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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-2005-116
Name of Additive:	Natuphos®
Active Substance(s):	3-phytase
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Date:	11/01/2006
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Date:	17/01/2006

EXECUTIVE SUMMARY

In the current application, authorisation is sought for Natuphos®.

The active agent is the enzyme 3-phytase produced by *Aspergillus niger*. The additive is already authorised in EU for the animal categories chickens for fattening, laying hens, piglets, pigs for fattening, sows and turkeys for fattening. The content of the active substance is expressed in terms of the enzyme activity FTU, where 1 FTU is the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate at pH 5.5 and 37°C in one minute. The current application is for the use of two products which are Natuphos® 5000 G (granulate) and Natuphos® 5000 L (liquid) with a minimum activity of 5000 FTU/g and 5000 FTU/ml, respectively.

The additive improves the utilisation of phosphorous in the feedingstuff by the target animal. The proposed dosages for the six categories of animals range from 250 to 700 FTU / kg feedingstuff, depending on the target animal.

For the determination of the phytase activity in the *feed additive,* in *premixes* and in *feedingstuff* the applicant proposes very similar analytical methods as published in a peer reviewed journal. The method is based on the principle that phytase releases inorganic phosphate from a substrate, which in the presence of molybdate/vanadate reagent forms a yellow complex. The yellow complex is measured with a spectrophotometer. The phytase activity of the sample is quantified against a phytase standard with defined activity. The applicant reported precision data of the methods for the determination of the phytase activity in all three matrices that were obtained via interlaboratory studies performed by the German Agricultural Analytical and Research Institutes (VDLUFA,Germany).

For the determination of the phytase activity in the *feed additive* the method proposed by the applicant has a limit of detection (LOD) of 45 FTU/kg and a limit of quantification (LOQ) of 90 FTU/kg. The same precision data were reported for the liquid and the granulated product, which were 2.5 % for the relative standard deviation for repeatability (RSD_r) and 4.9 % for the relative standard deviation for reproducibility (RSD_R). Acceptable values for the accuracy were reported, since the relative recovery rate for the solid formulation varied between 98 and 102 % and for the liquid formulation the relative recovery rate was 94 %. The method is considered suitable for the intended purpose.

For the determination of the phytase activity in *mineral premixtures*, the reported values for the LOD and LOQ were 45 FTU/kg and 90 FTU/kg, respectively. The reported precision

data were 4.9% for the RSD_r and 8.4% for the RSD_R. Accuracy data were derived from the analysis of four samples, revealing a relative recovery rate ranging from 93 to 101 %. The method performance characteristics are considered acceptable and the method is therefore considered suitable for the intended purpose.

For the determination of the phytase activity in *feedingstuff*, the obtained LOD of 45 FTU/kg and LOQ of 90 FTU/kg are acceptable, considering the lowest target level of the enzyme activity in feedingstuffs for laying hens and turkeys for fattening, which is 250 FTU/kg. The reported precision was 6.7 % for RSD_r and 11.1 % for RSD_R. Accuracy data were derived from the analysis of four chicken feedingstuff samples, revealing a relative recovery rate ranging from 97 to 103 %. Furthermore, the method has been adopted as AOAC Official Method (2000.12) and has been fully ring trial validated obtaining values for the RSD_r ranging from 2.5 to 8.6% and values for the RSD_R ranging from 14.0 to 27.6%. Therefore, the method is considered suitable for the intended purpose.

Several other very similar analytical methods for the determination of phytase activity in *feedingstuffs* exist and have also been ring trial validated. These include a method developed by FEFANA (European Association of Feed Additive Manufacturers) which has been validated according to IUPAC guidelines. It is an *absolute* method in contrast to the *relative* method proposed in the dossier, because it quantifies the samples against a phosphate standard and not an enzyme standard. In addition, the validation showed that the FEFANA method can be applied to the analysis of samples regardless of the specific phytase product present in the feedingstuffs. The RSD_r varied between 3.1 and 13.0% and the RSD_R varied between 5.2 and 14.2%. Both method performance characteristics are considered acceptable. This method, which is currently under evaluation to become a standard of the European Committee for Standardisation (CEN) is therefore recommended by the CRL for official control purposes.

No further testing or validation are required.

KEYWORDS

Phytase, Natuphos®, feedingstuff additive, zootechnical additive

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1. BACKGROUND

The name of the additive is Natuphos®. The active agent is the enzyme 3-phytase (EC 3.1.3.8), E1600 produced by the genetically modified micro-organism *Aspergillus niger* CBS 101672. The additive is already authorised in EU for the animal categories chickens for fattening, laying hens, piglets, pigs for fattening, sows and turkeys for fattening. The current application (*cf.* EFSA-Q-2005-116) is for the use of two products which are Natuphos® 5000 G (granulate) and Natuphos® 5000 L (liquid), having a minimum activity of 5000 FTU/g and 5000 FTU/ml, respectively.

