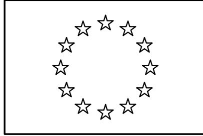

EUROPEAN COMMISSION
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Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives Authorisation

CRL
Feed Additives
Authorisation

D08/FSQ/CVH/GS/(2006) D/15162

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

Dossier related to:	**EFSA-Q-2006-004**
Name of Additive:	**Hemicell® Feed Enzyme**
Active Substance(s):	**β-D-mannanase (EC 3.2.1.78)**
Rapporteur Laboratory:	**National Research Institute of Animal Production, National Feed Laboratory, Lublin, Poland**
Report prepared by:	**Waldemar Korol** **31/05/06**
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EXECUTIVE SUMMARY

In the current application, *cf.* EFSA-Q-2006-004, authorisation is sought for Hemicell® Feed Enzyme under the category zootechnical additives, group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Hemicell® Feed Enzyme (liquid preparation) as a digestibility enhancer for chickens for fattening (broilers). The active ingredient is the β-D-mannanase enzyme (EC 3.2.1.78) produced by aerobic fermentation of a non-GMO strain of *Bacillus lentus* (ATTCC 55045). The active substance of the additive catalyses hydrolytic breakdown of mannan-containing hemicelluloses.

The activity of β-D-mannanase is expressed in terms of enzyme units U, where 1 U is the amount of the enzyme which liberates 0.72 microgram of reducing sugar (mannose equivalents) per minute from a mannan-containing substrate (locust bean gum) at pH 7.5 and 40 °C. According to the applicant, Hemicell® Feed Enzyme has a minimum guaranteed activity of 720 MU/l, where 1 million U (MU) is the amount of the enzyme which liberates 0.72 gram of reducing sugar under the same pH and temperature conditions. The additive is intended to be included into feedingstuffs obtaining a minimum β-D-mannanase activity of 79200 U/kg of feed.

For the determination of the β-D-mannanase activity in the *feed additive*, the applicant proposes an in-house developed method which is based on the principle that β-D-mannanase hydrolyses a mannan-containing substrate. The released sugars (mainly D-mannose) reduce 3,5-dinitrosalicylic acid to a yellow-orange coloured 3-amino-5-nitrosalicylic acid which is measured on a spectrophotometer using a D-mannose standard curve. The obtained method performance characteristics include a limit of quantification (LOQ) of 0.005 MU/l, and a relative standard deviation for repeatability (RSD_r) of 3.9 %. The method has also been tested by a second laboratory obtaining analytical results that are close to the applicant's results. Therefore, the method is considered suitable for the intended purpose.

For the determination of active substance in *feedingstuffs,* the same in-house developed spectrophotometric method as for the additive is proposed with some modifications to allow for detection of low activity levels. The provided performance characteristics include a limit of detection (LOD) of 28000 U/kg feed, a LOQ of 46000 U/kg feed, a RSD_r of 14.0 % and an

average recovery of 104 %. The method has also been tested by a second laboratory obtaining analytical results that are close to the applicant's results. Taking into account the target application level of at least 79200 U/kg feed and the acceptable values of performance parameters, the proposed method is considered suitable for official control of the activity of β-D-mannanase in feedingstuffs samples in the frame of the sought authorisation, i.e. in target feed samples (feedingstuffs for chickens for fattening) at the target activity level of the enzyme (79200 U /kg feed).

Further testing and validation is not considered necessary.

KEYWORDS

Hemicell® Feed Enzyme, β-D-mannanase, *Bacillus lentus,* digestibility enhancer, chickens for fattening

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1. BACKGROUND

Hemicell® Feed Enzyme is a feed additive for which authorisation is sought under the category zootechnical additives, group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. It contains β-D-mannanase (EC 3.2.1.78) as active agent produced by aerobic fermentation of a non-GMO strain of *Bacillus lentus* (ATTCC 55045).

The current application is for the use of Hemicell® Feed Enzyme as liquid preparation, which has a minimum guaranteed activity of 720 MU/l.

The intended use (*cf.* EFSA-Q-2006-004) is as digestibility enhancer for chickens for fattening by including the feed additive into feedingstuffs at a minimum dosage of 79200 U/kg.

