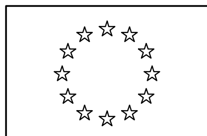

EUROPEAN COMMISSION

JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
European Union Reference Laboratory for Feed Additives

Ref. Ares(2012)1038702 - 06/09/2012

JRC.D.5/CvH/PRO/mds/ARES(2012)

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

Dossier related to:	**FAD-2010-0175 - CRL/100144** **FAD-2010-0211 – CRL/100145**
Name of Additives:	***Kemzyme Plus Dry*** **(E1620)** ***Kemzyme Plus Liquid*** **(E1621)**
Active Substance(s):	**- endo-1,3(4)-beta-glucanase (EC 3.2.1.6);** **- endo-1,4-beta-glucanase (EC 3.2.1.4);** **- alpha-amylase (EC 3.2.1.1);** **- endo-1,4-beta-xylanase (EC 3.2.1.8); and** **- bacillolysin (EC 3.4.24.28)**
Rapporteur Laboratory:	**European Reference Laboratory for Feed Additives (EURL-FA)**
Report prepared by:	**Piotr Robouch & Roberto Molteni**
Report revised by: Date:	**Stefano Bellorini (EURL-FA)** **05/09/2012**
Report approved by: Date:	**Christoph von Holst** **05/09/2012**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) and 10(2) for *Kemzyme Plus Dry* (FAD-2010-0175) and *Kemzyme Plus Liquid* (FAD-2010-0211), 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, chickens reared for laying, laying hens, turkeys for fattening, turkeys reared for breeding, other minor poultry for fattening, other minor poultry for laying, and weaned piglets (FAD-2010-0175, only).

According to the Applicant, *Kemzyme Plus Dry* is a preparation containing the five active substances: *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase (cellulase)*, *alpha-amylase*, *endo-1,4-beta-xylanase* and *bacillolysin (protease)* with a guaranteed minimum enzyme activity of 2350; 18000, 400, 35000 and 1700 U/g, respectively. *Kemzyme Plus Liquid* is a preparation containing the first four active substances listed above (does not include *bacillolysin*) with a guaranteed minimum enzyme activity of 10000, 310000, 400 and 210000 U/g, respectively.

The Applicant used the enzyme activity units as defined in the Commission Regulations (EC) No 1270/2009:

- One *endo-1,3(4)-beta-glucanase* unit (U) is the amount of enzyme which liberates 0.0056 micromoles of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 7.5 and 30 °C;
- One *endo-1,4-beta-glucanase* unit (U) is the amount of enzyme which liberates 0.0056 micromoles of reducing sugars (glucose equivalents) from carboxymethyl-cellulose per minute at pH 4.8 and 50 °C;
- One *alpha-amylase* unit (U) is the amount of enzyme which liberates 1 micromole of glucose from a cross-linked starch polymer per minute at pH 7.5 and 37 °C.
- One *endo- 1,4-beta-xylanase* unit (U) is the amount of enzyme which liberates 0.0067 micromoles of reducing sugars (xylose equivalents) from birchwood xylan per minute at pH 5.3 and 50 °C; and
- One *bacillolysin* unit (U) is the amount of enzyme which solubilises one microgram of azo-casein substrate per minute at pH 7.5 and 37 °C.

Kemzyme Plus Dry is intended to be used in *premixtures* and in complementary or complete *feedingstuffs* for the above mentioned poultry species, with the following proposed minimum enzyme activities for *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin*: 588, 4500, 100, 8750 and 425 U/kg *feedingstuffs*,

respectively. These minimum activities are to be doubled for piglets (weaned up to 35 kg). *Kemzyme Plus Liquid* is intended to be used in complementary or complete *feedingstuffs*, with the following proposed minimum enzyme activities for *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase* and *endo-1,4-beta-xylanase*: 500, 15000, 20 and 10500 U/kg *feedingstuffs*, respectively.

The Applicant submitted five single laboratory validated and further verified colorimetric methods, based on:

- the enzymatic hydrolysis of glucanase on the barley beta-glucan substrate at pH 7.5 and 30 ^{o}C, for the determination of *endo-1,3(4)-beta-glucanase* in both *feed additives;*
- the enzymatic hydrolysis of cellulase on the carboxylmethylcellulose at pH 4.8 and 50 ^{o}C, for the determination of *endo-1,4-beta-glucanase* in both *feed additives;*
- the formation of water soluble dyed fragments produced by the action of amylase on azurine cross-linked starch polymer substrates at pH 7.5 and 37 ^{o}C, for the determination of *alpha-amylase* in both *feed additives;*
- the enzymatic hydrolysis of xylanase on the birchwood xylane substrate at pH 5.3 and 50 ^{o}C, for the determination of *endo-1,4-beta-xylanase* in both *feed additives;* and
- the release of azo-dye resulting from the action of protease on the azo-casein substrate at pH 7.5 and 37 ^{o}C, for the determination of *bacillolysin* in the *feed additive Kemzyme Plus Dry*, only.

The following performance characteristics derived from the validation and verifications studies were reported: - a relative standard deviations for repeatability (RDS_r) ranging from 0.2 to 6.9 %; - a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 2.7 to 9 %; and - recovery rates (R_{Rec}) ranging from 77 to 122%. Based on the satisfactory experimental evidence available the EURL recommends for official control the five colorimetric methods submitted by the Applicant for the determination of *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin* in *Kemzyme Plus Dry* and/or *Kemzyme Plus Liquid*.

