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Community Reference Laboratory for Feed Additives Authorisation

CRL Feed Additives Authorisation

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**EFSA-Q-2005-224**
Name of Additive:	**Biogalactosidase®**
Active Subtance(s):	**α-D-galactosidase (EC 3.2.1.22)**
Rapporteur Laboratory:	**Laboratory of the General Directorate of Fair Trading, Consumer Affairs and Fraud Control (DGCCRF), Rennes, France**
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Date:	**07/04/2006**

EXECUTIVE SUMMARY

In the current application authorisation is sought for Biogalactosidase® 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 Biogalactosidase® as a digestibility enhancer for pigs for fattening. Biogalactosidase® is an enzyme preparation to be marketed in two forms: Biogalactosidase 1000P® is a microgranular brown powder using wheat flour as carrier and Biogalactosidase® is a liquid formulation based on a mixture of glycerol and water. The active substance of Biogalactosidase 1000P® and Biogalactosidase® is α-D-galactosidase, which is produced by a genetically modified strain of *Saccharomyces cerevisiae* CBS 615.94.

According to the nomenclature of the International Union of Biochemistry and Molecular Biology (IUBMB) and the Enzyme Commission (EC), α-D-galactosidase has the register number EC 3.2.1.22.

The activity of α-D-galactosidase is expressed in enzyme units (U). According to the applicant, one U is defined as the enzyme activity required to produce one micromole of p-nitrophenol (pNP) per minute at the specified assay conditions. Biogalactosidase® has a guaranteed minimum activity of 475 U/ml and Biogalactosidase 1000P® of 950 U/g. The feed additive is intended to be mixed into premixtures and/or compound feedingstuffs to obtain enzyme activity levels of 25 to 200 U/kg in compound feedingstuffs.

For the determination of the enzyme activity of α-D-galactosidase in the *feed additive*, the applicant proposes a spectrophotometric method developed and validated in-house. The method is based on the fact that α-D-galactosidase catalyses the hydrolysis of para-nitrophenyl-α-D-galactopyranoside (pNPG) to yield D-galactose and pNP. The pNP turns yellow after addition of sodium carbonate and under alkaline conditions and is measured spectrophotometrically at 405 nm. The limit of quantification (LOQ) is 1 U/ml for the liquid and 1 U/g for the solid product. This method is considered suitable for official control in the field of application that is sought.

For the determination of the enzyme activity of α-D-galactosidase in *premixtures*, the applicant proposes the same method as the one applied for the feed additive. The method's performance characteristics include a relative standard deviation for repeatability (RSD_r) of 4.8 % for a sample with a target enzyme activity of 20 U/g, and of 3.1 % for a sample with a target enzyme activity of 200 U/g The LOQ is identical to the LOQ calculated for determination of the activity of α-D-galactosidase in Biogalactosidase 1000P®, which is 1 U/g. These performance characteristics are considered acceptable and the method is considered suitable for official control in the field of application that is sought.

For the detection and determination of the enzyme activity of α-D-galactosidase in *feedingstuffs*, the applicant proposes a modified version of the method applied for the analysis of the feed additive and premixtures. The main modification is that a larger sample quantity and less pNPG substrate are used for the analysis. The method's performance characteristics include a RSD_r of 7.8 % for a sample with a target enzyme activity of 30 U/kg, and of 5.5 % for a sample with a target enzyme activity of 200 U/kg. The limit of detection (LOD) for the method is 2 U/kg of feedingstuffs and the LOQ is 5 U/kg. These performance characteristics are considered acceptable and the method is considered suitable for official control in the field of application that is sought.

Further testing or validation is not considered necessary.

KEYWORDS

Biogalactosidase®, α-D-galactosidase, *Saccharomyces cerevisiae* CBS 615.94, digestibility enhancer, pigs for fattening.

