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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-2010-0189** **CRL/100005**
Name of Feed Additive:	**Rovabio Excel**
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)**
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EXECUTIVE SUMMARY

In the current application authorisation is sought under articles 4(1) and 10(2) for *Rovabio Excel,* 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 two active substances: *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 *Penicillium funiculosum* Pf 8/403 (IMI 378536). The product is intended to be marketed in different formulations as: (i) liquid (*Rovabio Excel LC* and *Rovabio Excel LC2*); and (ii) solid (non-coated: *Rovabio Excel AP* and coated: *Rovabio Excel AP T-FLEX*). Liquid formulations have a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 7500 U/ml (≈ 1075 DNS units/ml) and an *endo-1,4-β-xylanase* activity of 5500 U/ml (≈ 800 DNS units/ml). Solid formulations have a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 30000 U/g (≈ 4300 DNS units/g) and an *endo-1,4-β-xylanase* activity of 22000 U/g (≈ 3200 DNS units/g).

The activity of *endo-1,3(4)-β-glucanase* and *endo-1,4-β-xylanase* is expressed in viscosimetric unit (U):

- one viscosimetric unit (U) is the amount of enzyme which hydrolyzes the substrate (barley betaglucan and wheat arabinoxylan, respectively), reducing the viscosity of the solution, to give a change in relative fluidity of 1 (dimensionless unit)/min at 30 ^{o}C and pH 5.5.

Alternatively, the activity of *endo-1,3(4)-β-glucanase* and *endo-1,4-β-xylanase* is expressed in DNS units (U):

- For glucanase, one "DNS unit" is the amount of enzyme which hydrolyzes the substrate barley betaglucan producing 1 µmole of glucose per minute at pH 5.0 and 50 °C;
- For xylanase, one "DNS unit" is the amount of enzyme which hydrolyzes birchwood xylan producing 1 µmole of xylose per minute at pH 4.0 and 50 °C.

Specifically, authorisation is sought for the use of *Rovabio Excel* for laying hens, turkeys for fattening, chickens for fattening, pigs for fattening, weaned piglets, ducks, guinea fowls, quails, geese, pheasants and pigeons. The *feed additive* is intended to be incorporated into *premixtures* and/or complete *feedingstuffs* with a minimum activity of 1100 U *endo-1,4-β-xylanase*/kg and 1500 U *endo-1,3(4)-β-glucanase*/kg. It is intended to be used in compound feed rich in non-starch polysaccharides, containing more than 50% cereals.

For the quantification of ***endo-1,3(4)-β-glucanase*** in the *feed additive*, the Applicant submitted a single laboratory validated and further verified method, based on colour formation of released sugar with 3,5-dinitrosalicylic acid (DNS). The assay is based on the enzymatic hydrolysis of the barley betaglucan at pH 5.0 and 50 °C. The following performance characteristics were derived from validation and verification studies: - a relative standard deviation for *repeatability* (RSD_r) ranging from 0.8 to 3 %, - a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 2.7 to 3.9 %, and - a *recovery* rate (R_{Rec}) ranging from 97 to 107 %.

For the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed a single laboratory validated and further verified viscosimetric method. *Endo-1,3(4)-β-glucanase* catalyses the hydrolysis of glycosidic bonds in the substrate (barley betaglucan) to yield glucose and consequently reduces the viscosity of sample solution. The decrease in viscosity of sample solution is determined using a falling ball viscosimeter at defined conditions (pH 5.5 and 30 °C). The following performance characteristics were derived from validation and verification studies:

- for the *feed additive*: - RSD_r ranging from 1.8 to 5.6 %, - RSD_{ip} ranging from 3.1 to 9.1 %, and - R_{Rec} ranging from 96.7 to 116.8 %; and
- for *premixtures* and *feedingstuffs*: - RSD_r ranging from 1.3 to 8.7 %, - RSD_{ip} ranging from 6.1 to 7.6 %, - R_{Rec} ranging from 81 to 124.6 %, and limits of detection (LOD) and quantification (LOQ) of 576 and 738 U/kg *feedingstuffs*.