Furtheremore, the Applicant submitted five single-laboratory validated and further verified methods (three colorimetric and two plate test methods), based on:

- the glucanase diffusion and the subsequent decolouring of the red agar medium due to the beta-glucan hydrolysis, for the determination of *endo-1,3(4)-beta-glucanase* in *premixtures* and *feedingstuffs;*

- the quantification of the water soluble dye fragments produced by the action of cellulase on azurine cross-linked water insoluble HE-cellulose substrate, for the determination of *endo-1,4-beta-glucanase* in *premixtures* and *feedingstuffs*;
- the formation of water soluble blue fragments produced by the action of alpha-amylase on azurine cross-linked insoluble blue-coloured starch polymer substrates, for the determination of *alpha-amylase* in *premixtures* and *feedingstuffs;*
- the quantification of water soluble dyed fragments produced by the action of xylanase on azurine cross-linked wheat arabinoxylan, for the determination of *endo-1,4-beta-xylanase* in *premixtures* and *feedingstuffs;* and
- the diffusion of protease in the azo-casein agar medium and the subsequent hydrolysis of casein, for the determination of *bacillolysin* in *premixtures* and *feedingstuffs* (containing *Kemzyme Plus Dry*, only).

The following performance characteristics derived from the validation and verifications studies were reported: - RDS_r ranging from 0.2 to 29 %; - RSD_{ip} ranging from 0.8 to 30 %; and R_{Rec} ranging from 72 to 123%. Furthermore, the EURL derived from the experimental data available limits of detections allowing the assessment of the minimum enzyme activities recommended by the Applicant. Based on the experimental evidence available, the EURL recommends for official control the colorimetric and plate test methods submitted by the Applicant for the determination of *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin* in *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

Kemzyme Plus Dry, Kemzyme Plus Liquid, endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase, bacillolysin, zootechnical, digestibility enhancers, chickens for fattening, chickens reared for laying, laying hens, turkeys for fattening, turkeys reared for breeding, other minor poultry for fattening, other minor poultry for laying, weaned piglets.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new use) and 10(2) (re-evaluation of an existing authorised additive) for *Kemzyme Plus Dry* (FAD-2010-0175) and *Kemzyme Plus Liquid* (FAD-2010-0211), 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 [1,2]. *Kemzyme Plus Dry* (E 1620) was already authorised under the Commission Regulations (EC) No 358/2005 and (EC) No 1270/2009, while *Kemzyme Plus Liquid* (E 1621) was already authorised under the Commission Regulations (EC) No 358/2005, (EC) No 1284/2006 and (EC) No 516/2010. The authorisation is sought for the use of the *feed additives* for chickens for fattening, chickens reared for laying, laying hens, turkeys for fattening, turkeys reared for breeding, other minor poultry for fattening, other minor poultry for laying and weaned piglets (FAD-2010-0175, only) [3,4].

According to the Applicant, *Kemzyme Plus Dry* is a preparation containing the following five active substances added to a 5% bentonite and 78% calcium carbonate carriers:

- *endo-1,3(4)-beta-glucanase* produced by Aspergillus aculeatus (CBS 589.94),
- *endo-1,4-beta-glucanase* produced by Trichoderma longibrachiatum (CBS 592.94),
- *alpha-amylase* produced by Bacillus amyloliquefaciens (DSM 9553),
- *endo- 1,4-beta-xylanase* produced by Trichoderma viride (NIBH FERM BP 4842),
- *bacillolysin* produced by Bacillus amyloliquefaciens (DSM 9554),

with a guaranteed minimum enzyme activity of 2350; 18000, 400, 35000 and 1700 U/g, respectively (see new-Annex-A [65]). *Kemzyme Plus Liquid* is a preparation containing the first four active substances listed above (does not include *bacillolysin*) with a guaranteed minimum enzyme activity of 10000, 310000, 400 and 210000 U/g, respectively (see new-Annex-A [66]). *Kemzyme Plus Liquid* consists of 100% enzyme preparations, as delivered by the suppliers; no other ingredients are included [4,6a,6b].

The Applicant used the same enzyme activity units [5b,6b] as defined in the Commission Regulations (EC) No 1270/2009:

- One *endo-1,3(4)-beta-glucanase* unit (U) is the amount of enzyme which liberates 0.0056 micromoles of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 7.5 and 30 °C;

- One *endo-1,4-beta-glucanase* unit (U) is the amount of enzyme which liberates 0.0056 micromoles of reducing sugars (glucose equivalents) from carboxymethyl-cellulose per minute at pH 4.8 and 50 °C;

- One *alpha-amylase* unit (U) is the amount of enzyme which liberates 1 micromole of glucose from a cross-linked starch polymer per minute at pH 7.5 and 37 °C.

- One *endo- 1,4-beta-xylanase* unit (U) is the amount of enzyme which liberates 0.0067 micromoles of reducing sugars (xylose equivalents) from birchwood xylan per minute at pH 5.3 and 50 °C; and

- One *bacillolysin* unit (U) is the amount of enzyme which solubilises one microgram of azo-casein substrate per minute at pH 7.5 and 37 °C.

