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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-2008-432
	FAD-2008-0022
	CRL/080011
Product name:	AveMix 02 CS and L
Active Substance(s):	Endo-1,4-beta-xylanase (EC 3.2.1.8)
	Endo-1,3(4)-beta-glucanase (EC 3.2.1.6)
	Pectinase (EC 3.2.1.15)
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EXECUTIVE SUMMARY

The current application authorisation is sought for *AveMix 02 CS and L* 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 *AveMix 02 CS and L* as a digestibility enhancer for piglets (weaned). The product is intended to be marketed as solid (*AveMix 02 CS*) and liquid (*AveMix 02 L*) formulations.

The active agents of *AveMix 02 CS and L* are (1) endo-1,4-β-*xylanase* produced by *Trichoderma reesei*, (2) endo-1,3(4)-β-*glucanase* produced by *Trichoderma reesei*, and (3) *pectinase* produced by *Aspergillus aculeatus*. The enzymatic activities are expressed in *xylanase* unit (XU), *betaglucanase* unit (BGU) and *pectinase* unit (PGLU), respectively. According to the applicant: (1) one XU-unit is the amount of enzyme which releases 1 µmol of reducing sugar (xylose equivalent) per minute from xylan of oat spelt at 50°C, pH = 4.8; (2) one BGU-unit is the amount of enzyme which releases 1 µmol of reducing sugar (cellobiose equivalent) per minute from betaglucan of barley at 50°C, pH = 5.0; and (3) one PGLU unit is the amount of enzyme which releases 1 µmol of reducing (glucose equivalent) sugar per minute from polymethylgalacturonic acid (pectin containing substrate) at 35°C and pH = 4.8.

The solid formulation (*AveMix 02 CS*) has an endo-1,4-β-*xylanase* activity of 21400 XU/g, an endo-1,3(4)-β-*glucanase* activity of 12300 BGU/g and a *pectinase* activity of 460 PGLU/g. The liquid formulation (*AveMix 02 L*) has an endo-1,4-β-*xylanase* activity of 10700 XU/g, an endo-1,3(4)-β-*glucanase* activity of 6150 BGU/g and a *pectinase* activity of 230 PGLU/g. Both formulations are intended to be mixed into *premixtures* and/or *feedingstuffs* to provide a minimum endo-1,4-β-*xylanase* activity of 2140 XU/kg, endo-1,3(4)-β-*glucanase* activity of 1230 BGU/kg and *pectinase* activity of 46 PGLU/kg in *feedingstuffs*.

Endo-1,4-β-*xylanase*: For the determination of the activity of endo-1,4-β-*xylanase* in the *feed additives* and *premixtures* a colorimetric method based on the formation of reducing sugar reacting with Dinitrosalicylic acid (DNS) is proposed. The method is in-house validated only for the *feed additives*, and the following performance characteristics were reported: -a recovery rate of 104%, - a relative standard deviation for repeatability (RSD_r) of 3%, - a

relative standard deviation for intermediate precision (RSD_R) of 5%. No validation data were provided by the applicant for the determination of the activity of endo-1,4-β-*xylanase* in the *premixtures.* For the determination of the activity of endo-1,4-β-*xylanase* in the *feedingstuffs* the applicant provided upon request from the CRL an in-house validated method based on the measurement of the rate of release of water soluble dyed fragments by endo-1,4-ß-*xylanase* from the dye cross-linked wheat arabinoxylan. The following performance characteristics were reported: - a recovery rate of 104%, - a RSD_r of 4%, - a RSD_R of 4%, - a limit of detection (LOD) and limit of quantification (LOQ) of 939 and 1878 XU/kg *feedingstuffs*, respectively.

