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

Capsozyme SB Plus

(FAD-2017-0067; CRL/160054)

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2017-0067 - CRL/160054**
Name of Product:	***Capsozyme SB Plus***
Active Agent (s):	**alpha-galactosidase (3.2.1.22)** **endo-1,4-beta-xylanase (3.2.1.8)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis (EURL-FA)** **29/03/2019**
Report approved by: Date:	**Christoph von Holst** **29/03/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4 (1) for a preparation of *alpha-galactosidase* and *endo-1,4-beta-xylanase* (*Capsozyme SB Plus)* under the category/functional group (4 a) "zootechnical additives"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive* for chickens and minor poultry species for fattening and chickens reared for laying.

According to the Applicant, *Capsozyme SB Plus* is a preparation containing the following two enzymes:

- *alpha-galactosidase* produced by *Aspergillus tubingensis* (ATCC SD6740) and
- *endo-1,4-beta-xylanase* produced by *Trichoderma longibrachiatum* (CBS 139997)

The Applicant expressed the *alpha-galactosidase* and *endo-1,4-beta-xylanase* activities in different units defined as follows:

- one unit of *alpha-galactosidase* activity (GALU) is defined as the amount of enzyme which degrades one micromole per minute of para-nitrophenyl-alpha-D-galactopyranoside at pH 5.5 and 37 °C; and
- one unit of *endo-1,4-beta-xylanase* activity (AXC) is the amount of enzyme, which liberates 0.058 micromoles per minute of reducing sugars, expressed as xylose equivalents, from a wheat arabinoxylan substrate at pH 4.7 and 30 °C.

According to the Applicant, *Capsozyme SB Plus* has a minimum enzyme activity of 40 GALU/g for *alpha-galactosidase* and of 50 AXC/g for *endo-1,4-beta-xylanase.* The product is intended to be incorporated directly in *feedingstuffs* or through *premixtures* with the following recommended enzyme activities in *feedingstuffs*: 14 GALU/kg for *alpha-galactosidase* and 18 AXC/kg for *endo-1,4-beta-xylanase*.

For the quantification of the active substances in the *feed additive, premixtures* and *feedingstuffs* the Applicant submitted different single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis of the correspondent substrates by *alpha-galactosidase* and *endo-1,4-beta-xylanase*. According to the results provided by the Applicant in the frame of the respective validation and verification studies, the EURL recalculated relative standard deviations for repeatability (RSD_r) and for intermediate precision (RSD_{ip}) ranging from 1.6 to 15 % and from 1.5 to 18 %, respectively.

Based on the performance characteristics available the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric methods for the quantification of *alpha-galactosidase* and *endo-1,4-beta-xylanase* 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Capsozyme SB Plus, alpha-galactosidase, endo-1,4-beta-xylanase, zootechnical additives, digestibility enhancers, chickens and minor poultry species for fattening and chickens reared for laying

1. BACKGROUND

In the current application authorisation is sought under Article 4 (1) (new *feed additive*) for *Capsozyme SB Plus*, under the category/functional group (4 a) "zootechnical additives"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003 [1-3] . The authorisation is sought for the use of the *feed additive* for chickens and minor poultry species for fattening and chickens reared for laying [1-3] .

According to the Applicant, *Capsozyme SB Plus* is a preparation containing the following two enzymes [4-5]:

- *alpha-galactosidase* produced by *Aspergillus tubingensis* (ATCC SD6740) and
- *endo-1,4-beta-xylanase* produced by *Trichoderma longibrachiatum* (CBS 139997)

The Applicant expressed the enzyme activities in different units defined as follows:

- one unit of *alpha-galactosidase* activity (GALU) is defined as the amount of enzyme which degrades one micromole per minute of para-nitrophenyl-alpha-D-galactopyranoside at pH 5.5 and 37 °C; and
- one unit of *endo-1,4-beta-xylanase* activity (AXC) is the amount of enzyme, which liberates 0.058 micromoles per minute of reducing sugars, expressed as xylose equivalents, from a wheat arabinoxylan substrate at pH 4.7 and 30 °C.