At first the Applicant expressed the condition of use in grams of product in tons of feed (equivalent to mg/kg) [5c, 6c]. Then the Applicant provided, upon request from EFSA, new "conditions of use" (see new-Annex-A [65,66]) for the two products. *Kemzyme Plus Dry* is intended to be used in *premixtures* and in complementary or complete *feedingstuffs* for the above mentioned poultry species, with the following proposed minimum enzyme activities for *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin*: 588, 4500, 100, 8750 and 425 U/kg *feedingstuffs*, respectively. These minimum activities are to be doubled for piglets (weaned up to 35 kg) (cf. Commission Regulation (EC) No 1270/2009). *Kemzyme Plus Liquid* is intended to be used in complementary or complete *feedingstuffs*, with the following proposed minimum enzyme activities for *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase* and *endo-1,4-beta-xylanase*: 500, 15000, 20 and 10500 U/kg *feedingstuffs*, respectively (cf. Commission Regulation (EC) No 516/2010).

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 *Kemzyme Plus Dry* and *Kemzyme Plus Liquid,* 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 feed additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive such as heavy metals (arsenic, cadmium, lead and mercury), dioxins, microbiological agents and mycotoxins are available from the respective European Union Reference Laboratories [7].

Description of the analytical methods for the determination of the active agents in the feed additive

For the determination of the five *active substances* in the two *feed additives*, the Applicant submitted five single-laboratory validated and further verified colorimetric methods described hereafter.

For the determination of *endo-1,3(4)-beta-glucanase* (glucanase) in both *feed additives* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released glucose with arsenomolybdate [8,9]. The assay is based on the enzymatic hydrolysis of glucanase on the barley beta-glucan substrate at pH 7.5 and 30 ^{o}C. The *feed additive* sample (5 g for the solid and 1 g for the liquid formulations) is extracted in 50 mL of aqueous phosphate buffer (pH 7.5) and centrifuged for 10 min to obtain a clear enzyme solution. The supernatant (1 mL) is placed into a test tube to which 1 mL of beta-glucan substrate 0.5 % is added. Each tube is shaken on a Vortex before incubation at 30 ^{o}C for 30 min. The Nelson's reagent (1 mL, containing $NaCO_3$, $NaHCO_3$, Na_2SO_4, $K_2C_4H_4O_6$, and $CuSO_4$) is added and the tubes are placed at 100 ^{o}C for 20 min to stop the reaction. Arsenomolybdate colour reagent (1 mL) is added to each tube and shaken for 5 min. All test tubes are cooled at room temperature and centrifuged. The content of glucanase is determined by colorimetry at 540 nm using a standard glucose (external) calibration curve. "Blank" samples (consisting of demineralised water) and the glucose calibration standard solutions undergo the same experimental procedure - without the addition of the feed additive.

For the determination of *endo-1,4-beta-glucanase* (cellulase) in both *feed additives* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released low molecular reduced carbohydrates (glucose & cellobiose) with ferrocyanide [14,15]. The assay is based on the enzymatic hydrolysis of cellulase on the Carboxyl-Methyl-Cellulose (CMC) substrate at pH 4.8 and 50 ^{o}C. At first, 3 mL of CMC substrate (in acetate buffer, pH 4.8) solution is preheated for 25 min at 90 ^{o}C and cooled afterwards to 50 ^{o}C. In the mean time, the *feed additive* sample (5 g for the solid and 1 g for the liquid formulations) is extracted in 50 mL of aqueous phosphate buffer (pH 7.5) and

centrifuged for 5 min to obtain a clear enzyme solution. The supernatant (1 mL) is placed into a test tube to which 3 mL of the preheated CMC substrate (at 50 ^{o}C) is added. Each tube is shaken on a Vortex before incubation at 50 ^{o}C for 20 min. The phosphate buffer solution (2 mL, pH = 13.3) is added to stop the reaction. After the addition of 3 mL of ferrocyanide colour reagent, each tube is placed in a water bath at 100 ^{o}C for 6 min. Finally, all tubes are placed in a cold water bath for 10 min and centrifuged. The content of cellulase is then determined by colorimetry at 420 nm using a standard glucose (external) calibration curve. "Blank" samples (consisting of demineralised water) and the glucose calibration standard solutions undergo the same experimental procedure - without the addition of the feed additive.

For the determination of *alpha-amylase* (amylase) in both *feed additives* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the formation of water soluble dyed fragments produced by the action of *alpha-amylase* on commercially available (Phadebas®, Megazyme) azurine cross-linked starch polymer substrates at pH 7.5 and 37 ^{o}C [20,21]. The *feed additive* sample (5 g for the solid and 1 g for the liquid formulations) is extracted in 50 mL of aqueous phosphate buffer (pH 7.5) and centrifuged for 5 min to obtain a clear enzyme solution. The supernatant (1 mL) is placed into a test tube to which 5 mL of phosphate buffer solution (pH 7.5) is added. Each tube is then preheated at 37 ^{o}C for 5 min. One Phadebas® tablet is added to each test tube and incubated at 37 ^{o}C for 15 min. Normal NaOH (1 mL) is added to each tube to stop the reaction. The content of amylase is determined by colorimetry at 620 nm and quantified against a reference alpha-amylase standard of know activity. "Blank" samples (consisting of demineralised water) undergo the same experimental procedure - without the addition of the feed additive.

For the determination of *endo- 1,4-beta-xylanase* (xylanase) in both *feed additives* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the colour formation of released xylose with arsenomolybdate [26,27]. The assay is based on the enzymatic hydrolysis of xylanase on the birchwood xylan substrate at pH 5.3 and 50 ^{o}C. The *feed additive* sample (5 g for the solid and 1 g for the liquid formulations) is extracted in 50 mL of aqueous phosphate buffer (pH 7.5) and centrifuged for 5 min to obtain a clear enzyme solution. The supernatant (1 mL) is placed into a test tube to which 1 mL of birchwood xylan substrate 0.5 % is added. Each tube is shaken on a Vortex before incubation at 50 ^{o}C for 15 min. The Nelson's reagent is added, and the tubes are placed at 100 ^{o}C for 20 min to stop the reaction. Arsenomolybdate colour reagent (1 mL) is then added to each tube and shaken for 5 min. Demineralised water (5 mL) is then added; all test tubes are cooled at room temperature and centrifuged. The content of xylanase is determined by colorimetry at 540 nm using a standard xylose (external) calibration curve. "Blank" samples (consisting of

demineralised water) and the xylose calibration standard solutions undergo the same experimental procedure - without the addition of the feed additive.

