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Community Reference Laboratory for Feed Additives

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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-2008-308
	FAD-2008-0011
	CRL/80004
Product name:	AveMix XG 10
Active Substance(s):	Endo-1,4-beta-xylanase (EC 3.2.1.8)
	Endo-1,3(4)-beta-glucanase (EC 3.2.1.6)
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Date:	23/01/2009
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EXECUTIVE SUMMARY

The current application authorisation is sought for *AveMix XG 10* 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 XG 10* as a digestibility enhancer for chickens for fattening. The product is intended to be marketed as solid and liquid formulations.

The active agents of *AveMix XG 10* are (1) endo-1,4-β-*xylanase* and (2) endo-1,3(4)-β-*glucanase* produced by the strain X-252 (MUCL 49755) and the strain A-34 (MUCL 49754)) of *Trichoderma reesei*, respectively. The enzymatic activities are expressed in *xylanase* unit (XU) and *betaglucanase* unit (BGU). According to the applicant 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, whereas 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^{o}C, pH = 5.0.

Both solid and liquid formulations of *Avemix XG10* have an endo-1,4-β-*xylanase* activity of 40 000 XU/g and an endo-1,3(4)-β-*glucanase* activity of 6 600 BGU/g. The product is intended to be mixed into *premixtures* and/or *feedingstuffs* to provide an endo-1,4-β-*xylanase* activity of 4 000 XU/kg *feedingstuffs* and an endo-1,3(4)-β-*glucanase* activity of 660 BGU/kg *feedingstuffs*.

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 4%, - a relative standard deviation for intermediate precision (RSD_R) of 5%, - a limit of detection (LOD) and a limit of quantification (LOQ) of 722 and 820 XU per gram of the *product*. No sufficient validation parameters have been provided 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 in a form of "Xylazyme AX tablet". The following performance characteristics were reported: - a recovery of 104%, - a RSD_r of 4%, - a RSD_R of 4%, - an LOD and LOQ of 939 and 1878 XU/kg feedingstuffs.

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 2%, - a RSD_R of 4%, - an LOD and LOQ of 100 and 116 BGU/g *product*. No sufficient validation parameters have been provided 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 of 109%, - a RSD_r of 6%, - a RSD_R of 5%, -an LOD and LOQ of 111 and 222 BGU/kg *feedingstuffs*.

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

Further testing or validation is not considered necessary.

KEYWORDS

AveMix XG 10, endo-1,4 β-*xylanase*, endo-1,3(4)-β-*glucanase, Trichoderma reesei,* digestibility enhancer, chickens for fattening.

1. BACKGROUND

AveMix XG 10 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 endo-1,4-β *xylanase* (EC 3.2.1.8)

and endo-1,3(4*)*-β-*glucanase* (EC 3.2.1.6) as the active agents [2], produced by the strain X-252 (MUCL 49755) and the strain A-34 (MUCL 49754)) of *Trichoderma reesei*, respectively. Both strains 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). According to the applicant, 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^{o}C, pH = 5.0 [2]. The product is intended to be marketed in solid (*AveMix XG 10*) *and* liquid (*AveMix XG 10 L*) formulations [4]. Both formulations have an endo-1,4 β-*xylanase* activity of 40 000 XU/g and an endo-1,3(4*)*-β-*glucanase* activity of 6 600 BGU/g [5]. The minimum target activities in *feedingstuffs* are (1) for endo-1,4 β-*xylanase* 4 000 XU per kg and (2) for endo-1,3(4*)*-β-*glucanase* in the *feedingstuffs* are of *feedingstuffs* and 660 BGU per kg of for chickens for fattening, respectively [5, 6].

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 XG 10*, (EFSA-Q-2008-308), 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 impurities in the *additive* (e.g. arsenic and heavy metals - cadmium, mercury and lead) are available at the respective Community Reference Laboratories [7].

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

Active agent 1: 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 [8]. 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 (1/4000). A sample-tube containing 0.5 ml sample solution and 1.0 ml substrate solution (2% xylan) together with a sample-blank tube containing 1.0 ml substrate solution (2% xylan) 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 homogenously mixed through a vortex. 0.5 ml sample solution is added just after the mixing to the sample-blank tube. Finally, a 0.5 ml standard addition (300 mg glucose/L) is added to all tubes. The tubes were then boiled for 10 min, cooled and centrifuged at 450 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® XG 10. The following performance characteristics by an in-house validation study were established for only *feed additives* [9]: - a recovery rate of 104%, -a relative standard deviation for repeatability (RSD_r) of 4%, - a relative standard deviation for intermediate precision (RSD_R) of 5%, - the limits of detection and quantification (LOD and LOQ) of 722 and 820 XU/g, respectively. These values are far below the declared levels of *xylanase* activity in the *feed additive* [9].

The applicant did not provide sufficient data upon request from the CRL on the assay protocol and its validation to demonstrate the suitability of the proposed method [8] to determine endo-1,4-β-*xylanase* in the *premixtures* for official control purposes.