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1. BACKGROUND

Biogalactosidase® 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 α-D-galactosidase as the active agent. The feed additive is presented in two different forms:

- Biogalactosidase®, which is a liquid formulation with a minimum activity of 475 U/ml
- Biogalactosidase 1000P®, which is a dry formulation with a minimum activity of 950 U/g

The feed additive is intended to be mixed into premixtures and/or compound feedingstuffs to obtain enzyme activity levels of 25 to 200 U/kg in compound feedingstuffs.

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, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the suitability of the methods of analysis and validation studies submitted in connection with Biogalactosidase®, *cf.* EFSA-Q-2005-224, was evaluated.

3. EVALUATION

The numbering system under this point refers to that of Section II of the "Guidelines for the assessment of additives in feedingstuffs, PART II: Enzymes and Micro-organisms (2.5 Control methods)", in the following referred as "the Guidelines".

Description of some analytical method listed under 2.5.1. of the Guidelines

Quantitative analysis of active substance in the feed additive

For the determination of the enzyme activity of α-D-galactosidase in the *feed additive*, the applicant proposes a spectrophotometric method that was developed and validated in-house. The method is based on the fact that α-D-galactosidase catalyses the hydrolysis of para-nitrophenyl-α-D-galactopyranoside (pNPG) to yield D-galactose and pNP. The pNP turns yellow after addition of sodium carbonate and under alkaline conditions and is measured spectrophotometrically at 405 nm. The activity of α-D-galactosidase is expressed in U/ml for the liquid product and U/g for the solid product. According to the applicant, one U is defined as the enzyme activity required to produce one micromole of p-nitrophenol (pNP) per minute at the specified assay conditions.

The sample is diluted with deionised water according to its expected enzyme activity range. 200 µl of the enzyme dilution is incubated at 37°C for 5 min with 1.8 ml of the substrate pNPG which has been prepared in 0.1 M acetate buffer adjusted at pH 5.0. The incubation is stopped by the addition of 4 ml of a 10 % sodium carbonate solution and the optical density of the reaction solution is measured at 405 nm. The enzyme activity of the sample is calculated by taking into account (1) the corrected absorbance which is the difference between the absorbance of the sample and the absorbance of the reagent blank, (2) the molar extinction coefficient of pNP, (3) the reaction time, (4) the enzyme dilution factors and (5) the sample weight.

The limits of quantification (LOQs) for the method were 1 U/g for the solid product and 1 U/ml for the liquid product, both of which are considered acceptable, taking into account the target enzyme activities in the products. Regarding the precision of the method, a relative standard deviation for repeatability (RSD_r) of 3.8 % was reported. Raw data and the design of validation experiments concerning the limit of detection (LOD) and LOQ have not been provided. Nevertheless, taking into account the acceptable performance characteristics and the fact that the applicant's method follows well known principles adapted from widely used methods to assay α-galactosidase in enzyme preparations derived from *Aspergillus niger* [1] and β-galactosidase [2, 3] that are found in the literature, this method is considered suitable for official control in the field of application that is sought.

Quantitative analysis of impurities

- Determination of heavy metals

Heavy metals are determined using an empirical semi-quantitative colorimetric method as described in the Guide to specifications of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO Food and Nutrition Paper (FNP) 5 rev.2). The method is based on the principle that heavy metals such as lead, mercury, cadmium and arsenic form colour compounds with hydrogen sulphide. The results are reported in total amounts of heavy metals present, expressed in terms of lead. The proposed method is commonly used and is considered suitable for the intended purpose. For the official control purposes, the CRL recommends to use a method based on atomic absorption spectroscopy.

- Determination of micro-organisms

Microbiological quality is ensured by investigating total viable count, coliforms, *E. coli*, *Salmonella* species and yeasts and moulds. Total viable count is performed using tryptone soya agar [US Pharmacopeia 21]. Coliforms are enumerated on violet red bile glucose agar [ISO 7402]. For determination of *Escherichia coli*, lactose broth and a Mac Conkey broth are used for pre-enrichment and a selective enrichment phases, respectively [US Pharmacopeia 21]. Yeasts and moulds are enumerated on Sabouraud dextrose agar [ISO 7954]. For detection of *Salmonella* species, lactose broth as well as selenite and tetrathionate broths are used for pre-enrichment and a selective enrichment phases in accordance with US Pharmacopeia 21. The proposed methods are commonly used and are considered suitable for the intended purpose.