For the determination of ***bacillolysin*** (protease) in the *feed additive* Kemzyme Plus Dry (only) the Applicant submitted a single-laboratory validated and further verified colorimetric method [32], based on the colour formation of the released azo-dye, derived from the Megazyme experimental protocol [33]. The assay is based on the release of azo-dye resulting from the action of protease on the azo-casein substrate at pH 7.5 and 37 ^{o}C. The *feed additive* sample (5 g) is placed in a test tube is extracted in 50 mL of aqueous phosphate buffer (pH 7.5) and centrifuged for 5 min to obtain a clear enzyme solution. The test tube is then preheated in a water bath at 37 ^{o}C for 5 min. Similarly, the azo-casein substrate solution is preheated to 37 ^{o}C for 10 min. The substrate solution (1 mL) is then added to the sample test tubes. Each tube is shaken on a Vortex before incubation at 37 ^{o}C for 15 min. Trichloroacetic acid 10 % solution (4 mL) is then added to each test tube and shaken to stop the reaction. All the test tubes are placed in a water bath for 50 ^{o}C for 15 min to allow the complete precipitation of proteins. Finally, the test tubes are centrifuged for 5 min and NaOH (2 M) is added to 3 mL of the sample aliquots. The content of protease is determined by colorimetry at 440 nm using a standard azo-casein (external) calibration curve. "Blank" samples (consisting of demineralised water) and the azo-casein calibration standard solutions undergo the same experimental procedure - without the addition of the feed additive.

For the determination of each of the five active substances in the two feed additives of interest the Applicant performed validation and verification studies. The reported performance characteristics are listed in Table 1. The EURL noticed that the verification results reported by Lab.3 were biased with a large scattered, compared to results from the Applicant, Lab.2, Lab.4 and Lab.6 [12,18,30]. Therefore, all of the values reported by Lab.3 were excluded from the compilation presented in Table 1.

Based on the satisfactory experimental evidence available the EURL recommends for official control the five colorimetry methods submitted by the Applicant for the determination of *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin* in *Kemzyme Plus Dry* and/or *Kemzyme Plus Liquid*.

Table 1: Performance characteristics of analytical methods for the determination of five active substances in two feed additives (*Kemzyme Plus Dry & Liquid*). Results obtained at high absorbance ranges are reported.

Kemzyme Plus Dry		**RSD_r (%)**		**RSD_{ip} (%)**		**R_{rec} (%)**
A.Substance	Activity (U/g)	Valid.	Verif.	Valid.	Verif.	Valid.
Glucanase	2600 - 3200	1.5 - 3.6 [10]	3.2 [12]	4.8 [10]	3.6 [12]	77 – 116 [10]
Cellulase	30300-47000	0.4 - 3.5 [16]	3.8 – 5.2 [18]	8.9 [16]	4.4 – 5.1 [18]	98 – 103 [16]
Amylase	620 - 800	0.2 – 3.9 [22]	2.6 - 3.0 [24]	5.6 [22]	2.9 [24]	96 – 115 [22]
Xylanase	22300-57300	1.5 – 6.9 [28]	1.2 – 1.8 [30]	7.6 [28]	7.1 – 8.1 [30]	91 - 103[28]
Protease	2300 - 3900	1.6 – 2.2 [34]	3.4 – 3.6 [35]	2.7 [34]	3.6 [35]	117 – 122 [34]
Kemzyme Plus Liquid		RSD_r (%)		RSD_{ip} (%)		R_{rec} (%)
A.Substance	Activity (U/g)	Valid.	Verif.	Valid.	Verif.	Valid.
Glucanase	14000-19500	2.2 – 7.1 [11]	2.7 [13]	5.3 [11]	3.4 [13]	64 - 99 [11]
Cellulase	292000-470000	0.8 – 2.6 [17]	2.7 – 6.3 [19]	6.0 [17]	3.1 – 6.1 [19]	108 - 119 [17]
Amylase	670 - 860	3.4 – 4.6 [23]	1.1 – 4.4 [25]	3.9 [23]	3.3 – 4.7 [25]	96 - 115 [23]
Xylanase	125000-192000	2.9 – 5.3 [29]	1.7 – 3.2 [31]	8.2 [29]	3.5 – 4.4 [31]	88 - 92 [29]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec} : recovery rate

Description of the analytical methods for the determination of the active agents in premixtures and feedingstuffs

For the determination of the five *active substances* of interest in *feedingstuffs*, the Applicant submitted five single-laboratory validated and further verified analytical methods based either on colorimetric or on plate test methods.

At first 100 g of feed sample is extracted in 1 L phosphate buffer (pH 6) during 45 min of continuous stirring. Few drops of Tween 20® are added to ensure complete extraction of the enzymes. The extract (20 mL) is centrifuged for 5 min and filtered to get a clear solution, later referred as "extracted sample". Several NRLs consider that the initial sample intake prescribed is too high and that it could be significantly reduced (i.e. 20 or 30 g of feed sample in 200 or 300 mL buffer solution, respectively).

