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

Dossier related to:	**FAD-2009-0006** **CRL/ 080018**
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)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA)** **Geel, Belgium**
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EXECUTIVE SUMMARY

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

Enzymatic activity of the active agents is expressed in "U" units, where:
- 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°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°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°C.

Avizyme 1505 has a minimum activity of 1500 U/g, 2000 U/g and 20000 U/g of *endo-1,4-β-xylanase*, *α-amylase* and *subtilisin*, respectively.

The applicant proposed the following activity ranges (minimum–maximum content) of *Avizyme 1505* in complete *feedingstuffs*: - from 150 to 300 U/kg for *endo-1,4-β-xylanase*, - from 200 to 400 U/kg for *α-amylase*; and from 2000 to 4000 U/kg for *subtilisin*.

For the determination of *endo-1,4-β-xylanase*, *α-amylase* and *subtilisin*, the applicant proposed single-laboratory validated methods, further verified by a second independent laboratory.

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposed colorimetric methods based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4 β-xylanase* on commercially available azurine cross-linked wheat arabinoxylan substrates. The analysis is carried out at pH 4.2 and 50°C. The rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard. The following method performance characteristics for *feedingstuffs* were recalculated by the CRL: - a relative standard deviation for *repeatability* (RSD_r) ranging from 3.2 to 4.2 %, - a relative standard deviation for

intermediate precision (RSD_{ip}) ranging from 4.2 to 5.3 %, - a *recovery rate* (R_{Rec}) of 97 %, and a limit of quantification (LOQ) of 133 U/kg which is well below the minimum activity proposed by the applicant.

For the determination of the activity of *α-amylase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposed colorimetric methods based on the quantification of water soluble dyed fragments produced by the action of *α-amylase* on commercially available azurine cross-linked starch polymer substrates. The analysis of *feed additive* is carried out at the pH 7.0 and 40°C, whereas the conditions of the analysis in *premixtures* and *feedingstuffs* are pH 6.3 and 37°C. The rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard. The following method performance characteristics for *feedingstuffs* were recalculated by the CRL: - RSD_r ranging from 5.1 to 5.6 %, - RSD_{ip} ranging from 6.6 to 8 %, - R_{Rec} = 98 %, and LOQ = 189 U/kg, which is well below the minimum activity proposed by the applicant.

For the determination of the activity of *subtilisin* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposed colorimetric methods based on the quantification of water soluble dyed fragments (azurine) produced by the action of *subtilisin* on commercially available cross-linked casein substrates. The analysis is carried out at pH 10.0 and 50°C; the rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard. The following method performance characteristics for *feedingstuffs* were recalculated by the CRL: - RSD_r ranging from 5.4 to 6.5 %, - RSD_{ip} ranging from 6.3 to 19 %, - R_{Rec} = 93 %, and LOQ 1920 U/kg, which is well below the minimum activity proposed by the applicant.

Based on the satisfactory performance characteristics mentioned above, the CRL recommends for official control the methods submitted by the applicant for the determination of *endo-1,4-β-xylanase*, *α-amylase* and *subtilisin* in the *feed additive*, *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

KEYWORDS

Avizyme 1505, endo-1,4 β-xylanase, α-amylase, subtilisin, *Trichoderma reesei, Bacillus amyloliquefaciens, Bacillus subtillis*, digestibility enhancer, laying hens

1. BACKGROUND

Avizyme 1505 is a product for which authorisation as *feed additive* is sought under the category "zootechnical additives", functional groups 4(a) "digestibility enhancers" according to Annex I of Regulation (EC) No 1831/2003 [1]. It contains three active agents [1,2]:

- *endo-1,4-β-xylanase* (E.C. 3.2.1.8), produced by a strain *Trichoderma reesei* (ATCC PTA 5588);
- *α-amylase* (E.C. 3.2.1.1), produced by a strain of *Bacillus amyloliquefaciens* (ATCC 3978);
- *subtilisin* (E.C. 3.4.21.62), produced by a strain of *Bacillus subtilis* (ATCC 2107);

All the strains have been deposited at the American Type Culture Collection (ATCC) in Manassas, VA, USA [3].

In the current application submitted according to Article 4(1) of Regulation (EC) No 1831/2003, the authorisation for *laying hens* is requested [1].

According to the applicant, enzymatic activity of the active agents is expressed in "U" units [2], where:

- 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.

Avizyme 1505 is a light brown fine granular powder, added to *complete feedingstuffs*, either directly or via *premixtures* [4]. The final product is carried on milled wheat flour [4].

Avizyme 1505 has a minimum activity of 1500 U/g, 2000 U/g and 20000 U/g of *endo-1,4-β-xylanase*, *α-amylase* and *subtilisin*, respectively [2].

