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

Dossier related to:	**FAD-2011-0049 - CRL/110008**
Name of Feed Additive:	**Hemicell**
Active Substance(s):	**Endo-1,4-β-mannanase (E.C. 3.2.1.78)**
Rapporteur Laboratory:	**National Research Institute of Animal Production, National Feed Laboratory, Lublin, Poland**
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Report approved by: Date:	**Christoph von Holst** **17/08/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Hemicell*, 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. According to the Applicant, the *feed additive* contains *endo-1,4-β-mannanase* (EC 3.2.1.78) as the active agent produced by the strain *Bacillus lentus* (ATCC 55045). The additive is intended to be marketed as a liquid enzyme preparation with a target *endo-1,4-β-mannanase* activity of minimum $7.2x10^8$ U/L.

The activity of *endo-1,4-β-mannanase* is expressed in mannanase units (U), where 1 U is the amount of enzyme which liberates 0.72 micrograms of reducing sugars (expressed as mannose equivalents) per minute from a mannan-containing substrate (locust bean gum) at pH 7.5 and 40 ^oC.

Specifically, authorisation is sought for the use of *Hemicell* for turkeys, laying hens, pigs, weaned piglets and all minor poultry and porcine species. The *feed additive* is intended to be used directly in complete *feedingstuffs* at minimum *endo-1,4-β-mannanase* activity of 79200 U/kg.

For the determination of the activity of *endo-1,4-β-mannanase* in the *feed additive* and *feedingstuffs,* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the reaction between reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS). The reducing sugars are produced by the action of *endo-1,4-β-mannanase* on a mannan-containing substrate at pH 7.5 and 40 ^oC. The following method performance characteristics were reported:

- for the *feed additive*, for concentration ranging from 0.69 to 1.12 $x10^9$ U/L: - a relative standard deviation for *repeatability* (RSD_r) ranging from 1.7 to 3.9 %; - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 2.8 to 3.6 %; and – a *recovery rate* (R_{Rec}) of 101 %;
- for *feedingstuffs,* for concentration ranging from 3.96 to 12 $x10^4$ U/kg: - a relative standard deviation for *repeatability* (RSD_r) ranging from 3.0 to 13.0 %; - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 4.6 to 14.0 %; and - a *recovery rate* (R_{Rec}) from 90.6 to 104%.

Furthermore, a limit of quantification (LOQ) was set by the EURL to the lowest enzyme activity measured by the Applicant: LOQ = 2000 U/kg, which is far below the minimum activity recommended by the Applicant in the conditions of use.

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method, based on the reaction between reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS) at pH 7.5 and 40 °C, for the determination of the activity of the *endo-1,4-β-mannanase* in the *feed additive* and *feedingstuffs*, within the concentration range covered by the experimental data.

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

Hemicell, *endo-1,4-β-mannanase*, *Bacillus lentus*, zootechnical additive, digestibility enhancers, turkeys, laying hens, pigs, weaned piglets, all minor poultry and porcine species.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use) for *Hemicell*, 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. According to the Applicant, the *feed additive* contains *endo-1,4-β-mannanase* (EC 3.2.1.78) as the active agent [1,2], produced by the strain *Bacillus lentus* (ATCC 55045). The strain was deposited at the "American Type Culture Collection" (ATCC) in Manassas, VA, USA [3].

The additive is intended to be marketed as liquid enzyme preparation, containing water, sorbitol, sodium chloride and sodium glutamate [4] with a target *endo-1,4-β-mannanase* activity of minimum $7.2x10^8$ U/L [2]

According to the Applicant [4] and to the Commission Regulation (EC) No 786/2007, the activity of *endo-1,4-β-mannanase* is expressed in mannanase units (U); where 1 U is the amount of enzyme which liberates 0.72 micrograms of reducing sugars (expressed as mannose equivalents) per minute from a mannan-containing substrate (locust bean gum) at pH 7.5 and 40 ^oC.

Specifically, authorisation is sought for the use of *Hemicell* for turkeys, laying hens, pigs, weaned piglets and all minor poultry and porcine species. The *feed additive* is intended to be

used directly in complete *feedingstuffs* (not in *premixtures*) [5] at a minimum *endo-1,4-β-mannanase* activity of 79200 U/kg [2].

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 *Hemicell*, 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 additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, mycotoxins and dioxins) are available from the respective European Union Reference Laboratories [6].

