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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-2008-692
FAD-2008-0043
CRL/080023

Product name: 3-Phytase (Natuphos)

Active Substance(s): 3-phytase (EC 3.1.3.8)

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EXECUTIVE SUMMARY

The current application authorisation is sought for *3-phytase* (*Natuphos*) under the category ‘zootechnical additives’, group 4(a), digestibility enhancer for pigs for fattening according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for *3-phytase* according to Article 13(3) of Regulation (EC) No 1831/2003.

The active agent of *Natuphos* is *3-phytase* produced by submerged fermentation of *Aspergillus niger* (CBS 101.672). One FTU-unit is the amount of *3-phytase* which liberates one μmol of inorganic phosphate per minute from sodium phytate at $\text{pH} = 5.5$ and $37\text{ }^{\circ}\text{C}$. The product is intended to be marketed in three forms, as powder (*Natuphos* 5000), as granulate (*Natuphos* 5000 G and 10000 G) and as liquid formulation (*Natuphos* 5000 L and 10000 L), containing, either 5000 or 10000 FTU/g or ml of *product*. These products are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain an enzyme activity level of minimum 100 FTU/kg in complete *feedingstuffs* for pigs for fattening.

For the determination of *3-phytase* activity in *feed additives*, *premixtures* and *feedingstuffs* the applicant proposes a colorimetric method measuring inorganic phosphate released by *3-phytase* from phytate substrate at $\text{pH} = 5.5$ and $37\text{ }^{\circ}\text{C}$. The released inorganic phosphate forms a yellow complex with an acidic molybdate/vanadate reagent, which is measured at 415 nm and quantified against a standard curve of phosphate for *feed additives*; and is based on a reference enzyme *Natuphos*, available from the applicant, for *premixtures* and *feedingstuffs*.

The applicant submitted the validation data regarding the analytical method determining *3-phytase* activity in *feed additives*, *premixtures* and *feedingstuffs*, which were obtained from collaborative studies organised by the Association of German Agricultural Analytical and Research Institutes (VDLUFA).

For the determination of *3-phytase* activity in *feed additives* the following performance characteristics were reported: - a relative standard deviation for repeatability (RSD_r) of 2.5 %, - a relative standard deviation for reproducibility (RSD_R) of 4.9 % and - recovery rate (RR) ranging from 98 to 102 %. Based on these acceptable performance characteristics the proposed method is

considered suitable for determination of *3-phytase* activity in *feed additives* for pigs for fattening for official control purposes in the frame of authorisation.

For the determination of *3-phytase* activity in *premixtures* the method performance characteristics obtained from *feedingstuffs* are applicable to the premixture samples which are diluted with ground corn meal and therefore behave as a matrix of *feedingstuffs*.

For the determination of *3-phytase* activity in *feedingstuffs* the performance characteristics of the VDLUFA method, obtained at 600 U/kg enzymatic activity, were: - a limit of detection (LOD) of 45 FTU/kg of *feedingstuffs*, - a limit of quantification (LOQ) of 90 FTU/kg of *feedingstuffs*, - a RSD_T ranging from 6.4 to 7.0 %, - a RSD_R ranging from 11.1 to 12.3 % and RR ranging from 98 to 103 %. Upon request from the CRL the applicant provided additional precision data obtained at lower enzyme activity levels close to the minimum level in *feedingstuffs* (100 FTU/kg). For enzyme activity levels between 100 and 500 FTU/kg of *feedingstuffs* the reported relative standard deviation for intermediate precision (RSD) ranges from 11.8 to 15.2 %.

Several other ring trial validated methods for the determination of *phytase* activity in *feedingstuffs* exist. These include a colorimetric method which is developed by FEFANA (European Association of Feed Additive Manufacturers) and validated on various *phytase* products (including *Natuphos* products). The method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN) and is very similar to the above mentioned VDLUFA method, but the quantification is based on the use of phosphate standard curve. The validation of draft CEN method included a *phytase* activity level ranging from 700 to 1500 U/kg of *feedingstuffs*, whereas the validation range of VDLUFA method was extended to cover the low *phytase* activities ranging from 100 to 500 FTU/kg of *feedingstuffs*. Therefore, the proposed VDLUFA method is found suitable for official controls for the determination of *phytase* activity at minimum proposed level (100 FTU/kg of *feedingstuffs*) in the frame of present authorisation.

Based on acceptable performance characteristics the applicant proposed VDLUFA method is recommended for official control purpose for the determination of *3-phytase* activity in *premixtures* and *feedingstuffs*.

Further testing or validation is not considered necessary.