Endo-1,3(4)-β-*glucanase*: For the determination of the activity of endo-1,3(4)-β-*glucanase* in the *feed additives* and *premixtures* a colorimetric method based on the formation of reducing sugar reacted with DNS is proposed. The method is in-house validated only for the *feed additives*, and the following performance characteristics were reported: -a recovery rate of 107%, - a RSD_r of 4%, - a RSD_R of 5%. No validation data were provided by the applicant for the determination of the activity of endo-1,3(4)-β-*glucanase* in the *premixtures.* For the determination of the activity of endo-1,3(4)-β-*glucanase* in the *feedingstuffs* the applicant provided upon request from the CRL an in-house validated method based on the measurement of the rate of release of water soluble dyed fragments by endo-1,3(4)-β-*glucanase* from the dye cross-linked barley glucan. The following performance characteristics were reported: - a recovery rate of 109%, - a RSD_r of 6%, - a RSD_R of 5%, -an LOD and LOQ of 111 and 222 BGU/kg *feedingstuffs*, respectively.

Pectinase: For the determination of the activity of *pectinase* in the *feed additives* and *premixtures* a colorimetric method based on the formation of reducing sugar reacted with DNS is proposed. The method is in-house validated only for the *feed additives*, and the following performance characteristics were reported: -a recovery rate of 105%, - a RSD_r of 5%, - a RSD_R of 14%. No validation data were provided by the applicant for the determination of the activity of *pectinase* in the *premixtures*. For the determination of the activity of *pectinase* in the *feedingstuffs* a viscosimetric method based on the measurement of reduced viscosity by the enzyme of a pectin substrate, which is then directly related to enzymatic activity. The method is in-house validated and the following performance

characteristics were reported: -a recovery rate of 113%, - a RSD_r of 9%, - a RSD_R of 9%, - an LOD and LOQ of 14 and 28 PGLU/kg *feedingstuffs*, respectively.

Based on these acceptable performance characteristics, the proposed methods are considered suitable for determination of endo-1,4-ß-*xylanase*, endo-1,3(4)-β-*glucanase* and *pectinase* activities in *feed additives* and *feedingstuffs* for official control purposes in the frame of authorisation. Since validation parameters of the methods for premixtures are not available, the CRL is unable to comment on the suitability of the proposed methods for this matrix.

Further testing or validation is not considered necessary.

KEYWORDS

AveMix 02 CS and L; endo-1,4 β-*xylanase*; endo-1,3(4)-β-*glucanase*; *pectinase*; *Trichoderma reesei*; *Aspergillus aculeatus*; digestibility enhancer; weaned piglets.

1. BACKGROUND

AveMix 02 CS and L 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]. The product contains three active agents [2]:

- endo-1,4-β *xylanase* (EC 3.2.1.8) produced by the strain X-252 (MUCL 49755) of *Trichoderma reesei*;
- endo-1,3(4)-β-*glucanase* (EC 3.2.1.6) produced by the strain A-34 (MUCL 49754) of *Trichoderma reese;,*
- *pectinase* (EC 3.2.1.15) produced by the strains derived from *Aspergillus aculeatus* (E1603).

The strains of *Trichoderma reesei* have been deposited at the Belgian Coordinated Collections of Microorganisms (BCCM) [3]. The activity of endo-1,4-β *xylanase* is expressed as *xylanase* unit (XU), where one XU-unit is the amount of enzyme which releases 1 µmol of reducing sugar (xylose equivalent) per minute from xylan of oat spelt at 50°C, pH = 4.8. The activity of endo-1,3(4)-β-*glucanase* is expressed as *betaglucanase* unit (BGU), where one

