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Community Reference Laboratory for Feed Additives

Feed Additives

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

Dossier related to:	**EFSA-Q-2006-025** **FAD-2005-0028**
Name of Additive:	**Quantum™ Phytase**
Active Substance(s):	**6-phytase (E.C. 3.1.3.26)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA)**
Report prepared by:	**Dalia Garalevičienė (CRL-FA)** **16/01/07**
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EXECUTIVE SUMMARY

In the current application authorisation is sought for Quantum™ Phytase under the category 'zootechnical additives', functional groups 4(a) 'digestibility enhancers' and 4(c) 'substances, which favourably affect the environment', according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Quantum™ Phytase as a digestibility enhancer and a substance, which favourably affects the environment, for chickens, ducks and turkeys for fattening, laying hens and weaned piglets. The additive is intended to be marketed as a powder (Quantum™ Phytase 2500 D) and as liquid formulation (Quantum™ Phytase 5000 L).

The active agent of Quantum™ Phytase is 6-phytase, produced by a strain of *Pichia pastoris* (DSM 15927). The enzymatic activity is expressed in phytase units (FTU). One FTU is the amount of enzyme which liberates one μmole of inorganic phosphate per minute from sodium phytate at pH 5.5 and 37°C. Quantum™ Phytase 2500 D and Quantum™ Phytase 5000 L have a target activity of 2500 FTU/g and 5000 FTU/ml of product. Quantum™ Phytase 2500 D is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas Quantum Phytase™ 5000 L is sprayed directly onto feed. Both formulations are used to obtain an enzyme activity level of 100 to 2700 FTU/kg in *feedingstuffs*.

For the determination of the activity of 6-phytase in the *feed additive,* the applicant proposes a method which measures the enzyme-catalysed formation of inorganic phosphate released from sodium phytate. The phosphate forms with molybdate and vanadate ions a coloured complex, which is measured on a spectrophotometer and quantified via a phosphate standard curve. The measurements are carried out at pH 5.5 and 37°C and therefore the activity is expressed in terms of FTU. Method performance characteristics include a relative standard deviation for reproducibility (RSD_R) of 3.4 % calculated from the results of two laboratories and recovery rates ranging from 80 to 101 %.

For the determination of the activity of 6-phytase in *premixtures,* the applicant proposes the same method as for the *feed additive,* but using a modified extraction buffer. The measurements are carried out at pH 5.5 and 37°C and the activity is expressed in terms of FTU/g. Method performance characteristics include a RSD_R of 5.5 % calculated from the results of two laboratories and recovery rates ranging from 80 to 94 %.

For the quantification of the activity of 6-phytase in *feedingstuffs,* the applicant proposes a different in-house developed method, performing the activity measurements at *pH 4.5* and *60°C* - and not at FTU conditions - , thereby introducing a new activity Unit: Quantum™ Phytase unit (QPU). One QPU is the amount of enzyme liberating one μmole of inorganic phosphate from sodium phytate per minute measured at 60°C and at a pH of 4.5. In the final

step of the analysis the measured QPUs are converted into FTUs by using an experimentally obtained conversion factor. However, the method protocol does not explicitly explain the determination of this conversion factor. The limit of detection of the method is 25 FTU/kg, the limit of quantification is 75 FTU/kg, the intermediate relative standard deviation for reproducibility (RSD_R) varies from 5.6 to 11.9 % and the recovery rate varies from 93 to 126 %. The obtained method performance characteristics are considered acceptable.

Since the CRL favours the use of inter-laboratory validated methods, the applicant applied, upon request of the CRL, a recently collaboratively validated method which has been developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) and which measures the enzyme activity at FTU conditions. This harmonised method is suitable for the determination of the activity of *various* phytase products in *feedingstuffs*. However, the experimental results revealed that the FTU values obtained with the FEFANA method were up to 15 % *lower* than the corresponding values of the applicant's method. Therefore the applicant proposed to use its in-house validated method for the determination of the Quantum™ Phytase activity in *feedingstuffs* and not the FEFANA method. The CRL agrees with this proposal but is concerned that the suggested approach of measuring the enzyme activity in *feedingstuffs* at QPU conditions (pH 4.5 and 60°C) followed by conversion from QPU units to FTU units, introduces additional uncertainty into the measurements. In addition the CRL considers that, for consistent analytical results, the enzyme activity in the *feed additive*, in *premixtures* and in *feedingstuffs* should be determined at the same conditions and expressed in the same units.

Therefore, the CRL recommends:

- to express the enzyme activity of 6-phytase, regardless of the matrix, in QPU;
- to *modify* the proposed register entry by expressing the target activity values in *feedingstuffs* as given in the "conditions of use" in terms of QPUs and not in terms of FTUs. In addition, the *definition* of the QPU needs to be included in the register entry instead of the definition of FTU;
- to employ the applicant's method in the frame of official controls for the determination of the enzyme activity of 6-phytase, in *feed additive, premixtures* and complete *feedingstuffs,* expressed in terms of QPUs, thereby excluding the employment of a conversion factor from QPU to FTU.

