EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

JRC.DG.D.6/CvH/DM/AG/ARES(2010)531664

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

Dossier related to:	**FAD-2009-0015** **CRL/ 090005**
Name of Additive:	**Endofeed ® DC**
Active Substance (s):	**Endo-1,3(4)-β-glucanase (EC 3.2.1.6)** **Endo-1,4-β-xylanase (E.C. 3.2.1.8)**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additives (CRL-FA),** **Geel, Belgium**
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Report approved by: Date:	**Christoph von Holst (CRL-FA)** **24/08/2010**

EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Endofeed® DC* under the category/functional group '4a': digestibility enhancers, according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Endofeed® DC* for chickens for fattening, laying hens, pigs for fattening and for minor avian and porcine species. The *feed additive* consists of two active agents, *endo-1,3(4)-β-glucanase* (EC 3.2.1.6) and *endo-1,4-β-xylanase* (EC 3.2.1.8), produced by the strain *Aspergillus niger* (NRRL 25541).

According to the applicant, enzymatic activity of the active agents is expressed in "U" units, where: (i) One U of *endo-1,3(4)-β-glucanase* is the amount of enzyme that liberates 1 µmol of reducing sugars (glucose equivalents) from oat β-glucan per minute at pH 4.0 and 30°C; and (ii) One U of *endo-1,4-β-xylanase* is the amount of enzyme that liberates 1 µmol of reducing sugars (xylose equivalents) from oat xylan per minute at pH 4.0 and 30°C.

Endofeed® DC has a minimum *endo-1,3(4)-β-glucanase* activity of 1100 U/g and an *endo-1,4-β-xylanase* activity of 1600 U/g, respectively. It is intended to be used in complete *feedingstuffs* with minimum activity of 138 U/kg *endo-1,3(4)-β-glucanase* and 200 U/kg *endo-1,4-β-xylanase*.

For the determination of the activity of *endo-1,3(4)-β-glucanase* in *feed additives* and *premixtures* the applicant proposes a single laboratory validated and further verified colorimetric method based on the quantification of the reducing sugars (glucose equivalents) released from barley β-glucan in the presence of 3,5-dinitrosalicilic acid (DNS) at pH 4.0 and 30°C. For the determination in *feedingstuffs* the applicant proposes a single laboratory validated and further verified colorimetric method based on depolymerisation of soluble fragments from azo-barley-glucan at pH 4.8 and 50°C. The activity of the sample is calibrated against reference enzyme standards with known activity determined at the definition conditions of the activity unit (pH 4.0 and 30°C). The following method performance characteristics were reported for the *feed additive*, *premixtures* and *feedingstuffs*: - a relative standard deviation for *repeatability* (RSD_r) ranging from 1.5 to 8.5 %, - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 6.3 to 11 %, - a *recovery rate* (R_{Rec}) ranging from 90 to 126 %; and a limit of detection (LOD) and a limit of quantification (LOQ) of 16 U/kg and 53 U/kg *feedingstuffs*, respectively. Furthermore, the applicant organised a ring-trial validation with four laboratories for the verification of the method when determining *endo-1,3(4)-β-glucanase in feedingstuffs* and a relative standard deviation for *reproducibility* (RSD_R) ranging from 11 - 17 % was reported.

For the determination of the activity of *endo-1,4-β-xylanase* in *feed additives* and *premixtures* the applicant proposes a single laboratory validated and further verified colorimetric method based on the quantification of the reducing sugars (glucose equivalents) released from oat xylan in the presence of 3,5-dinitrosalicilic acid (DNS) at pH 4.0 and 30°C. For the *feedingstuffs* the applicant proposes single laboratory validated and further verified colorimetric method based on depolymerisation of soluble fragments from 2% azo-xylan solution in the presence of 96% ethanol at pH 4.8 and 50°C. The activity of the sample is calibrated against reference enzyme standards with known activity determined at the definition conditions of the activity unit (pH 4.0 and 30°C). The following method performance characteristics were reported the *feed additive*, *premixtures* and *feedingstuffs*: - RSD_r ranging from 0.8 to 6.7 %, - RSD_{ip} ranging from 6.4 to 7.7 %, - R_{Rec} ranging from 90 to 121 %; and an LOD and LOQ of 18 U/kg and 60 U/kg *feedingstuffs*, respectively. Furthermore, the applicant organised a ring-trial validation with four laboratories for the verification of the method when determining *endo-1,4-β-xylanase in feedingstuffs* and an RSD_R ranging from 5 - 12 % was reported.

Based on the satisfactory performance characteristics mentioned above, the CRL recommends for official control the validated and further verified analytical methods submitted by the applicant for the determination of the activity of *endo-1,3(4)-β-glucanase* and *endo-1,4-β-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) is not considered necessary.