BGU-unit is the amount of enzyme which releases 1 µmol of reducing sugar (cellobiose equivalent) per minute from betaglucan of barley at 50°C, pH = 5.0. The activity of *pectinase* is expressed as *pectinase* PGLU unit, where one PGLU unit is the amount of enzyme which releases 1 µmol of reducing (glucose equivalent) sugar per minute from polymethylgalacturonic acid (pectin containing substrate) at 35°C and pH = 4.8 [4]. The product is intended to be marketed in solid (*AveMix 02 CS*) *and* liquid (*AveMix 02 L*) formulations. The solid formulation has an endo-1,4 β-*xylanase* activity of 21400 XU/g, an endo-1,3(4*)*-β-*glucanase* activity of 12300 BGU/g and an *pectinase* activity of 460 PGLU/g. The liquid formulation has an endo-1,4 β-*xylanase* activity of 10700 XU/g, an endo-1,3(4*)*-β-*glucanase* activity of 6150 BGU/g and an *pectinase* activity of 230 PGLU/g [5]. The minimum target activities in *feedingstuffs* for weaned piglets are (1) 2140 XU/kg for endo-1,4 β-*xylanase,* (2) 1230 BGU/kg for endo-1,3(4*)*-β-*glucanase* and (3) 46 PGLU/kg for *pectinase* [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 *AveMix 02 CS and L*, (EFSA-Q-2008-432), 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 qualitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the *additive* (e.g. arsenic and heavy metals - cadmium, mercury and lead) are available at the respective Community Reference Laboratories [6].

Description of the analytical methods for the determination of the active agent 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 additives* and *premixtures* the applicant proposes a colorimetric method based on the formation of reducing sugar reacted with Dinitrosalicylic acid (DNS), where the colour change is proportional to xylose equivalents measured at 550 nm [7]. Approximately 1 g of feed additive is added with 80 ml citrate buffer (0.05 M, pH = 4.8), stirred for 30 min, filtered and diluted (1/1500 for solid product or 1/700 for liquid product). A sample-tube containing 0.5 ml sample solution and 1.0 ml substrate solution (2% xylan, Sigma n° 0376) together with a sample-blank tube containing 1.0 ml substrate solution are incubated at 50 ^{o}C for 15 min. A blank-tube containing 1.5 ml buffer and a standard-tube containing 1.0 ml buffer and 0.5 ml xylose solution (0.5 g xylose in 50ml buffer) are prepared without incubation. 4 ml DNS solution is added to all tubes, and homogenised by mixing. 0.5 ml sample solution is added just after the mixing to the sample-blank tube. Finally, a 0.5 ml standard solution (300 mg glucose/L) is added to all tubes. The tubes are then boiled for 10 min, cooled and centrifuged at 4500 rpm for 10 min. The supernatants are then taken for the absorbance readings at 550 nm using a spectrophotometer. A xylose standard curve is prepared to determine the activity of endo-1,4-β-*xylanase* in *Avemix 02 CS and L*. The method was in-house validated by the applicant and performance characteristics provided only for *feed additive* upon the CRL request are [8]: - a recovery rate of 104%, -a relative standard deviation for repeatability (RSD_r) of 3%, - a relative standard deviation for intermediate precision (RSD_R) of 5%.

The applicant did not provide the validation data requested by the CRL for the determination of endo-1,4-β-*xylanase* in the *premixtures*. Hence, the CRL could not evaluate the suitability of the proposed method [7] for official control purposes.

For the determination of the activity of endo-1,4-β-*xylanase* in the *feedingstuffs* an colorimetric method is proposed, based on the measurement of the rate of release of water soluble dyed fragments by endo-1,4-ß-*xylanase* from the dye cross-linked wheat arabinoxylan in a form of "Xylazyme AX tablet" from Megazyme is proposed [9]. An enzyme standard addition is used: 5.0 g feed sample is extracted in 50 ml acetic acid (0.1 M) for 10 min at 22^{o}C

