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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

Axtra® XAP 104 TPT

(FAD-2017-0053; CRL/170049)

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-2017-0053 - CRL/170049**
Name of Product :	***Axtra® XAP 104 TPT***
Active Agent (s):	**Endo-1,4-beta-xylanase (3.2.1.8)** **alfa-amylase (3.2.1.1)** **protease (3.4.21.62)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Stefano Bellorini (EURL-FA)** **17/05/2018**
Report approved by: Date:	**Christoph von Holst** **18/05/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4 (1) for *Axtra® XAP 104 TPT* under the category/functional group (4 a) "zootechnical additive"/"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 for fattening and reared for laying, laying hens and all minor poultry species.

According to the Applicant, *Axtra® XAP 104 TPT* is a preparation containing *endo-1,4-β-xylanase, α-amylase* and *protease.* The Applicant expressed the enzyme activities in different units defined as follows:

- one unit of *endo-1,4-β-xylanase* activity (U_X) is the amount of enzyme, which liberates 0.48 micromoles per minute of reducing sugars, expressed as xylose equivalents, from a wheat arabinoxylan substrate at pH 4.2 and 50 °C;
- one unit of *α-amylase* activity (U_A) is the amount of enzyme required to release, in the presence of an excess of α-glucosidase, 0.20 micromoles per minute of glucosidic linkages, expressed as p-nitrophenol equivalents, from a maltoheptasoide substrate at pH 8.0 and 40 °C; and
- one unit of *protease* activity (U_P) is the amount of enzyme which liberates 2.3 micrograms per minute of phenolic compounds, expressed as tyrosine equivalents, from a casein substrate at pH 10.0 and 50 °C

According to the Applicant, *Axtra® XAP 104 TPT* has a guaranteed minimum enzyme activity of 20000 U_X/g *endo-1,4-β-xylanase,* 2000 U_A/g *α-amylase* and 4000 U_P/g *protease.* The product is intended to be incorporated directly in *feedingstuffs* or through *premixtures* with the following proposed minimum enzyme activities in *feedingstuffs*: 1000 U_X/kg for *endo-1,4-β-xylanase;* 100 U_A/kg for *α-amylase* and 2000 U_P/kg for *protease*.

For the quantification of the active substances in the *feed additive, premixtures* and *feedingstuffs* the Applicant submitted three single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis:

- by xylanase of an azurine cross-linked wheat arabinoxylan substrate at pH 4.2 and 50 °C for the determination of *endo-1,4-β-xylanase*;
- by amylase of an azurine cross-linked starch polymer substrate at pH 8.0 and 40 °C for the determination of *α-amylase*; and
- by protease of a dyed cross-linked casein substrate at pH 10.0 and 50 °C for the determination of *protease*.

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 the three enzymes 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

Axtra® XAP 104 TPT, *endo-1,4-β-xylanase*, *α-amylase*, *protease,* zootechnical, digestibility enhancers, chickens for fattening and reared for laying, laying hens and minor poultry species

1. BACKGROUND

In the current application authorisation is sought under Article 4 (1) (new *feed additive*) for *Axtra® XAP 104 TPT*, under the category/functional group (4 a) "zootechnical additive"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003 [1][2][3]. The authorisation is sought for the use of the *feed additive* for chickens for fattening and reared for laying, laying hens and minor poultry species [1][2][3].

According to the Applicant, *Axtra® XAP 104 TPT* is a preparation containing the following three enzymes [4][5]:

- *endo-1,4-β-xylanase* produced by *Trichoderma reesei* (ATCC PTA-5588)
- *α-amylase* produced by *Bacillus licheniformis* (ATCC SD-6525) and
- *protease* produced by *Bacillus subtillis* (ATCC SD-2107)

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

- one unit of *endo-1,4-β-xylanase* activity (U_X) is the amount of enzyme, which liberates 0.48 micromoles per minute of reducing sugars, expressed as xylose equivalents, from a wheat arabinoxylan substrate at pH 4.2 and 50 °C;
- one unit of *α-amylase* activity (U_A) is the amount of enzyme required to release, in the presence of an excess of α-glucosidase, 0.20 micromoles per minute of glucosidic linkages, expressed as p-nitrophenol equivalents, from maltoheptasoide substrate at pH 8.0 and 40 °C; and
- one unit of *protease* activity (U_P) is the amount of enzyme which liberates 2.3 micrograms per minute of phenolic compounds, expressed as tyrosine equivalents, from a casein substrate at pH 10.0 and 50 °C.