For the determination of *endo-1,3(4)-beta-glucanase* (glucanase) in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified plate test method, based

on the glucanase diffusion and the subsequent decolouring of the red agar medium due to the beta-glucan hydrolysis [36,37]. At first the agar substrate is prepared as follows: 30 mL of beta-glucan is added to 100 mL of phosphate buffer (pH 6) and stirred at 70 ^{o}C for 15 min. After the addition of 4 mg of Congo Red, the solution is boiled for 5 min. Agar (1 g) is then added and the solution is boiled again for 10 min. The gel substrate is then cooled to 50 ^{o}C and 25 mL aliquots are poured on Petri dishes and cooled to room temperature. Several 5 mm holes are made in the gel throughout the Petri dish. Aliquots of the extracted sample (70 µL) are placed in the holes of the agar plates and incubated for 17 h at 37 ^{o}C. HCl (1N) is poured on the plate to stop the reaction. The diameter of the clear zone is measured and compared to the external calibration curve to derive the "added" or "total" glucanase activity (cf. discussion about calibrations).

For the determination of *endo-1,4-beta-glucanase* (cellulase) in *feedingtuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of the water soluble dye fragments produced by the action of cellulase on commercially available (Cellazyme C®, Megazyme) azurine cross-linked water insoluble HE-cellulose substrate [40,41]. An aliquot of the extracted sample (2 mL) is added to 1 mL acetate buffer (pH 4.8) in a test tube. One Cellazyme C® tablet is added and the solution is incubated for 3 h. Ethanol (4 mL) is added to stop the reaction and the solution is let to rest for 20 min at room temperature before centrifugation for 3 min and filtration to get a clear solution. The rate of dye release is measured colorimetrically at 585 nm and quantified using the external calibration curve to derive "added" or "total" cellulase activity (cf. discussion about calibrations).

For the determination of *alpha-amylase* (amylase) in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the formation of water soluble blue fragments produced by the action of *alpha-amylase* on commercially available (Phadebas®, Megazyme) azurine cross-linked insoluble blue-coloured starch polymer substrates [44,45]. An aliquot of the extracted sample (1 mL) is placed into a test tube to which 5 mL of phosphate buffer solution (pH 7.5) is added. Each tube is then preheated at 37 ^{o}C for 5 min. One Phadebas® tablet is added to each test tube and incubation at 37 ^{o}C for 15 min. Fifteen minutes later NaOH (1M, 1 mL) is added to each tube to stop the reaction and the solution is centrifuged for 5 min. The content of amylase is determined by colorimetry at 620 nm using the external calibration curve to derive "added" or "total" amylase (cf. discussion about calibrations).

For the determination of *endo- 1,4-beta-xylanase* (xylanase) in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified colorimetric method, based on the quantification of water soluble dyed fragments produced by the action of xylanase on commercially available (Xylazyme AX®, Megazyme) azurine cross-linked wheat arabinoxylan [48,49]. An aliquot of the extracted sample (2 mL) is placed into a test tube to which 1 mL of acetate buffer solution (pH 4.8) is added. One Xylazyme AX® tablet is added to each test tube and incubated at 50 ^{o}C for 3 h. Ethanol (4 mL) is added to each tube to stop the reaction, and the solution is let to rest for 20 min at room temperature before centrifugation for 5 min. The content of xylanase is determined by colorimetry at 585 nm using the external calibration curve to derive "added" or "total" xylanase (cf. discussion about calibrations).

For the determination of *bacillolysin* (protease) in *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified plate test method [52], based on the diffusion of protease in the azo-casein agar medium and the subsequent decolouring due to the hydrolysis of casein. At first the agar substrate is prepared as follows: 3 g of gelatine is added to 0.35 g of azo-casein, dissolved in 36 mL tris-HCl-buffer (pH 7.4). A saturated solution of $CaCO_3$ (14 mL) is then added and the solution is heated to 50 ^{o}C. Another solution is prepared with 1 g Agar and 0.05 thimerosal, dissolved in 50 mL deionised water, brought to boiling and cooled to 50 ^{o}C. The two solutions are then mixed. Aliquots of this solution (25 mL) are poured on Petri dishes and cooled to room temperature. Several 5 mm holes are made in the gel throughout the Petri dish. Aliquots of the extracted sample (70 µL) are placed in the holes of the agar plates and incubated for 21 h at 37 ^{o}C. Acetic acid (1.25 M) is poured on the plate to stop the reaction. The diameter of the clear zone is measured and compared to the external calibration curve to derive the "added" or "total" protease activity (cf. discussion about calibrations).

External Calibration

All the experimental protocols described above use external calibrations with Kemzyme standard solutions used as enzyme standards. The corresponding enzyme activities are characterised - at the same "reference" conditions (pH and temperature) used for the definition of the enzyme activity units - applying the methods for the determination of the *active substances* in the *feed additives* recommended above. *Kemzyme Plus Liquid* extracts are prepared diluting 0.4 g of the liquid formulation in 100 mL phosphate buffer (pH 6) and stirring for 5 min. *Kemzyme Plus Dry* extracts are prepared adding 2 g of the solid formulation and few drops of Tween 20® to 100 mL phosphate buffer (pH 6) solution, and stirring for 15 min.

