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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-2007-020** **FAD-2006-0039**
Product name:	**Avizyme 1505**
Active Substances:	**Endo-1,4-β-xylanase (EC 3.2.1.8)** **α-amylase (E.C. 3.2.1.1)** **Subtilisin (E.C. 3.4.21.62)**
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Date:	**09/10/2007**
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Date:	**10/10/2007**

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Avizyme 1505* under the category 'zootechnical additives', group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Avizyme 1505* as a digestibility enhancer for chickens for fattening and ducks for fattening. The product is intended to be marketed as a granular powder formulation.

The active agents of Avizyme 1505 are 1) endo-1,4-β-xylanase, produced by a strain of *Trichoderma reesei* (ATCC PTA 5588), 2) α-amylase, produced by a strain of *Bacillus amyloliquefaciens* (ATCC 3978) and 3) subtilisin, produced by a strain of *Bacillus subtilis* (ATCC 2107). Enzymatic activity of the active agents is expressed in units (U):

- One U of endo-1,4-β-xylanase is the amount of enzyme that liberates 0.5 µmol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50^{o}C;
- One U of α-amylase is the amount of enzyme that liberates 1 µmol of glucosidic linkages per minute from a water insoluble cross-linked starch polymer substrate at pH 6.5 and 37^{o}C;
- One U of subtilisin is the amount of enzyme that liberates 1 µmol of phenolic compound (tyrosine equivalents) per minute from a casein substrate at pH 7.5 and 40^{o}C.

The product has a target activity of 1500 U endo-1,4-β-xylanase/g, 2000 U α-amylase/g and 20000 U subtilisin/g. *Avizyme 1505* is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of 75 to 300 U endo-1,4-β-xylanase/kg, 100 to 400 U α-amylase/kg and 1000 to 4000 U subtilisin/kg in *feedingstuffs.*

In general, the methods proposed for the determination of the activity of the active agents in different matrices are based on quantification of dyed compounds produced by enzymatic action of commercially available substrates. Enzymatic activity of the samples is calculated using reference enzyme standards, available from the applicant upon request, of which the activity is obtained applying the conditions described by the definitions of units. When analysing *feedingstuffs,* calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzymes. In the case that identical blank feed samples are *not* available, a standard addition technique is employed. The applicant introduced some adaptations to the protocols provided by the suppliers of substrates. All modified methods have been single-laboratory validated and showed acceptable performance characteristics such as limit of detection, limit of quantification and relative standard deviation for repeatability.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a method based on the quantification (λ = 590 nm) of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on cross-linked wheat xylan substrates. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 5.3 and 50°C on a cross-linked oat spelt xylan. Analyses are carried out at pH 4.0 and 40°C (*feed additive*), at pH 5.3 and 40°C (*premixtures*) and at pH 4.2 and 50°C (*feedingstuffs*).

For the determination of the activity of α-amylase in the *feed additive,* the applicant proposes a method based on the quantification (λ = 405 nm) of free *p*-nitrophenol produced by the action of α-amylase on blocked *p*-nitrophenyl maltoheptaoside at pH 5.6 and 37°C. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 6.5 and 37°C. For the analysis of the activity of α-amylase in *premixtures* and *feedingstuffs,* quantification (λ = 620 nm) of dyed oligomers produced by the action of α-amylase on azurine-crosslinked starch at pH 6.4 and 37°C is proposed.

For the determination of the activity of subtilisin in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes a method based on the quantification (λ = 590 nm) of *dyed oligomers* produced by the action of subtilisin on azurine-cross linked casein. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured by quantification of *phenolic compounds* released from casein at pH 7.5 and 40°C. Analyses are carried out at pH 10 and 50°C (*feed additive* and *feedingstuffs*) and at pH 8.0 and 40°C (*premixtures*).

Though the methods proposed by the applicant are based on well known principles and show acceptable performance characteristics, the CRL is concerned that the suggested approach of measuring the enzymatic activity at *different* conditions in various matrices compared to the conditions described by the definitions of units and to the conditions of the determination of the activity of reference enzymes, introduces additional uncertainty into the measurements. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions described by the definitions of units;
- that the minimum activity of endo-1,4-β-xylanase, specified in the register entry (75 U/kg) is replaced by the limit of quantification of the method, which is 500 U/kg;
- that the minimum activity of α-amylase, specified in the register entry (100 U/kg) is replaced by the limit of quantification of the method, which is 160 U/kg;