For the quantification of ***endo-1,4-β-xylanase*** in the *feed additive*, the Applicant submitted a single laboratory validated and further verified method, based on colour formation of released sugar with 3,5-dinitrosalicylic acid (DNS). The assay is based on the enzymatic hydrolysis of the birchwood xylan at pH 4.0 and 50 °C. The following performance characteristics were derived from validation and verification studies: - RSD_r ranging from 0.4 to 3.5 %, - RSD_{ip} ranging from 2.7 to 2.9 %, and - R_{Rec} ranging from 95 to 106.8 %.

For the quantification of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposed a single laboratory validated and further verified viscosimetric method. *Endo-1,4-β-xylanase* catalyses the hydrolysis of xylosidic bonds in the wheat arabinoxylan substrate to yield xylose and consequently reduces the viscosity of sample solution. The decrease in viscosity of sample solution is determined using a falling ball viscosimeter at defined conditions (pH 5.5 and 30 °C). The following performance characteristics were derived from validation and verification studies:

- for the *feed additive*: - RSD_r ranging from 1.2 to 3.7 %, - RSD_{ip} ranging from 3.6 to 6.1 %, and - R_{Rec} ranging from 95.2 to 109.5 %;
- for *premixtures*: - RSD_r ranging from 2.1 to 6 %, - RSD_{ip} ranging from 3.2 to 5.8 %, and - R_{Rec} ranging from 79.5 to 102.1 %; and
- for *feedingstuffs*: - RSD_r ranging from 1.6 to 6.4 %, - RSD_{ip} ranging from 3.3 to 6.1 %, - R_{Rec} ranging from 89.3 to 120 %, and LOD and LOQ of 571 and 706 U/kg *feedingstuffs*, respectively.

Based on the satisfactory performance characteristics mentioned above, the EURL recommends for official control the single laboratory validated and further verified methods submitted by the Applicant, within the concentration range covered by the experimental data:

(i) viscosimetric methods, for the quantification of the activity of total *endo-1,3(4)-β-glucanase* and *endo-1,4-β-xylanase* in the *feed additive*, *premixtures** and *feedingstuffs*;* (*applying standard addition) and

(ii) DNS methods, for the quantification of total *endo-1,3(4)-β-glucanase* and *endo-1,4-β-xylanase* in the *feed additive.*

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

Rovabio Excel, *endo-1,3(4)-β-glucanase*, *endo-1,4-β-xylanase*, *Penicillium funiculosum*, zootechnical additive, digestibility enhancers, laying hens, turkeys for fattening, chickens for fattening, pigs for fattening, weaned piglets, ducks, guinea fowls, quails, geese, pheasants, pigeons

1. BACKGROUND

In the current application authorisation is sought under articles 4(1) (new target species) and 10(2) (re-evaluation of additives already authorised) for *Rovabio Excel,* 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 following Commission Regulations: - (EC) No 1259/2004 for broilers, - (EC) No 943/2005 for layers, - (EC) No 1206/2005 for pigs for fattening and - (EC) No 322/2009 for weaned piglets and ducks for fattening. According to the Applicant, the *feed additive* contains two active substances: *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 *Penicillium funiculosum* Pf 8/403 (IMI 378536). The strain has been deposited at the International Mycological Institute (IMI) in Surrey, UK [3]. The product is intended to be marketed in different formulations [2, 3] as:

- liquid (*Rovabio excel LC* and *Rovabio Excel LC2*); and
- solid (non-coated: *Rovabio Excel AP* and coated: *Rovabio Excel AP T-FLEX*).

Wheat flour is the main carrier of the solid formulation, while water+sorbitol is the one for the liquid formulation. Liquid formulations have a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 7500 U/ml (≈ 1075 DNS units/ml) and an *endo-1,4-β-xylanase* activity of 5500 U/ml (≈ 800 DNS units/ml) [2]. Solid formulations have a guaranteed minimum *endo-1,3(4)-β-glucanase* activity of 30000 U/g (≈ 4300 DNS units/g) and an *endo-1,4-β-xylanase* activity of 22000 U/g (≈ 3200 DNS units/g) [2].

The activity of *endo-1,3(4)-β-glucanase* and *endo-1,4-β-xylanase* is expressed in viscosimetric unit (U):

- one viscosimetry unit (U) is the amount of enzyme which hydrolyzes the substrate (barley betaglucan and wheat arabinoxylan, respectively), reducing the viscosity of the solution, to give a change in relative fluidity of 1 (dimensionless unit)/min at 30 ^{o}C and pH 5.5 [3].

