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

Optiphos®
(FAD-2016-0019; CRL/150024)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2016-0019 - CRL/150024**
Name of Feed Additive:	***Optiphos®***
Active Agent (s):	**6-phytase**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA)** **11/10/2016**
Report approved by: Date:	**Christoph von Holst** **17/10/2016**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 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. This feed additive is currently authorised for avian and porcine species. In the frame of this application authorisation is sought for its use in salmonidae and other fin fish. The active agent is *6-phytase*, produced by *Komagataella pastoris*.

The enzymatic activity of the active agents is expressed in "OTU" units, where "one OTU is the amount of enzyme that catalyses the release of one micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 citrate buffer at 37 °C, measured as the blue phosphorus-molybdate complex color at 820 nm".

The *feed additive* is intended to be marketed as two granulate formulations (*G* 4000 and *CT* 4000) and one liquid formulation (*L* 8000*)* with minimum activities of 4000 OTU/g, and of 8000 OTU/g for the granulated and liquid formulations respectively. The Applicant proposed a minimum activity for *6-phytase* in complete *feedingstuffs* of 250 OTU/kg for salmonidae and other fin fish.

For the quantification of phytase activity in *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the same single-laboratory validated and further verified colorimetric method evaluated by the EURL in the frame of the dossier FAD-2010-0008, based on the quantification of the inorganic phosphate released by the enzyme from the sodium phytate. Supplementary experimental results were provided for feedingstuffs for rainbow trout. Based on the satisfactory performance characteristics available the EURL recommends for official control the colorimetric method mentioned above for the quantification of *phytase* activity in the *feed additive*, *premixtures* 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

6-phytase, *Optiphos*®, digestibility enhancers, salmonidae and other fin fish

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use) for *Optiphos®* under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1]. This feed additive is currently authorised for avian and porcine species [2]. In the frame of this application authorisation is sought for its use in salmonidae and other fin fish [1][3].

According to the Applicant, *6-phytase* is the active agent of *Optiphos®* produced by *Komagataella pastoris* [2]. The *phytase* enzymatic activity is expressed in OTU/g units, where "one OTU is the amount of enzyme that catalyses the release of one micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 citrate buffer at 37 °C, measured as the blue phosphorus-molybdate complex color at 820 nm" [2].

The *feed additive Optiphos®* is intended to be marketed as two granulate (*G 4000* and *CT 4000*) and one liquid (*L 8000*) formulations [3], where:

- *Optiphos® G 4000* is composed by pregelatinised starch, wheat meal and *6-phytase*, with a minimum activity of 4000 OTU/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 OTU/g; and
- *Optiphos® L 8000* is composed by sucrose, sodium benzoate, purified water and *6-phytase*, with a minimum activity of 8000 OTU/g [4].

The Applicant proposed a minimum activity for *6-phytase* in complete *feedingstuffs* of 250 OTU/kg for salmonidae and other fin fish [3].

Note: The analytical methods for the quantification of *Optiphos®* in the relevant matrices were already evaluated by the EURL in the frame of the dossier FAD 2010-0008 [5].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. microbiological agents, arsenic, cadmium, lead, mercury, mycotoxins and dioxins) are available from the respective European Union Reference Laboratories [6].

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

For the quantification of the activity of *6-phytase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted the same single-laboratory validated [7] and further verified [8] colorimetric method, referred in the Commission Implementing Regulation (EU) 2016/348 [2] based on the quantification of the inorganic phosphate released by the enzyme from the sodium phytate (phytic acid dodecasodium salt, $C_6H_6O_{24}P_6Na_{12}$) [9].

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. Diafiltration removes approximately 85-88 % of background sample salts while retaining 95-100 % of *phytase* activity. The filtered solution is diluted and an aliquot is incubated at pH 5.5 and 37 ^{o}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 spectrophotometry at 820 nm. The released inorganic phosphate is quantified against a phosphate standard [9]. The relevant performance characteristics calculated by EURL [10][11] are presented in Table 1.

This method was further applied with minor experimental modifications by a second laboratory in the frame of a verification study to quantify the *6-phytase* activity in rainbow trout *feedingstuffs* supplemented with *Optiphos®* [12]. The performance characteristics calculated by the EURL [13] were similar to those determined in the previous studies, demonstrating the suitability of the proposed method (Table 1).

