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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

Amylofeed®
(FAD-2010-0353; CRL/100264)

European Union Reference Laboratory
Feed Additives

European Union Reference Laboratory

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

Dossier related to:	**FAD-2010-0353 - CRL/100264**
Name of Additives:	**Amylofeed® (E1612)**
Active Substance(s):	**- endo-1,3(4)-beta-glucanase (EC 3.2.1.6);** **- endo-1,4-beta-xylanase (EC 3.2.1.8);** **- alpha-amylase (EC 3.2.1.1)**
Rapporteur Laboratory:	**European Reference Laboratory for Feed Additives (EURL-FA)**
Report prepared by:	**Johanna Keltti (EURL-FA)**
Report revised by: Date:	**Piotr Robouch (EURL-FA)** **21/02/2013**
Report approved by: Date:	**Christoph von Holst** **22/02/2013**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) and 10(2) for *Amylofeed® (E1612)* 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. *Amylofeed®* was already authorised under the Commission Regulations (EC) No 1453/2004. The authorisation is sought for the use of the *feed additive* for weaned piglets and young minor porcine species.

According to the Applicant, *Amylofeed®* is a preparation containing the following three active substances added to a 60 % dried, ground barley malt sprouts carrier: *endo-1,3(4)-beta-glucanase*; *endo-1,4-beta-xylanase* and *alpha-amylase*; with a guaranteed minimum enzyme activity of 275, 400 and 3100 U/g, respectively.

The Applicant defined the enzyme activity units as follows:

- One *endo-1,3(4)-beta-glucanase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 4.0 and 30 ºC;
- One *endo- 1,4-beta-xylanase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from rye arabinoxylan per minute at pH 4.0 and 30 ºC; and
- One *alpha-amylase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from wheat starch per minute at pH 5.0 and 30 ºC.

Amylofeed® is intended to be used in *premixtures* and/or complete *feedingstuffs* for the above mentioned species, with the following proposed minimum enzyme activities for *endo-1,3(4)-beta-glucanase, endo-1,4-beta-xylanase* and *alpha-amylase*: 138, 200 and 1550 U/kg *feedingstuffs*, respectively.

For the determination of the activity of *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-xylanase* in the *feed additive, premixtures and feedingstuffs* the Applicant provided experimental data and the same analytical methods that were previously evaluated and recommended by EURL in the report on *Endofeed® DC* (FAD-2009-0015). The recommendations provided therein are considered appropriate for the *Amylofeed®* dossier under evaluation.

For the determination of the activity of *alpha-amylase* in the *feed additive* the Applicant proposed a single laboratory validated and further verified colorimetric method based on the enzymatic reaction of amylase on wheat starch substrate in the presence of 3,5-dinitrosalicylic acid (DNS) at pH 4.0 and 30 ºC.

For the determination of the activity of *alpha-amylase* in the *feedingstuffs* the Applicant proposed a single laboratory validated and further verified colorimetric Ceralpha method (Megazyme; AOAC 2002.01), a colorimetric method measuring depolymerised soluble fragments released by action of the *amylase* on oligosaccharide "non-reducing-end blocked p-nitrophenyl maltoheptaoside".

The Applicant presented the following performance characteristics, derived from the validation and verification studies, for the determination of alpha-amylase in the *feed additive* and *feedingstuffs*:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2 to 10 %;
- a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 4.5 to 15 %;
- a recovery rate (R_{Rec}) ranging from 99 to 120 %; and
- a limit of quantification of 350 U/kg *feedingstuffs*.

For the determination of the enzyme activities in *premixtures*, the Applicant suggested to dilute *premixture* samples with blank feeds (such as heat treated wheat flour) and to analyse it applying the method for *feedingstuffs* presented above.

Based on the satisfactory experimental evidence available the EURL recommends for official control the two single-validated and further verified colorimetric methods submitted by the Applicant for the determination of *alpha-amylase* 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

Amylofeed®, endo-1,3(4)-beta-glucanase, endo-1,4-beta-xylanase, alpha-amylase, zootechnical additives, digestibility enhancers, weaned piglets, young minor porcine species.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use) and 10(2) (re-evaluation of an existing authorised additive) for *Amylofeed® (E1612)* under the category/functional group 4(a) "zootechnical additives"/"digestibility enhancers", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. *Amylofeed® (E1612)* was already authorised for weaned piglets under the Commission Regulations (EC) No 1453/2004. The authorisation is sought for the use of the *feed additive* for weaned piglets and young minor porcine species [2].