Alternatively, the activity of *endo-1,3(4)-β-glucanase* and *endo-1,4-β-xylanase* is expressed in DNS units (U):

- For glucanase, one "DNS unit" is the amount of enzyme which hydrolyzes the substrate barley betaglucan producing 1 µmole of glucose per minute at pH 5.0 and 50 °C [3],

- For xylanase, one "DNS unit" is the amount of enzyme which hydrolyzes birchwood xylan producing 1 µmole of xylose per minute at pH 4.0 and 50 °C [3].

Specifically, authorisation is sought for the use of *Rovabio Excel* for laying hens, turkeys for fattening, chickens for fattening, pigs for fattening, weaned piglets, ducks, guinea fowls, quails, geese, pheasants and pigeons [2]. The *feed additive* is intended to be incorporated into *premixtures* and/or complete *feedingstuffs* with a minimum activity of 1100 U *endo-1,4-β-xylanase*/kg and 1500 U *endo-1,3(4)-β-glucanase*/kg [2]. It is intended to be used in compound feed rich in non-starch polysaccharides, containing more than 50% cereals [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 *Rovabio Excel*, 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 [4].

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

Endo-1,3(4)-β-glucanase

Colorimetric method: For the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive*, the Applicant submitted a single laboratory validated and further verified method, based on colour formation of released sugar with 3,5-dinitrosalicylic acid (DNS) [5]. One glucanase

unit corresponds to the liberation of one µmole of glucose equivalent per minute at pH 5.0 and 50 °C. The assay is based on the enzymatic hydrolysis of the barley betaglucan. The enzyme (0.6 g for *Rovabio Excel AP* and 0.9 g for *Rovabio Excel LC* formulations) is extracted at room temperature under magnetic stirring, for 10 minutes for *AP* formulations, for 30 minutes for *AP T-FLEX* formulations and manually for *LC* formulations. If necessary, the extract is diluted in 0.01M sodium acetate buffer solution (pH 5.5) [5]. The substrate (1.75 mL of 1.5% betaglucan solution) is equilibrated at 50 °C for at least 5 minutes. Then 250 µL of extracted sample is added and incubated at 50 °C for 10 minutes. After 10 minutes, the reaction is stopped by adding 2 mL of DNS solution. The tubes are then placed into a 95 °C water bath for 15 minutes and 10 ml of water is added. The reaction products are determined by colorimetry at 540 nm using a standard glucose calibration curve. The performance characteristics determined during method validation [6] and verification [7] studies are summarized in Table 1.

Viscosimetric method, general: For the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed a single laboratory validated and further verified viscosimetric method [8, 9, 26]. *Endo-1,3(4)-β-glucanase* catalyses the hydrolysis of glycosidic bonds in the substrate (barley betaglucan) to yield glucose and consequently reduces the viscosity of sample solution. The decrease in viscosity of sample solution is determined using a falling ball viscosimeter at defined conditions. The glucanase activity is quantified against a linear regression curve prepared from enzyme standards with known activities resolved in pure solutions (external calibration). One unit of *endo-1,3(4)-β-glucanase* activity is equivalent to the quantity of enzyme that hydrolyzes the substrate, thus reducing the solution viscosity, in order to change the relative fluidity by one unit per minute without dimension, at pH 5.5 and 30 °C.

Viscosimetric method, feed additive: For the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive* [8], the enzyme (0.6 g for *AP* and 0.9 g for *LC* formulations) is extracted at room temperature under magnetic stirring, for 10 minutes for *AP* formulations, for 30 minutes for *AP T-FLEX* formulations and manually for *LC* formulations. ~~If necessary,~~ The extract is diluted in 0.01M sodium acetate buffer solution (pH 5.5). Substrate (1 mL 1% barley betaglucan solution) and 3 mL of water are placed in a tube, sonicated for 5 seconds and equilibrated for minimum 5 minutes at 30 °C. Then 1 mL of the extracted sample is added and the analysis is carried out [8].

Viscosimetric method, premixture: For the quantification of *endo-1,3(4)-β-glucanase* in *premixtures* [26] the Applicant performed, upon request by the EURL, a solid dilution of the premixtures samples with a blank feed and demonstrated the applicability of the method for

the *feedingstuffs* described below. The obtained performance characteristics are summarized in Table 1.