KEYWORDS

Natuphos, *3-phytase*, *Aspergillus niger*, digestibility enhancer, pigs for fattening

1. BACKGROUND

3-phytase (*Natuphos*) is a product for which authorisation as feed additive is sought under the category ‘zootechnical additives’, functional groups ‘digestibility enhancers’ for pigs for fattening, according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorisation is sought for *3-phytase* according to Article 13(3) of Regulation (EC) No 1831/2003 [1]. The additive has already been authorised according to Regulation (EC) No 243/2007 for piglets, pigs for fattening, chickens for fattening and ducks [2].

The active agent of *Natuphos* is *3-phytase* (EC 3.1.3.8) produced by submerged fermentation of *Aspergillus niger* (CBS 101.672) deposited at the Centraalbureau voor Schimmelcultures (CBS) under the number CBS 101.672 (NPH54) in the Netherlands [3]. The activity of *3-phytase* is expressed in FTU (phytase) unit, which is equivalent to U (phytase) unit in the indicated reference (VDLUFA method). According to the applicant, one FTU-unit is the amount of *3-phytase* which liberates one μmol of inorganic phosphate per minute from sodium phytate at $\text{pH} = 5.5$ and $37\text{ }^\circ\text{C}$ [3]. The product is intended to be marketed in three forms [2]:

- as powder formulation (*Natuphos 5000*) with a target *3-phytase* activity of 5000 FTU/g,
- as granulate formulation (*Natuphos 5000 G* and *10000 G*) with a target *3-phytase* activity of 5000 FTU/g and 10000 FTU/g, respectively,
- as a liquid formulation (*Natuphos 5000 L* and *10000 L*) with a target *3-phytase* activity of 5000 FTU/ml and 10000 FTU/ml, respectively.

These products are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain enzyme activity level of minimum 100 FTU/kg in complete *feedingstuffs* for pigs for fattening [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 of the European Parliament and of the Council as regards the duties and the tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, 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 *Natuphos*, (EFSA-Q-2008-692), and their suitability to be used for official controls in the frame of 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* (e.g. arsenic and heavy metals - cadmium, mercury and lead) are available at the respective Community Reference Laboratories [5].

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

For the determination *3-phytase* activity in the *feed additive, premixtures and feedingstuffs* the applicant proposes a colorimetric method measuring inorganic phosphate released by *3-phytase* from phytate substrate at pH = 5.5 and 37 °C [6]. The released inorganic phosphate forms a yellow complex with an acidic molybdate/vanadate reagent, which is measured at 415 nm and quantified against a standard curve of phosphate for *feed additives*; and is based on a reference enzyme *Natuphos*, available from the applicant, for *premixtures and feedingstuffs* [6]. The method has been developed and described by Engelen *et al.*, [8] and validated through a collaborative study organised by VDLUFA [9].

For the determination of *3-phytase* activity in *feed additives*, either liquid or solid products are suspended in acetate buffer (pH = 5.5). The extract is filtered and diluted and then incubated with the phytate substrate at 37 °C. After 30 minutes incubation the reaction is stopped with an acidic molybdate/vanadate reagent. The yellow formed complex is measured with a

spectrophotometer at a wavelength of 415 nm against a standard curve of phosphate [9]. Upon request from the CRL the applicant confirmed the use of an absolute method for the determination of *feed additives*. The method performance characteristics reported by the VDLUFA collaborative study [9] were: - a relative standard deviation for repeatability (RSD_r) of 2.5 % and - a relative standard deviation for reproducibility (RSD_R) of 4.9 % for granulated products at an enzyme activity level of 21360 U/g [11]. Recovery rate (RR) determined by the applicant by adding enzyme solution after suspension of *Natuphos* 5000 samples ranges from 98 to 102 % [11].

Based on acceptable performance characteristics, the proposed method is recommended for official control purposes for the determination of *3-phytase* activity in *feed additives* in the frame of authorisation.

For the determination of *premixtures* the applicant proposed a modification of the VDLUFA-method [9]: the premixture samples are diluted with ground tapioca or corn meal, containing no phytase activity. The resulting samples are therefore treated as *feedingstuffs* [12] and the validation data obtained from the samples of *feedingstuffs* is applicable for these *premixtures* samples.

