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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

Hemicell®
(FAD-2014-0001; CRL/130033)

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-2014-0001 - CRL/130033**
Name of Feed Additive:	**Endo-1,4-β-mannanase (E.C. 3.2.1.78)**
Active Agent (s):	**Endo-1,4-β-mannanase (E.C. 3.2.1.78)**
Rapporteur Laboratory:	**National Research Institute of Animal Production, National Laboratory for Feedingstuffs, Lublin, Poland**
Report prepared by:	**Waldemar Korol (NIARP-NLF)**
Report checked by: Date:	**Zigmas Ezerskis and Piotr Robouch (EURL-FA)** **12/08/2015**
Report approved by: Date:	**Christoph von Holst** **12/08/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *endo-1,4-β-mannanase* (EC 3.2.1.78), 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. The *feed additive* is already authorised under the Commission Regulation (EC) No 786/2007 for chickens for fattening and Commission Implementing Regulation (EU) No 103/2013. According to the Applicant, the *feed additive* contains the active substance *endo-1,4-β-mannanase* (EC 3.2.1.78) produced by the *Paenibacillus lentus* strain.

The *feed additive* is intended to be marketed as solid (*Hemicell®HT*) or liquid (*Hemicell®HT-L*) enzyme preparations, containing a minimum *endo-1,4-β-mannanase* activity of $1.6x10^8$ U/kg and $5.9x10^8$ U/L, respectively. According to the Applicant, the activity of *endo-1,4-β-mannanase* is expressed in units (U), where 1 U is the amount of *endo-1,4-β-mannanase* enzyme which liberates 0.72 micrograms of reducing sugars (mannose equivalents) per minute from mannan-containing substrate (locust bean gum) at pH 7.0 and 40 °C. Specifically, authorisation is sought for the use of *Hemicell®HT* and *Hemicell®HT-L* for a variety of chickens, turkeys and pig species. The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* (*Hemicell®HT* only) at a recommended minimum *endo-1,4-β-mannanase* activity of 32000 U/kg complete *feedingstuffs*.

For the quantification of the *endo-1,4-β-mannanase* activity in the *feed additive* and *feedingstuffs*, the Applicant submitted two single-laboratory validated and further verified colorimetric methods, based on the reaction of reducing sugars (mannose equivalent) released by the action of *endo-1,4-β-mannanase* on a mannan-containing substrate (locust bean gum, LBG) in the presence of 3,5-dinitrosalicylic acid (DNS).

The following performance characteristics of the methods were reported in the frame of validation and verification studies for *endo-1,4-β-mannanase* activity in the *feed additive and feedingstuffs* ranging from 245 to 690 and 0.015 to 10 mega ($x10^6$) units (MU)/kg, respectively: - a relative standard deviation for *repeatability* (RSD_r) ranging from 0.7 to 12.4 %; - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 4.2 to 12.4 %; and - a *recovery rate* (R_{Rec}) ranging from 91 to 126 %. Furthermore, the Applicant reported the limit of quantification (LOQ) of 15000 U/kg *feedingstuffs*, which is below the minimum activity recommended by the Applicant in the conditions of use.

For the quantification of *endo-1,4-β-mannanase* activity in *premixtures* the Applicant diluted premixture samples with blank feed and applied the above mentioned method for *feedingstuffs*. Two vitamin/mineral *premixtures* for poultry and swine were analysed in the

frame of homogeneity studies and a relative standard deviation for *repeatability* (RSD_r) ranging from 15.8 to 17.2% was reported for *endo-1,4-β-mannanase* activity ranging from 50 to 59 MU/kg *premixtures*.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis and the reaction of reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS), for the quantification of total *endo-1,4-β-mannanase* activity 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

Endo-1,4-β-mannanase, *Hemicell*, *Paenibacillus lentus*, zootechnical additives, digestibility enhancers, piglets (weaned), pigs, chickens and turkeys for fattening, chickens reared for laying, turkeys reared for breeding, minor porcine and poultry species

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use of a feed additive already authorised) for *endo-1,4-β-mannanase* (EC 3.2.1.78), under the category / functional group 4(a) "zootechnical additives / digestibility enhancers" [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003. The *feed additive* is already authorised under the Commission Regulation (EC) No 786/2007 for chickens for fattening and Commission Implementing Regulation (EU) No 103/2013.