The applicant proposed the following activity ranges (minimum–maximum content) of *Avizyme 1505* in complete *feedingstuffs*: - from 150 to 300 U/kg for *endo-1,4-β-xylanase*, - from 200 to 400 U/kg for *α-amylase*, and from 2000 to 4000 U/kg for *subtilisin*.

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Avizyme 1505*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective Community Reference Laboratories [5].

Description of the analytical methods for the determination of the active agents in the feed additive, premixtures and feedingstuffs

Endo-1,4-β-xylanase

For the determination of the activity of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposed colorimetric methods [6,7] based on the quantification of water soluble dyed fragments produced by the action of *endo-1,4 β-xylanase* on commercially available azurine cross-linked wheat arabinoxylan substrates. The analysis is carried out at pH 4.2 and 50^{o}C; the rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard. The activity of the reference enzyme standard is measured at the definition conditions of the activity (pH 5.3 and 50^{o}C on a cross-linked oat spelt xylan). The analysis in *feed additive*, *premixtures* and *feedingstuffs* have the same standard operating procedures, with minor modifications introduced in feed analysis (i.e. incubation time). Furthermore, the *premixtures* is diluted with heat treated wheat flour to obtain an activity that is in the normal range of detection for feed assay. These single-laboratory validated methods [8,9] were further verified by a second independent laboratory [10-12]. The performance characteristics presented in Table 1 were recalculated by CRL [13] using the experimental data provided by the applicant [8-9,14].

Table 1 Performance characteristics of analytical methods for the determination of the activity of *endo-1,4-β-xylanase* in *feed additive, premixtures* and *feedingstuffs.* Precision values were recalculated by the CRL [13] based on the experimental validation [8,9] and verification [14] data provided.

	RSD_r, % [13]		RSD_{ip}, % [13]		LOD U/kg [8,9]	LOQ U/kg [8,9]	Recovery rate % [8,9]
	Validation	Verification	Validation	Verification			
Feed additive	9.5	4.1	9.5	4.1	—	—	103
Premixture	3.2	6.8	5.3	6.8	320	1064	95
Feedingstuffs	3.2	4.2	5.3	4.2	40	133	97

RSD_r,and RSD_{ip}: relative standard deviation for *repeatability* and for *intermediate precision*;
LOD and LOQ: limit of detection and quantification;
Target values ranging from 22158 to 28259 U/g *feed additive* and from 1093 to 1332 U/kg *feedingstuffs*

α-amylase

For the determination of the activity of *α-amylase* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposed colorimetric methods [15,16] based on the quantification of water soluble dyed fragments produced by the action of *α-amylase* on commercially available azurine cross-linked starch polymer substrates. The analysis of *feed additive* is carried out at pH 7.0 and 40°C; the rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard. The activity of the reference enzyme standard is measured at definition conditions of the activity (pH 6.5 and 37°C on a cross-linked starch polymer).

The analysis of *premixtures* and *feedingstuffs* are carried out at pH 6.3 and 37°C, measured on a spectrophotometer at 620 nm. The analysis in *feed additive*, *premixtures* and *feedingstuffs* have the same standard operating procedures, only minor modification are introduced in feed analysis (i.e. wavelength, pH); furthermore, the *premixtures* is diluted with heat treated wheat flour to obtain an activity that is in the normal range of detection for feed assay. These single-laboratory validated methods [17,18] were further verified by a second independent laboratory [19-21]. The performance characteristics presented in Table 2 were recalculated by CRL [13] using the experimental data provided by the applicant [14,17-18].

Table 2 Performance characteristics of analytical methods for the determination of the activity of *α-amylase* in *feed additive, premixtures* and *feedingstuffs.* Precision values were recalculated by the CRL [13] based on the experimental validation [17,18] and verification [14] data provided.

	RSD_r, % [13]		RSD_{ip}, % [13]		LOD U/kg [17,18]	LOQ U/kg [17,18]	Recovery rate % [17,18]
	Validation	Verification	Validation	Verification			
Feed additive	4	6.6	7	7.5	—	—	104
Premixture	5.6	8.8	8	9.1	456	1512	98
Feedingstuffs	5.6	5.1	8	6.6	57	189	98

RSD_r,and RSD_{ip}: relative standard deviation for *repeatability* and for *intermediate precision*;
LOD and LOQ: limit of detection and quantification;
Target values ranging from 8233 to 10268 U/g *feed additive*, and ranging from 1402 to 1817 U/kg *feedingstuffs*;

Subtilisin

For the determination of the activity of *subtilisin* in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposed colorimetric methods [22,23] based on the quantification of water soluble dyed fragments (azurine) produced by the action of *subtilisin* on commercially available cross-linked casein substrates. The analysis is carried out at pH 10.0 and 50°C; the rate of dye release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard. The activity of the reference enzyme standard is measured at the definition conditions of the activity (pH 7.5 and 40°C on cross-linked casein). The analysis in *feed additive*, *premixtures* and *feedingstuffs* have the same standard operating procedures, only minor modification are introduced in feed analysis (i.e. incubation time). Furthermore, the *premixtures* is diluted with heat treated wheat flour to obtain an activity that is in the normal range of detection for feed assay. These single-laboratory validated methods [24,25] were further verified by a second independent laboratory [26-28]. The performance characteristics presented in Table 3, were recalculated by CRL [13] based on the experimental data provided by the applicant [14,24-25].