Capsozyme SB Plus has a minimum enzyme activity of 40 GALU/g for *alpha-galactosidase* and 50 AXC/g for *endo-1,4-beta-xylanase* [6]. The product is intended to be incorporated directly in *feedingstuffs* or through *premixtures* with the following minimum enzyme activities in *feedingstuffs*: 14 GALU/kg for *alpha-galactosidase* and 18 AXC/kg for *endo-1,4-beta-xylanase* [3, 7].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL 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 *Capsozyme SB Plus* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of ***alpha-galactosidase*** in the *feed additive, premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released 4-nitrophenol [8]. The assay is based on the enzymatic reaction of *alpha-galactosidase* on the para-nitrophenyl-alpha-D-galactopyranoside substrate at pH 5.5 and 37 °C.

The *feed additive* sample (2 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride dihydrate (0.0147 %), the solution is stirred for 20 minutes at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.5 ml) is placed into a test tube together with 1 ml of the para-nitrophenyl-alpha-D-galactopyranoside substrate (0.12 mM) and incubated at 37 °C for 15 minutes.

The *premixture* sample (20 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and bovine serum albumin (BSA) (1.2 %), the solution is then stirred for 20 minutes at room temperature. Low activity premixtures (< 5 GALU/g) require an additional desalting step through a PD10 column. The eluted solution (0.5 ml) is placed into a test tube together with 1 ml of the para-nitrophenyl-alpha-D-galactopyranoside substrate (0.12 mM) and incubated at 37 °C for 15 minutes.

The sample of *feedingstuffs* (10 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride dihydrate (0.0147 %), the solution is stirred for 30 minutes at room temperature and centrifuged at 1800 to 2000 g for 10 minutes. The obtained solution is passed through a PD10 column. The eluted solution (0.5 ml) is placed into a test tube together with 1 ml of the para-nitrophenyl-alpha-D-galactopyranoside substrate (0.12 mM) and incubated at 37 °C for 60 minutes.

After incubation, 2.5 ml of a 0.0625 M borax-sodium hydroxide buffer solution at pH 9.7 is added to all samples of the *feed additive*, *premixtures* and *feedingstuffs*, allowed to cool down at least for 15 minutes and centrifuged at 2000 g for 5 minutes. The absorbance of the solution is then measured against water at 405 nm using a 4-nitrophenol external calibration curve [8].

For the quantification of ***endo-1,4-beta-xylanase*** in the *feed additive* and *premixtures* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released xylose with dinitrosalycilic acid (DNS) [10]. The assay is based on the enzymatic hydrolysis by xylanase of the wheat arabinoxylan substrate at pH 4.7 and 30 °C.

For the *feed additive* and *premixtures*, aliquots of 2 and 20 g, respectively, are mixed with 0.25 M acetate buffer (pH 4.5), containing Tween (0.01 %) and bovine serum albumin (BSA) and further stirred for 20 minutes. An aliquot (0.05 ml) of the solution is placed into a test tube, together with 0.45 ml of wheat arabinoxylan 1 % solution in 0.1 M acetate buffer (pH 4.7), and incubated at 30 ^{o}C for 20 minutes [10].

The reaction is stopped by adding 0.75 ml of a dinitrosalycilic acid (DNS) solution (10 g/l). Then all the samples are covered with aluminium foil and placed in a boiling water bath for 10 minutes. Afterwards the samples are quickly cooled down to room temperature with cold water, diluted with deionised water and mixed again. Finally, the absorbance is measured against a blank at 540 nm. External calibration is performed with water solutions of a commercial xylose standard.

For the *feedingstuffs*, the Applicant proposed a different method [11] involving the extraction of the enzyme from the feed before action on an azo-xylan substrate. Feed aliquots of 20 g are mixed with 100 ml of 50 mM citrate-Na-phosphate buffer at pH 4.8 for 30 minutes followed by 10 minutes centrifugation. An aliquot (0.5 ml) of the supernatant was mixed with the same volume of a 1 % substrate solution and incubated at 50 ^{o}C for 3 h. The reaction is stopped by adding 2 ml of ethanol. After 30 minutes, the samples were centrifuged for 10 minutes. Finally, the absorbance of the supernatant is measured at 585 nm. Calibration is performed with blank feed fortified with the *feed additive* (*Capsozyme SB Plus*) with a known enzyme activity expressed in AXC units [12]. The calibrants are submitted in parallel to the same analytical procedure. As the blank feed is normally not available for the control laboratories the EURL requested to the Applicant to replace the blank feed with heat-treated wheat. Consequently, following the EURL request, the Applicant provided evidence proving the suitability of both calibration approaches [13].