According to the Applicant, the *feed additive* contains the active substance *endo-1,4-β-mannanase* (EC 3.2.1.78) produced by the *Paenibacillus lentus* strain [2,3].

The *feed additive* is intended to be marketed as solid (*Hemicell®HT*) or liquid (*Hemicell®HT-L*) enzyme preparations [3]. *Hemicell®HT* contains linseed meal, calcium carbonate, mineral oil and *endo-1,4-β-mannanase* with a minimum activity of $1.6x10^8$ U/kg; while *Hemicell®HT-L* contains water, sorbitol, sodium chloride, sodium glutamate and *endo-1,4-β-mannanase* with a minimum activity of $5.9x10^8$ U/L [3].

According to the Applicant, the activity of *endo-1,4-β-mannanase* is expressed in units (U), where 1 U is the amount of *endo-1,4-β-mannanase* enzyme which liberates 0.72 micrograms

of reducing sugars (mannose equivalents) per minute from mannan-containing substrate (locust bean gum) at pH 7.0 and 40 °C [3].

Specifically, authorisation is sought for the use of *Hemicell®HT* and *Hemicell®HT-L* for chickens and turkeys for fattening, chickens reared for laying, turkeys reared for breeding, piglets (weaned), pigs for fattening, minor porcine and poultry species [2]. The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* (*Hemicell®HT* only) at a recommended minimum *endo-1,4-β-mannanase* activity of 32000 U/kg complete *feedingstuffs* [2,3].

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 *endo-1,4-β-mannanase* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

Qualitative and quantitative 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, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [4].

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

For the quantification of the *endo-1,4-β-mannanase* activity in the *feed additive* and *feedingstuffs*, the Applicant submitted two single-laboratory validated and further verified colorimetric methods [5,6], based on the reaction of reducing sugars (mannose equivalent) released by the action of *endo-1,4-β-mannanase* on a mannan-containing substrate (locust bean gum, LBG) in the presence of 3,5-dinitrosalicylic acid (DNS).

For the quantification of *endo-1,4-β-mannanase* activity in Hemicell®HT-L samples (1g) are diluted with Glycine buffer (pH 8.5) containing Bovine Serum Albumin (BSA) and pre-incubated at 40 °C for 15 minutes [5]. Hemicell®HT samples (10 g) are extracted in distilled

water, shaken at 250 RPM for 40 minutes at room temperature, an aliquot diluted with Glycine buffer with BSA (pH 8.5) and pre-incubated under the same conditions as Hemicell®HT-L. All samples are then incubated with LBG substrate at 40 °C for 10 min. DNS is added to the incubated and non-incubated samples to stop the reaction. Samples are further reacted for 5 to 15 minutes in boiling bath and centrifuged, then cooled to room temperature. The absorbance of the yellow-orange coloured solutions is measured spectrophotometrically at 550 nm. The endo-1,4-β-mannanase activity is derived from the difference in absorbance of the incubated and non-incubated with LBG aliquots. The quantification is performed using *endo-1,4-β-mannanase* enzyme external reference standard calibration curve [5]. The reference standards undergo the standard operating procedure described above. The certified enzymatic activity of the reference standard is expressed in units at pH 7.0 and 40 °C, thus ensuring the traceability of the determined *endo-1,4-β-mannanase* activities expressed in U (defined at pH 7.0 and 40 °C) even though the enzyme activity measurements were carried out at different experimental conditions.

For the quantification of *endo-1,4-β-mannanase* activity in *feedingstuffs* the sample (10 g) is first ground and extracted in distilled water, shaken for 40 min and centrifuged [6]. An aliquot of the supernatant is incubated with LBG mannose substrate in Tris buffer (pH 7.0) at 40 °C for 60 min. Aliquots of the incubated samples (for 5-15 min or 50-60 min) are reacted with DNS at 100 °C for 5 to 15 min, centrifuged, then cooled to room temperature. The absorbance of the yellow-orange coloured solutions is measured spectrophotometrically at 550 nm. The difference in absorbance of the two sample aliquots (ΔOD) with different incubation times is proportional to the total *endo-1,4-β-mannanase* activity. Quantification is performed using mannose external standard calibration curve [6].