According to the Applicant, *Amylofeed®* is a preparation containing the following three active substances added to a 60 % dried, ground barley malt sprouts carrier: - *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-xylanase* produced by *Aspergillus niger (phoenicis)* (NRRL 25541) and *alpha-amylase* produced *by Aspergillus oryzae* (ATCC 66222); with a guaranteed minimum enzyme activity of 275, 400 and 3100 U/g, respectively [2, 3].

While reviewing the information provided by the Applicant the EURL identified an inconsistency related to the definition of the activity units of the various enzymes of interest. Upon request for clarification by the EURL, the Applicant clarified the enzyme activity units [4] as follows:

- One *endo-1,3(4)-beta-glucanase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 4.0 and 30 ºC;

- One *endo- 1,4-beta-xylanase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from rye arabinoxylan per minute at pH 4.0 and 30 ºC; and

- One *alpha-amylase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from wheat starch per minute at pH 5.0 and 30ºC.

Amylofeed® is intended to be used in *premixtures* and/or complete *feedingstuffs* for the above mentioned species, with the following proposed minimum enzyme activities for *endo-1,3(4)-beta-glucanase, endo-1,4-beta-xylanase* and *alpha-amylase*: 138, 200 and 1550 U/kg *feedingstuffs*, respectively [2], which corresponds to 500 g of *Amylofeed®* / ton *feedingstuffs*.

The EURL has previously evaluated the analytical methods of the *Endofeed® DC* dossier (cf. report FAD-2009-0015), which has been submitted from the same applicant as the current one. Furthermore, *Endofeed®* contains two enzymes, namely *endo-1,3(4)-beta-glucanase* and *endo-1,4-beta-xylanase* [6], which are also present in *Amylofeed®*.

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 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 *Amylofeed®*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Qualitative and quantitative composition of impurities in the feed 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 European Union Reference Laboratories [5].

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

For the determination of the activity of *endo-1,3(4)-beta-glucanas* and *endo-1,4-beta-xylanase* in the *feed additive, premixtures and feedingstuffs* the Applicant provided experimental data and the same analytical methods that were previously evaluated and recommended by EURL in the report on *Endofeed® DC* (FAD-2009-0015). The recommendations provided therein are considered appropriate for the *Amylofeed®* dossier under evaluation [6].

For the determination of the activity of *alpha-amylase* in the *feed additive* the Applicant proposed a single laboratory validated and further verified colorimetric method based on the

quantification of the reducing sugars (glucose equivalents) released from the wheat starch substrate [7]. Test samples (25 mg) are extracted in 0.1 M acetate buffer (pH 5.0) and incubated with the 0.5 % wheat starch substrate solution at 30 ºC for a maximum of 10 min. The reaction is stopped by adding 3,5-dinitrosalicylic acid (DNS). This sample treatment is also applied to the amylase standard solution (containing 2700 U/g) and to the blank samples (free of any amylase). The amylase content is then determined by colorimetry at 540 nm and quantified against the amylase standard with known activity. The performance characteristics obtained in the frame of the validation [7] and verification [8] studies are presented in Table 1.

Based on the satisfactory experimental evidence available the EURL recommends for official control the colorimetry method submitted by the Applicant for the determination of *alpha-amylase* in *Amylofeed®*.

For the determination of the activity of *alpha-amylase* in the *feedingstuffs* the Applicant proposed a single laboratory validated and further verified colorimetric Ceralpha method (Megazyme; AOAC 2002.01) based on the quantification of the reducing sugars (glucose equivalents) released from the substrate [9]. Test samples (3 g) are extracted in malate extraction buffer (pH 5.4) and incubated with the "non-reducing-end blocked p-nitrophenyl maltoheptaoside" oligosaccharide substrate solution at 40 ºC for a total of 20 min. The reaction is stopped by adding stopping reagent from alpha-amylase kit. The colour change produced is proportional to the amount of reduced sugars released, which itself is proportional to the activity of the enzymes present in the samples. This sample treatment is also applied to blank feed samples (such as heat treated wheat flour, free of any amylase) spiked with different amounts of *Amylofeed®* solutions, with known amylase content. The amylase content is then determined by colorimetry at 400 nm, quantified using the matrix-matched calibration curve and the results are expressed in U/kg. The traceability of the results to the amylase units (U) is ensured by the *Amylofeed®* spike solution characterised against the amylase standard (see *feed additive* method, described above).