- and that the minimum activity of subtilisin, specified in the register entry (1000 U/kg) is replaced by the limit of quantification of the method, which is 2000 U/kg.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as described by the definitions of units, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

KEYWORDS

Avizyme 1505, endo-1,4 β-xylanase, α-amylase, subtilisin, *Trichoderma reesei, Bacillus amyloliquefaciens, Bacillus subtillis*, digestibility enhancer, chickens for fattening, fattening ducks

1. BACKGROUND

Avizyme 1505 is a product for which authorisation as feed additive is sought under the category 'zootechnical additives', functional groups 'digestibility enhancers', according to Annex I of Regulation (EC) No 1831/2003 [1]. It contains 1) endo-1,4-β-xylanase (E.C. 3.2.1.8), produced by a strain *Trichoderma reesei* (ATCC PTA 5588), 2) α-amylase (E.C. 3.2.1.1), produced by a strain of *Bacillus amyloliquefaciens* (ATCC 3978) and 3) subtilisin (E.C. 3.4.21.62), produced by a strain of *Bacillus subtilis* (ATCC 2107) as the active agents [2]. The producer strains have been deposited at the American Type Culture Collection (ATCC) in Manassas, VA, USA [3].

Enzymatic activity of the active agents is expressed in units (U) [4]:

- One U of endo-1,4-β-xylanase is the amount of enzyme that liberates 0.5 µmol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50^{o}C;
- One U of α-amylase is the amount of enzyme that liberates 1 µmol of glucosidic linkages per minute from a water insoluble cross-linked starch polymer substrate at pH 6.5 and 37^{o}C;
- One U of subtilisin is the amount of enzyme that liberates 1 µmol of phenolic compound (tyrosine equivalents) per minute from a casein substrate at pH 7.5 and 40^{o}C.

The product has a target activity of 1500 U endo-1,4-β-xylanase/g, 2000 U α-amylase/g and 20000 U subtilisin/g. *Avizyme 1505* is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of 75 to 300 U endo-1,4-β-xylanase/kg, 100 to 400 α-amylase/kg and 1000 to 4000 U subtilisin/kg in *feedingstuffs* [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 *Avizyme 1505*, *cf.* EFSA-Q-2007-020, 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 quantitative composition of impurities in the additive

For the determination of arsenic and heavy metals (cadmium, mercury and lead), microbiological agents and mycotoxins, the applicant proposes well known methods published and approved by national standard offices [5] and therefore considered suitable for intended purposes. For official controls, various internationally accepted 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 agents in the feed additive, premixtures and feedingstuffs

In general, the methods proposed for the determination of the activity of the active agents in different matrices are based on quantification of dyed compounds produced by enzymatic action of commercially available substrates. Enzymatic activity of the samples is calculated using reference enzyme standards, available from the applicant upon request, of which the activity is obtained applying the conditions described by the definitions of units [4]. When analysing *feedingstuffs,* calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzymes. In the case that identical blank feed samples are *not* available, a standard addition technique is employed. The applicant introduced some adaptations to the protocols provided by the suppliers of substrates. All modified methods have been single-laboratory validated [6, 7, 8, 9]

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a method based on the quantification (λ = 590 nm) of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on cross-linked wheat xylan substrates. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 5.3 and 50^{o}C on a cross-linked oat spelt xylan. Analyses are carried out at pH 4.0 and 40^{o}C (*feed additive*), at pH 5.3 and 40^{o}C (*premixtures*) and at pH 4.2 and 50^{o}C (*feedingstuffs*) [6, 7, 8]. For the determination of the activity in *Avizyme 1505*, the method performance characteristics include a limit of detection (LOD) of 1.2 U/g, limit of quantification (LOQ) of 1.5 U/g products and a relative standard deviation for repeatability

(RSD$_r$) of 4.4% [6]. For *premixtures*, a LOD is 13.0 U/g, a LOQ is 19.3 U/g, an RSD$_r$ is 3.5% and recovery rate is 96.4% [9]. For *feedingstuffs*, a LOD of 285 U/kg, a LOQ of 530 U/kg, an RSD$_r$ of 7.5% and a recovery rate of 97% were obtained [9].