and then centrifuged. Three samples tubes, each containing 10 ml feed extract are added with 0, 0.5 and 1.0 ml *xylanase* standard solution prepared from the solid formulation of *AveMix 02 CS* with a declared activity of 21400 XU/g. 0.5 ml aliquots of these sample tubes and a blank tube containing only 0.5 ml buffer are then incubated at 50 °C for 3 min. One "Xylazyme AX" tablet is added to each tube and further incubated at 50 °C for 30 min. The reaction is stopped by the addition of 3 ml trizma solution (2%, e.g. Sigma), and the absorbance of the formed colour is measured at 590 nm [9]. The *xylanase* activity of *AveMix 02 CS* used for standard addition is measured by the method described earlier [7]. The same formulation of feed additive (liquid or powder) used for the preparation of *feedingstuffs* should be used for the calibration, and therefore made available by the applicant to the official control laboratories. Upon request from the CRL the applicant submitted an in-house validation study [10] of a similar product "*AveMix XG 10*" containing the same endo-1,4-β-*xylanase* present in *AveMix 02 CS*. The following performance characteristics were reported for a target *xylanase* activity ranging from 3173 to 14348 XU/kg *feedingstuffs*: - a recovery of 104%, - a RSD_r of 4%, - a RSD_R of 4% [10]. Furthermore, the applicant determined LOD and LOQ analysing feed samples spiked with a low dosage of "*AveMix XG 10"* to be 939 and 1878 XU/kg *feedingstuffs*, respectively [10]. These values are below the minimum recommended endo-1,4-β-*xylanase* activity level of 2140 XU/kg in *feedingstuffs* [4].

Endo-1,3(4)-β-*glucanase*

For the determination of the activity of endo-1,3(4)-β-*glucanase* in the *feed additives* and *premixtures* the applicant proposes a colorimetric method based on the formation of reducing sugar reacted with DNS, where the colour change is proportional to cellobiose equivalent, measured at 550 nm [11]. Approximately 1 g of feed additive is added with 80 ml acetate buffer (0.1 M pH = 5.0), stirred for 30 min, filtered and diluted (1/1000 for solid formulation and 1/400 for liquid formulation). A sample-tube containing 0.5 ml sample solution and 1.0 ml substrate solution (1% beta-glucan, barley-medium viscosity n°206) together with a sample-blank tube containing 1.0 ml substrate solution are incubated at 50 °C for 10 min. A blank-tube containing 1.5 ml buffer and a standard-tube containing 1.0 ml buffer and 0.5 ml cellobiose solution (1 g cellobiose in 50 ml acetate buffer) are prepared without incubation. All tubes are added with 4 ml DNS solution and homogenised by mixing. 0.5 ml sample solution is added to the sample-blank tube just after mixing. Finally, 0.5 ml

standard addition (300 mg glucose/L) is added to all tubes. The tubes are then boiled for 10 min, cooled and added 10 ml water. The supernatants are taken for the absorbance readings at 550 nm using a spectrophotometer. A glucose standard curve is prepared to determine the activity of endo-1,3(4)-β-*glucanase* in *Avemix 02 CS and L*. The method was in-house validated by the applicant and performance characteristics provided for the *feed additive* upon the CRL request are [12]: - a recovery rate of 107%, - a RSD_r of 4%, - a RSD_R of 5%.

The applicant did not provide the validation data requested by the CRL for the determination of endo-1,3(4)-β-*glucanase* in the *premixtures*. Hence, the CRL could not evaluate the suitability of the proposed method [11] for official control purposes.