In the original dossier the Applicant applied the matrix matched calibration using untreated feeds, containing naturally occurring enzymes with various endogenous enzyme activities [36,40,44,48,52]. Increasing amount of extracts (i.e. 0.25; 0.5; 0.75 and 1 mL) - originating from one of the Kemzyme formulations - were spiked to the untreated feed. When analysing the feed samples, the interpolation of this calibration curve provides the "added" enzyme activity in the *feedingstuffs*. This approach is reasonable only when untreated feed samples are available – which is rarely the case during an official control. The performance characteristics reported in Tables 2 and 3 were obtained using this type of calibration.

Upon request from the EURL, the Applicant re-interpreted the experimental data available as if a standard addition approach was applied [54], in which increasing amounts of the Kemzyme calibration extracts would have been added to already "treated" feed. The extrapolation of the resulting calibration curve to the "zero" diameter or "zero" absorbance (for the plate method or the absorbance one, respectively) provides the "total" enzyme activity (= endogenous + "added") in the *feedingstuffs*. The Applicant amended the standard operating procedures [55-59]. The best results were obtained for finished feed samples treated with *Kemzyme Plus Liquid* (Stability study, Tab.7,8 [60-64]), while highly scattered plots were presented for feeds spiked with the solid (dry) formulation.

The EURL identified an alternative, based on the matrix matched approach already suggested by the Applicant. Instead of spiking untreated feeds with increasing amounts of characterised Kemzyme standard solutions, enzyme-free feed (such as heat-treated whole grain wheat flour) could be used as "blank" samples to spike and construct the calibration curve. The interpolation of this calibration curve would provide accurate values of the "total" enzyme activities in the treated feed sample. The performance characteristics are expected to be similar to those presented in Tables 2 and 3.

Table 2: Performance characteristics of analytical methods for the determination of five active substances in feedingstuffs, spiking untreated feed with 100 to 750 g/T of Kemzyme Plus Dry were used. The enzyme activities (expressed in U/g) were derived using the following batch composition: 4040, 26148, 496, 60119 and 2651 U/g of Kemzyme Plus Dry (cf. Tab.2 [5]), for *glucanase, cellulase, amylase, xylanase* and *protease*, respectively.

	Activity (*)	**RSD_r (%)**		**RSD_{ip} (%)**		**R_{rec} (%)**	
A.Substance	(U/g)	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.
Glucanase	0.4-3	1.5–8.7 [12,38]	18 [12]	5.5-8 [12,38]	17.5 [12]	85-107 [12,38]	107 [12]
Cellulase	2.6-20	2.4–7.4 [18,42]	10 [18]	5.8–9.7 [18,42]	9.4 [18]	78–97 [18,42]	82 [18]
Amylase	0.05-0.37	12-29 [24,46]	11–13 [24]	16-30 [24,46]	12-24 [24]	93-109 [24,46]	79-88 [24]
Xylanase	6-45	2.9–13 [30,50]	5.3 [30]	10–11 [50]	7.9 [30]	72-122 [30,50]	98 [30]
Protease	0.3-2.0	3.3–7.7 [35,53]	13-23 [35]	5.8-10 [35,53]	17-23 [35]	98-109 [35,53]	103–123 [35]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec} : recovery rate

Table 3: Performance characteristics of analytical methods for the determination of five active substances in feedingstuffs, spiking untreated feed with 50 to 500 g/T of Kemzyme Plus Liquid were used. The enzyme activities (expressed in U/g) were derived using the following batch composition: 10789, 375300, 496 and 221336 U/g of Kemzyme Plus Liquid (cf. Tab.2 [6]), for *glucanase*, *cellulase*, *amylase* and *xylanase*, respectively.

	Activity (*)	**RSD_r (%)**		**RSD_{ip} (%)**		**R_{rec} (%)**	
A.Substance	(U/g)	Valid.	Verif.	Valid.	Verif.	Valid.	Verif.
Glucanase	0.5-5.4	0.2–8.7 [13,39]	18 [13]	0.8-8 [13,39]	17.5 [13]	85-107 [13,39]	107 [13]
Cellulase	19-188	4.7-8.2 [19,43]	10 [19]	6.3–9.7 [19,43]	9.4 [19]	78–97 [19,43]	82 [19]
Amylase	0.03-0.25	12-19 [25,47]	11–13 [25]	10-16 [25,47]	12-24 [25]	93-109 [25,47]	79-88 [25]
Xylanase	11-111	4.4-9.1 [31,51]	5.3 [31]	6.5-7.2 [51]	7.9 [31]	72-122 [31,51]	98 [31]

RSD_r and RSD_{ip}: relative standard deviation for *repeatability* and *intermediate precision*; R_{rec} : recovery rate

The Applicant performed validation and verification studies for the determination of each of the five active substances in *feedingstuffs*. The reported performance characteristics are listed in Table 2. As for limits of detection or quantification, the Applicant reported unrealistic values, larger than several reported analytical results. Instead of that the EURL over-estimated the Limits Of Quantification (LOQ) by setting them equal to the lowest enzyme activities measured: LOQ = 404; 2635; 50; 6012; 265 U/kg *feedingstuffs* for *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin*, respectively. Assuming that the limits of detection (LOD) are equal to one third of the LOQs, we derive for the five enzymes listed above: LOD = 135, 878, 17, 2004 and 88 U/kg *feedingstuffs*, respectively. Knowing that the lowest minimum enzyme activities in feedingstuffs are requested for Kemzyme Plus Dry (587, 1000, 100, 5000, 1212 U/kg, respectively) the over-estimated LODs indicate that the submitted methods allow the detection of all the enzymes of interest in *feedingstuffs*, thus allowing to assess whether the minimum recommended values are respected. However, experimental results below the limits of quantification are to be expected with large measurement uncertainties.