Axtra® XAP 104 TPT has a guaranteed minimum enzyme activity of 20000 U_X/g *endo-1,4-β-xylanase*, 2000 U_A/g *α-amylase* and 4000 U_P/g *protease* [3][6]. The *feed additive* is intended to be incorporated through *premixtures* or directly into *feedingstuffs* at minimum enzyme activities in *feedingstuffs* of 1000 U_X/kg for *endo-1,4-β-xylanase;* 100 U_A/kg for *α-amylase* and 2000 U_P/kg for *protease* [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 *Axtra® XAP 104 TPT* 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 ***endo-1,4-β-xylanase*** in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of the water soluble dyed fragments produced by the action of *xylanase* on a commercially available azurine cross-linked wheat arabinoxylan substrate (Xylazyme tablets) at pH 4.2 and 50 °C [9].

For the *feed additive*, aliquots of 1 g are mixed with 100 ml of 0.2 M acetate buffer (pH 4.2), stirred for 20 min and filtered. An aliquot (0.1 ml) of the filtrate is diluted with the acetate buffer (0.4 ml), a Xylazyme tablet is added and then incubated at 50 ^oC for 10 min.

For the *premixtures*, a solid dilution with heat treated wheat is initially carried out in order to obtain the appropriate activity. Two aliquots (10 g) of the diluted *premixtures* are mixed with 100 ml of 0.2 M acetate buffer (pH 4.2), stirred for 10 min and filtered. An aliquot (0.1 ml) of the filtrate is diluted with the acetate buffer (0.4 ml), a Xylazyme tablet is added and then incubated at 50 ^oC during 60 min.

For the *feedingstuffs*, two aliquots of 5 g are mixed with 50 ml of 0.2 M acetate buffer (pH 4.2) stirred for 10 min and filtered. An aliquot (0.1 ml) of the filtrate is then diluted with

the acetate buffer (0.4 ml), a Xylazyme tablet is then added, and incubated at 50 °C during 60 min.

The reaction is stopped by adding 5 ml of a stop solution (TRIS solution 2 %). Samples are vigorously mixed, let cool down for 5 min and mixed again. Finally, the solutions are centrifuged and the absorbance is measured against a blank at 590 nm. External calibration is performed with xylanase standards prepared on blank feed sample (for *premixtures* and *feedingstuffs*) or on the acetate buffer (for the *feed additive*) and using a reference standard with a known enzyme activity expressed in U_X and available from the Applicant upon request. The calibrants are submitted in parallel to the same analytical procedure than the respective sample's supernatants. Tables 1 and 2 present the performance characteristics reported by the Applicant based on experimental data obtained in the frame of the validation [10] and verification [11] studies. Additionally, the Applicant reported a limit of quantification (LOQ) of 185 U_X/kg *feedingstuffs*.

For the quantification of ***α-amylase*** in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of the water soluble dyed fragments released by the hydrolysis of the glucosidic linkages by α-amylase on a commercially available azurine cross-linked starch polymer substrate (Amylazyme tablets) at pH 8.0 and 40 °C [12].

For the *feed additive*, aliquots of 1 g are mixed with 100 ml of 0.1 M TRIS buffer (pH 8.0) containing $CaCl_2$ (5 mM), stirred for 20 min, filtered and diluted with the same buffer to reach the appropriate activity. An Amylazyme tablet is then added to 1 ml of the diluted solution, and incubated at 40 °C during 10 min.

For the *premixtures*, a solid dilution with cereal is initially carried out in order to obtain the appropriate activity. Then two 10 g aliquots of the diluted *premixtures* are mixed with 100 ml of 0.1M TRIS buffer (pH 8.0) containing $CaCl_2$ (5 mM), stirred for 10 min and filtered. An aliquot (0.5 ml) of the filtrate is then diluted with the buffer (0.5 ml), an Amylazyme tablet is added, and incubated at 40 °C during 60 min.

