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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

Fra Octazyme C Dry
(FAD-2014-0028; CRL/140021)

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-2014-0028 - CRL/140021**
Name of Product:	***Fra Octazyme C Dry***
Active Agent (s):	**Endo-1,4-β-xylanase (3.2.1.8)** **Endo-1,3(4)-β-glucanase (3.2.1.6)** **Endo-1,4-β-glucanase (3.2.1.4)** **Manan-endo-1,4-β-mannosidase (3.2.1.78)** **Pectinase** **α-galactosidase (3.2.1.22)** **Protease (3.4.21.62)** **α-amylase (3.2.1.1)**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA)** **07/11/2016**
Report approved by: Date:	**Christoph von Holst** **08/11/2016**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4 (1) for *Fra Octazyme C Dry* under the category/functional group 4 (a) "zootechnical additive"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additives* for chickens for fattening and weaned piglets.

According to the Applicant, *Fra Octazyme C Dry* is a preparation containing eight enzymes with a guaranteed minimum activity of 160000 BXU/g for *endo-1,4-β-xylanase;* 20000 BU/g for *endo-1,3(4)-β-glucanase;* 3200 U_G/g for *endo-1,4-β-glucanase;* 1000 U_M/g for *manan-endo-1,4-β-mannosidase;* 2100 U_P/g for *pectinase;* 80 GALU/g for *α-galactosidase;* 1500 U_{PR}/g for *protease,* and 10000 U_A/g for *α-amylase.*

The Applicant expressed the enzyme activities in different units defined as follows:

- one unit of *endo-1,4-β-xylanase* activity (BXU) is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as xylose equivalents, from the beechwood xylan substrate at pH 5.3 and 50 °C;
- one unit of *endo-1,3(4)-β-glucanase* activity (BU) is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as glucose equivalents, from the barley beta-glucan substrate at pH 4.8 and 50 °C;
- one unit of *endo-1,4-β-glucanase* activity (U_G) is the amount of enzyme which catalyses the production of one micromole per minute of reducing sugars, expressed as glucose equivalents, from carboxymethylcellulose substrate at pH 5.5 and 40 °C;
- one unit of *manan-endo-1,4-β-mannosidase* activity (U_M) is the amount of enzyme that produces reducing sugars having a reducing power corresponding to one micromole of mannose per minute from galactomannan substrate at pH 7.0 and 50 °C;
- one unit of *pectinase* activity (U_P) is the amount of enzyme, which liberates one micromole per minute of reducing sugars, expressed as glucose equivalents, from the orange polygalacturonic acid substrate at pH 4.5 and 40 °C;
- one unit of *α-galactosidase* activity (GALU) is defined as the amount of enzyme which degrades one micromole per minute of ρ-nitrophenyl α-D-galactopyranoside at pH 5.5 and 37 °C;
- one unit of *protease* activity (U_{PR}) is defined as the amount of enzyme which liberates one microgram per minute of phenolic compound, expressed as tyrosine equivalents from the casein substrate at pH 7.5 and 50 °C; and

- one unit of *α-amylase* activity (U_A) is the amount of enzyme which catalyses the production of one micromole per minute of reducing sugars, expressed as glucose equivalents, from the wheat starch substrate at pH 5.5 and 40 °C.

Fra Octazyme C Dry is intended to be incorporated directly in *feedingstuffs* for chickens and weaned piglets with the following proposed minimum enzyme activities in *feedingstuffs:* 8000 BXU/kg for *endo-1,4-β-xylanase;* 1000 BU/kg for *endo-1,3(4)-β-glucanase;* 160 U_G/kg for *endo-1,4-β-glucanase;* 50 U_M/kg for *manan-endo-1,4-β-mannosidase;* 105 U_P/kg for *pectinase;* 4 GALU/kg for *α-galactosidase;* 75 U_{PR}/kg for *protease,* and 500 U_A/kg for *α-amylase*.