Viscosimetric method, feedingstuffs: For the quantification of added *endo-1,3(4)-β-glucanase* in *feedingstuffs* [9], approximately 10 g of ground feed is weighed into a 250 mL conical flask. Then a 100 mL of MES/SDS buffer solution (pH 6.0) is slowly added. In the case of feeds made with T-FLEX, 50 g of ground feed is weighed and 500 mL of buffer solution is added. The sample is placed into a cold water bath and stirred for 30 minutes. Then, 5 ml of this solution is transferred to a new tube, and centrifuged for 10 minutes at 7000 rpm. Supernatant is filtered and filtrated solution is diluted in 0.01M buffer acetate solution (pH 5.5). Substrate (1 mL of 1% barley betaglucan) and 3 mL of water are placed in a tube, sonicated for 5 seconds and equilibrated for minimum 5 minutes at 30 °C. After incubation 1 mL of the extracted sample is added and the viscosimetric method for the analysis of *feed additive* is carried out [7]. This experimental protocol is applied to treated feedingstuff sample (in which the enzyme was added) and to an untreated control sample (matrix matched blank, with no added enzyme). The activity of the added *endo-1,3(4)-β-glucanase* is determined by subtracting the activity of the control sample from the total activity. Such blank correction is only possible when control samples are available and allows for the determination of the added enzyme activity. When no control samples are available, the analysis delivers the total activity.

The performance characteristics determined during method validation [10, 11] and verification [12, 13] studies are summarized in Table 1. Furthermore, the Applicant reported LOD and LOQ of 576 and 738 U/kg *feedingstuffs*.

The EURL considers the viscosimetry method submitted by the Applicant suitable for official control to determine the total *endo-1,3(4)-β-glucanase* activity in *feedingstuffs* (and *premixtures*).

Based on the performance characteristics presented, the EURL recommends for official control the following single laboratory validated and further verified methods submitted by the Applicant, within the concentration range covered by the experimental data:

(i) viscosimetric method, for the quantification of total *endo-1,3(4)-β-glucanase* in the *feed additive*, *premixtures** and *feedingstuffs**, (*applying standard addition)

(ii) DNS method, for the quantification of total *endo-1,3(4)-β-glucanase* in the *feed additive.*

Table 1: Summary of method performance characteristics for the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive, premixtures* and *feedingstuffs* containing 9000-67000, 323-408 and 1.0-2.2 U/g, respectively.

	RSD_r (%)		RSD_{ip} (%)		Recovery (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
Feed Additive (DNS)	1.0 -3.0 [6]	0.8-2.7 [7]	3.9 [6]	2.7 [7]	97-107 [6]	98.9-106.8 [7]
Feed Additive (V)	1.8-3.9 [10]	5.6 [12]	3.1[10]	9.1 [12]	96.7-116.8 [10]	94-114 [12]
Premixtures (V)	-	7-8.6[26]	-	7.6 [26]	-	81–116 [26]
Feedingstuffs (V)	1.3-4.6 [11]	2.5-8.7 [13]	6.1 [11]	6.8 [13]	88.2-124.6 [11]	82.3-100 [13]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*, respectively.
V - viscosimetric method
DNS- colorimetric DNS method

Endo-1,4-β-xylanase

Colorimetric method: For the quantification of *endo-1,4-β-xylanase* in the *feed additive*, the Applicant submitted a single laboratory validated and further verified method, based on colour formation of released sugar with 3,5-dinitrosalicylic acid (DNS) [14]. One xylanase unit corresponds to the liberation of one µmole of xylose equivalent per minute at pH 4.0 and 50 °C. The assay is based on the enzymatic hydrolysis of the birchwood xylan. The enzyme (0.6 g for *AP* and 0.9 g for *LC* formulations) is extracted in water at room temperature under magnetic stirring, for 10 minutes for *AP* formulations, for 30 minutes for *AP T-FLEX* formulations and manually for *LC* formulations. If necessary, the extract is diluted [14]. The substrate (1.75 mL of 1,5% birchwood xylan solution) is placed into a tube and equilibrated at 50 °C for at least 5 minutes. Then 250 µL of extracted sample is added and incubated at 50 °C for 10 minutes. The reaction is stopped by adding 2 mL of DNS solution. The reaction products are determined by colorimetry at 540 nm after reaction of the reducing group with 3,5-dinitrosalicylic acid, using a standard xylose calibration curve. The calculated enzymatic activity is then expressed in xylose equivalents. The performance characteristics determined during validation [15] and verification [16] studies are summarized in Table 2.