In the case that the "conditions of use" in the final register entry will be expressed in terms of FTUs and not – as proposed by the CRL – in terms of QPUs, the CRL recommends the FEFANA method to be used for official controls.

Further testing or validation is not considered necessary.

KEYWORDS

Quantum™ Phytase, 6-phytase, *Pichia pastoris*, digestibility enhancer

BACKGROUND

Quantum™ Phytase is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional groups 'digestibility enhancers' and 'substances, which favourably affect the environment', according to Annex I of Regulation (EC) No 1831/2003 [1]. It contains 6-phytase as the active agent [2], produced by a strain *Pichia pastoris* (DSM 15927) [3], which has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (D.S.M.Z.) in Braunschweig, Germany. The activity of 6-phytase is expressed as phytase units (FTU). According to the applicant, one FTU is the amount of enzyme which liberates one μmole of inorganic phosphate per minute from sodium phytate at pH 5.5 and 37°C. The additive is marketed in two forms [4]:

- Quantum™ Phytase 2500 D, which is a solid formulation with a target phytase activity of 2500 FTU/g;
- Quantum™ Phytase 5000 L, which is a liquid formulation with a target phytase activity of 5000 FTU/ml.

According to EFSA-Q-2006-025, Quantum™ Phytase is intended to be mixed into premixtures and/or complete feedingstuffs to obtain enzyme activity levels of 500 to 2500 FTU/kg in complete feedingstuffs for chickens for fattening, 125 to 2000 FTU/kg in complete feedingstuffs for laying hens, 250 to 2000 FTU/kg in complete feedingstuffs for fattening ducks, 125 to 2700 FTU/kg in complete feedingstuffs for turkeys for fattening and 100 to 2500 FTU/kg in complete feedingstuffs for weaned piglets [5,6].

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 Quantum™ Phytase (*cf.* EFSA-Q-2006-025) and their suitability to be used for official controls in the frame of authorisation, were evaluated.

EVALUATION

Identification/Characterisation of the feed additive

Quantitative and quantitative composition of impurities in the additive

For the determination of arsenic, heavy metals (cadmium, mercury and lead), microbiological agents and mycotoxins, the applicant proposes well known methods published and approved by Joint FAO/WHO Expert Committee on Food Additives [7], applied by external accredited laboratories [8-10] and therefore considered suitable for intended purposes. 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 and premixtures

For the determination of the activity of 6-phytase in the *feed additive* and *premixtures*, the applicant proposes a *modified* version of a colorimetric method described by Engelen et al. [11]. The method is based on the fact that 6-phytase catalyses the hydrolysis of sodium phytate to yield an inorganic phosphate. The inorganic phosphate released is complexed with molybdate and vanadate ions and the coloured product is measured on a spectrophotometer at a wavelength of 415 nm. Main deviation from the published method, as proposed by the applicant, refers to the fact that calibration is done against the phosphate standard curve instead of a standardised commercial phytase sample.

When analysing the *feed additive*, five replicates of the analysis are performed for each batch of phytase. In brief, 1.0 g of either dry or liquid additive are extracted in sodium acetate buffer and incubated with sodium phytate for 60 minutes (37^{o}C, pH 5.5). Following, enzymatic reaction is stopped and absorbance of a coloured complex is measured at 415 nm. The activity of phytase, based on the concentration of released phosphate, is calculated using a phosphate standard calibration curve. The transferability of the method has been checked between two laboratories on both formulations of Quantum™ Phytase, obtaining a relative standard deviation for reproducibility (RSD_R) of 3.4 % and recovery rates from 80 to 101 % [12].

When analysing *premixtures* the same method as for *feed additive* is proposed by the applicant, just a modified buffer, supplemented by ethylenediaminetetraacetic acid (EDTA) disodium salt dehydrate, bovine serum albumin and dibasic sodium phosphate, is used for the extraction of dry Quantum™ Phytase from *premixtures*. The transferability of the method has been checked between two laboratories, obtaining a RSD_R of 5.5 % and recovery rates from 80 to 94 % [13].

Besides the applicant's proposed method, which follows well known principles, there are published methods for the determination of the activity of phytase in the *feed additive* and in *premixtures*. These methods have been validated in interlaboratory studies. The Association of German Agricultural Analytical and Research Institutes (VDLUFA) proposed a colorimetric method, applicable for determination of phytase's activity in *feed additive* (RSD_R of 4.9 %) [14] and a similar method, applicable for the determination of phytase's activity in *feedingstuffs* (RSD_R of about 12 %) and *premixtures* (RSD_R of 8.4%) [15]. However, as Quantum™ Phytase has not been included in the validation studies for VDLUFA methods, the suitability of the methods for the analysis of this particular feed additive cannot be evaluated.