The Applicant submitted eight single-laboratory validated and further verified colorimetric methods for the quantification of the active substances in the *feed additive* and *feedingstuffs*, based on the enzymatic hydrolysis:

- of xylanase on the beechwood xylan substrate at pH 5.3 and 50 °C for the determination of *endo-1,4-β -xylanase*;
- of glucanase on the barley beta-glucan substrate at pH 4.8 and 50 °C for the determination of *endo-1,3(4)-β-glucanase;*
- of cellulase on the carboxymethylcellulose substrate at pH 5.5 and 40 °C for the determination of *endo-1,4-β-glucanase;*
- of mannanase on the locust bean gum substrate at pH 7.0 and 50 °C for the determination of *manan-endo-1,4-β-mannosidase;*
- of pectinase on the orange polygalacturonic acid substrate at pH 4.5 and 40 °C for the determination of *pectinase;*
- of *α-galactosidase* on the ρ-nitrophenyl α-D-galactopyranoside substrate at pH 5.5 and 37 °C for the determination of *α-galactosidase;*
- of protease on casein substrate at pH 7.5 and 50 °C for the determination of *protease*; or
- of amylase on the wheat starch substrate at pH 5.5 and 40 °C for the determination of *α-amylase*.

Based on the performance characteristics available the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric methods for the quantification of the eight enzymes in the *feed additive.*

Due to the lack of suitable experimental data, the EURL cannot evaluate the proposed method for the quantification of *protease* in *feedingstuffs*. Furthermore, the EURL considers the proposed single-laboratory validated and further verified colorimetric methods suitable i) for the quantification of *endo-1,4-β-xylanase* and *manan-endo-1,4-β-mannosidase* at the minimum activities proposed by the Applicant and ii) for the quantification of

endo-1,3(4)-β-glucanase, *endo-1,4-β-glucanase*, *pectinase*, *α-galactosidase*, and *α-amylase* at activity levels above their respective limits of quantification (LOQ) in *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

Fra Octazyme C Dry, endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase, manan-endo-1,4-β-mannosidase, pectinase, α-galactosidase, protease, α-amylase, zootechnical, digestibility enhancers, chickens for fattening, weaned piglets

1. BACKGROUND

In the current application authorisation is sought under article 4 (1) (new feed additive) for *Fra Octazyme C Dry*, under the category/functional group 4 (a) "zootechnical additive"/"digestibility enhancers", according to the classification system of Annex 1 of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for the use of the *feed additive* for chickens for fattening and weaned piglets [2].

According to the Applicant, *Fra Octazyme C Dry* is a preparation containing the following eight enzymes [3]:

- *endo-1,4-β-xylanase* produced by *Trichoderma citrinoviride* (DSM 17470)
- *endo-1,3(4)-β-glucanase* produced by *Trichoderma citrinoviride* (DSM 17470)
- *endo-1,4-β-glucanase* produced by *Trichoderma citrinoviride* (DSM 17470)
- *manan-endo-1,4-β-mannosidase* produced by *Trichoderma citrinoviride* (DSM 17470)
- *pectinase* produced by *Aspergillus niger* agg (DSM 27958)
- *α-galactosidase* produced by *Aspergillus niger agg* (DSM 27958)
- *protease* produced by *Bacillus licheniformis* (DSM 27949)
- *α-amylase* produced by *Bacillus amyloliquefaciens* (DSM 27497)

with a guaranteed minimum enzyme activity of 160000 BXU/g; 20000 BU/g; 3200 U_G/g; 1000 U_M/g; 2100 U_P/g; 80 GALU/g; 1500 U_{PR}/g and 10000 U_A/g, respectively [2].

The Applicant expressed the enzymes activity in different units defined as follows:

- one unit of *endo-1,4-β-xylanase* activity (BXU) is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as xylose equivalents, from the beechwood xylan substrate at pH 5.3 and 50 °C;