For the determination of the activity of α-amylase in the *feed additive,* the applicant proposes a method based on the quantification (λ = 405 nm) of free *p*-nitrophenol produced by the action of α-amylase on blocked *p*-nitrophenyl maltoheptaoside at pH 5.6 and 37°C [6]. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 6.5 and 37°C. For the analysis of the activity of α-amylase in *premixtures* and *feedingstuffs,* quantification (λ = 620 nm) of dyed oligomers produced by the action of α-amylase on azurine-crosslinked starch at pH 6.4 and 37°C is proposed [7, 8, 13]. For the determination of the activity in *Avizyme 1505*, method performance characteristics include a LOD of 0.04 U/ml, a LOQ of 0.13 U/ml and an RSD$_r$ of 4.3%. For *premixtures*, an RSD$_r$ is 5.1% and recovery rate is 107%. For *feedingstuffs*, a LOD of 85 U/kg, a LOQ of 162 U/kg, an RSD$_r$ of 5.6% and a recovery rate of 104% were obtained [9].

For the determination of the activity of subtilisin in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes a method based on the quantification (λ = 590 nm) of dyed oligomers produced by the action of subtilisin on azurine-cross linked casein [6, 7, 8]. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured by quantification of *phenolic compounds* released from casein at pH 7.5 and 40°C. Analyses are carried out at pH 10 and 50°C (*feed additive* and *feedingstuffs*) and at pH 8.0 and 40°C (*premixtures*). For the *Avizyme 1505*, method performance characteristics include a LOD of 1.0 U/g, a LOQ of 1.6 U/g and an RSD$_r$ of 5.2%. For *premixtures*, a LOD is 0.5 U/g, a LOQ is 1.8 U/g, an RSD$_r$ is 5.1 % and recovery rate is 100%. For *feedingstuffs*, a LOD of 921 U/kg, a LOQ of 2001 U/kg, an RSD$_r$ of 4.0% and a recovery rate of 99% were obtained [9].

There are several published and ring-trial validated methods for the determination of the activity of one active agent (endo-1,4-β-xylanase) in different matrices. When measuring the activity of xylanase in a *product*, Bailey *et al*. (1992) reported RSD$_R$ values of about 17% to 30%, depending on the substrate used [10]. Cosson *et al.* (1999) obtained RSD$_r$ of 4.6 to 11.9% for the assay of xylanase in *feedingstuffs* using a dyed xylan substrate [11]. König *et al*. (2002) tested xylanase *products* in four laboratories using a wheat arabinoxylan and measuring the amount of released reducing sugars. The obtained within-laboratory RSD$_R$ varied from 4.4 to 5.3% [12]. However, none of these studies were performed on *Avizyme 1505*. For this reason, their applicability to the analysis of *Avizyme 1505* cannot be evaluated. Nevertheless, the obtained precision data reported in this dossier can be compared with corresponding data from the above mentioned studies and are considered acceptable.

4. CONCLUSIONS AND RECOMMENDATIONS

The proposed methods for the determination of the enzyme activity in the various matrices show an acceptable performance profile. However, the different methods apply different analytical conditions, whereas a harmonised system would be required. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the feed additive, in premixtures and in feedingstuffs is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions described by the definitions of units;
- that the minimum activity of endo-1,4-β-xylanase, specified in the register entry (75 U/kg) is replaced by the limit of quantification of the method, which is 500 U/kg;
- that the minimum activity of α-amylase, specified in the register entry (100 U/kg) is replaced by the limit of quantification of the method, which is 160 U/kg;
- and that the minimum activity of subtilisin, specified in the register entry (1000 U/kg) is replaced by the limit of quantification of the method, which is 2000 U/kg.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as described by the definitions of units, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Avizyme 1505* 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. 1831/021-2006.

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

[3] Main dossier, Section II, Reference B 10.

[4] Annex III. Proposal of Register entry.

[5] Main dossier, Section II, Subject 2, Item 1.4.

[6] Main dossier, Section II, Reference B 21.

[7] Main dossier, Section II, Reference B 23.

[8] Main dossier, Section II, Reference B 22.

[9] Supplementary information received upon request of EFSA (letter dated 26/02/2007).
[10] Bailey, M.J. *et al.* Journal of Biotechnology, 23 (1992) 257-270.
[11] Cosson, T. *et al*. Animal Feed Science and Technology, 77 (1999) 345-353.
[12] König, J. *et al*. Anal. Bioanal. Chem., 374 (2002) 80-87.
[13] Supplementary information received upon request of the CRL (e-mail dated 21/09/2007).

7. RAPPORTEUR LABORATORY

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

8. ACKNOWLEDGEMENTS

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- Plantedirektoratets Laboratorium, Lyngby, Denmark.
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- Sächsische Landesanstalt für Landwirtschaft, Leipzig, Germany.
- Central Institute for Supervising and Testing in Agriculture, Praha, Czech Republic.