Table 1 presents the performance characteristics based on experimental data obtained by the Applicant in the frame of the validation [14-15] and verification [16-17] studies. Additionally,

the Applicant reported limits of quantification (LOQ) of 4.9 AXC and 5.0 GALU/kg *feedingstuffs* for *endo-1,4-beta-xylanase* and *alpha-galactosidase,* respectively.

Furthermore experimental data for *premixtures* obtained by the Applicant in the frame of the stability studies [20] led to similar precisions (recalculated by the EURL [18-19]).

Table 1: Performance characteristics of analytical methods for the determination of *endo-1,4-beta-xylanase* and *alpha-galactosidase* in the *feed additive* (*Capsozyme SB Plus*) and in *feedingstuffs.*

	Matrix	Mean activity (Unit/kg)		RSD_r (%)*		RSD_{ip} (%)*		R_{rec} (%)	
Active Substance		Valid.	Verif.	Valid.	Verif.	Valid.	Verif.	Val.	Verif.
alpha-galactosidase [14, 16]	FS	19	18	15	15	18	18	98	95
	FA	33000	40000	1.6	5.9	7.0	7.1	100	100
endo1,4-β-xylanase [15, 17]	FS	22.2	23.7	6.6	4.8	8.5	5.0	108	97
	FA	56000	53000	1.9	1.5	3.3	1.5	99	107

RSD_r: relative standard deviation for *repeatability;* RSD_{ip}: relative standard deviation for *intermediate precision*; R_{rec}: recovery rate; Valid.: validation; Verif.: verification; (*) Recalculated by the EURL

Based on the performance characteristics available, the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric methods for the quantification of *endo-1,4-beta-xylanase* and *alpha-galactosidase* in the *feed additive, premixtures* and *feedingstuffs.*

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not considered necessary by the EURL.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the single laboratory validated and further verified colorimetric methods submitted by the Applicant for the quantification of *alpha-galactosidase* and *endo-1,4-beta-xylanase* in the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *alpha-galactosidase* in the *feed additive*, *premixtures* and *feedingstuffs*:

- colorimetric method based on the enzymatic reaction of *alpha-galactosidase* on the para-nitrophenyl-alpha-D-galactopyranoside substrate

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

- colorimetric method based enzymatic reaction of *endo-1,4-beta-xylanase* i) on a wheat arabinoxylan substrate (for the *feed additive* and *premixtures*) and ii) on an azo-xylan substrate (for *feedingstuffs*)

One unit of *alpha-galactosidase* activity (GALU) is defined as the amount of enzyme which degrades one micromole per minute of para-nitrophenyl-alpha-D-galactopyranoside at pH 5.5 and 37 °C.

One unit of *endo-1,4-beta-xylanase* activity (AXC) is the amount of enzyme, which liberates 0.058 micromoles per minute of reducing sugars, expressed as xylose equivalents, from a wheat arabinoxylan substrate at pH 4.7 and 30 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Capsozyme SB Plus* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] Application, Reference SANTE/E5: FORW. APPL. 1831-0055-2017
[2] *Application, Annex 1 (Submission No:1511796463996-2156)
[3] *Application, Proposal for Register Entry – Annex A
[4] *Technical dossier, Section II: 2.2 Characterisation of the active substance(s)/agent(s)
[5] *Technical dossier, Section II-Annex_II_20
[6] *Technical dossier, Section II: 2.1 Identity of the additive
[7] *Technical dossier, Section II: 2.5 Conditions of use
[8] *Technical dossier, Section II-Annex_2.6.1a
[9] *Technical dossier, Section II-Annex 2.6.1b
[10] Supplementary information, Annex 2a & 2b
[11] Supplementary information, Annex 2c

[12] Supplementary information, Annex 1

[13] Supplementary information, Annex 3

[14] *Technical dossier, Section II- Annex 2.6.1e & 2.6.1.g (val galact FA &FS)

[15] Supplementary information, Annex 3c & 3d

[16] *Technical dossier, Section II- Annex 2.6.1.i & 2.6.1.k (verf galact FA &FS)

[17] Supplementary information, Annex 3a & 3b

[18] Supplementary information, ANOVA_galactosidase.pdf

[19] Supplementary information, ANOVA_xylanose.pdf

[20] *Technical dossier, Section II: 2.4.1 Stability

*Refers to Dossier no: FAD-2017-0067

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)