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Community Reference Laboratory – Feed Additives Authorisation

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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-2005-281**
Name of Additive:	**Rovabio™ PHY AP/LC**
Active Agent(s):	**3-phytase (E.C. 3.1.3.8)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additive Authorisation, IRMM, Geel, Belgium (CRL-FAA)**
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Date:	**11/08/2006**
Report approved by:	**Christoph von Holst (CRL-FAA)**
Date:	**16/08/2006**

EXECUTIVE SUMMARY

In the current application authorisation is sought for ROVABIO™ PHY under the category zootechnical additives, digestibility enhancers, group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use ROVABIO™ PHY for chickens for fattening, laying hens, weaned piglets and fattening pigs, according to EFSA-Q-2005-281. ROVABIO™ PHY is an enzyme preparation, available in powder form (ROVABIO™ PHY AP) and in liquid form (ROVABIO™ PHY LC). The enzyme is produced by *Penicillium funiculosum* 4.05 b (CBS 111443). The feed additive has a target activity of minimum 2500 RPU/g for ROVABIO™ PHY AP and of minimum 1000 RPU/ml for ROVABIO™ PHY LC, where 1 RPU is the amount of enzyme that releases 1 μM inorganic ortho-phosphate per minute from sodium phytate as substrate at pH 5.5 and 37° C. It is intended to be mixed into compound feedingstuffs to a level of 250 RPU/kg.

For the determination of the enzyme activity of 3-phytase, in the *feed additive,* ROVABIO™ PHY, a colorimetric method is proposed by the applicant. The principle of the method is that after diluting the feed additive and performing an enzyme kinetics test in the presence of sodium phytate, the amount of phosphate released is measured via the formation of a phosphomolybdate complex by reduction of iron (II). The amount of phosphate released is read off a calibration curve constructed using inorganic phosphate. The method is considered suitable for official control purposes.

For the determination of the enzyme activity in *premixtures* and in *feedingstuffs* the applicant proposes an adapted version of the colorimetric method applied for feed additives. In the adapted version an initial step of extraction of the active agent from the premixture or feedingstuff is included.

When tested on *premixtures* for chicken and piglet feed the method's performance characteristics include relative repeatability standard deviation (RSD_r) values of 1.1-4.2 %; and recovery rates between 34 and 67 %.
When tested on *feedingstuffs* (chicken and piglet feed) the method's performance characteristics include RSD_r values of 1.0-7.6 %; and recovery rates between 100 and 131 %.

The applicant's method follows well known principles for the determination of phytase activity in various matrices, and transferability of the method has been verified by testing the method with a second laboratory. It should be noted though that other analytical methods for the determination of phytase activity in premixtures and feedingstuffs exist, which have been validated in inter-laboratory studies. These include a method proposed by the Association of German Agricultural Analytical and Research Institutes (*Bestimmung der Phytaseaktivität in Futtermitteln und Vormischungen (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] which resulted in a relative between-laboratory standard deviation for reproducibility (RSD_R) of about 12 % for feedingstuffs and 8.4 % for a mineral premixture. Another method [Engelen et al. (2001) J. AOAC Int., 84, 629-633] obtained RSD_R values ranging from 14.0 to 27.6 % for feedingstuffs. However, data regarding ROVABIO^{TM} pertaining to these two methods was not submitted by the applicant.

The European Association of Feed Additive Manufacturers (FEFANA) developed a method, suitable for the analysis of all phytase products currently authorised within the EU, which follows the same principle as the applicant's method, in order to allow for the measurement of phytase activity in feedingstuffs, regardless of the specific phytase product used. The FEFANA method has been validated in an inter-laboratory study which was performed on feedingstuffs containing different 3- or 6- phytase products. 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). For these reasons, the CRL asked the applicant to compare the proposed in-house method with the inter-laboratory validated FEFANA method for chicken and piglet feed. The applicant provided results, showing a comparison between the proposed in-house method and a, to some extent modified, FEFANA method (different sample weight, different extraction solutions, buffer used during detection stage instead of water). With the modified FEFANA method, performance characteristics comparable to the method proposed by the applicant were obtained. While it is likely that the FEFANA method would be suitable for official control purposes for determining the enzyme activity of ROVABIO^{TM} PHY in feedingstuffs, the data provided by the applicant concerns a *modified* version of the FEFANA method. For this reason, the CRL has no evidence of the suitability of the FEFANA method

for official control purposes for this particular feed additive. Taking into account these facts, for determination of the enzyme activity of ROVABIO™ PHY in feedingstuffs, the CRL recommends the applicant's own method for official control purposes.

