/g. *Optiphos L 8000* is composed by sucrose, sodium benzoate, purified water and *6-phytase*, with a minimum activity of 8000 FTU/mL.

The Applicant proposed two minimum activities for *6-phytase* in complete *feedingstuffs*:
- 125 FTU/kg for chickens for fattening, other birds for fattening, chickens reared for laying, laying hens, other laying birds, pigs for fattening and sows; - 250 FTU/kg for turkeys for fattening, turkeys reared for breeding and piglets (weaned).

For the determination of the activity of *6-phytase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposed a single-laboratory validated and further verified colorimetric method based on the quantification of the inorganic phosphate released by the enzyme from the sodium phytate (phytic acid dodecasodium salt, $C_6H_6O_{24}P_6Na_{12}$). Released inorganic phosphate is determined measuring the absorbance of the blue phosphomolybdate complex by a spectrophotometry at 820 nm. The released inorganic phosphate is quantified against a phosphate standard. The following method performance characteristics were recalculated by the EURL, based on the experimental data provided by the Applicant:

For the *feed additive*:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 1.07 to 1.85 %;
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 2.54 to 4.42 %;
- a *recovery rate* (R_{Rec}) ranging from 97 to 105 %.

For *premixtures* and *feedingstuffs*:

- RSD_r ranging from 4.98 to 6.96 %;
- RSD_{ip} ranging from 4.98 to 7.27 %;
- R_{Rec} ranging from 95 to 105 %.

Furthermore, the Applicant calculated a limit of detection (LOD) and quantification (LOQ) for *feedingstuffs* of 8.9 and 29.8 FTU/kg, respectively; both are well below the minimum activity proposed by the Applicant.

Aware of the existence of the ISO 30024 standard method the EURL requested the Applicant to apply this method to *Optiphos*. The Applicant reported precisions (RSD_r and RSD_{ip}) of the order of 6%, and measured activity values about three times higher than those expected. Such a bias could be due to differences in the experimental protocols of the two methods investigated. The EURL emphasizes that due to this bias, the *phytase* activity measurements obtained with Applicant's method on this specific product cannot be compared with *phytase* activity measurements of other *phytase* products, for which the ISO standard is applicable.

Taking into account the international effort of harmonisation of the analytical method for *phytase* determination the EURL cannot presently recommend for official control any of the two methods mentioned above. However complying with article 10 (Commission Regulation (EC) No 378/2005) the EURL considers necessary the organisation of an inter-laboratory comparison to determine an accurate conversion factor (f)¹ allowing the applicability of the ISO method in the frame of official control when determining *phytase* (*Optiphos*) in the *feed additive*, *premixtures* and *feedingstuffs*.

Furthermore the EURL considers that the Applicant should provide a different abbreviation for *optiphos* *phytase* activity unit in the registry entry. This will avoid confusion with the *phytase* activity unit (FTU) defined by the ISO method.

¹ $f = \frac{\text{phytase activity determined by ISO 30024}}{\text{phytase activity determined by Applicant method}}$

KEYWORDS

Optiphos, 6-phytase, *Pichia pastoris*, digestibility enhancers, chickens and turkeys for fattening, laying hens, chickens reared for laying, turkeys reared for breeding, other birds for fattening and laying, weaned piglets, pigs for fattening and sows

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) for *Optiphos* under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1]. Authorisation is sought for chickens and turkeys for fattening, laying hens, chickens reared for laying, turkeys reared for breeding, other birds for fattening and laying, weaned piglets, pigs for fattening and sows [2].

The active agent is 6-*phytase* (E.C. 3.1.3.26), produced by the strain *Pichia pastoris* [3]. The strain is deposited at the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany [4].

According to the Applicant, enzymatic activity of the active agents is expressed in FTU units [3], where: one FTU is defined as the amount of enzyme that catalyzes the release of 1.0 micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 buffer at 37°C.

The *feed additive* is intended to be marketed in two granulate formulations (*Optiphos G 4000* and *Optiphos CT 4000*) and one liquid formulation (*Optiphos L 8000*) [2].

Optiphos G 4000 is composed by pregelatinised starch, wheat meal and 6-*phytase*, with a minimum activity of 4000 FTU/g. *Optiphos CT 4000* is composed by pregelatinised starch, wheat meal, distilled monoglyceride, palm oil, corn grid and 6-*phytase*, with a minimum activity of 4000 FTU/g. *Optiphos L 8000* is composed by sucrose, sodium benzoate, purified water and 6-*phytase*, with a minimum activity of 8000 FTU/mL [2].

The Applicant proposed two minimum activities for 6-*phytase* in complete *feedingstuffs*: - 125 FTU/kg for chickens for fattening, other birds for fattening, chickens reared for laying, laying hens, other laying birds, pigs for fattening and sows; - 250 FTU/kg for turkeys for fattening, turkeys reared for breeding and piglets (weaned) [2].

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 *Optiphos*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

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 such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective European Union Reference Laboratories [5].