The Applicant did not provide any experimental data for the determination of the five *active substances* in *premixtures*, but he suggested to dilute *premixtures* samples with "blank" feed (such as enzyme-free feed or a heat-treated whole grain wheat flour), and to analyse the resulting sample applying the above mentioned methods for *feedingstuffs*.

Based on the satisfactory experimental evidence available the EURL recommends for official control the five colorimetric methods submitted by the Applicant for the determination of *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin* in *premixtures* and *feedingstuffs;* using calibration standards prepared by spiking Kemzyme Dry or Liquid feed additives of known enzymatic activity into enzyme-free feeds.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of these authorisations the EURL recommends for official control the ten single laboratory validated and further verified methods submitted by the Applicant for the determination of *endo-1,3(4)-beta-glucanase, endo-1,4-beta-glucanase, alpha-amylase, endo-1,4-beta-xylanase* and *bacillolysin* in *Kemzyme Plus Dry* and/or *Kemzyme Plus Liquid, premixtures* and *feedingstuffs* (eight colorimetric methods and two test plate methods).

Furthermore, the EURL recommends using a "blank" feed (enzyme-free or heat-treated whole grain wheat flour) for the matrix-matched external calibration and for the solid dilution of premixtures samples.

Recommended text for the register entry (analytical method)

For the determination of *endo-1,3(4)-beta-glucanase* in the *feed additives*:

- colorimetric method based on the enzymatic hydrolysis of glucanase on the barley beta-glucan substrate at pH 7.5 and 30 ^{o}C.

For the determination of *endo-1,4-beta-glucanase* in the *feed additives*:

- colorimetric method based on the enzymatic hydrolysis of cellulase on the carboxylmethylcellulose at pH 4.8 and 50 ^{o}C

For the determination of *alpha-amylase* in the *feed additives*:

- colorimetric method based on the formation of water soluble dyed fragments produced by the action of amylase on azurine cross-linked starch polymer substrates at pH 7.5 and 37 ^{o}C

For the determination of *endo-1,4-beta-xylanase* in the *feed additives*:

- colorimetric method based on the enzymatic hydrolysis of xylanase on the birchwood xylane substrate at pH 5.3 and 50 ^{o}C

For the determination of *bacillolysin* in the *feed additive*: (*Kemzyme Plus Dry*, only)

- colorimetric method based on the release of azo-dye resulting from the action of protease on the azo-casein substrate at pH 7.5 and 37 ^{o}C

For the determination of *endo-1,3(4)-beta-glucanase* in *premixtures* and *feedingstuffs*:

- plate test method based on the glucanase diffusion and the subsequent decolouring of the red agar medium due to the beta-glucan hydrolysis

For the determination of *endo-1,4-beta-glucanase* in *premixtures* and *feedingstuffs*:

- colorimetric method based on the quantification of the water soluble dye fragments produced by the action of cellulase on azurine cross-linked water insoluble HE-cellulose substrate

For the determination of *alpha-amylase* in *premixtures* and *feedingstuffs*:

- colorimetric method based on the formation of water soluble blue fragments produced by the action of amylase on azurine cross-linked insoluble blue-coloured starch polymer substrates

For the determination of *endo-1,4-beta-xylanase* in *premixtures* and *feedingstuffs*:

- colorimetric method based on the quantification of water soluble dyed fragments produced by the action of xylanase on azurine cross-linked wheat arabinoxylan

For the determination of *bacillolysin* in *premixtures* and *feedingstuffs*: (for *Kemzyme Plus Dry*, only)

- test plate method based on the diffusion of protease in the azo-casein agar medium and the subsequent hydrolysis of casein

One *endo-1,3(4)-beta-glucanase* unit (U) is the amount of enzyme which liberates 0.0056 micromoles of reducing sugars (glucose equivalents) from barley beta-glucan per minute at pH 7.5 and 30 °C;

One *endo-1,4-beta-glucanase* unit (U) is the amount of enzyme which liberates 0.0056 micromoles of reducing sugars (glucose equivalents) from carboxymethyl-cellulose per minute at pH 4.8 and 50 °C;

One *alpha-amylase* unit (U) is the amount of enzyme which liberates 1 micromole of glucose from a cross-linked starch polymer per minute at pH 7.5 and 37 °C.

One *endo- 1,4-beta-xylanase* unit (U) is the amount of enzyme which liberates 0.0067 micromoles of reducing sugars (xylose equivalents) from birchwood xylan per minute at pH 5.3 and 50 °C; and

One *bacillolysin* unit (U) is the amount of enzyme which solubilises one microgram of azo-casein substrate per minute at pH 7.5 and 37 °C.