Table 3 Performance characteristics of analytical methods for the determination of the activity of *subtilisin* in *feed additive, premixtures* and *feedingstuffs.* Precision values were recalculated by the CRL [13] based on the experimental validation [24,25] and verification [14] data provided.

	RSD_r, % [13]		RSD_{ip}, % [13]		LOD U/kg [24,25]	LOQ U/kg [24,25]	Recovery rate % [24,25]
	Validation	Verification	Validation	Verification			
Feed additive	2.7	6.2	8.4	7.5	—	—	93
Premixture	6.5	9.2	19	27.6	6560	15400	93
Feedingstuffs	6.5	5.4	19	6.3	820	1920	93

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and for *intermediate precision*;
LOD and LOQ: limit of detection and quantification;
Target values ranging from 86173 to 109106 U/g *feed additive*, and ranging from 2829 to 4779 U/kg *feedingstuffs*;

Based on the above mentioned performance characteristics, the CRL recommends for official control, the method submitted by the applicant, for the determination of *endo-1,4-beta-xylanase, α-amylase*, *subtilisin* in the *feed additive*, *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the CRL recommends for official control the validated and verified method, submitted by the applicant, for the determination of *endo-1,4-β-xylanas, α-amylase* and *subtilisin* in *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the determination of *endo-1,4-β-xylanase* in *feed additive*, *premixtures* and *feedingstuffs*:

- colorimetric method based on the quantification of water soluble dyed fragments (azurine) produced by the action of *endo-1,4 β-xylanase* on cross-linked wheat arabinoxylan substrates.

For the determination of *α-amylase* in *feed additive*, *premixtures* and *feedingstuffs*:

- colorimetric method based on the quantification of water soluble dyed fragments (azurine) produced by the action of *α-amylase* on cross-linked starch polymer substrates.

For the determination of *subtilisin* in *feed additive*, *premixtures* and *feedingstuffs*:

- colorimetric method based on the quantification of water soluble dyed fragments (azurine) produced by the action of *subtilisin* on cross-linked casein substrates.

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. 1831/0004-2009

[2] *Application, Proposal for Register Entry

[3] *Technical dossier, Section II – Annex_II_B16_Strain deposit certificate.pdf

[4] *Technical dossier, Section II – 2.1.4. Purity

[5] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[6] *Technical dossier, Section II – Annex_II_B34_XYL MoA in product (N).pdf

[7] *Technical dossier, Section II – Annex_II_B37_XYL MoA in feed.pdf

[8] *Technical dossier, Section II – Annex_II_B40_XYL VAL in product.pdf

[9] *Technical dossier, Section II – Annex_II_B43_ XYL VAL in feed.pdf

[10] *Technical dossier, Section II – Annex_II_B46_XYL VER in product.pdf

[11] *Technical dossier, Section II – Annex_II_B49_XYL VER in premix.pdf

[12] *Technical dossier, Section II – Annex_II_B52_XYL VER in feed.pdf

[13] *Additional Information – Precision data as recalculated by the CRL.xls

[14] *Supplementary information provided by the applicant upon request CRL

[15] *Technical dossier, Section II – Annex_II_B35_AMYL MoA in product.pdf

[16] *Technical dossier, Section II – Annex_II_B38_AMYL MoA in feed.pdf

[17] *Technical dossier, Section II – Annex_II_B41_AMYL VAL in product.pdf

[18] *Technical dossier, Section II – Annex_II_B44_AMYL VAL in feed.pdf

[19] *Technical dossier, Section II – Annex_II_B47_AMYL VER in product.pdf

[20] *Technical dossier, Section II – Annex_II_B50_AMYL VER in premix.pdf

[21] *Technical dossier, Section II – Annex_II_B53_AMYL VER in feed.pdf

[22] *Technical dossier, Section II – Annex_II_B36_PRO MoA in product.pdf

[23] *Technical dossier, Section II – Annex_II_B39_PRO MoA in feed.pdf

[24] *Technical dossier, Section II – Annex_II_B42_PRO VAL in product.pdf

[25] *Technical dossier, Section II – Annex_II_B45_PRO VAL in feed.pdf

[26] *Technical dossier, Section II – Annex_II_B48_PRO VER in product.pdf

[27] *Technical dossier, Section II – Annex_II_B51_PRO VER in premix.pdf

[28] *Technical dossier, Section II – Annex_II_B54_PRO VER in feed.pdf

* Refers to Dossier No. FAD-2009-0006

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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- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava (SK)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)