- one unit of *endo-1,3(4)-β-glucanase* activity (BU) is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as glucose equivalents, from the barley beta-glucan substrate at pH 4.8 and 50 °C;
- one unit of *endo-1,4-β-glucanase* activity (U_G) is the amount of enzyme which catalyses the production of one micromole per minute of reducing sugars, expressed as glucose equivalents, from carboxymethylcellulose substrate at pH 5.5 and 40 °C;
- one unit of *manan-endo-1,4-β-mannosidase* activity (U_M) is the amount of enzyme that produces reducing sugars having a reducing power corresponding to one micromole of mannose per minute from galactomannan substrate at pH 7.0 and 50 °C;
- one unit of *pectinase* activity (U_P) is the amount of enzyme, which liberates one micromole per minute of reducing sugars, expressed as glucose equivalents, from the orange polygalacturonic acid substrate at pH 4.5 and 40 °C;
- one unit of *α-galactosidase* activity (GALU) is defined as the amount of enzyme which degrades one micromole per minute of ρ-nitrophenyl α-D-galactopyranoside at pH 5.5 and 37 °C;
- one unit of *protease* activity (U_{PR}) is defined as the amount of enzyme which liberates one microgram per minute of phenolic compound, expressed as tyrosine equivalents from the casein substrate at pH 7.5 and 50 °C; and
- one unit of *α-amylase* activity (U_A) is the amount of enzyme which catalyses the production of one micromole per minute of reducing sugars, expressed as glucose equivalents, from the wheat starch substrate at pH 5.5 and 40 °C.

Fra Octazyme C Dry is intended to be incorporated directly into *feedingstuffs* with the following minimum enzyme activities: 8000 BXU/kg for *endo-1,4-β-xylanase;* 1000 BU/kg for *endo-1,3(4)-β-glucanase;* 160 U_G/kg for *endo-1,4-β-glucanase;* 50 U_M/kg for *manan-endo-1,4-β-mannosidase;* 105 U_P/kg for *pectinase*; 4 GALU/kg for *α-galactosidase;* 75 U_{PR}/kg for *protease*, and 500 U_A/kg for *α-amylase* [2]. These activity contents correspond exactly to the dosage recommended by the Applicant, i.e 50 g of *Fra Octazyme C Dry* per ton of complete feed.

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *Fra Octazyme C Dry* 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, mycotoxins 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 ***endo-1,4-β -xylanase*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released xylose with dinitrosalycilic acid (DNS) [5][6]. The assay is based on the enzymatic hydrolysis of xylanase on the beechwood xylan substrate at pH 5.3 and 50 °C.

The *feed additive* sample (2 g) is extracted with 0.05 M citrate buffer (pH 5.3) with 1.2 % bovine serum albumine (BSA), the solution is stirred for 30 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.1 ml) is placed into a test tube together with 0.9 ml of the beechwood xylan substrate (1.0 % and pH 5.3) and shaken on a Vortex before incubation at 50 °C for 5 min.

The *feedingstuffs* sample (10 g) is extracted with 0.05 M citrate buffer (pH 5.3) with 1.2 % bovine serum albumine (BSA), the solution is stirred for 30 min at room temperature and centrifuged at 1800 - 2000 g for 10 min. The obtained solution is passed through a PD10 column. The eluted solution (0.1 ml) is placed into a test tube together with 0.9 ml of the beechwood xylan substrate (1.0 % and pH 5.3) and shaken on a Vortex before incubation at 50 °C for 120 min.

After incubation 1.5 ml of a DNS solution (10 g/l) is added to all samples (*feed additive* and *feedingstuffs*), mixed and placed in boiling water for 5 min. All test tubes are then cooled at room temperature with cold water. The content of *xylanase* is determined by colorimetry at 540 nm using a standard xylose (external) calibration curve. The xylose calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [5].

For the quantification of ***endo-1,3(4)-β-glucanase*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method,

based on the colour formation of released glucose with DNS [7][8]. The assay is based on the enzymatic hydrolysis of glucanase on the barley beta-glucan substrate at pH 4.8 and 50 °C. The *feed additive* sample (2 g) is extracted with 0.1 M acetate buffer (pH 4.8), the solution is stirred for 20 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.05 ml) is placed into a test tube together with 0.45 ml of the beta-glucan substrate (1.0 % w/v and pH 4.8) and shaken on a Vortex before incubation at 50 °C for 10 min.