The performance characteristics obtained in the frame of the validation [10] and verification [11] studies are presented in Table 1. Furthermore, the Applicant determined a limit of quantification (LOQ) of 350 U/kg [10].

For the determination of *alpha-amylase* in *premixtures*, the Applicant suggested to dilute *premixture* samples with blank feeds (such as heat treated wheat flour) and to analyse it applying the method for *feedingstuffs* presented above [12].

Based on the satisfactory experimental evidence available the EURL recommends for official control the colorimetry methods submitted by the Applicant for the determination of *alpha-amylase* in *premixtures and feedingstuffs.*

Table 1: Performance characteristics of analytical methods for the determination of *alpha-amylase* in *feed additive* (FA) and *feedingstuffs* (FS).

		RSD_r (%)		**RSD_{ip} (%)**		**R_{rec} (%)**	
	Activity	Validation	Verification	Validation	Verification	Validation	Verification
FA	3100-6360 U/g	3.3 [7]	2.6 [8]	8.0 [7]	4.5 [8]	102-120 [7]	99 [8]
FS	450-4360 U/kg	2-10 [10]	7.1 [11]	12 [10]	14.6 [11]	108-114 [10]	117 [11]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec} : recovery rate

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 determination of *endo-1,3(4)-beta-glucanase, endo-1,4-β-xylanase* and *alpha-amylase in Amylofeed® feed additive, premixtures* and *feedingstuffs*.

For the determination of *alpha-amylase* in *premixtures*, the Applicant suggested to dilute *premixture* samples with blank feeds (such as heat treated wheat flour), and to analyse it applying the method for *feedingstuffs.*

Recommended text for the register entry (analytical method)

Determination of *endo-1,3(4)-beta-glucanase* in the *feed additive*:

- Colorimetric method based on the enzymatic reaction of glucanase on barley beta-glucan substrate in the presence of 3,5-dinitrosalicylic acid (DNS) at pH 4.0 and 30 ºC

Determination of *endo-1,4-β-xylanase* in the *feed additive*:

- Colorimetric method based on the enzymatic reaction of xylanase on rye arabinoxylan substrate in the presence of DNS at pH 4.0 and 30 ºC

Determination of *alpha-amylase* in the *feed additive*:

- Colorimetric method based on the enzymatic reaction of amylase on wheat starch substrate in the presence of DNS at pH 5.0 and 30 ºC

Determination of the active substances in *premixtures* and *feedingstuffs*:

- Colorimetric method measuring depolymerised soluble fragments released by action of glucanase on azo-barley-glucan
- Colorimetric method measuring depolymerised soluble fragments released by action of *endo-1,4-β-xylanase* on azo-xylan
- Colorimetric method measuring depolymerised soluble fragments released by action of *amylase* on oligosaccharide "non-reducing-end blocked p-nitrophenyl maltoheptaoside"

One *endo-1,3(4)-beta-glucanase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 4.0 and 30 ºC;

One *endo- 1,4-beta-xylanase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from rye arabinoxylan per minute at pH 4.0 and 30 ºC; and

One *alpha-amylase* unit (U) is the amount of enzyme which liberates 1 micromole of reducing sugars (glucose equivalents) from wheat starch per minute at pH 5.0 and 30 ºC.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference sample of *Amylofeed®* 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] *Reference SANCO/D/2 Forw. Appl. 1831/00186(10017)-2010

[2] *Application, Proposal for Register Entry

[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; methods of analysis

[4] *Supplementary information - NewDefinitionsUnits-PenTec-Amylofeed

[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] FAD-2009-0015 - JRC.DG.D.6/CvH/DM/AG/ARES(2010)531664 http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2009-0015.pdf

[7] *Technical dossier, Section II - Annex_II_6_1_1

[8] *Supplementary information - Annex_II_6_1_5_Updated

[9] *Technical dossier, Section II - Megazyme_2000

[10] *Supplementary information - Annex_II__6_1_2_Updated
[11] *Supplementary information - Annex_II__6_1_11
[12] *Technical dossier, Section II - Annex_II_6_1_3
*Refers to Dossier No. FAD-2010-0353

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
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