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis submitted in connection with Hemicell® Feed

Enzyme, *cf.* EFSA-Q-2006-004, and their suitability to be used for official controls, were evaluated.

3. EVALUATION

The numbering system under this point refers to that of Part II of the “Guidelines for the assessment of additives in feedingstuff, Part II: Enzymes and Microorganism (2.5 Control methods)”, adopted by the Scientific Committee on Animal Nutrition on 22 October 1999, in the following referred as “the Guidelines”.

Description of some of the methods listed under items 2.5.1 of the Guidelines

Determination of heavy metal

Graphite furnace atomic absorption spectroscopy methods are proposed for the determination of heavy metals (arsenic, lead, cadmium). Fluorine is determined by an ion-selective electrode method after oxygen flask combustion. Mercury is analysed by flameless atomic absorption spectroscopy (cold vapour technique). All the methods are considered suitable for their purposes.

Determination of micro-organisms

Aerobic and anaerobic mesophilic bacteria contained in the enzyme product were analysed by colony count technique on agar according to the Food and Drug Administration (FDA) Bacteriological Analytical Manual, 7th ed. (1992) and the American Public Health Association (APHS) (2001) standard method, respectively. Isolation and confirmation of *Salmonella* was performed according to the AOAC (Association of Offical Agricultural Chemists) Official Method 991.14. *E.coli*, *Listeria monocytogenes, Staphylococcus aureus,* moulds and yeasts were analyzed according to the FDA Bacteriological Analytical Manual, 7th ed. (1992). The proposed methods are commonly used and therefore considered suitable for the intended purposes. However, for official control purposes the corresponding ISO/CEN standards are recommended by the CRL.

Determination of mycotoxins

For determination of deoxynivalenol (DON), fumonisin FB_1, FB_2, FB_3, ochratoxin A, zearalenone and T-2 toxins the applicant proposed the Neogen tests based on enzyme linked immunosorbent assay (ELISA) technique which are considered suitable for the intended purpose. However, for official control of ochratoxin A, zearalenone, T-2 and DON fully ring trial validated methods that are available at the CRL are recommended.

For aflatoxin B_1 the applicant proposed an high performance liquid chromatography (HPLC) method according to Directive 92/95 with the limit of quantification (LOQ) equal to 10 μg/kg which is considered suitable for the intended purpose. However, taking into account that the above directive is no longer in force, a fully ring trial validated method, available at the CRL is recommended.

Quantitative analysis of the activity of β-D-mannanase in the additive

For the determination of β-D-mannanase activity in the additive, the applicant provided an in-house developed spectrophotometric method [1] which has been also tested against a viscosimetric method recommended by the National Research Council (NRC) [2]. The proposed method is based on reduction of 3,5-dinitrosalicylic acid (DNS) reagent with reducing sugars to produce a stable yellow-orange coloured 3-amino-5-nitrosalicylic acid solution which is measured spectrophotometrically at 550 nm. The reducing sugars are produced by the action of β-D-mannanase (the active substance in Hemicell® Feed Enzyme) on a mannan-containing substrate, namely locust bean gum (LBG). The activity of β-D-mannanase is expressed in terms of enzyme units (U), where 1 U is the amount of the enzyme which liberates 0.72 microgram of reducing sugar (mannose equivalents) per minute from a mannan-containing substrate (LBG) at pH 7.5 and 40 °C. As regards the content of active agent in the feed additive, the enzyme activity is expressed as MU/l, where 1 million U (MU) is the amount of the enzyme which liberates 0.72 gram of reducing sugar per minute under the same pH and temperature conditions. The enzyme activity is not expressed in International Units because an available international standard method does not exist. The concentration of reducing sugar yield is calculated from the D-mannose standard curve based on the relationship between the absorbance and the D-mannose concentration within linear range of the assay. Finally, the activity is calculated taking into account the enzyme dilution factor and the reaction time.