The *feedingstuffs* sample (10 g) is extracted with 0.1 M acetate buffer (pH 4.8), the solution is stirred for 30 min at room temperature and centrifuged at 1800-2000g for 10 min. The obtained solution is passed through a PD10 column. The eluted solution (0.05 ml) is placed into a test tube together with 0.45 ml of the beta-glucan substrate (1.0 % w/v and pH 4.8) and shaken on a Vortex before incubation at 50 °C for 240 min.

After incubation 0.5 ml of a DNS solution (10 g/l) is added to all samples (*feed additive* and *feedingstuffs*), mixed and placed in boiling water for 5 min. All test tubes are then cooled at room temperature with cold water and 5 ml of deionised water is added. The content of glucanase is determined by colorimetry at 540 nm using a standard glucose (external) calibration curve. The glucose calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [7].

For the quantification of ***endo-1,4-β-glucanase*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released glucose with DNS [9][10]. The assay is based on the enzymatic hydrolysis of cellulase on the carboxymethylcellulose substrate at pH 5.5 and 40 °C.

The *feed additive* sample (2 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride (1 mM) and the solution is stirred for 20 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.05 ml) is placed into a test tube together with 0.45 ml of the carboxymethylcellulose substrate (3.75 % and pH 5.5) and shaken on a Vortex before incubation at 40 °C for 20 min.

The *feedingstuffs* sample (10 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride (1 mM) and the solution is stirred for 30 min at room temperature, centrifuged at 1800-2000 g for 10 min. The obtained solution is passed through a PD10 column. The eluted solution (0.05 ml) is placed into a test tube together with 0.45 ml of the carboxymethylcellulose substrate (3.75 % and pH 5.5) and shaken on a Vortex before incubation at 40 °C for 240 min.

After incubation 0.75 ml of a DNS solution (10 g/l) is added to all samples (*feed additive* and *feedingstuffs*), mixed and placed in boiling water for 20 min. All test tubes are cooled at room temperature with cold water and 1 ml of deionised water is added. The content of cellulase is determined by colorimetry at 540 nm using a standard glucose (external) calibration curve. The glucose calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [9].

For the quantification of ***manan-endo-1,4-β-mannosidase*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released reducing sugars with DNS [11][12]. The assay is based on the enzymatic hydrolysis of mannanase on the galactomannan substrate at pH 7.0 and 50 °C.

The *feed additive* sample (2 g) is extracted with 0.05 M 3,3-dimethyl-glutarate sodium hydroxide buffer (pH 7.0) containing Tween 20 (0.01 %), the solution is stirred for 20 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.05 ml) is placed into a test tube together with 0.45 ml of the galactomannan substrate (0.3 % Locust Beam Gum at pH 7.0 (LBG)) and incubated at 50 °C for 20 min.

The *feedingstuffs* sample (10 g) is extracted with 0.05 M 3,3-dimethyl-glutarate sodium hydroxide buffer (pH 7.0) containing Tween 20 (0.01 %), the solution is stirred for 30 min at room temperature and centrifuged at 1800-2000 g for 10 min. The obtained solution is passed through a PD10 column. The eluted solution (0.05 ml) is placed into a test tube together with 0.45 ml of the galactomannan substrate (LBG) and incubated at 50 °C for 240 min.

After incubation 0.75 ml of a DNS solution (10 g/l) is added to all samples (*feed additive* and *feedingstuffs*), mixed and placed in boiling water for 5 minutes. All test tubes are cooled at room temperature with cold water. The content of mannanase is determined by colorimetry at 540 nm using a standard mannose (external) calibration curve. The mannose calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [11].

For the quantification of ***pectinase*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released reducing sugars, expressed as glucose equivalents, with DNS [13][14]. The assay is based on the enzymatic hydrolysis of pectinase on the orange polygalacturonic acid substrate at pH 4.5 and 40 °C.

The *feed additive* sample (2 g) is extracted with 0.25 M acetate buffer (pH 4.5) containing Tween 20 (0.01 %), the solution is stirred for 20 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.5 ml) is placed into a test tube together

with 0.5 ml of the pectin substrate (Orange Polygalacturonic Acid 0.5 % at pH 4.5 (OPA)) and incubated at 40 °C for 30 min.