For the *feedingstuffs* two aliquots of 5 g are mixed with 50 ml of 0.1M TRIS buffer (pH 8.0) containing $CaCl_2$ (5 mM), stirred for 10 min and filtered. An aliquot (0.5 ml) of the filtrate is then diluted with the buffer (0.5 ml), an Amylazyme tablet is added, and finally incubated at 40 °C during 60 min.

The reaction is stopped by adding 10 ml of a stop solution (TRIS solution 2 %). The samples are vigorously mixed, let cool down for 5 min and centrifuged. The absorbance of the resulting solutions is finally measured against a blank at 590 nm. External calibration is performed with amylase standards prepared on blank feed sample (for *premixtures* and

feedingstuffs) or on the TRIS buffer (for the *feed additive*) using a reference standard with a known enzyme activity expressed in U_A and available from the Applicant upon request. The calibrants are submitted in parallel to the same analytical procedure than the respective sample's supernatants. Tables 1 and 2 present the performance characteristics reported by the Applicant based on experimental data obtained in the frame of the validation [13] and verification [14] studies. Additionally, the Applicant reported a limit of quantification (LOQ) of 74 U_A/kg *feedingstuffs*.

For the quantification of ***protease*** in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of the water soluble dyed fragments released by the hydrolysis of protease on a commercially available dyed cross-linked casein substrate (Protazyme tablets) at pH 10.0 and 50 °C [15].

For the *feed additive*, aliquots of 1 g are mixed with 100 ml of 0.2 M TRIS buffer (pH 10.0), stirred for 20 min, filtered and diluted with the same buffer to reach the appropriate activity. An aliquot (1.0 ml) of the filtrate is then diluted with the buffer (1.0 ml), a Protazyme tablet is added and incubated at 50 ^{o}C during 20 min.

For the *premixtures*, a solid dilution with wheat is initially carried out in order to obtain the appropriate activity. Then, two 10 g aliquots of the diluted *premixtures* are mixed with 50 ml of 0.2 M TRIS buffer (pH 10.0), stirred for 10 minutes, centrifuged and filtered. An aliquot (1.0 ml) of the filtrate is then diluted with the buffer (1.0 ml), a Protazyme tablet is added and incubated at 50 ^{o}C during 60 min.

For the *feedingstuffs* two aliquots of 5 g are mixed with 50 ml of 0.2 M TRIS buffer (pH 10.0), stirred for 10 min, centrifuged and filtered. An aliquot (1.0 ml) of the filtrate is then diluted with the buffer (0.5 ml), a Protazyme tablet is added and incubated at 50 ^{o}C during 60 min.

Table 1: Performance characteristics of analytical methods for the determination of three enzymes in the *feed additive* (*Axtra® XAP 104 TPT*).

	Mean activity (Unit/g)		**RSD_r (%)**		**RSD_{ip} (%)**		**R_{rec} (%)**	
Active Substance	**Valid.**	**Verif.**	**Valid.**	**Verif.**	**Valid.**	**Verif.**	**Val.**	**Verif.**
endo1,4-β-xylanase [10][11]	15682	17375	9.5	6.2	11.1	7.0	100	111
α-amylase [13][14]	5583	5623	3.3	3.6	8.8	3.8	100	101
protease [16]	17273	17897	3.1	6.4	7.4	6.9	100	104

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

Table 2: Performance characteristics of analytical methods for the determination of three enzymes in *premixtures*

	Mean activity (Unit/kg)		RSD_r (%)		RSD_{ip} (%)		R_{rec} (%)	
Active Substance	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.	Val.	Verif.
endo1,4-β-xylanase [10][11]	128138	120407	3.9	6.6	6.1	6.6	100	94
α-amylase [13][14]	52450	55073	7.2	11.8	8.8	11.8	101	107
protease [18][19]	203093	159790	8.7	9.8	14.7	21.0	100	79