The method performance characteristics derived from validation [7-9] and verification studies [10-12] are presented in Table 1. In addition, the Applicant reported the limit of quantification (LOQ) of 15000 U/kg *feedingstuffs* [9], which is below the minimum activity recommended by the Applicant in the conditions of use [2].

For the quantification of *endo-1,4-β-mannanase* activity in *premixtures* the Applicant diluted premixture samples with blank feed [3] and applied the above mentioned method for *feedingstuffs* [6]. Two vitamin/mineral *premixtures* for poultry and swine were analysed in the frame of homogeneity studies and a relative standard deviation for *repeatability* (RSD_r) ranging from 15.8 to 17.2% was reported for *endo-1,4-β-mannanase* activity ranging from 50 to 59 MU/kg *premixtures* [3].

Table 1: Performance characteristics for the quantification of *endo-1,4-β-mannanase* total activity in the *feed additive*: *Hemicell®HT* (dry) & *Hemicell®HT-L* (liquid) and *feedingstuffs* (FS)

	Feed additive (Hemicell® HT)		*Feed additive (Hemicell® HT-L)*		*Feedingstuffs*	
	Validation	Verification	Validation	Verification	Validation	Verification
Activity, MU/kg	245 – 249		675 – 690(*)		0.015 – 10	
RSD_r, %	0.9 – 2.2	1.8 – 3.2	1.6 – 4.2	0.7 – 4.0	0.04 – 12.4	1.9 – 18.6
RSD_{ip}, %	5.7	6.1	4.2	6.5	2.3 – 24.1	3.3 – 36.9
R_{Rec}, %	93	94	96	98	93 – 111(**)	91 – 126(**)
Reference	[8]	[11]	[8]	[11]	[9]	[12]

MU: mega (x10^6) units; RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*, respectively; R_{Rec}: a recovery rate; (*) MU/L; (**) recalculated after background subtraction.

For the quantification of *endo-1,4-β-mannanase* in the *feed additive* the Applicant submitted an alternative method using the automatized liquid handling system [13], for which similar performance characteristics were submitted [14,15].

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis and the reaction of reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS), for the quantification of *endo-1,4-β-mannanase* total activity 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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control two single-laboratory validated and further verified colorimetric methods, based on the enzymatic hydrolysis and the reaction of reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS), to quantify the total activity of *endo-1,4-β-mannanase* in the *feed additive*, *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- colorimetric methods based on enzymatic hydrolysis and the reaction of reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS)

One unit of *endo-1,4-β-mannanase* activity (U) is the amount of the enzyme which liberates 0.72 micrograms of reducing sugars (mannose equivalents) from a mannan-containing substrate (locust bean gum) per minute at pH 7.0 and 40 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Hemicell®HT* and *Hemicell®HT-L* 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 SANCO/G1: Forw. Appl. 1831/0001-2014

[2] *Application, Proposal to Register Entry – Annex A

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

[4] 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

[5] *Technical dossier, Section II – Annex II_6_1_1

[6] *Technical dossier, Section II – Annex II_6_1_4

[7] *Technical dossier, Section II – Annex II_6_1_2

[8] *Technical dossier, Section II – Annex II_6_1_2 (updated)

[9] *Technical dossier, Section II – Annex II_6_1_5 (updated)

[10] *Technical dossier, Section II – Annex II_6_1_3

[11] *Technical dossier, Section II – Annex II_6_1_3 (updated)

[12] *Technical dossier, Section II – Annex II_6_1_6 (updated)

[13] *Technical dossier, Section II – Annex II_6_1_8

[14] *Technical dossier, Section II – Annex II_6_1_7

[15] *Technical dossier, Section II – Annex II_6_1_7 (updated)

*Refers to Dossier no: FAD-2014-0001

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was National Research Institute of Animal Production, National Laboratory for Feedingstuffs in Lublin, Poland. 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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- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen, Jena (DE)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid (ES)[1]
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)[2]
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft, Nossen (DE)[3]

Name and address according to Regulation (EC) No 885/2009:

[1] Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid.
[2] Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes.
[3] Sächsische Landesanstalt für Landwirtschaft. Fachbereich 8 – Landwirtschaftliches Untersuchungswesen, Leipzig.