Description of the analytical methods for the determination of the active agent in the feedingstuffs

For the determination of the enzyme activity in *feedingstuffs* the applicant proposes an *in-house* developed method that also measures the enzyme-catalysed formation of inorganic phosphate released from sodium phytate. However, the enzymatic reaction is carried out at pH 4.5 and at 60°C and not at FTU conditions (pH 5.5 and 37°C), since the specific 6-phytase as present in Quantum™ Phytase acts optimally at these modified conditions, reflecting its specific thermostability. According to the applicant, the modified conditions improve the specificity of the method, since phytase from other sources present in the sample is not active at these conditions. Thermostable phytase such as Quantum™ Phytase can be added to the feed *before* pelleting, which might render the extraction of the enzyme more difficult due to starch gelatinisation caused by the high feed-processing temperatures [17]. Therefore sodium borate extraction buffer is utilised to ensure efficient recovery of the enzyme from a feed, which has been processed at higher temperatures.

Since the measurements are carried out at pH 4.5 and 60°C, the unit for phytase activity is defined as Quantum™ Phytase unit (QPU) which is the amount of enzyme liberating one µmole of inorganic phosphate from sodium phytate per minute at 60°C and pH 4.5. Enzymatic activity is thus expressed in terms of QPU/kg *feedingstuffs*. For harmonisation of units, the applicant proposes to convert measured enzymatic activity from QPU/kg to FTU/kg, using fortified feed samples and calculating a matrix-dependent ratio factor. On average, 5.66-fold more phosphate is liberated at 60°C when compared to 37°C, and the conversion factor to be applied varies from 4.8 to 6.5 [16][19].

4.5 g of a ground feed sample are extracted with sodium borate buffer for 30 minutes. The supernatant containing the enzyme is incubated with the sodium phytate substrate for 60 min. at 60°C. Afterwards ammonium molybdate-vanadate in diluted nitric acid is added and the

absorbance is measured on a spectrophotometer at 415 nm [18]. Finally the measured QPUs are transferred into FTUs, but the method protocol does not explicitly explain the determination of this conversion factor to be applied in the respective measurements.

The protocol of the method and the results of the in-house validation study have been submitted by the applicant [18][19]. The method performance characteristics include a limit of detection (LOD) of 25 FTU/kg, a limit of quantification (LOQ) of 75 FTU/kg, a relative standard deviation for repeatability (RSD_r) between 6.6 and 12.4 %, intermediate RSD_R between 5.6 and 11.9 % and recovery rates ranging from 93 to 126 % [19]. The obtained method performance characteristics are considered acceptable.

In general the CRL favours the use of inter-laboratory validated methods. There are several published and ring-trial validated methods for the determination of the activity of phytase in the feedingstuffs, following the same principle as the applicant's method. It must be, however, noted that these methods measure enzymatic activity at pH 5.5 and temperature 37°C, thus at the conditions and definition of FTUs.

The Association of German Agricultural Analytical and Research Institutes (VDLUFA) proposed a colorimetric method [15] demonstrating a RSD_R of about 12 % when analysing phytase activity in feedingstuffs. Engelen et al. [11] reported RSD_R values between 14.0 and 27.6% for feedingstuffs. On behalf of the European Association of Feed Additive Manufacturers (FEFANA), a harmonised method has been developed [20] and collaboratively validated [21]. This method is applicable for the measurement of phytase activity in feedingstuffs, containing any of phytase products (E 1600, E 1614, E 1640) currently authorised within the EU. The obtained values for the RSD_R, ranging from 5 to 14%, are considered acceptable for the intended use. This method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN).

Therefore the CRL asked the applicant to compare the proposed in-house validated method with the FEFANA harmonised method [20] for feedingstuffs. The applicant provided results, showing a comparison between its in-house method and the harmonised method, applied on mash and pelleted feed [17]. With the harmonised method, phytase's activity was on average 5% less in pellets produced at 70°C and 15% less in pellets produced at 85°C, compared to the applicant's method. According to the applicant, there were two main reasons for the sub-optimal results, namely (1) the insufficient extraction of 6-phytase from pelleted feed using *water* instead of *borate* buffer, as proposed in applicant's method and (2) the fact that the analytical conditions of the harmonised method (pH 5.5 and 37°C) are different compared to the optimal activity conditions of Quantum™ Phytase (pH 4.5 and 60°C) [17]. As a consequence, the applicant recommended using its in-house validated method for the determination of active agent in the feedingstuffs and not the FEFANA method.