The *feedingstuffs* sample (10 g) is extracted with 0.25 M acetate buffer (pH 4.5) containing Tween 20 (0.01 %), the solution is stirred for 30 min at room temperature, centrifuged at 1800-2000 g for 10 min. The obtained solution is passed through a PD10 column. The eluted solution (0.5 ml) is placed into a test tube together with 0.5 ml of the pectin substrate (OPA) and incubated at 40 °C for 120 min.

After incubation 1.5 ml of a DNS solution (10 g/l) is added to all samples (*feed additive* and *feedingstuffs*), mixed and placed in boiling water for 5 min. After that all test tubes are cooled at room temperature with cold water and further centrifuged at 2000 g for 5 min. The content of pectinase is determined by colorimetry at 540 nm using a standard glucose (external) calibration curve. The glucose calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [13].

For the quantification of ***α-galactosidase*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released 4-nitrophenol [15][16]. The assay is based on the enzymatic reaction of *α-galactosidase* on the ρ-nitrophenyl α-D-galactopyranoside substrate at pH 5.5 and 37 °C.

The *feed additive* sample (2 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride dihydrate (0.0147 %), the solution is stirred for 20 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.5 ml) is placed into a test tube together with 1 ml of the ρ-nitrophenyl α-D-galactopyranoside substrate (0.12 mM) and incubated at 37 °C for 15 min.

The *feedingstuffs* sample (10 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride dihydrate (0.0147 %), the solution is stirred for 30 min at room temperature and centrifuged at 1800-2000 g for 10 min. The obtained solution is passed through a PD10 column. The eluted solution (0.5 ml) is placed into a test tube together with 1 ml of the ρ-nitrophenyl α-D-galactopyranoside substrate (0.12 mM) and incubated at 37 °C for 60 min.

After incubation 2.5 ml of a 0.0625 M borax-sodium hydroxide buffer solution at pH 9.7 is added to all samples (*feed additive* and *feedingstuffs*), allowed to cool at least for 15 min and centrifuged at 2000 g for 5 min. The content of *α-galactosidase* is determined by colorimetry at 540 nm using a standard 4-nitrophenol (external) calibration curve. The 4-nitrophenol calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [15].

For the quantification of ***protease*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released tyrosine with Follin's reagent [17][18]. The assay is based on the enzymatic hydrolysis of protease on casein substrate at pH 7.5 and 50 °C.

The *feed additive* sample (2 g) is extracted with a 0.02 M sodium chloride solution containing Tween 20 (0.01 %), the solution is stirred for 10 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.54 ml) is placed into a test tube together with 1.6 ml of the casein substrate (1.2 % and pH 7.5) and incubated at 50 °C for 30 min.

The *feedingstuffs* sample (10 g) is extracted with a 0.02 M sodium chloride solution containing Tween 20 (0.01 %), the solution is stirred for 30 min at room temperature and centrifuged at 1800-2000 g for 10 min. The obtained solution is passed through a PD10 column. The eluted solution (0.54 ml) is placed into a test tube together with 1.6 ml of the casein substrate (1.2 % and pH 7.5) and incubated at 50 °C for 30 min.

After incubation 2.0 ml of a trichloroacetic acid solution (18.8 g/l) is added to all samples (*feed additive* and *feedingstuffs*), allowed to cool at least for 15 min and further filtered or centrifuged at 2000 g for 10 min. The supernatant (0.5 ml) is mixed with 2.5 ml of sodium carbonate solution 0.55 M followed by 0.5 ml of Folli's reagent. The tubes are then incubated at 50 ^{o}C for another 20 min. The content of protease is determined by colorimetry at 660 nm using a standard tyrosine (external) calibration curve. The tyrosine calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [17].

For the quantification of ***α-amylase*** in the *feed additive* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released glucose with DNS [19][20]. The assay is based on the enzymatic hydrolysis of amylase on the wheat starch substrate at pH 5.5 and 40 °C.

