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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:** **FAD-2007-0002**  
**EFSA-Q-2007-041**

**Product name:** **Natuphos<sup>®</sup>**

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

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## EXECUTIVE SUMMARY

In the current application authorisation is sought for *Natuphos*® under the category 'zootechnical additives' and the functional group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Natuphos*® as a digestibility enhancer for ducks.

The active agent of *Natuphos*® is 3-phytase, produced by a strain of *Aspergillus niger* (CBS 101.672). Enzymatic activity is expressed in FTU (phytase) units. One FTU is defined as the amount of enzyme that liberates one  $\mu\text{mol}$  of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37°C. The product is intended to be marketed in three forms, as a powder (*Natuphos*® 5000), as a granulate (*Natuphos*® 5000 G and 10000 G) and as liquid formulation (*Natuphos*® 5000 L and 10000 L), containing, as indicated in the trade names, either 5000 or 10000 FTU/g. The products are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain enzyme activity level of minimum 300 FTU/kg to recommended 750 FTU/kg in complete *feedingstuffs*.

For the determination of the activity of 3-phytase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a colorimetric method, based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37°C by the enzyme 3-phytase. Released phosphate forms with molybdate and vanadate ions a coloured complex that is measured with a spectrophotometer at 415 nm and quantified against a standard curve based on the reference enzyme *Natuphos*®, available from the applicant upon request.

The applicant reported precision data of the methods for the determination of the phytase activity in all three matrices that were obtained via inter-laboratory studies performed by the German Agricultural and Research Institutes (VDLUFA, Germany).

For the determination of the 3-phytase activity in the *feed additive*, method performance characteristics include a limit of detection (LOD) of 45 FTU/kg, a limit of quantification (LOQ) of 90 FTU/kg, a relative standard deviation for repeatability ( $\text{RSD}_r$ ) of 2.5% and a relative standard deviation for reproducibility ( $\text{RSD}_R$ ). The values for the recovery rate ranged from 98 to 102%. The inter-laboratory study of the method for the determination of 3-phytase activity in *premixtures* revealed an  $\text{RSD}_r$  of 4.9% and an  $\text{RSD}_R$  of 8.4%. Since the obtained method performance characteristics are acceptable, the methods are considered suitable for the official control purposes in the frame of authorisation.

The validation of the method for the determination of the phytase activity in *feedingstuffs* showed values for the LOD, LOQ and the recovery rate that were comparable to those of the methods for the determination of the phytase activity in the *feed additive* and in *premixtures*. The  $\text{RSD}_r$  was 6.9%, and the  $\text{RSD}_R$  was 11.1%, obtained in an inter-laboratory study organised by VDLUFA. Therefore, the method is considered suitable for official control purposes.

Several other methods for the determination of phytase activity in *feedingstuffs* exist and have also been ring trial validated. These include a colorimetric method developed by FEFANA (European Association of Feed Additive Manufacturers) which has been validated according to the IUPAC guidelines on various phytase products and obtaining values for the RSD<sub>R</sub> of 5 to 14%. In contrast to the method proposed by the applicant, the quantification is based on phosphate standard curve and not on the reference enzyme. Based on acceptable performance characteristics, this method, which is currently under evaluation to become a standard of the European Committee for Standardisation (CEN), is considered suitable for official control purposes and therefore recommended by the CRL for determination of enzymatic activity in *feedingstuffs*.

Further testing or validation is not considered necessary.

## KEYWORDS

*Natuphos*®, 3-phytase, *Aspergillus niger*, digestibility enhancer, ducks

## 1. BACKGROUND

*Natuphos*® is a product for which authorisation is sought under the category 'zootechnical additives' and the functional group 'digestibility enhancers', according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. *Natuphos*® contains 3-phytase (E.C. 3.1.3.8) as the active agent [2], produced by a microorganism *Aspergillus niger*, which is deposited at the Centraalbureau voor Schimmelcultures (CBS) under the number CBS 101.672 (NPH54) in Utrecht, the Netherlands.

The activity of 3-phytase is expressed in FTU (phytase) units. According to the applicant, one FTU is defined as the amount of enzyme which liberates one µmol of inorganic phosphate per minute from sodium phytate at pH 5.5 and 37°C. The product is intended to be marketed in three forms, as a powder (*Natuphos*® 5000), as granulate (*Natuphos*® 5000 G and 10000 G) and as liquid formulation (*Natuphos*® 5000 L and 10000 L), containing, as indicated in the trade names, at least 5000 or 10000 FTU/g, respectively [2]. The products are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain enzyme activity level of minimum 300 FTU/kg to recommended 750 FTU/kg in complete *feedingstuffs* [3].

3-phytase (*Natuphos*), produced by *Aspergillus niger* CBS 101.672, has already been authorised under number '4a 1600' as a feed additive for piglets, pigs for fattening and chickens for fattening [4].

## 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*®, cf. EFSA-Q-2007-041, and their suitability to be used for official controls in the frame of authorisation, were evaluated.

### 3. EVALUATION

#### ***Identification/Characterisation of the feed additive***

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

For the determination of arsenic [5], heavy metals [5] and microbiological agents [6], the applicant proposes analytical methods based on well known techniques and therefore considered suitable for intended purposes except of an old-fashioned semi-quantitative method. For official controls various standard methods based on the same analytical techniques and routinely applied by official control authorities are available and recommended by the CRL.