Viscosimetric method, general: For the quantification of *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant proposed a single laboratory validated and further verified viscosimetric method [17-19]. *Endo-1,4-β-xylanase* catalyses the hydrolysis of xylosidic bonds in the wheat arabinoxylan substrate to yield xylose and consequently reduces the viscosity of sample solution. The decrease in viscosity of sample solution is determined using a falling ball viscosimeter at defined conditions. The xylanase activity is quantified against a linear regression curve prepared from enzyme standards with known activities resolved in pure solutions (external calibration). One unit of *endo-1,4-β-xylanase* activity is equivalent to the quantity of enzyme that hydrolyzes the substrate, thus

reducing the solution viscosity, in order to change the relative fluidity by one unit per minute without dimension, at pH 5.5 and 30 °C.

Viscosimetric method, feed additive: For the quantification of *endo-1,4-β-xylanase* in the *feed additive* [17], the enzyme (0.6 g for *AP* and 0.9 g for *LC* formulations) is extracted in water at room temperature under magnetic stirring, for 10 minutes for *AP* formulations, for 30 minutes for *AP T-FLEX* formulations and manually for *LC* formulations. If necessary, the extract is diluted. Substrate (4.8 mL of 0.25% wheat arabinoxylan solution) is placed in a tube, sonicated for 5 seconds and equilibrated for minimum 5 minutes at 30 °C. Then 0.2 mL of the extracted sample is added and the analysis is carried out [17].

Viscosimetric method, premixtures: For the quantification of *endo-1,4-β-xylanase* in *premixtures* [18], approximately 4.5 g of premix is placed in 100 mL of 1M sodium acetate buffer solution (pH 5.5). The solution is extracted for 30 minutes at room temperature under continuous magnetic stirring. The solution is filtrated and further diluted in sodium acetate buffer. The viscosimetric method for analysis of the *feed additive* is followed [17].

Viscosimetric method, feedingstuffs: For the quantification of added *endo-1,4-β-xylanase* in the *feedingstuffs* [19], approximately 10 g of ground feed is weighed into a 250 mL conical flask. Then a 100 mL of MES/SDS buffer solution (pH 6.0) is slowly added. In the case of feeds made with T-FLEX, 50 g of ground feed is weighed and 500 mL of buffer solution is added. The sample is placed into a cold water bath and stirred for 30 minutes. This solution is centrifuged for 10 minutes at 7000 rpm and filtered. The supernatant is placed into a tube. Substrate (3.2 mL of 0.3% wheat arabinoxylan solution) is placed in a tube, sonicated for 5 seconds and incubated for minimum 5 minutes at 30 °C. After incubation 0.8 mL of the extracted sample is added and the viscosimetric method for the analysis of *feed additive* is carried out [17]. This experimental protocol is applied to treated feedingstuff sample (in which the enzyme was added) and to an untreated control sample (matrix matched blank, with no added enzyme). The activity of the added *endo-1,4-β-xylanase* is determined by subtracting the activity of the control sample from the total activity. Such blank correction is only possible when control samples are available and allows for the determination of the added enzyme activity. When no control samples are available, the analysis delivers the total activity.

The performance characteristics determined during method validation [20-22] and verification [23-25] studies are summarized in Table 2. Furthermore, the Applicant reported limits of detection (LOD) and quantification (LOQ) of 571 and 706 U/kg *feedingstuffs*, respectively.

The EURL considers the viscosimetry method submitted by the Applicant suitable for official control to determine the total *endo-1,4-β-xylanase* activity in *feedingstuffs* (and *premixtures*).

Table 2: Summary of method performance characteristics for the quantification of *endo-1,4-β-xylanase* in the *feed additive, premixtures* and *feedingstuffs* containing 5500-30000, 75-2600 and 0.5-1.8 U/g, respectively.