Regarding identification and characterisation of the additive, methods pertaining to the enumeration and detection of micro-organisms, identification of the strain including DNA profile for *Penicillium funiculosum* 4.05 b (CBS 111443), and determination of mycotoxins are provided.

No further testing or validation is required.

KEYWORDS

ROVABIO™, phytase, enzyme activity, digestibility enhancer

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1. BACKGROUND

ROVABIO™ PHY is a feed additive for which authorisation is sought under the category zootechnical additives, digestibility enhancers, group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use ROVABIO™ PHY for chickens for fattening, laying hens, weaned piglets and fattening pigs. The feed additive contains 3-phytase as the active ingredient. The intended use (cf. EFSA-Q-2005-281) of the current application is to enhance the digestibility in the above mentioned animal species, by mixing the feed additive into compound feedingstuffs to a level of 250 RPU/kg.

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 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 suitability of the methods of analysis and validation studies submitted in connection with ROVABIO™ PHY, *cf.* EFSA-Q-2005-281, was evaluated.

3. EVALUATION

The numbering system under this point refers to that of Guidelines for the assessment of additives in feedingstuffs, PART II: Enzymes and Micro-organisms (the Guidelines).

Description of the methods used for the determination of the criteria listed (cf. pt. 2.5.1 of the Guidelines)

Quantitative analysis of active substance in the feed additive

For the determination of the enzyme activity of 3-phytase in the feed additive, a colorimetric method is proposed by the applicant. The principle of the method is that after appropriate dilution of the phytase and after performing an enzyme kinetics test in the presence of sodium phytate, the amount of phosphate released is measured via the formation of a phosphomolybdate complex by reduction of iron (II) measured at 700 nm (when using a spectrophotometer) or at 690 nm (when using a microplate reader). The amount of phosphate released is read off a calibration curve constructed using inorganic phosphate. Validation studies for the method were carried out individually for both ROVABIO^{TM} PHY AP and ROVABIO^{TM} PHY LC. They showed the following performance characteristics: RSD_r values of 1.6-5.3 %; linearity ($r^2 \geq 0.999$); recovery rates between 97 and 101 %, defined as "measured relative to expected" enzyme activity level; and a specificity of 100%. For specificity, this was determined by confirming the lack of enzyme activity when measuring three blank samples. The applicant's method follows well known principles for the determination of phytase activity in various matrices and is considered acceptable for official control purposes. It should be noted that a ring trial validated method exists, which was published by the Association of German Agricultural Analytical and Research Institutes (VDLUFA) [VDLUFA METHODENBUCH III, 4. Erg. 1997, 27.1.1] However, no data was submitted on ROVABIO^{TM} for this method.

Identification/Characterisation of the feed additive

Methods pertaining to the enumeration and detection of micro-organisms; identification of the strain including DNA profile for *Penicillium funiculosum* 4.05 b (CBS 111443) and determination of mycotoxins were provided.

The proposed methods are commonly used and are considered acceptable.

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

For the determination of the enzyme activity in premixtures and in feedingstuffs the applicant proposes an adapted version of the colorimetric method applied for feed additives. In the adapted version an initial step of extraction of the active agent from the premixture or feedingstuff is included. Specifically, 5-10 g of sample is stirred with 50 ml sodium acetate buffer for 30 minutes at about 4° C. 10 ml of the extract is transferred into centrifugal tubes for centrifugation at 5000 rpm for 10 minutes at 4° C. 0.5 ml of supernatant is transferred to test tubes, which are equilibrated for 5 minutes in a 37 ° C water-bath. Defined quantities of sodium phytate and trichloroacetic acid solutions, the latter being a stopping agent, are added at specified times. Centrifugation at 5000 rpm for 10 minutes at 4 ° C follows. 4 ml from each test tube is transferred to another test tube. 4 ml of colour reagent is added. The solution is allowed to react for 30 minutes, after which optical density is measured at 700 nm. Validation studies for the method were carried out individually for both ROVABIO^{TM} PHY AP and in ROVABIO^{TM} PHY LC and showed the following performance characteristics: When tested on premixtures for chicken and piglet feed the method's performance characteristics include RSD_r values of 1.1-4.2 %; linearity in the 75-125% level interval (r^2 = 0.976); recovery rates of 34 % (chicken premixture) and 67 % (piglet premixture); and a specificity of 100%. Regarding the low recovery rates, in the CRL's opinion, to reduce the effect of possibly interfering factors originating from the premixtures, it might be considered to perform the measurements only *after* dilution of the premixture into feedingstuff (see recovery rates when measuring in feedingstuffs).