The *feed additive* sample (2 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride (1 mM), the solution is stirred for 20 min at room temperature, let stand for few minutes and diluted appropriately. The supernatant (0.05 ml) is placed into a test tube together with 0.45 ml of the wheat starch substrate (0.5 % and pH 5.5) and incubated at 40 °C for 20 min.

The *feedingstuffs* sample (10 g) is extracted with 0.25 M acetate buffer (pH 5.5) containing Tween 20 (0.01 %) and calcium chloride (1 mM), the solution is stirred for 30 min at room temperature and centrifuged at 1800-2000 g for 10 min. The obtained solution is then passed through a PD10 column. The eluted solution (0.05 ml) is placed into a test tube together with 0.45 ml of the wheat starch substrate (0.5 % and pH 5.5) and incubated at 40 °C for 60 min.

Table 1: Performance characteristics of analytical methods for the determination of eight enzymes in five lots of the *feed additive* (*Fra Octazyme C Dry*) [21].

	Mean Activity (Unit/g)		RSD_{ip} (%)		R_{rec} (%)
Active Substance	**Valid.**	**Verif.**	**Valid.**	**Verif.**	**Verif.**
endo1,4-β-xylanase	184783	193113	11	5	105
endo-1,3(4)-β-glucanase	22431	22568	9	4	101
endo-1,4-β-glucanase	3516	3598	3	2	102
manan-endo-1,4-β-mannosidase	1028	1065	9	4	104
pectinase	2529	2485	3	2	98
α-galactosidase	79	80	6	6	101
protease	1415	1417	12	5	100
α-amylase	11001	10819	10	5	98

RSD_{ip}: relative standard deviation for *intermediate precision*; R_{rec}: recovery rate;
Valid.: validation; Verif: verification

After incubation 0.75 ml of a DNS solution (10 g/l) is added to all samples (*feed additive* and *feedingstuffs*), and placed in boiling water for 20 min. All test tubes are then cooled at room temperature with cold water and 1 ml of deionised water is added and mixed well on a Vortex. The content of amylase is determined by colorimetry at 540 nm using a standard glucose (external) calibration curve. The glucose calibration standard solutions undergo the sample treatment described above - without the addition of feed samples [19].

The Applicant performed validation and verification studies for the analysis of each of the investigated enzymes in the *feed additive* and *feedingstuffs*.

Satisfactory performance characteristics were provided for the quantification of the eight enzymes in the *feed additive*. A few unexpectedly high recoveries (up to 300 %) were reported for *endo-1,4-β-glucanase*, *pectinase*, *protease* and *α-amylase* in the frame of the verification study. Furthermore large relative standard deviations for repeatability and intermediate precision (up to 27 %) were reported for *protease* in the frame of the verification study. Upon request of the EURL, the Applicant provided new data on different lots of the *feed additive* analysed by a second laboratory [21] that led to acceptable performance characteristics for the eight enzymes (Table 1).

Based on the performance characteristics available the EURL recommends for official control the proposed single-laboratory validated and further verified colorimetric methods for the quantification of the eight enzymes in the *feed additive*.

Table 2: Performance characteristics of analytical methods for the determination of eight enzymes in *feedingstuffs*.

		Tested activity (Unit/kg)		RSD_r (%)		RSD_{ip} (%)		R_{rec} (%)	LOQ (Unit/kg)	Min Activity (Unit/kg)
Active Substance	**Ref**	**Valid.**	**Verif.**	**Valid.**	**Verif.**	**Valid.**	**Verif.**	**Verif.**		**[2]**
endo1,4-β-xylanase	[22]	70513	66000	6	6	5	5	97	1100	8000
endo-1,3(4)-β-glucanase	[23]	5180	5600	7	12	9	10	91	1819*	1000
endo1,4-β-glucanase	[24]	572	368	5	12	4	13	67	334*	160
manan-endo-1,4-β-mannosidase	[25]	70	73	8	16	7	23	88	51	50
pectinase	[26]	1404	1310	8	8	9	10	101	1220*	105
α-galactosidase	[27]	29	31	6	3	4	3	99	27*	4
protease	[28]	-	-	-	-	-	-	-	-*	75
α-amylase	[28]	4325	4930	7	8	6	4	116	4936*	500