	RSD_r (%)		RSD_{ip} (%)		Recovery (%)	
	Validation	Verification	Validation	Verification	Validation	Verification
Feed Additive (DNS)	0.4-2.6 [15]	1.0-3.5 [16]	2.7 [15]	2.9 [16]	95-104 [15]	98.9-106.8 [16]
Feed Additive (V)	1.7-3.7 [20]	1.2-3.7 [23]	3.6 [20]	6.1 [23]	95.4-102.6 [20]	95.2-109.5 [23]
Premixtures (V)	2.1-4.9 [21]	2.4-6.0 [24]	3.2-3.8 [21]	4.6-5.8 [24]	79.6-102.1 [21]	79.5-100.7 [24]
Feedingstuffs (V)	1.6-4.2 [22]	2.6-6.4 [25]	4.7-5.0 [22]	3.3-6.1 [25]	89.3-120 [22]	96.1-109.1 [25]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*, respectively.
V - Viscosimetric method
DNS- DNS method

Based on the performance characteristics presented, the EURL recommends for official control the following single laboratory validated and further verified methods submitted by the Applicant, within the concentration range covered by the experimental data:

(i) viscosimetric method, for the quantification of total *endo-1,4-β-xylanase* in the *feed additive*, *premixtures* and *feedingstuffs**, (*applying standard addition)

(ii) DNS method, for the quantification of total *endo-1,4-β-xylanase* in the *feed additive.*

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:

- the single laboratory validated and further verified colorimetric (DNS) method based on the enzymatic hydrolysis of barley betaglucan at pH = 5.0 and 50 °C for the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive;*
- the single laboratory validated and further verified method based on decrease of viscosity produced by action of *endo-1,3(4)-β-glucanase* on the glucan substrate barley betaglucan at pH = 5.5 and 30 °C for the quantification of *endo-1,3(4)-β-glucanase* in the feed *additive, premixtures* and *feedingstuffs*,

- the single laboratory validated and further verified colorimetric (DNS) method based on the *endo-1,4-β-xylanase* hydrolysis of birchwood xylan at pH = 4.0 and 50 °C for the quantification of *endo-1,4-β-xylanase* in the *feed additive*, and
- the single laboratory validated and further verified method based on the decrease of viscosity produced by action of *endo-1,4-β-xylanase* on the xylan-containing substrate (wheat arabinoxylan) at pH = 5.5 and 30 °C for the quantification of *endo-1,4-β-xylanase* in the *feed additive, premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive, premixtures* and *feedingstuffs*:

- viscosimetric method based on decrease in viscosity produced by action of *endo-1,3(4)-β-glucanase* on the glucan substrate barley betaglucan at pH = 5.5 and 30 °C.

Alternatively, for the quantification of *endo-1,3(4)-β-glucanase* in the *feed additive:*

- colorimetric (DNS) method based on the enzymatic hydrolysis of barley betaglucan at pH = 5.0 and 50 °C.

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

- viscosimetric method based on decrease in viscosity produced by action of *endo-1,4-β-xylanase* on the xylan containing substrate (wheat arabinoxylan) at pH = 5.5 and 30 °C.

Alternatively for the quantification of *endo-1,4-β-xylanase* in the *feed additive:*

- colorimetric (DNS) method based on the *endo-1,4-β-xylanase* hydrolysis of birchwood xylan at pH = 4.0 and 50 °C

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Rovabio Excel* 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/D/2 Forw. Appl. 1831/00118-2010
[2] *Application, Proposal for 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_53
[6] *Technical dossier, Section II-Annex_II_61
[7] *Technical dossier, Section II-Annex_II_69
[8] *Technical dossier, Section II-Annex_II_52
[9] *Technical dossier, Section II-Annex_II_57
[10] *Technical dossier, Section II-Annex_II_60
[11] *Technical dossier, Section II-Annex_II_65
[12] *Technical dossier, Section II-Annex_II_68
[13] *Technical dossier, Section II-Annex_II_73
[14] *Technical dossier, Section II-Annex_II_51
[15] *Technical dossier, Section II-Annex_II_59
[16] *Technical dossier, Section II-Annex_II_67
[17] *Technical dossier, Section II-Annex_II_50
[18] *Technical dossier, Section II-Annex_II_54
[19] *Technical dossier, Section II-Annex_II_56
[20] *Technical dossier, Section II-Annex_II_58
[21] *Technical dossier, Section II-Annex_II_62
[22] *Technical dossier, Section II-Annex_II_64
[23] *Technical dossier, Section II-Annex_II_66
[24] *Technical dossier, Section II-Annex_II_70
[25] *Technical dossier, Section II-Annex_II_72
[26] *Supplementary information

*Refers to Dossier No. FAD-2010-0189

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was The Danish Plant Directorate, Lyngby, Denmark. 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.

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