Based on the performance characteristics provided the EURL recommends for official control the colorimetric methods mentioned above for the quantification of *phytase* activity in the *feed additive*, *premixtures* and *feedingstuffs*.

Table 1 Performance characteristics of analytical methods for the quantification of the activity of *6-phytase* in the *feed additive (FA), premixtures (PM)* and *feedingstuffs (FS).* Precision values were recalculated by the EURL [10][11] based on the experimental data provided by the Applicant*, obtained in the frame of the validation (Val) and verification (Ver) studies* [7][8][12].

	Activity (OTU/g)	RSD_r (%)*		RSD_{ip} (%)*		R_{Rec} (%)		LOQ (OTU/g)	
		Val [7]	Ver [8]	Val [7]	Ver [8]	Val [7]	Ver [8]	Val [7]	Ver [8]
FA	4000	1.1	1.7	2.5	4.0	96-105	100	-	-
PM	100	5.4	6.4	7.3	6.4	100-103	103	7	5
FS	0.25/0.5	4.1/6.1	-/4.8	4.1/6.1	-/4.8	95-103	97	0.02	0.03
FS [12]	0.5		4.6		4.6		106		0.11

RSD_r & RSD_{ip}: relative standard deviation for *repeatability* & *intermediate-precision,* respectively;
R_{Rec}: *recovery* rate (%);
* Recalculated by EURL [10][11]

Upon the EURL request, the Applicant provided in the frame of a previous dossier FAD-2010-0008 [5] experimental data showing that the application of the ISO 30024 standard method for the determination of the *phytase* activity in *Optiphos®* products leads to an overestimation of the *phytase* activity [5]. As stated in the previous evaluation report the EURL considers the current ISO 30024 standard method not suitable for the determination of *phytase* activity in samples containing *Optiphos®*. The EURL is currently investigating the conversion factor between the "Optiphos" unit and the "international phytase" unit defined in the above mentioned ISO standard that would allow the application of the ISO method for determining *phytase* activity in matrices containing the *Optiphos®* products.

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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for official control the in-house validated and further verified colorimetric method based on the enzymatic reaction of *phytase* on the phytate for the quantification of *phytase* activity in the *feed additive*, *premixtures* and *feedingstuffs*, in compliance with what is indicated in the Commission Implementing Regulation (EU) 2016/348 [2].

Recommended text for the register entry (analytical method)

For the quantification of *phytase a*ctivity in the *feed additive*, premixtures and feedingstuffs:

- colorimetric method based on the enzymatic reaction of *phytase* on the phytate

One *phytase* unit (OTU) is the amount of enzyme that catalyses the release of one micromole of inorganic phosphate per minute from 5.1 mM sodium phytate in pH 5.5 citrate buffer at 37 °C, measured as the blue phosphorus-molybdate complex color at 820 nm

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] *Application, Reference SANCO/G1: Forw. Appl. 1831/0018-2016

[2] Commission Implementing Regulation (EU) 2016/348 amending Implementing Regulation (EU) No 98/2012 as regards the minimum content of the preparation of 6-phytase (EC 3.1.3.26) produced by *Komagataella pastoris* (DNS 23036) as a feed additive for pigs for fattening (holder of authorisation Huvepharma EOOD)

[3] *Application, Proposal for Register Entry – Annex A

[4] *Technical dossier, Section II Identity, characterisation and conditions of use of the additive; methods of analysis – 2.1.3 Qualitative and quantitative composition

[5] EURL Evaluation Reports https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0008.pdf

[6] 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

[7] *Technical dossier, Section II, Annexes, References II.28, II.29 & II.30

[8] *Technical dossier, Section II, Annexes, References II.35, II.36 & II.37

[9] *Technical dossier, Section II, Annex, Reference II.55

[10] *Supplementary information, EURL_Optiphos_val.pdf

[11] *Supplementary information, EURL_Optiphos_ver.pdf

[12] *Technical dossier, Section II, Annex, Reference II.59

[13] *Supplementary information, EURL_Optiphos_fish_ver.pdf

*Refers to Dossier no: FAD-2016-0019

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
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