For the determination of the activity of endo-1,3(4)-β-*glucanase* in the *feedingstuffs* a colorimetric method based on the measurement of the rate of release of water soluble dyed fragments by endo-1,3(4)-β-*glucanase* from the dye cross-linked barley glucan from Megazyme is proposed [13]. A *betaglucanase* standard addition is used: Three feed samples of 25 g are added with 0, 50 and 100 ppm of *AveMix 02 CS* standard solution (stabilised by a buffer and added with 0.1% bovine serum albumine) with an endo-1,3(4)-β-*glucanase* activity of 12300 BGU/g. The samples are extracted in acetic acid (40 mM, pH = 4.6) for 30 min, filtered and diluted 3 times with a buffer. The feed extracts (0.5 ml) and a blank containing 0.5 ml buffer are then incubated with 0.5 ml substrate (Azo-barley glucan available from Megazyme) at 40 ^{o}C for 210 min. The reaction is stopped by adding 3 ml of the stopping reagent (industrial methylated spirits/methoxyethanol) and centrifuged. The formed colour is measured at 590 nm using a spectrophotometer. The endo-1,3(4)-β-*glucanase* activity of the enzyme used for standard addition is measured by the method described earlier [11]. The same form of feed additive (liquid or powder) used for the preparation of *feedingstuffs* should be used for the calibration, and therefore made available by the applicant to the official control laboratories. Upon request from the CRL, the applicant submitted an in-house validation study [14], of a similar product "*AveMix XG 10*" containing the same endo-1,3(4)-β-*glucanase* as in the *AveMix 02 CS*. The following performance characteristics were reported for a target activity ranging from 518 to 2406 BGU/kg *feedingstuffs*: - a recovery of 109%, - a RSD_r of 6% and - a RSD_R of 5% [14]. The applicant determined LOD and LOQ analysing feed samples spiked with a low dosage of "*AveMix XG 10"* to be 111 and 222 BGU/kg of

feedingstuffs, respectively [14]. These values are far below the minimum recommended endo-1,3(4)-β-*glucanase* activity level of 1230 BGU/kg in *feedingstuffs*.

Pectinase

For the determination of the activity of *pectinase* in the *feed additives* and *premixtures* the applicant proposes a colorimetric method based on the formation of reducing sugar reacted with DNS, where the colour change is proportional to glucose equivalent, measured at 550 nm [15]. Approximately 1 g of feed additive is added with 80 ml citrate buffer (0.1 M pH = 4.8), stirred for 30 min, filtered and diluted (1/60 for solid formulation and 1/40 for liquid formulation). A sample-tube containing 0.5 ml sample solution and 1.0 ml substrate solution (1% pectin, Sigma P-2157) together with a sample-blank tube containing 1.0 ml substrate solution are incubated at 35 ^{o}C for 20 min. A blank-tube containing 1.5 ml buffer and a standard-tube containing 1.0 ml buffer and 0.5 ml glucose standard solution (1.1 g glucose monohydrate in 50 ml citrate buffer) are prepared without incubation. All tubes are added with 4 ml DNS solution and homogenised by mixing. 0.5 ml sample solution is added to the sample-blank tube just after the mixing. The tubes are then boiled for 10 min, cooled and centrifuged at 4500 rpm for 10 min. The supernatants are taken for the absorbance readings at 550 nm using a spectrophotometer. A glucose standard curve is prepared to determine the activity of *pectinase* in *Avemix 02 CS and L*. The method was in-house validated by the applicant and the performance characteristics provided for the *feed additive* upon request from the CRL [16] are: - a recovery rate of 105%, - a RSD_r of 5%, - a RSD_R of 14%.

The applicant did not provide the validation data requested by the CRL for the determination of *pectinase* in the *premixtures*. Hence, the CRL could not evaluate the suitability of the proposed method [15] for official control purposes.

For the determination of the activity of *pectinase* in the *feedingstuffs* a viscosimetric method is proposed, based on the measurement of reduced viscosity by the enzyme of a pectin substrate and proportional to the pectinase activity [17]. A 25 g feed sample is extracted in 250 ml acetic acid (0.1 M, pH = 4.0) at 22 ^{o}C for 30 min. The extract is centrifuged for 5 min at 4300 rpm. A *pectinase* standard addition is used. Four tubes, each containing 30 ml pectin solution (1.67% pectin) and 3 ml feed extract are added with 0.0, 0.05, 0.10 and 0.20 ml