The method has been validated in-house by the applicant [3] in accordance with harmonized guidelines for single-laboratory validation published by IUPAC [4]. All required parameters, including applicability, selectivity, calibration and linearity, accuracy, precision, range, limit of detection (LOD), limit of quantification (LOQ), robustness, and reproducibility were considered. The provided performance characteristics include a LOD of 0.0034 MU / l product, a LOQ of 0.0048 MU / l product and a relative repeatability standard deviation (RSD_r) of 3.9 %. The working range of method was indicated as 0.0015-1000 MU/l product. Taking into account the target level of application which is equal to 720 MU enzyme/l additive and the acceptable value of performance parameters, the proposed method is fit for the intended purpose.

Description of the qualitative and quantitative analytical methods for routine control of the active substance in premixtures and feedingstuffs (cf. pt. 2.5.2 of Guidelines)

Hemicell® Feed Enzyme is applied directly to feed pellets using a suitable spray application system, after dilution of 110 ml of the additive with 890 ml clean water.

For the determination of β-D-mannanase in *feedingstuffs,* the applicant proposed the same in-house developed spectrophotometric method [1] with some modifications in order to detect lower activity levels. As regards the content of active agent in the feedingstuffs, the enzyme activity is expressed as U/kg feed.

The protocol foresees the extraction of 10 g of grinded feed sample with 100 ml water. However, it is recommended by the CRL to increase the sample amount to 20 g and water volume to 200 ml, considering potential heterogeneity of the test material. Consequently, a 500 ml shake flask should be recommended in point 12.4c of the protocol and 200 ml water in point 12.4d (Annex II.2.1.3.2M).

An aliquot of 0.6 ml centrifuged feed extract is incubated with 3.0 ml of the LBG substrate solution for exactly 45 minutes. The reaction is stopped by the addition of 1.5 ml of DNS reagent working solution and, after placing tubes in a boiling water bath for exactly 5 minutes, the yellow-orange colour is measured in the spectrophotometer at 550 nm against water blank tube. The reducing sugar yield in mannose equivalent (in g/ml) is calculated from the standard D-mannose curve which in turn leads to the determination of the enzyme activity in the feed sample.

The protocol involves the parallel and identical treatment of four categories of experimental tubes: D-mannose, blank, control and enzyme solution. All experiments must be conducted in

duplicate or preferably in triplicate. The relative percent difference for duplicates and triplicates measurement of absorbance values was proposed as an element of internal quality assurance.

The method was validated in-house by the applicant [3] in accordance with harmonized guidelines for single-laboratory validation published by IUPAC [4]. The provided performance characteristics include: (a) a LOD of 28000 U/kg feed and a LOQ of 46000 U/kg feed, where LOD and LOQ values have been defined as the mean results of the sample blank measurements plus 3 times [LOD] and 6 times [LOQ] the standard deviation, (b) a RSDr of 14 % (15.5 % with a target dose of 39600 U/kg feed [n=30]; 13.5 % with a target dose of 79000 U/kg feed [n=162]; 12.5 % with a target dose of 118800 U/kg feed [n=30]), and (c) an average recovery rate of 104.0 %. The working range of the method is from 46000 U/kg feed to 950000 U/kg feed (12 times the proposed dose). Transferability of the method has been confirmed by applying the protocol in a second laboratory obtaining analytical results that are close to the applicants' results.

Following the CRL request, the applicant provided further details on the measurement of the background noise due to reducing sugars possibly already present in the feed, prior to the enzymatic measurement [5]. The applicant proposed two separate measurements, applying almost the same analytical procedure: 1) determination of the enzyme activity in the feed sample where the substrate LBG solution was used and (2) determination of the reducing sugar content in the same feed sample without enzymatic reaction (baseline sample), where only Tris buffer solution without the substrate was used (Annex II.2.13.2M point 13.3 b). Accordingly, suitable corrections in the method protocol were introduced by the applicant (Annex II.2.13.2M, point 6.1 page 8, point 13.2 page 20, point 13.3 page 21, point 14.3 page 23). The possible influence of background noise on the precision of the method was consequently assessed. Ten recent and independent broiler feed samples (using nine or ten replicates) were analysed and compared with baseline feed samples (without addition of LBG substrate) taken from the same replicates [6]. The mean baseline sample enzyme activity was 5300 U/kg indicating a low background level of reducing sugars in the feed and the repeatability standard deviation for the analytical results was 1600 U/kg. The mean enzyme activity of the corresponding feed samples was 107000 U/kg and the repeatability standard deviation of the analytical results was 12800 U/kg. After subtracting the baseline sample enzyme activity from the feed sample enzyme activity obtaining the final value of the activity of β-mannanase in feed of 101700 U/kg, the repeatability standard deviation (SD) was 13200