For the determination of the activity of endo-1,4-β-*xylanase* in the *feedingstuffs* 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 in a form of "Xylazyme AX tablet" available from Megazyme is provided upon request from the CRL [10]. The analytical protocol foresees the use of enzyme standard addition technique: 5.0 gram of feed sample is extracted in 50 ml acetic acid (0.1M) for 10 min at 22^{o}C. Three samples tubes containing 10 ml feed extract are added with 0, 0.5 and 1.0 ml xylanase standard solution (62876 XU *xylanase* activity per ml), respectively. 0.5 ml aliquots of three sample tubes together with a blank tube containing only 0.5 ml buffer are then incubated at 50 ^{o}C for 3 min. One Xylazyme AX tablet (cross linked wheat arabinoxylan) is added to each tube and further incubated at 50 ^{o}C for 30 min. The reaction is stopped by the addition of a trizma solution, and the absorbance of the formed colour is measured at 590 nm. The *xylanase* activity of the enzyme which is used for standard addition is measured by the method described earlier [8]. The applicant determined LOD and LOQ analysing feedingstuffs spiked with a low dosage (10 ppm) of *AveMix XG 10* to be 939 and 1878 XU/kg of *feedingstuffs* [10]. The following performance characteristics were reported: -a recovery of 104%, - a RSD_r of 4%, - a RSD_R of 4% [10].

Active agent 2: 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 citrate buffer (0.05 M pH = 5.0), stirred for 30 min, filtered and diluted (1/500). A sample-tube containing 0.5 ml sample solution and 1.0 ml substrate solution (1% beta-glucan) together with a sample-blank tube containing 1.0 ml substrate solution (1% beta-glucan) are incubated at 50 ^{0}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 homogenously mixed through a vortex. 0.5 ml sample solution is added to the sample-blank tube just after the mixing. Finally, 0.5 ml standard addition (300 mg glucose/L) is added to all tubes. The tubes were then boiled for 10 min, cooled and centrifuged at 450 rpm for 10 min.

The supernatants are taken for the absorbance readings at 550 nm using a spectrophotometer. A standard curve is prepared to determine the activity of endo-1,3(4)-β-*glucanase* in Avemix® XG 10. The following performance characteristics were established by an in-house validation study only for the *feed additive* [12]: - a recovery rate of 107%, - a RSD_r of 2%, - a RSD_R of 4%, - an LOD and LOQ of 100 and 116 BGU/g of *product*. These values are far below the declared levels of *betaglucanase* activity in the *feed additive* [12].

The applicant did not provide sufficient data upon request from the CRL on the assay protocol and its validation to demonstrate the suitability of the proposed method [11] to determine endo-1,3(4)-β-*glucanase* in the *premixtures* for official control purposes.

For the determination of the activity of endo-1,3(4)-β-*glucanase* in the *feedingstuffs* 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 available from Megazyme is provided upon request from the CRL [13]. The analytical protocol is based on the use of a *betaglucanase* standard addition solution. Three feed samples of 25 g are added with 0, 50 and 100 ppm of *AveMix XG 10* standard addition solution (stabilised in buffer and added 0.1% bovine serum albumine) with a *betaglucanase* activity of 10640 BGU per gram. 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 is then incubated with 0.5 ml substrate (barley glucan 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 *betaglucanase* activity of the enzyme which is 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 to the official control laboratories. The applicant investigated LOD and LOQ analysing feedingstuffs spiked with a low dosage (10 ppm) of *AveMix XG 10*, respectively 111 and 222 BGU/kg [13]. The following performance characteristics were reported: - a recovery of 109%, - a RSD_r of 6% and - a RSD_R of 5%, - an LOD and LOQ of 111 and 222 BGU/kg *feedingstuffs,* respectively determined with low dosage *AveMix XG 10* [13].

Based on these acceptable performance characteristics, the proposed methods are considered suitable for determination of endo-1,4-ß-*xylanase* and endo-1,3(4*)*-β-*glucanase* 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 XG 10* authorisation, the CRL recommends the applicant proposed methods for determination of endo-1,4 β-*xylanase*, endo-1,3(4*)*-β-*glucanase* in *feed additives* and *feedingstuffs* (not in *premixtures*) for chicken for fattening for 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.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *AveMix XG 10* 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. 1831/011-2008.

[2] * Section II_Identity.pdf. Item 2.1.3.

[3] * Section II_Identity.pdf: Items 2.1.6 and 2.2.2.

[4] * Section II_Identity.pdf: Item 2.1.5.

[5] * Annex III.pdf. Proposal of Register entry.

[6] * Section II_Identity.pdf: Item 4.1.

[7] 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

[8] * Section II\Annexes Sect II. annex II.5.1.1

[9] * Supplementary information. annex II.5.1.9

[10] * Supplementary information. annex II.5.2.2_04.12.08

[11] * Section II\Annexes Sect II. annex II.5.1.2

[12] * Supplementary information. annex II. II.5.1.10

[13] * Supplementary information. annex II.5.2.1_12.12.08

*Refers to Dossier number: FAD-2008-0011

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, DK.
- Państwowy Instytut Weterynaryjny, Puławy, PL.
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