pectinase standard solution (3 g *Avemix 02 CS* in 100 ml water, diluted to 1/100). 30 ml pectin solution (1.67% pectin) was added to the blank tube containing 3 ml of water. All tubes are then incubated at 50 ^{o}C for 4 h. After incubation the viscosity of samples are measured at 50^{o}C at 100 rpm for 1 min using a digital Brookfield viscometer (model DVII with needle n^{o}1). The viscosities, measured as centipoises (cPs), corrected for the viscosity of the blank sample are used as a measure for the *pectinase* activity determined by the method described earlier [15], where the quantification is performed by a calibration curve of a reference *pectinase* with known activity. The same form of feed additive (liquid or powder) used for the preparation of *feedingstuffs* should be used for the calibration, and therefore made available by the applicant to the official control laboratories. Upon request from the CRL the applicant submitted an in-house validation study. The performance characteristics were determined at a target activity ranging from 25 to 63 PGLU/kg *feedingstuffs.* The recovery rate was determined to be 113% [18]. The CRL recalculated the precision to be $RSD_r = RSD_R = 9\%$ [19]. The applicant determined LOD and LOQ analysing feed samples spiked with a low dosage of "*AveMix 02 CS"* to be 14 and 28 PGLU/kg *feedingstuffs*, respectively [18]. These values are below the minimum recommended *pectinase* activity level of 46 PGLU/kg in *feedingstuffs* [4].

Based on these acceptable performance characteristics, the proposed methods are considered suitable for determination of endo-1,4-ß-*xylanase*, endo-1,3(4)-β-*glucanase* and *pectinase* activities in *feed additives* and *feedingstuffs* (not in *premixtures*) for official control purposes in the frame of authorisation.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of *AveMix 02 CS and L* authorisation, the CRL recommends the applicant proposed methods for determination of endo-1,4 β-*xylanase*, endo-1,3(4)-β-*glucanase* and *pectinase* in *feed additives* and *feedingstuffs* (not in *premixtures*) for weaned piglets official control purposes.

Further testing or validation is not considered necessary.

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

Characterisation of the active substances in the *feedingstuffs*:

- Colorimetric method measuring water soluble dye released by action of endo-1,4-β-*xylanase* from dye cross-linked wheat arabinoxylan substrate;
- Colorimetric method measuring water soluble dye released by action of endo-1,3(4)-β-*glucanase* from dye cross-linked barley betaglucan substrate;
- Viscosimetric method based on a decrease of viscosity produced by action of *pectinase* on the pectin-containing substrate, polymethylgalacturonic acid.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *AveMix 02 CS and L* 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] *Application/Reference SANCO/D/2 Forw. Appl. 1831/022-2008.

[2] * Technical dossier/Section II_Identity.pdf. Item 1.3, Item 2.1, Item 2.3.

[3] * Technical dossier/Section II/Annexes: Annex II.131

[4] * Application/Annex III.pdf. Proposal of Register entry.

[5] * Technical dossier/Section II_Identity.pdf: Item 4.1.

[6] 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 Community reference laboratories, Official Journal of the European Union L 136

[7] * Technical dossier/Section II/Annexes Sect II. annex II.5.1.1

[8] * Supplementary information. Annex II.5.1.9_13.02.09

[9] * Technical dossier/Section II/Annex II.5.2.2

[10] *Supplementary information. Annex II.5.2.2_13.02.09

[11] * Technical dossier/Section II/ Annex II.5.1.2

[12] * Supplementary information. Annex II. II.5.1.10_13.02.09

[13] * Technical dossier/Section II/ Annex II.5.2.1

[14] * Supplementary information. Annex II.5.2.1_13.02.09

[15] * Technical dossier/Section II/ Annex II.5.1.3

[16] * Supplementary information. Annex II.5.1.11_13.02.09

[17] * Technical dossier/Section II/ Annex II.5.2.7

[18] * Supplementary information. Annex II.5.2.7_13.02.09

[19] *Rapporteur Laboratory Report/Rapporteur Notes_Precision.doc

*Refers to Dossier number: FAD-2008-0022

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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