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

For the determination of the activity of *endo-1,4-β-mannanase* in the *feed additive* and *feedingstuffs,* the Applicant submitted a colorimetric method [7-11], based on the reaction between reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS). The reducing sugars are produced by the action of *endo-1,4-β-mannanase* on a mannan-containing substrate, locust bean gum (LBG), at pH 7.5 and 40 ^{o}C. The method has already been evaluated by the EURL [12]. However, the Applicant submitted recent experimental data obtained in the frame of validation and verification studies [7, 10-11].

The *feed additive* samples are diluted with Tris buffer (pH 7.5) and pre-incubated at 40 °C for 15 to 20 minutes. The *feedingstuffs* samples (10 g) are first grinded and extracted in distilled water, shaken at 300 RPM for 30 minutes at room temperature, and pre-incubated under the same conditions as feed additive samples [7]. All the samples are incubated with LBG mannose substrate at 40 ^{o}C for 45 min. The reaction is stopped by the addition of DNS, which

together with reducing sugars forms a stable yellow-orange coloured solution. The samples are then incubated for additional 5 minutes in boiling bath, centrifuged for 10 minutes at 2700 RPM and the absorbances of the sample solutions are measured spectrophotometrically at 550 nm, using water as the blank. The quantification is done by using external calibration, using standard D-mannose curve [7].

The method performance characteristics are presented in Table 1. Additionally, a limit of quantification (LOQ) was set by the EURL, to the lowest enzyme activity measured by the Applicant: LOQ = 2000 U/kg, which is far below the minimum activity recommended by the Applicant in the conditions of use. Furthermore, the Applicant performed additional experiments [8,9] showing that interferences from the low level of reducing sugars - potentially present in the feed prior to enzymatic reaction - are negligible [7].

Based on the performance characteristics presented, the EURL recommends for official control the single-laboratory validated and further verified colorimetric method, based on the reaction between reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS) at pH 7.5 and 40 °C, for the determination of the activity of the *endo-1,4-β-mannanase* in the *feed additive* 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.

Table 1: Performance characteristics for the quantification of *endo-1,4-β-mannanase* in the *feed additive (FA)* and *feedingstuffs (FS)*

	Activity	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
		Validation	Verifcation	Validation	Verification	Validation	Verification
FA	$(0.69\text{-}1.12)\times10^9$ U/L	3.9 [7]	1.7 [10]	3.6 [7]	2.8 [10]	-	101 [10]
FS	$(3.96\text{-}12)\times10^4$ U/kg	13 [7]	3.0 [11]	14 [7]	4.6 [11]	104 [7]	90.6 [11]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision,* respectively.
R_{Rec}: a 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 method, submitted by the Applicant, based on the reaction between reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS) at pH 7.5 and 40 °C, to determine the activity of *endo-1,4-β-mannanase* in the *feed additive* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- colorimetric method based on the reaction between reducing sugars (mannose equivalent) with 3,5-dinitrosalicylic acid (DNS) at pH 7.5 and 40 °C.

One unit of activity (U) is the amount of the enzyme which liberates 0.72 microgram of reducing sugars (mannose equivalents) from a mannan-containing substrate (locust bean gum) per minute at pH 7.5 and 40 ^{o}C.

Note: The EURL suggest a wording different from the one included in Commission Regulation (EC) No 786/2007 in order to implement a harmonized description for enzyme activity methods.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Hemicell* 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. 1831/0001-2012
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II, Annex_II_2_1_2_4
[4] *Technical dossier, Section II, 2.1.3 Qualitative and quantitative composition
[5] *Technical dossier, Section II, 2.4.1. Stability
[6] 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
[7] *Technical dossier, Section II, Annex_II_6.1.1 Mannanase assay and method validation
[8] *Technical dossier, Section II, Annex_II_6.1.4 Method validation Nov 2005
[9] *Technical dossier, Section II, Annex_II_6.1.5 Method validation Jun 2006

[10] *Technical dossier, Section II, Annex_II_6.1.2 Additive verification
[11] *Technical dossier, Section II, Annex_II_6.1.3 Feed verification
[12] #FAD-2005-0027 - D08/FSQ/CVH/GS/(2006) D/15162
*Refers to Dossier No. FAD-2011-0049
#http://irmm.jrc.ec.europa.eu/EURLs/EURL_feed_additives/authorisation/evaluation_reports/

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was the 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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