CONCLUSIONS AND RECOMMENDATIONS

Based on the insufficient accuracy of the results when applying the harmonised FEFANA method at FTU conditions (pH 5.5 and 37°C) to the determination of Quantum™ Phytase in feedingstuffs, the applicant recommended using its in-house validated method for the determination of the enzyme activity. The CRL agrees with this proposal but is concerned that the suggested approach of measuring the enzyme activity in *feedingstuffs* at QPU conditions (pH 4.5 and 60°C), followed by conversion from QPU units to FTU units, introduces additional uncertainty into the measurements. In addition the CRL considers that, for consistent analytical results, the enzyme activity in the *feed additive*, in *premixtures* and in *feedingstuffs* should be determined at the same conditions and expressed in the same units.

Therefore, the CRL recommends:

- to express the enzyme activity of 6-phytase, regardless of the matrix, in QPU;
- to *modify* the proposed register entry by expressing the target activity values in *feedingstuffs* as given in the "conditions of use" in terms of QPUs and not in terms of FTUs. In addition, the *definition* of the QPU needs to be included in the register entry instead of the definition of FTU;
- to employ the applicant's method in the frame of official control for the determination of the enzyme activity of 6-phytase, in *feed additive, premixtures* and complete *feedingstuffs,* expressed in terms of QPUs, thereby excluding the employment of a conversion factor from QPU to FTU;

In the case that the "conditions of use" in the final register entry will be expressed in terms of FTUs and not – as proposed by the CRL – in terms of QPUs, the CRL recommends the FEFANA method to be used for official controls.

Further testing or validation is not considered necessary.

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

Depending on the phytase units included in the register entry the CRL recommends the following method descriptions.

If "conditions of use" are expressed in terms of QPUs:

- Colorimetric method based on reaction of vanadomolybdate on inorganic phosphate produced by action of 6-phytase on a phytate-containing substrate (sodium phytate) at pH 4.5 and 60°C. One Quantum Phytase unit (QPU) is defined as the amount of enzyme that liberates one μmole of inorganic phosphate from sodium phytate per minute at pH 4.5 and 60°C.

If "conditions of use" are expressed in terms of FTUs:

- Colorimetric method based on reaction of vanadomolybdate on inorganic phosphate produced by action of 6-phytase on a phytate-containing substrate (sodium phytate) at pH 5.5 and 37°C. One phytase unit (FTU) is defined as the amount of enzyme that liberates one μmole of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37°C.

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of Quantum™ Phytase have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

REFERENCES

[1] Reference SANCO/D/2 Forw. Appl. 1831/001-2006.

[2] Main dossier, Section II, Subject 2, Item 2.1.

[3] Main dossier, Section II, Subject 2, Item 2.4.

[4] Main dossier, Section II, Subject 1, Item 1.1.

[5] Main dossier, Section II, Subject 2. Items 4.1-4.2.

[6] Annex III, Proposal of Register entry.

[7] Main dossier, Section II, Appendix II-16.

[8] Main dossier, Section II, Appendix II-2.

[9] Main dossier, Section II, Appendix II-4.

[10] Main dossier, Section II, Appendix II-5.

[11] Engelen AJ, van der Heeft FC, Randsdorp PH, Somers WA, Schaefer J, van der Vat BJ. Determination of phytase activity in feed by a colorimetric enzymatic method: collaborative interlaboratory study. J AOAC Int. 2001, 84, 629-33.

[12] Main dossier, Section II, Appendices II-33 and II-34.

[13] Main dossier, Section II, Appendix II-36.

[14] *Bestimmung der Phytaseaktivität in Enzymstandardmaterialien und Enzym- präparaten (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; 4th Auxiliary supply 1997; VDLUFA ISBN 3-922712-66-7, in German.

[15] *Bestimmung der Phytaseaktivität in Futtermittelen und Vormishungen (Determination of the phytase activity in feedingstuffs and premixtures)* Method book III of VDLUFA "The chemical analysis of feedingstuffs"; Method Number 27.1.2; 4th Auxiliary supply 1997; VDLUFA ISBN 3-922712-66-7, in German.

[16] Main dossier, Section II, Appendix II-35.

[17] Supplementary information on comparison of FEFANA's and applicant's method, requested by the CRL-FA (September 2006).

[18] Supplementary information, Annex 3, requested by the CRL-FA (December 2006).

[19] Supplementary information, Annex 5, requested by the CRL-FA (December 2006).

[20] CEN-method draft: Animal feedingstuffs – Determination of phytase activity; Working document N 410 of CEN TC 327.

[21] Gisele Gizzi and Christoph von Holst (2005). Validation study on a new method for a determination of phytase activity in feed: Results from an interlaboratory study conducted according to the IUPAC harmonised protocol. European Commission, DG JRC, IRMM, Geel.

RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Community Reference Laboratory for Feed Additives, IRMM, Geel, Belgium.

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