When tested on feedingstuffs (chicken and piglet feed) the method's performance characteristics include RSD_r values of 1.0-7.6 %; linearity in the 50-150% level interval (r^2 > 0.990); recovery rates between 100 and 131 %; and a specificity of 100%.
Although the applicant's methods follow well known principles for the determination of phytase activity in various matrices, several other analytical methods for the determination of phytase activity in premixtures and feedingstuffs exist, which have been validated in

inter-laboratory studies. These include a method proposed by the Association of German Agricultural Analytical and Research Institutes (*Bestimmung der Phytaseaktivität in Futtermitteln und Vormischungen (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] which resulted in a relative between-laboratory standard deviation for reproducibility (RSD_R) of about 12 % for feedingstuffs and 8.4 % for a mineral premixture. Another method [Engelen et al. (2001) J. AOAC Int., 84, 629-633] obtained RSD_R values ranging from 14.0 to 27.6 % for feedingstuffs. However, data regarding ROVABIO^{TM} pertaining to these two methods was not submitted by the applicant. The European Association of Feed Additive Manufacturers (FEFANA) developed a method, suitable for the analysis of all phytase products currently authorised within the EU, which follows the same principle as the applicant's method, in order to allow for the measurement of phytase activity in feedingstuffs, regardless of the specific phytase product used. The FEFANA method has been validated in an inter-laboratory study which was performed on feedingstuffs containing different 3- or 6- phytase products. 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). For these reasons, the CRL asked the applicant to compare the proposed in-house method with the inter-laboratory validated FEFANA method for chicken and piglet feed. The applicant provided results, showing a comparison between the proposed in-house method and a, to some extent modified, FEFANA method (different sample weight, different extraction solutions, buffer used during detection stage instead of water). With the modified FEFANA method, performance characteristics comparable to the method proposed by the applicant were obtained (RSD_r < 5.0% for both methods; linearity for both methods; recovery rates of 77-96 % for the modified FEFANA method and 100-131% for applicant's own method).

While it is likely that the FEFANA method would be suitable for official control purposes for determining the enzyme activity of ROVABIO^{TM} PHY in feedingstuffs, the data provided by the applicant concerns a *modified* version of the FEFANA method. For this reason, the CRL has no evidence of the suitability of the FEFANA method for official control purposes for this particular feed additive. Taking into account these facts, for

determination of the enzyme activity of ROVABIO^{TM} PHY in feedingstuffs, the CRL recommends the applicant's own method for official control purposes.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant's method follows well known principles for the determination of phytase activity in various matrices, and transferability was verified by testing of the method by another laboratory. Several other analytical methods for the determination of phytase activity in premixtures and feedingstuffs exist, which have been validated in inter-laboratory studies, and in general the CRL favours the use of such methods. For these reasons, the CRL asked the applicant to compare the proposed in-house method with the inter-laboratory validated FEFANA method for chicken and piglet feed. The applicant provided results, showing a comparison between the proposed in-house method and a, to some extent modified, FEFANA method. While it is likely that the FEFANA method would be suitable for official control purposes for determining the enzyme activity of ROVABIO^{TM} PHY in feedingstuffs, the data provided by the applicant concerns a *modified* version of FEFANA's method. For this reason, the CRL has no evidence of the suitability of the FEFANA method for official control purposes for this particular feed additive. Taking into account these facts, for determination of the enzyme activity of ROVABIO^{TM} PHY in feedingstuffs, the CRL recommends the applicant's own method for official control purposes. The CRL also recommends that work is carried out to demonstrate comparability between the applicant's method and the FEFANA method.

No further testing or validation is required.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

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

6. REFERENCES

[1] *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

[2] Engelen et al. (2001) J. AOAC Int., 84, 629-633

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

[5] 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

7. RAPPORTEUR LABORATORY

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