U/kg, which was only slightly increased compared to the enzyme determination without baseline correction (SD = 12800 U/kg). Therefore, these intra-assay data confirmed that the low level of reducing sugars typically present in feeds did not significantly change the precision of the method and contribute only to a small part (less than 10 %) to the overall activity measured from feed samples containing the enzyme at the target level. Taking into account the target level of application which is minimum 79200 U enzyme /kg feed and the acceptable value of performance parameters, the proposed method is fit for the purpose of official controls to determine the activity of β-D-mannanase in feedingstuffs samples in the field of application that is sought.

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant submitted the same spectrophotometric control method for the determination of β-D-mannanase activity in the feed additive and in feedingstuffs. This method is based on the principle that β-D-mannanase hydrolyses a mannan-containing substrate and the resulting sugars (mainly D-mannase) reduce 3,5-dinitrosalicylic acid (DNS) reagent to produce stable yellow-orange colour 3-amino-5-nitrosalicylic acid solution which is measured spectrophotometrically at 550 nm.

For the measurements of the activity of β-D-mannanase in feedingstuffs the applicant proposed an extraction of 10 g of the feed sample. It is recommended to increase the sample amount to 20 g considering potential heterogeneity of the test material.

Upon request of the CRL the applicant submitted further details in the method protocol concerning the baseline sample measurement.

Acceptable performance characteristics were provided, therefore the method is considered suitable for official controls in feedingstuffs at the proposed activity level.

Recommendation

Proposed text for the register entry, fourth column (Composition, chemical formula, description, analytical method):

Analytical method: Reducing sugar assay for β-D-mannanase by colorimetric reaction of dinitrosalicylic acid reagent on reducing sugar yield.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, three samples of Hemicell® Feed Enzyme (liquid form) were sent to the Community Reference Laboratory for Feed Additives Authorisation. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Hsiao, H-Y. (October 2005) Reducing Sugar Assay. Analytical method for β-D-mannanase in Hemicell® Feed Enzyme (liquid or dry presentations) or feed samples containing Hemicell® Feed Enzyme, by colorimetric reaction of dinitrosalicylic acid reagent on reducing sugar yield (Annex II.2.1.3.2.M).

[2] NRC (1981) National Research Council, Food and Nutrition Board, "Food Chemicals Codex", National Academy Press, 1981, pp. 490-491.

[3] Hsiao, H-Y. (2005) Analytical method validation. Reducing sugar assay for β-D-mannanase, used for the identification and quantification of β-D-mannanase in Hemicell® Feed Enzyme or feeds containing Hemicell® Feed Enzyme (Annex II.2.5.2).

[4] IUPAC Technical Report (2002). Thompson, M. Ellison, S.R. and Wood, R. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis. Pure and Applied Chemistry. 74 (5), 835-855.

[5] Hsiao, H-Y. (May 2006) Reducing Sugar Assay. Analytical method for β-D-mannanase in Hemicell® Feed Enzyme (liquid or dry presentations) or feed samples containing Hemicell® Feed Enzyme, by colorimetric reaction of dinitrosalicylic acid reagent on reducing sugar yield (Annex II.2.1.3.2.M).

[6] Hsiao, H-Y. (June 2006) Analytical method validation. Reducing sugar assay for β-D-mannanase, used for the identification and quantification of β-D-mannanase in Hemicell® Feed Enzyme or feeds containing Hemicell® Feed Enzyme (Annex II.2.5.2).

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the National Research Institute of Animal Production, National Feed Laboratory in Lublin, Poland