KEYWORDS

Endofeed® DC, endo-1,3(4)-β-glucanase, endo-1,4-β-xylanase*, Aspergillus niger,* digestibility enhancers, chickens for fattening, laying hens, pigs for fattening, minor avian and porcine species

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new use) and 10(2) for *Endofeed® DC* under the category/functional group '4a': digestibility enhancers, according to Annex I of Regulation (EC) No 1831/2003 [1]. The feed additive is already authorised by Regulation (EC) No 255/2005. Specifically, authorisation is sought for the use of *Endofeed® DC* for chickens for fattening, laying hens, pigs for fattening and for minor avian and porcine species. *Endofeed® DC* is a dry, brown granular powder. It consists of two active agents,

endo-1,3(4)-β-glucanase (EC 3.2.1.6) and *endo-1,4-β-xylanase* (EC 3.2.1.8) [2], produced by the strain *Aspergillus niger* (NRRL 25541). The strain has been deposited at the Northern Regional Research Laboratory of US Department of Agriculture (NRRL) in Illinois, USA [3]. According to the applicant, enzymatic activity of the active agents is expressed in "U" units [4], where:

- One U of *endo-1,3(4)-β-glucanase* is the amount of enzyme that liberates 1 µmol of reducing sugars (glucose equivalents) from oat β-glucan per minute at pH 4.0 and 30°C; and
- One U of *endo-1,4-β-xylanase* is the amount of enzyme that liberates 1 µmol of reducing sugars (xylose equivalents) from oat xylan per minute at pH 4.0 and 30°C.

Endofeed® DC has a minimum *endo-1,3(4)-β-glucanase* activity of 1100 U/g and an *endo-1,4-β-xylanase* activity of 1600 U/g [2]. It is intended to be used in complete *feedingstuffs* with minimum activity of 138 U/kg *endo-1,3(4)-β-glucanase* and 200 U/kg *endo-1,4-β-xylanase* [2], respectively.

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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application or group of applications. For this dossier, the methods of analysis submitted in connection with *Endofeed® DC*, 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 Community Reference Laboratories [5].

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

Endo-1,3(4)-β-glucanase

For the determination of the activity of *endo-1,3(4)-β-glucanase* the applicant proposes a single laboratory validated and further verified colorimetric method for *feed additive* [6] and *premixtures* [7] based on the quantification of the reducing sugars (glucose equivalents) released from barley β-glucan. Test samples and reference samples (available upon request from the applicant) are extracted in 0.1 M acetate buffer (pH 4.0) and incubated with 0.4% β-glucan solution at 30°C for a total of 10 minutes. The reaction is stopped by adding 3,5-dinitrosalicilic acid (DNS). The colour change produced is proportional to the amount of reduced sugar released and to the activity of *endo-1,3(4)-β-glucanase* present in the sample. The optical density is measured on a spectrophotometer at 540 nm and quantified against a reference enzyme standard available upon request from the applicant. Additional control samples (standard enzyme diluted with barley sprouts) are analyzed together with premixture samples to monitor the accuracy of the measurement results. The method performance characteristics derived from the validation [7,8] and verification [9,10] studies are presented in Table 1.

For the determination of the activity of *endo-1,3(4)-β-glucanase* in *feedingstuffs,* the applicant proposes another ring-trial validated (by four laboratories) colorimetric method, based on depolymerisation of soluble fragments from azo-barley-glucan in the presence of precipitating solution [11]. Test samples and spiked blank samples are extracted from the feed with a 50 mM citrate phosphate buffer (pH 4.8) and incubated with azo-barley-glucan at 50°C for 3 hours. The reaction is stopped by adding 96% ethanol. The enzyme-substrate reaction produces a colour, the intensity of which is measured on a spectrophotometer at 585 nm. The activity of the sample is calibrated against blank feed spiked with enzymes with known activity determined at the definition conditions of the activity unit (pH 4.0 and 30°C). When the blank feed is not available, the use of standard addition technique is suggested.

The method performance characteristics derived from the ring trial study [11] are presented in Table 1. Furthermore a limit of detection (LOD) and a limit of quantification (LOQ) were derived by the CRL, based on the data provided by the applicant [11]:

- LOD = 16 U/kg and LOQ = 53 U/kg *feedingstuffs.*

Based on the satisfactory performance characteristics mentioned above, the CRL recommends for official control the analytical methods submitted by the applicant for the determination of *endo-1,3(4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs*.

Table 1: Method performance characteristics for the determination of *endo-1,3(4)-β-glucanase* in *feed additive* (FA), *premixtures* (PM) *and feedingstuffs* (FA)

	RSD_r (%)		RSD_{ip} (%)		R_{Rec} (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	8.1 [7]	2.3 [9]	6.3 [7]	9.8 [9]	94-119 [7]	90-104 [9]
PM	1.5-4.1 [8]	5.5 [10]	11 [8]	np	104 [8]	105 [10]
FS	5.0-8.5 [11]		RSD_R = 11-17 [11]		106 [11]	115-126 [11]

RSD_r, RSD_{ip}, RSD_R - relative standard deviation for *repeatability*, *intermediate precision* and *reproducibility*
R_{Rec} – recovery rate
np – not provided