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 tasks of the Community Reference Laboratory concerning applications for authorisations of feedingstuff 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 suitability of the control methods submitted in connection with Natuphos®, *cf.* EFSA-Q-2005-116, was evaluated.

3. EVALUATION

The numbering system under this point refers to the report of the Scientific Committee on Animal Nutrition on the revision of the guidelines for the assessment of additives in animal nutrition, adopted on 22 October 1999 (Guidelines for the assessment of additives in feedingstuff Part II: Enzymes and Microorganisms).

Description of some of the methods listed under 2.5.1 of the Guidelines

Quantitative analysis of the active agent in the feed additive

For the determination of the enzyme activity in the feed additive the applicant proposes a photometric method which is based on the principle that phytase releases phosphate from the substrate phytate [1]. The amount of phosphate is measured with a spectrophotometer and the content of the enzyme in the samples is expressed in terms of its activity. By definition, 1 FTU is the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate at pH 5.5 and 37°C in one minute.

The sample (1.25-2.0 g) is extracted with acetate buffer (pH=5.5) containing $CaCl_2$. The suspension is diluted with acetate buffer. This extract containing the enzyme is incubated with sodium phytate for 60 minutes liberating inorganic phosphate from the substrate. The incubation is stopped by adding acid molybdate/vanadate reagent which at the same time produces a coloured complex with the phosphate produced. The absorbance of the yellow complex, which is a measure for the amount of phosphate, is measured at a wavelength of 415 nm. A standard curve is prepared by subjecting various amounts of phytase with a defined activity to the same procedure, measuring the corresponding amount of phosphate and plotting the measured absorbance against the respective activity of the standard solutions. Endogenous phospate is determined by analysing the target samples in the same way, but adding the stop solution prior to the addition of the substrate (“Blank samples”). The measured absorbances of the Blank samples are subtracted from the corresponding absorbances of the samples that underwent the whole analytical procedure and the obtained differences of the absorbance are used to calculate the phytase activity of the samples from the standard curve.

The limit of detection (LOD) is 45 FTU/kg and the limit of quantification (LOQ) is 90 FTU/kg. These limits are the same for all methods, regardless of whether the enzyme activity is measured in the feed additive, premixtures or feedingstuffs.

Identical precision data were reported for the liquid and the granulated product, which were 2.5 % for the relative standard deviation for repeatability (RSD_r) and 4.9 % for the relative standard deviation for reproducibility (RSD_R) obtained via an interlaboratory study. The validation procedure for the experiments has not been described in detail, but reference is made to the method collection of German Agricultural Analytical and Research Institutes (VDLUFA, Germany) [2]. The accuracy for the analysis of the solid formulation expressed in terms of the relative recovery rate varied from 98.% to 102 %, obtained on the determination of the phytase activity of four samples of Natuphos® 5000 G. The recovery values were obtained with addition of enzyme solutions after suspension of samples. A more rigorous recovery experiment would be performed by adding standard to the dry enzyme product. For Natuphos® 5000L, 94.5% recovery was obtained with a double determination of addition of enzyme after dilution. The method is considered fit for the intended purpose.

Description of qualitative and quantitative methods for routine control of the active agents in premixtures and feedingstuffs (cf. pt. 2.5.2 of the Guidelines)

For the determination of the phytase activity in *premixtures* the same method as described above is proposed, but slightly modified to make it applicable to the analysis of this type of matrix. In short, 2 g of the sample are added to 200 g of corn or tapioca meal and a suspension is prepared with an acetate buffer solution (pH 5.5). The mixture is stirred for another 50 min and the suspension is filtered. An aliquot of the diluted extract is subjected to the actual determination of the enzyme activity applying the same procedure as for the analysis of the feed additive. The recovery values obtained with addition of enzyme solutions after suspension of samples were 93-101% for four samples of mineral premixtures. A more rigorous recovery experiment would be performed by adding standard to the dry premixture.

The method has been validated by an interlaboratory study, obtaining for the RSD_r a value of 4.9 % and for the RSD_R a value of 8.4 %. The dossier did not contain details on the validation exercise but exclusively the reference [3]. The method is considered suitable for the indented purpose.