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

The reaction is stopped by adding 5 ml of a stop solution (Tri-sodium-phosphate 20 g/l). The samples are then placed in an ice-water bath for 10 s, vigorously mixed and centrifuged. The absorbance of the resulting solutions is finally measured against a blank at 590 nm. External calibration is performed with protease standards prepared on blank feed sample (for *premixtures* and *feedingstuffs*) or on the assay buffer (for the *feed additive*) using a reference standard with a known enzyme activity expressed in U_P and available from the Applicant upon request. The calibrants are submitted in parallel to the same analytical procedure than the respective sample's supernatants. Tables 1, 2 and 3 present the performance characteristics reported by the Applicant based on experimental data obtained in the frame of the validation and verification [16][17][18][19] studies. Additionally, the Applicant reported a limit of quantification (LOQ) of 1289 U_P/kg *feedingstuffs*.

The Applicant performed validation and verification studies for the analysis of each of the investigated enzymes in relevant matrices leading to acceptable performance characteristics for the quantification of the three target enzymes in the *feed additive* (Table 1), in *premixtures* (Table 2) and in *feedingstuffs* (Table 3).

Table 3: Performance characteristics of analytical methods for the determination of three enzymes in *feedingstuffs*.

	Mean activity (Unit/kg)		RSD_r (%)		RSD_{ip} (%)		R_{rec} (%)		LOQ (Unit/kg)	Min. activity
Active Substance	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.	Valid.	
endo1,4-β-xylanase [10][11]	3064	1772	3.9	5.8	6.1	4.0	93-95	97	185	1000
α-amylase [13][14]	299	257	8,5	7.1	10.5	6.6	99	97	74	100
protease [17][19]	2173	1981	8.7	5.8	11.4	9.3	93-98	92	1289	2000

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec}: recovery rate; Valid.: validation; Verif.: verification; LOQ: limit of quantification;

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 the three enzymes 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)

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)

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 by the EURL.

4. CONCLUSIONS AND RECOMMENDATIONS

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

Recommended text for the register entry (analytical method)

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

- colorimetric method, based on the enzymatic hydrolysis by xylanase of an azurine cross-linked wheat arabinoxylan substrate at pH 4.2 and 50 °C

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

- colorimetric method based on the enzymatic hydrolysis by amylase of an azurine cross-linked starch polymer substrate at pH 8.0 and 40 °C

For the quantification of *protease* in *feed additive* and *premixtures* and *feedingstuffs*:

- colorimetric method based on the enzymatic hydrolysis by protease of a dyed cross-linked casein substrate at pH 10.0 and 50 °C.

One unit of *endo-1,4-β-xylanase* activity (U_X) is the amount of enzyme, which liberates 0.48 micromoles per minute of reducing sugars, expressed as xylose equivalents, from a wheat arabinoxylan substrate at pH 4.2 and 50 °C.

One unit of α-amylase activity (U_A) is the amount of enzyme required to release, in the presence of an excess of α-glucosidase, 0.20 micromoles per minute of glucosidic linkages, expressed as p-nitrophenol equivalents, from a maltoheptasoide substrate at pH 8.0 and 40 °C.

One unit of protease activity (U_P) is defined as the amount of enzyme which liberates 2.3 micrograms per minute of phenolic compounds, expressed as tyrosine equivalents from a casein substrate at pH 10.0 and 50 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Axtra® XAP 104 TPT* 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. 1831-0042-2017
[2] *Application, Annex 1 (Submission No: 1501775849052-2114)
[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_II_41; II_42 & II_43
[9] *Technical dossier, Section II-Annex_II_44 & II_45
[10] *Technical dossier, Section II-Annex_II_46; II_47 & II_48
[11] *Technical dossier, Section II-Annex_II_49; II_50 & II_51
[12] *Technical dossier, Section II-Annex_II_52 & II_53
[13] *Technical dossier, Section II-Annex_II_54; II_55 & II_56
[14] *Technical dossier, Section II-Annex_II_57; II_58 & II_59
[15] *Technical dossier, Section II-Annex_II_60 & II_62
[16] Supplementary information, Annex S-1
[17] Supplementary information, Annex S-2
[18] Supplementary information, Annex S-3

*Refers to Dossier no: FAD-2017-0053

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

The following National Reference Laboratories contributed to this report:

- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)