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec}: recovery rate; Valid.: validation; Verif.: verification; LOQ: limit of quantification set as the minimum activity analysed; * LOQ > Min. Activity, does not allow to enforce the minimum activity proposed by the Applicant in *feedingstuffs*

Satisfactory performance characteristics (for precision and recovery) were provided for the quantification of most of the eight enzymes in the *feedingstuffs*. The Applicant proposed as minimum dosage 50 g of the *feed additive* per ton of complete feed which corresponds to a minimum activity of 8000 BXU/kg for *endo-1,4-β-xylanase;* 1000 BU/kg for *endo-1,3(4)-β-glucanase;* 160 U_G/kg for *endo-1,4-β-glucanase;* 50 U_M/kg for *manan-endo-1,4-β-mannosidase;* 105 U_P/kg for *pectinase*; 4 GALU/kg for *α-galactosidase;* 75 U_{PR}/kg for *protease*, and 500 U_A/kg for *α-amylase* in *feedingstuffs* [2]. This approach led to very low activity levels for several enzymes. However, the Applicant did not provide experimental evidence of the applicability of the proposed methods at the minimum activity suggested for several enzymes. The EURL reviewed all the experimental data presented in the dossier and set the limit of quantification (LOQ) to the lowest activity analysed by the Applicant (Table 2). Consequently the proposed colorimetric methods are not suitable to monitor the minimum levels in *feedingstuffs* suggested by the Applicant for *endo-1,3(4)-β-glucanase*; *endo-1,4-β-glucanase*; *α-galactosidase, pectinase,* and *α-amylase.* Moreover, due to the lack of suitable experimental data, the EURL cannot evaluate the method proposed by the Applicant for the quantification of *protease* in *feedingstuffs*.

Based on the performance characteristics available the EURL considers the proposed single-laboratory validated and further verified colorimetric methods suitable i) for the quantification of *endo-1,4-β -xylanase* and *manan-endo-1,4-β-mannosidase* at the minimum activities proposed by the Applicant and ii) for the quantification of *endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase, pectinase, α-galactosidase,* and *α-amylase* at activity levels above their respective limits of quantification (LOQ) in *feedingstuffs*.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the eight single laboratory validated and further verified colorimetric methods submitted by the Applicant for the quantification of *endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase, manan-endo-1,4-β-mannosidase*, *pectinase*, *α-galactosidase*, *protease*, and *α-amylase* in the *feed additive* and for the enforcement of the proposed minimum activities of *endo-1,4-β-xylanase* and *manan-endo-1,4-β-mannosidase* in *feedingstuffs.*

Recommended text for the register entry (analytical method)

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

- colorimetric method based on the enzymatic hydrolysis of xylanase on the beechwood xylan substrate at pH 5.3 and 50 °C

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

- colorimetric method based on the enzymatic hydrolysis of glucanase on the barley beta-glucan substrate at pH 4.8 and 50 °C

For the quantification of *endo-1,4-β-glucanase* in the *feed additive*:

- colorimetric method based on the enzymatic hydrolysis of cellulase on the carboxymethylcellulose substrate at pH 5.5 and 40 °C

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

- colorimetric method based on the enzymatic hydrolysis of mannanase on the galactomannan substrate at pH 7.0 and 50 °C

For the quantification of *pectinase* in the *feed additive*:

- colorimetric method based on the enzymatic hydrolysis of pectinase on the orange polygalacturonic acid substrate at pH 4.5 and 40 °C

For the quantification of *α-galactosidase* in the *feed additive*:

- colorimetric method based on the enzymatic reaction of *α-galactosidase* on the ρ-nitrophenyl α-D-galactopyranoside substrate at pH 5.5 and 37 °C

For the quantification of *protease* in *feed additive*:

- colorimetric method based on the enzymatic hydrolysis of protease on casein substrate at pH 7.5 and 50 °C

For the quantification of *α-amylase* in *feed additive*:

- colorimetric method based on the enzymatic hydrolysis of amylase on the wheat starch substrate at pH 5.5 and 40 °C

One unit of *endo-1,4-β-xylanase* activity (BXU) is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as xylose equivalents, from the beechwood xylan substrate at pH 5.3 and 50 °C.