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

For the determination of the activity of *6-phytase* in the *feed additive, premixtures* and *feedingstuffs*, the Applicant proposed a single-laboratory validated colorimetric method based on the quantification of the inorganic phosphate released by the enzyme from the sodium phytate (phytic acid dodecasodium salt, $C_6H_6O_{24}P_6Na_{12}$) [3]. Citrate buffer pH 5.5 is used to extract the *feed additive, premixtures* and *feedingstuffs* samples. Before centrifuging or filtering, the *premixtures* and *feedingstuffs* extracts are diafiltered through a 10000 dalton exclusion limit membrane, to reduce levels of salts and other interfering small molecules [3]. Diafiltration removes approximately 85-88 % of background sample salts while retaining 95-100 % of *phytase* activity [3]. The filtered solution is diluted and an aliquot is incubated at pH 5.5 and 37 °C for 15 min. The reaction is stopped by adding a trichloroacetic (TCA) solution. Released inorganic phosphate is determined measuring the absorbance of the blue phosphomolybdate complex by a spectrophotometry at 820 nm. The released inorganic phosphate is quantified against a phosphate standard.

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)*. Precision values were recalculated by the EURL [12] based on the experimental data provided by the Applicant [6-11].

	RSD _r , % [12]		RSD _{ip} , % [12]		LOD [15]	LOQ [15]	R _{rec} % [6-11]
	Validation	Verification	Validation	Verification			
<i>Feed additive</i>	1.07	1.85	2.54	4.42	---	---	97-105
<i>Premixture</i>	6.96	6.38	7.27	6.38	4.17	13.9	96-105
<i>Feedingstuffs</i>	6.13	4.98	6.13	4.98	8.9	29.8	95-103

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and for *intermediate precision*.

R_{rec}: recovery rate

LOD and LOQ: limit of detection and quantification (expressed in FTU/g and FTU/kg for PM and FS, respectively).

Target values of 4000, 102 and 0.5 FTU/g for FA, PM and FS, respectively

The single-laboratory validated method [6-8] was further verified by a second independent laboratory [9-11]. The performance characteristics presented in Table 1 were recalculated by EURL [12] using the experimental data provided by the Applicant, related to the granular formulations [6-11].

Aware of the existence of the ISO 30024 standard method [13] the EURL requested the Applicant to apply this method to *Optiphos*. The Applicant reported acceptable precisions (RSD_r and RSD_{ip}) of the order of 6%, and measured activity values about three times higher than those expected [14]. Such a bias could be due to differences in the experimental protocols of the two methods investigated. The EURL emphasizes that due to this bias, the *phytase* activity measurements obtained with Applicant's method on this specific product cannot be compared with *phytase* activity measurements of other *phytase* products, for which the ISO standard is applicable.

Taking into account the international effort of harmonisation of the analytical method for *phytase* determination the EURL cannot presently recommend for official control any of the two methods mentioned above. However complying with article 10 (Commission Regulation (EC) No 378/2005) the EURL considers necessary the organisation of an inter-laboratory comparison to determine an accurate conversion factor (f)² allowing the applicability of the

² $f = \frac{\text{phytase activity determined by ISO 30024}}{\text{phytase activity determined by Applicant method}}$

ISO method in the frame of official control when determining *phytase* (Optiphos) in the *feed additive, premixtures* and *feedingstuffs*.

Furthermore the EURL considers that the Applicant should provide a different abbreviation for optiphos phytase activity unit in the registry entry. This will avoid confusion with the phytase activity unit (FTU) defined by the ISO method.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL requested the Applicant to apply the internationally agreed ISO 30024 method for the determination of *phytase* to Optiphos. The Applicant reported precisions of the order of 6%, and measured activity values about three times higher than those expected. The EURL emphasizes that due to this bias, the *phytase* activity measurements obtained with Applicant's method on this specific product cannot be compared with *phytase* activity measurements of other *phytase* products, for which the ISO standard is applicable.

Taking into account the international effort of harmonisation of the analytical method for *phytase* determination the EURL cannot recommend for official control any of the two methods mentioned above. However complying with article 10 (Commission Regulation (EC) No 378/2005) the EURL considers necessary the organisation of an inter-laboratory comparison to determine an accurate conversion factor (*f*) allowing the applicability of the ISO method in the frame of official control when determining *phytase* (Optiphos) in the *feed additive, premixtures* and *feedingstuffs*.

Furthermore the EURL considers that the Applicant should provide a different abbreviation for optiphos phytase activity unit in the registry entry. This will avoid confusion with the phytase activity unit (FTU) defined by the ISO method.

Recommended text for the register entry (analytical method)

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

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Optiphos* 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] *Technical dossier, Section II – Reference_II_14_Safe deposit DSMZ Pichia
 - [5] 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
 - [6] *Technical dossier, Section II – Reference_II_28 validation for feed additive
 - [7] *Technical dossier, Section II – Reference_II_29 validation for feedingstuffs
 - [8] *Technical dossier, Section II – Reference_II_30 validation for premixtures
 - [9] *Technical dossier, Section II – Reference_II_35 verification for feed additive
 - [10] *Technical dossier, Section II – Reference_II_36 verification for feedingstuffs
 - [11] *Technical dossier, Section II – Reference_II_37 verification for premixtures
 - [12] *Additional Information – Precision data as recalculated by the EURL
 - [13] ISO 30024, Animal feeding stuffs – Determination of phytase activity
 - [14] *Supplementary information provided by the Applicant upon request EURL – Reply *Optiphos* in feed by FEFANA method
 - [15] *Supplementary information provided by the Applicant upon request EURL – Reply questions 19/01/2011
- * Refers to Dossier No. FAD-2010-0008

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was European Union 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) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer (DE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
- Univerza v Ljubljani, Veterinarska fakulteta, Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava (SK)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)