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Kemzyme Plus Dry* and *Kemzyme Plus Liquid* 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] *Reference SANCO/D/2 Forw. Appl. 1831/00113(10035)-2010
[2] #Reference SANCO/D/2 Forw. Appl. 1831/00129(10037)-2010
[3] *Application, Proposal for Register Entry
[4] #Application, Proposal for Register Entry
[5] *Technical dossier, Section II: (a) 2.1.1 Identity; (b) 2.1.3 Composition; (c) 2.5.1 Conditions of use;
[6] #Technical dossier, Section II: (a) 2.1.1 Identity; (b) 2.1.3 Composition; (c) 2.5.1 Conditions of use;
[7] 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
[8] *Technical dossier, Section II-Annex_II_40 (glucanase/FA SOP)
[9] #Technical dossier, Section II-Annex_II_35 (glucanase/FA SOP)
[10] *Technical dossier, Section II-Annex_II_45 (glucanase/FA Valid.)
[11] #Technical dossier, Section II-Annex_II_39 (glucanase/FA Valid.)
[12] *Technical dossier, Section II-Annex_II_50 (glucanase/FA&FS Verif.)
[13] #Technical dossier, Section II-Annex_II_43 (glucanase/FA&FS Verif.)
[14] *Technical dossier, Section II-Annex_II_41 (cellulase/FA SOP)
[15] #Technical dossier, Section II-Annex_II_36 (cellulase/FA SOP)
[16] *Technical dossier, Section II-Annex_II_46 (cellulase/FA Valid.)
[17] #Technical dossier, Section II-Annex_II_40 (cellulase/FA Valid.)
[18] *Technical dossier, Section II-Annex_II_51 (cellulase/FA&FS Verif.)
[19] #Technical dossier, Section II-Annex_II_44 (cellulase/FA&FS Verif.)
[20] *Technical dossier, Section II-Annex_II_42 (amylase/FA SOP)
[21] #Technical dossier, Section II-Annex_II_37 (amylase/FA SOP)
[22] *Technical dossier, Section II-Annex_II_47 (amylase/FA Valid.)
[23] #Technical dossier, Section II-Annex_II_41 (amylase/FA Valid.)
[24] *Technical dossier, Section II-Annex_II_52 (amylase/FA&FS Verif.)
[25] #Technical dossier, Section II-Annex_II_45 (amylase/FA&FS Verif.)
[26] *Technical dossier, Section II-Annex_II_44 (xylanase/FA SOP)
[27] #Technical dossier, Section II-Annex_II_38 (xylanase/FA SOP)
[28] *Technical dossier, Section II-Annex_II_49 (xylanase/FA Valid.)
[29] #Technical dossier, Section II-Annex_II_42 (xylanase/FA Valid.)
[30] *Technical dossier, Section II-Annex_II_54 (xylanase/FA&FS Verif.)
[31] #Technical dossier, Section II-Annex_II_46 (xylanase/FA&FS Verif.)
[32] *Technical dossier, Section II-Annex_II_43 (protease/FA SOP)
[33] Megazyme S-AZCAS 12/07 - *Endo-protease using azo-casein*
www.megazyme.com/downloads/en/data/S-AZCAS.pdf (last visited on 03/08/2012)

[34] *Technical dossier, Section II-Annex_II_48 (protease/FA Valid.)
[35] *Technical dossier, Section II-Annex_II_53 (protease/FA&FS Verif.)
[36] *Technical dossier, Section II-Annex_II_55 (glucanase/FS SOP)
[37] #Technical dossier, Section II-Annex_II_47 (glucanase/FS SOP)
[38] *Technical dossier, Section II-Annex_II_60 (glucanase/FS Valid.)
[39] #Technical dossier, Section II-Annex_II_51 (glucanase/FS Valid.)
[40] *Technical dossier, Section II-Annex_II_56 (cellulase/FS SOP)
[41] #Technical dossier, Section II-Annex_II_48 (cellulase/FS SOP)
[42] *Technical dossier, Section II-Annex_II_61 (cellulase/FS Valid.)
[43] #Technical dossier, Section II-Annex_II_52 (cellulase/FS Valid.)
[44] *Technical dossier, Section II-Annex_II_57 (amylase/FS SOP)
[45] #Technical dossier, Section II-Annex_II_49 (amylase/FS SOP)
[46] *Technical dossier, Section II-Annex_II_62 (amylase/FS Valid.)
[47] #Technical dossier, Section II-Annex_II_53 (amylase/FS Valid.)
[48] *Technical dossier, Section II-Annex_II_59 (xylanase/FS SOP)
[49] #Technical dossier, Section II-Annex_II_50 (xylanase/FS SOP)
[50] *Technical dossier, Section II-Annex_II_63 (xylanase/FS Valid.)
[51] #Technical dossier, Section II-Annex_II_54 (xylanase/FS Valid.)
[52] *Technical dossier, Section II-Annex_II_58 (protease/FS SOP)
[53] *Technical dossier, Section II-Annex_II_63 (protease/FS Valid.)
[54] *Supplementary information – 00 Kemzyme Plus Dry
[55] *Supplementary information – 01 Procedure xylanase
[56] *Supplementary information – 02 Procedure glucanase
[57] *Supplementary information – 03 Procedure cellulase
[58] *Supplementary information – 04 Procedure amylase
[59] *Supplementary information – 05 Procedure protease
[60] *Supplementary information – 06 Total activity xylanase
[61] *Supplementary information – 07 Total activity glucanase
[62] *Supplementary information – 08 Total activity cellulase
[63] *Supplementary information – 09 Total activity amylase
[64] *Supplementary information – 10 Total activity protease
[65] *Supplementary information – new-Annex-A
[66] #Supplementary information – new-Annex-A

*Refers to Dossier No. FAD-2011-0175

#Refers to Dossier No. FAD-2010-0211

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the European Union 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:

- Fødevarestyrelsen (FVST), Ringsted, DK
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha, CZ
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim, DE
- Państwowy Instytut Weterynaryjny (NVRI), Puławy, PL
- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala, SE
- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky (UKSUP), Bratislava, SK

Furthermore, TLL (Thüringer Landesanstalt für Landwirtschaft, Abteilung Untersuchungswesen. Jena, DE) agreed with the proposed methods for the enzyme activity determination in the feed additives.