One unit of *endo-1,3(4)-β-glucanase* activity (BU) is the amount of enzyme, which liberates one nanomole per second of reducing sugars, expressed as glucose equivalents, from the barley beta-glucan substrate at pH 4.8 and 50 °C.

One unit of *endo-1,4-β-glucanase* activity (U_G) is the amount of enzyme which catalyses the production of one micromole per minute of reducing sugars, expressed as glucose equivalents, from carboxymethylcellulose substrate at pH 5.5 and 40 °C.

One unit of *manan-endo-1,4-β-mannosidase* activity (U_M) is the amount of enzyme that produces reducing sugars having a reducing power corresponding to one micromole of mannose per minute from galactomannan substrate at pH 7.0 and 50 °C.

One unit of *pectinase* activity (U_P) is the amount of enzyme, which liberates one micromole per minute of reducing sugars, expressed as glucose equivalents, from the orange polygalacturonic acid substrate at pH 4.5 and 40 °C.

One unit of *α-galactosidase* activity (GALU) is defined as the amount of enzyme which degrades one micromole per minute of ρ-nitrophenyl α-D-galactopyranoside at pH 5.5 and 37 °C.

One unit of *protease* activity (U_{PR}) is defined as the amount of enzyme which liberates one microgram per minute of phenolic compound, expressed as tyrosine equivalents from the casein substrate at pH 7.5 and 50 °C.

One unit of *α-amylase* activity (U_A) is the amount of enzyme which catalyses the production of one micromole per minute of reducing sugars, expressed as glucose equivalents, from the wheat starch substrate at pH 5.5 and 40 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Fra Octazyme C Dry* 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. 1831/0043-2014
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II: 2.2 Characterisation of the active substance(s)/agent(s)
[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_2.6.1f
[6] *Technical dossier, Section II-Annex_2.6.1i1 & 2.6.1i2
[7] *Technical dossier, Section II-Annex_2.6.1g
[8] *Technical dossier, Section II-Annex_2.6.1l1 & 2.6.1l2
[9] *Technical dossier, Section II-Annex_2.6.1h
[10] *Technical dossier, Section II-Annex_2.6.1n1 & 2.6.1n2
[11] *Technical dossier, Section II-Annex_2.6.1c
[12] *Technical dossier, Section II-Annex_2.6.1j1 & 2.6.1j2
[13] *Technical dossier, Section II-Annex_2.6.1a
[14] *Technical dossier, Section II-Annex_2.6.1o1 & 2.6.1o2

[15] *Technical dossier, Section II-Annex_2.6.1b
[16] *Technical dossier, Section II-Annex_2.6.1p1 & 2.6.1p2
[17] *Technical dossier, Section II-Annex_2.6.1e
[18] *Technical dossier, Section II-Annex_2.6.1m1 & 2.6.1m2
[19] *Technical dossier, Section II-Annex_2.6.1d
[20] *Technical dossier, Section II-Annex_2.6.1k1 & 2.6.1k2
[21] *Supplementary information, FAD-2014-0028_SIn_Add 281015 Answers 160725.pdf
[22] *Technical dossier, Section II-Annex_2.6.1i3 & 2.6.1i4
[23] *Technical dossier, Section II-Annex_2.6.1l3 & 2.6.1l4
[24] *Technical dossier, Section II-Annex_2.6.1n3 & 2.6.1n4
[25] *Technical dossier, Section II-Annex_2.6.1j3 & 2.6.1j4
[26] *Technical dossier, Section II-Annex_2.6.1o3 & 2.6.1o4
[27] *Technical dossier, Section II-Annex_2.6.1p3 & 2.6.1p4
[28] *Technical dossier, Section II-Annex_2.6.1m3 & 2.6.1m4
[29] *Technical dossier, Section II-Annex_2.6.1k3 & 2.6.1k4

*Refers to Dossier no: FAD-2014-0028

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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