Endo-1,4-β-xylanase

For the determination of the activity of *endo-1,4-β-xylanase* the applicant proposes a single laboratory validated and further verified colorimetric method for *feed additive* [6] and *premixtures* [7] based on the quantification of the reducing sugars (glucose equivalents) released from oat xylan. Test samples and reference samples (available upon request from the applicant) are extracted in 0.1 M acetate buffer (pH 4.0) and incubated with 0.5% xylan solution at 30°C for a total of 10 minutes. The reaction is stopped by adding 3,5-dinitrosalicilic acid (DNS). The colour change produced is proportional to the amount of reduced sugar released and to the activity of *endo-1,4-β-xylanase* present in the sample. The optical density is measured on a spectrophotometer at 540 nm and quantified against a reference enzyme standard available upon request from the applicant. Additional control samples (standard enzyme diluted with barley sprouts) are analyzed together with premixture samples to monitor the accuracy of the measurement results. The method performance characteristics derived from the validation [7,8] and verification [9,10] studies are presented in Table 2.

For the determination of the activity of *endo-1,4-β-xylanase* in *feedingstuffs,* the applicant proposes another ring-trial validated (by four laboratories) colorimetric method, based on depolymerisation of soluble fragments from azo-xylan in the presence of precipitating solution [11]. Test samples and spiked blank samples are extracted from the feed with a 50 mM citrate phosphate buffer (pH 4.8) with 2% azo-xylan solution at 50°C for 4 hours. The reaction is stopped by adding 96% ethanol. The enzyme-substrate reaction produces a colour, the intensity of which is measured on a spectrophotometer at 585 nm. The activity of the sample is calibrated against blank feed spiked with enzymes with known activity determined at the definition conditions of the activity unit (pH 4.0 and 30°C). When the blank feed is not available, the use of standard addition technique is suggested.

Table 2: Method performance characteristics for the determination of *endo-1,4-β-xylanase* in *feed additive* (FA), *premixtures* (PM) *and feedingstuffs* (FS)

	RSD_r, %		RSD_{ip}, %		R_{Rec} (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
FA	6.7 [7]	2.7 [9]	6.4 [7]	7.5 [9]	97-121 [7]	94-110 [9]
PM	1.7-3.9 [8]	4.3 [10]	7.7 [8]	np	100 [8]	90 [10]
FS	0.8-5.4 [11]		RSD_R = 5–12 % [11]		108 [11]	94-103 [11]

RSD_r, RSD_{ip}, RSD_R - relative standard deviation for *repeatability*, *intermediate precision* and *reproducibility*
R_{Rec} – recovery rate
np – not provided

The method performance characteristics derived from the ring trial study [11] are presented in Table 2. Furthermore an LOD and an LOQ were derived by the CRL, based on the data provided by the applicant [11]:

- LOD = 18 U/kg and (LOQ) = 60 U/kg *feedingstuffs*.

Based on the satisfactory performance characteristics presented in Table 2, the CRL recommends for official control the analytical methods submitted by the applicant for the determination of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the CRL recommends for official control the colorimetric methods submitted by the applicant to determine the activities of *endo-1,3(4)-β-glucanase* and *endo-1,4-β-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 in accordance with article 10 of Commission Regulation (EC) No 378/2005 is not considered necessary.

Recommended text for the register entry (analytical method)

Characterisation of the active substances in the *feed additive* and *premixtures*:

– Colorimetric method measuring reducing sugars (glucose equivalents) released by action of *endo-1,3(4)-β-glucanase* on barley β-glucan substrate in the presence of 3,5-dinitrosalicilic acid (DNS).

- Colorimetric method measuring reducing sugars (glucose equivalents) released by action of *endo-1,4-β-xylanase* on oat xylan substrate in the presence of DNS.

Characterisation of the active substances in *feedingstuffs*:

- Colorimetric method measuring depolymerised soluble fragments released by action of *endo-1,3(4)-β-glucanase* on azo-barley-glucan.
- Colorimetric method measuring depolymerised soluble fragments released by action of *endo-1,4-β-xylanase* on azo-xylan.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Endofeed® DC* have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Application/Ref:SANCO/D/2: Forw. Appl. 1831/013-2009
[2] *Application, Proposal for Register Entry, Annex A
[3] *Technical dossier, Section II, Annex_II_2_1_2a
[4] *Technical dossier, Section II, Identity, characterisation & conditions of use of the additive
[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] *Technical Dossier, Section II, Annex_II_6_1a
[7] *Technical Dossier, Section II, Annex_II_6_1c_eng_version
[8] *Technical Dossier, Section II, Annex_II_6_1b_eng_version
[9] *Technical Dossier, Section II, Annex_II_6_1e
[10] *Technical Dossier, Section II, Annex_II_6_1g
[11] *Technical Dossier, Section II, Annex_II_6_1d_eng_version
* Refers to Dossier No. FAD-2009-0015

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Community 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

The following National Reference Laboratories contributed to this report:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)