For the determination of the phytase activity in *feedingstuffs* 200 g of the sample are extracted with acetate buffer pH 5.50 containing $CaCl_2$ and analysed as described above. The reported values for the LOD were 45 FTU/kg and for the LOQ 90 FTU/kg. These values were obtained by analysing 10 blank feedingstuff samples without an incubation step and calculating the mean and the standard deviation of these responses. The LOD and LOQ were calculated by adding three times and ten times of the standard deviation to the mean response,

respectively. These obtained values for the LOD and LOQ were acceptable, considering the lowest level of use for the additives (250 FTU/kg in complete feedingstuff for laying hens and turkeys for fattening). The method has been validated by an interlaboratory study, obtaining for the RSD_r a value of 6.7 % and for the RSD_R a value of 11.1 %. The dossier did not contain details on the validation exercise, but exclusively the reference [3]. The recovery values obtained with addition of enzyme solutions after suspension of samples were 97.5-100% for four pig feedingstuff samples and 97.5 – 102 % for four chicken feedingstuff samples. A more rigorous recovery experiment would be performed by adding standard to the dry feedingstuff. Furthermore, the method is an AOAC Official Method (2000.12) and has been fully validated according to AOAC guidelines. RSD_r 2.5-8.6% and RSD_R 14.0-27.6% were obtained [4]. Therefore the method is considered fit for the intended purpose.

For the determination of phytase activity in *feedingstuff*, a recently issued CEN/TC 327 draft [5] of a similar method as the applicant's method is available which has been developed by the European Association of Feed Additive Manufacturers (FEFANA) and validated according to the IUPAC harmonised protocol. This method quantifies the enzyme activity against a phosphate standard. It is therefore an *absolute* method in contrast to the *relative* method proposed in the dossier, because it quantifies the samples against a phosphate standard and not an enzyme standard. In addition the validation showed that the FEFANA method can be applied to the analysis of samples regardless of the specific phytase product present in the feedingstuffs. The method has been validated with five feed samples containing all the phytase products currently commercially available ranging from 750 to 1500 FTU/kg. The values for the RSD_r varied between 3.1 and 13.0% and the values for the RSD_R varied between 5.2 and 14.2% [6]. The CRL recommends this method for *official control* purposes.

CHECK LIST FOR SUBMITTED METHODS

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned in Part I (1.- A. Premixtures) accompanied by information on:				
	- Sampling Method used				
	- Percentage Recovery	X			
	- Specificity				
	- Accuracy	X			
	- Precision	X			
	- Limits of detection	X			
	- Limits of quantification	X			
	- Validation procedure used	X			Without information on design of validation experiments, exclusively reference
1.2	Is/Are the method(s) mentioned in Part I (1.- A. Animal feedingstuff) accompanied by information on:				
	- Sampling Method used				
	- Percentage Recovery	X			
	- Specificity				
	- Accuracy	X			
	- Precision	X			
	- Limits of detection	X			
	- Limits of quantification	X			
	- Validation procedure used				Without information on design of validation experiments, exclusively reference

N/A: Not applicable

4. CONCLUSIONS AND RECOMMENDATIONS

The control methods for the determination of phytase activity in the feed additive, premixtures and feedingstuffs submitted by the applicant are based on well known and accepted principles.

The method for the determination of phytase activity in the feed additive is considered suitable for the intended purpose even if a detailed description of the validation procedure is not provided.

The method of analysis of phytase activity in premixtures and feedingstuffs has been validated and obtained acceptable performance characteristics. Therefore is considered suitable for the purpose.

However, for *official control* of phytase activity in feedingstuffs, the FEFANA method is recommended since it is an *absolute* method and the validation data showed its applicability regardless the specific phytase product present in the feedingstuffs.

No further testing or validation are needed.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

The applicant provided the Community Reference Laboratory with the required reference samples. The dossier has been made available to the CRL by EFSA. The documentation is constructed by 2 correspondences and 65 technical annexes. The latter describe the safety, genealogy, taxonomy and methods of analysis.

6. REFERENCES

[1] Engelen et al. Simple and rapid determination of phytase activity. *Journal of AOAC International* vol. 77, No. 3, pp. 760-764. 1994

[2] *Bestimmung der Phytaseaktivität in Enzymstandardmaterialien und Enzympräparaten* (Determination of the phytase activity in enzyme standard materials and enzyme preparations). Method book III of VDLUFA „The chemical analysis of feedingstuffs“; Method Number 27.1.1 ; 4-th Auxiliary supply 1997 ; VDLUFA ISBN 3-922712-66-7, in German

[3] *Bestimmung der Phytaseaktivität in Futtermitteln und Vormischungen (Determination of the phytase activity in feedstuffs and premixes)* Method book III of VDLUFA „The chemical analysis of feedingstuffs) Method Number 27.1.2 ; 4-th Auxiliary supply 1997; VDLUFA ISBN 3-922712-66-7, in German

[4] Engelen et al. Determination of Phytase Activity in Feed by a Colorimetric Enzymatic Method: Collaborative Interlaboratory Study. *Journal of AOAC International*, vol. 84, No. 3, pp. 629-633. 2001

[5] CEN-method draft: Animal feedingstuffs– Determination of phytase activity; Working document N 347 of CEN TC 327.

[6] 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.