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

For the determination of the activity of 3-phytase in the *feed additive, premixtures and feedingstuffs* the applicant proposes a colorimetric method based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37°C by the 3-phytase. The reaction is stopped by adding of molybdate/vanadate reagent, afterwards forming a coloured complex with released inorganic phosphate. This complex is then measured on a spectrophotometer at 415 nm and enzymatic activity is quantified against a standard curve based on the reference *Natuphos*® enzyme available from the applicant upon request. The method has been developed and described by Engelen *et al.* [7] and validated in the ring trial organised by the German Agricultural and Research Institutes (VDLUFA) [8].

When analysing *feed additive*, either liquid or solid product is suspended in acetate buffer containing CaCl<sub>2</sub> and Tween (pH 5.5). The extract is diluted and filtered and then general assay conditions are followed [7][9]. The limit of detection (LOD) and limit of quantification (LOQ) of the method is 45 and 90 FTU/kg, respectively. The VDLUFA inter-laboratory study [8] with fourteen laboratories [10] and conducted on granulated products revealed a relative standard deviation for repeatability (RSD<sub>r</sub>) of 2.5% and a relative standard deviation for reproducibility (RSD<sub>R</sub>) of 4.9%. The enzymatic recovery, when analysed in four *in-house* experiments by adding enzyme solution after suspension of *Natuphos*® 5000 samples [8], varied from 98 to 102%. Based on acceptable performance characteristics, the method is considered suitable for official control purposes.

When analysing *premixtures*, 100 g of ground sample are mixed with a suspension of 600 g corn or tapioca meal and extracted in acetate buffer (pH 5.5) supplemented by calcium chloride and Tween. After stirring and filtering, general assay conditions are followed [11]. The same values for the LOD and LOQ as mentioned above apply. The VDLUFA inter-laboratory study [8] with fourteen laboratories [10] revealed an RSD<sub>r</sub> of 4.9% and an RSD<sub>R</sub> of 8.4%. Enzymatic recovery, when analysed in four *in-house* experiments, varied from 93 to

101%. Based on acceptable performance characteristics, the method is considered suitable for official control purposes.

For the quantification of the activity of 3-phytase in *feedingstuffs*, 100 g of feed sample are ground [12] and extracted in acetate buffer (pH 5.5) supplemented by calcium chloride and Tween. After stirring and filtering, general assay conditions are followed [9][12]. The same values for the LOD and LOQ as mentioned above apply. The VDLUFA inter-laboratory study [8] with fourteen laboratories [10] revealed an  $RSD_r$  of 6.9% and an  $RSD_R$  of 11%. Enzymatic recovery, when analysed in four *in-house* experiments on *feedingstuffs* for chickens and pigs, varies from 98 to 103%. Taking into account the target enzyme activity level of minimum 300 FTU/kg to recommended 750 FTU/kg of complete *feedingstuffs* and the acceptable values of performance characteristics, the proposed method is considered suitable for official control purposes.

Several other methods for the determination of phytase activity in *feedingstuffs* exist and have also been ring trial validated. These include a colorimetric method developed by FEFANA (European Association of Feed Additive Manufacturers) [13] which has been validated according to the IUPAC guidelines on various phytase products and obtaining values for the  $RSD_R$  ranging from 5 to 14% [14]. The principles of the FEFANA and the applicant's method are very similar, but the FEFANA method utilises a phosphate standard for quantification, whereas the quantification of the applicant's method is based on a reference enzyme. Based on acceptable performance characteristics, this method, which is currently under evaluation to become a standard of the European Committee for Standardisation (CEN), is considered suitable for official control purposes and therefore recommended by the CRL for determination of enzymatic activity in *feedingstuffs*.

#### 4. CONCLUSIONS AND RECOMMENDATIONS

For the quantification of the activity of 3-phytase in the *feed additive, premixtures and feedingstuffs*, the applicant proposes ring-trial validated colorimetric methods based on well known principles, demonstrating acceptable performance characteristics and which are considered suitable for official control. For the determination of the 3-phytase activity in *feedingstuffs*, the CRL, however, recommends another ring trial validated method which is similar to the applicant's methods but is applicable to different phytase products.

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 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. 1831/01-2007.
- [2] REG08\_Section II.
- [3] Annex III. Proposal of Register entry.
- [4] 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.
- [5] 5 Annexes of section II: REG\_42.1 to REG\_42.5.
- [6] 8 Annexes of section II: REG\_43 to REG\_45 and REG\_46.1 to REG\_46.5.
- [7] Engelen *et al.*(1994). Simple and rapid determination of phytase activity. Journal of AOAC International, Vol. 77(3), p. 760-764.
- [8] Annex of section II: REG32\_ValidationReport\_PM\_0857\_01e.
- [9] Annex of section II: REG32\_Phytase-Activity\_PM\_0857\_01e.
- [10] Supplementary information submitted upon request of the CRL-FA (received 25 June 2007).
- [11] Annex of section II: REG39\_Phytase\_mineral\_premix\_PM\_0860\_01e.
- [12] Annex of section II: REG38\_Phytase\_feed\_PM\_0858\_01e.
- [13] CEN-method draft: Animal feedingstuffs - Determination of phytase activity; Working document N 410 of CEN TC 327.
- [14] Gisele Gizzi and Christoph von Holst (2005). Validation study on a new method for the determination of phytase activity in feed: Results from an interlaboratory study conducted according to the IUPAC harmonised protocol. European Commission, DG JRC, IRMM, Geel, Belgium.

## 7. RAPPORTEUR LABORATORY

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

## **8. ACKNOWLEDGEMENTS**

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- Agricultural Institute of Slovenia, Ljubljana, Slovenia.
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Germany.
- Laboratory of the Government Chemist, Teddington, United Kingdom.