For the quantification of *3-phytase* activity in *feedingstuffs* a feed sample of 200 g is extracted in acetate buffer (pH 5.5) without any calcium chloride. After stirring, filtering and diluting the samples are incubated for 60 minutes with the phytate substrate at 37 °C. After stopping the reaction with an acidic molybdate/vanadate reagent the samples are measured with a spectrophotometer at a wavelength of 415 nm against a standard curve based on a reference *Natuphos* enzyme, available from the applicant upon request [13]. The method performance characteristics, obtained in the VDLUFA collaborative study [9] at an enzyme activity level of 600 U/kg of *feedingstuffs* were: - a limit of detection (LOD) of 45 FTU/kg of *feedingstuffs*, - a limit of quantification (LOQ) of 90 FTU/kg of *feedingstuffs*, - a RSD_r ranging from 6.4 to 7.0 % and - a RSD_R ranging from 11.1 to 12.3 %. RR determined by the applicant on *feedingstuffs* ranges from 98 to 103 % [11]. Upon request from the CRL the applicant provided additional precision data obtained at lower enzyme activity levels close to the minimum level in *feedingstuffs* (100 FTU/kg). For enzyme activity levels between 100 and 500 FTU/kg the reported relative standard deviation for intermediate precision (RSD) ranges from 11.8 to 15.2 % [14].

Several other ring trial validated methods for the determination of *phytase* activity in *feedingstuffs* exist. These include a colorimetric method which is developed by FEFANA (European Association of Feed Additive Manufacturers) and validated on various phytase products (including *Natuphos* products). The method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN) and is very similar to the above mentioned VDLUFA method, but the quantification is based on the use of phosphate standard curve [15]. The validation of draft CEN method included a phytase activity level ranging from 700 to 1500 U/kg of *feedingstuffs* [16], whereas the validation range of VDLUFA method was extended to cover the low phytase activities ranging from 100 to 500 FTU/kg of *feedingstuffs*. Therefore, the proposed VDLUFA method is found suitable for official controls for the determination of *3-phytase* activity at minimum proposed level (100 FTU/kg of *feedingstuffs*) in the frame of present authorisation.

Based on acceptable performance characteristics the applicant proposed VDLUFA method is recommended for official control purpose for the determination of *3-phytase* activity in *premixtures* and *feedingstuffs*.

4. CONCLUSIONS AND RECOMMENDATIONS

For the quantification of *3-phytase* activity in various matrices, the applicant proposes ring-trial validated colorimetric methods based on well known principles and demonstrating acceptable performance characteristics. The analytical VDLUFA-methods for the determination of *3-phytase* activity in *feed additive*, in *premixture* and in *feedingstuffs* are recommended by the CRL for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

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

Colorimetric method based on reaction of vanadomolybdate on inorganic phosphate produced by action of *3-phytase* on a phytate-containing substrate (sodium phytate) at pH = 5.5 and 37 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Natuphos* 5000, *Natuphos* 5000 G and 10000 G and *Natuphos* 5000 L and *Natuphos* 10000 L, have been sent to the Community Reference Laboratory for Feed Additives.

The dossier has been made available to the CRL by EFSA.

6. REFERENCES

- [1] * Reference SANCO/D/2 Forw. Appl. 1831/028-2008
- [2] * Commission Regulation (EC) No 243/2007 concerning the authorisation of 3-phytase (*Natuphos*) as a feed additive. Official Journal of the European Union L 73/4, 13.3.2007
- [3] * Technical dossier Section II: identity, characterisation and condition of use of the additive.
- [4] * Annex III. Proposal of Register entry.
- [5] COMMISSION REGULATION (EC) No 776/2006 amending Annex VII to Commission 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
- [6] * Annex of Section II: REG 32 to 37
- [7] * Annex of section II: REG32_Phytase-Activity_PM_0857_01e
- [8] Engelen *et al.*(1994). Simple and rapid determination of phytase activity. Journal of AOAC International, Vol. 77(3), p. 760-764.
- [9] Bestimmung der Phytaseaktivität in Enzymstandardmaterialien und Enzympräparaten, 27.1.1. VDLUFA-Methodenbuch III, 4. Erg. 1997
- [10] Bestimmung der Phytaseaktivität in Futtermitteln und Vormischungen, 27.1.2. VDLUFA-Methodenbuch III, 4. Erg. 1997
- [11] *Annex of section II: REG32_ValidationReport_PM_0857_01e
- [12] * Annex of section II: REG39_Phytase_mineral_premix_PM_0860_01e
- [13] * Annex of section II: REG38_Phytase_feed_PM_0858_01e.
- [14] * Supplementary information from the applicant
- [15] CEN-method final draft: Animal feedingstuffs - Determination of phytase activity ISO/FDIS 30024:008 (E)

- [16] Gisele G. Thyregod P., von Holst C., Bertin G., Vogel K., Faurschou-Isaksen M., Betz R., Murphy R., Brandt Andersen B.: " Determination of Phytase Activity in Feed: Interlaboratory Study" J AOAC International (2008) Vol. 91, No. 2 259-267

* Refers to Dossier number: FAD-2008-0043

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Austrian Agency for Health and Food Safety, Vienna, Austria

8. ACKNOWLEDGEMENTS

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- Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany
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