Description of the quantitative analytical methods for routine control of the active substance in premixtures under 2.5.2 of the Guidelines

For the determination of enzyme activity of α-D-galactosidase in the *premixtures,* the same in-house developed spectrophotometric method as for the feed additive is proposed.

The precision of the method was calculated using two different samples, obtaining a relative standard deviation for repeatability (RSD_r) of (1) 4.8 % for a sample containing a target enzyme activity of 20 U/g and (2) 3.1 % for a sample containing a target enzyme activity of 200 U/g. The LOQ is identical with the LOQ calculated for determination of the activity of α-D-galactosidase in Biogalactosidase 1000P®, which is 1 U/g. Though some raw data and information on the design of some of the validation experiments were missing, the method is considered suitable for official control in the field of application that is sought, for the same reasons as elaborated in the method chapter on the analysis of the *feed additive.*

Description of the quantitative analytical methods for routine control of the active substance in feedingstuffs under 2.5.2 of the Guidelines

For the determination of the enzyme activity of α-D-galactosidase in *feedingstuffs* and in order to allow for detection of the relatively low enzyme activity levels in feedingstuffs, the applicant proposed a modified version of the method applied for the analysis of the feed additive and premixtures. The main modification is that a larger sample quantity and less pNPG substrate are used for the analysis.

The protocol foresees the extraction of 5.0 g of ground feed sample with 0.1 M acetate buffer adjusted at pH 5.0. However, the CRL recommends to increase the sample amount to 20 g, to take into account potential heterogeneity of the test material. An aliquot of 0.5 ml of centrifuged extract is incubated at 37^{o}C for 5 minutes with 0.45 ml of the substrate pNPG which has been prepared in a solution of 0.1 M sodium acetate. The incubation is stopped by the addition of 1 ml of a 10 % sodium carbonate solution and the optical density of coloured pNP is measured at 405 nm. The enzyme activity is calculated taking into consideration the same details as for the analysis of the feed additive and premixtures.

Precision values of the method were calculated by the rapporteur laboratory and based on results of replicate analysis of pelleted feed delivered by the applicant. The RSD_r calculated from the analytical results of 10 different lots containing the enzyme with a target activity of 65 U/kg was 3.1 % and the corresponding RSD_r calculated from the results from 5 different lots with a target enzyme activity of 45 U/kg was 8.3 %. For both sample sets the experiments also confirmed acceptable values for the percentage of measured to target enzyme activity, which were, on average, above 90 %. In addition, the applicant reported on the results of experiments where two extracts from two pelleted samples with different target enzyme activity were prepared and 20 aliquots from each extract were subjected to analysis. The relative standard deviation in this case was 7.8 % for a sample containing 30 U/kg and 5.5 % for a sample containing 200 U/kg. The estimation of the LOD and LOQ - as calculated by the rapporteur laboratory - was based on the results from the analysis of 20 extracts from 1 blank feed samples conducted by applicant. The LOD corresponding to the average of the analytical results from the blank sample plus three times the standard deviation was 2 U/kg and the LOQ corresponding to the average of the analytical results from the blank sample plus ten times the standard deviation was 5 U/kg. Moreover, the results of experiments conducted on pelleted feedingstuff with various enzyme activities of α-D-galactosidase confirmed the method's linearity (R^2 = 0.9931) in the range of 10-50 U/kg.

Taking into account (1) the LOQ of 5 U/kg which is well below the minimum level of the enzyme activity in the feedingstuffs of 25 U/kg, (2) the acceptable data for the precision and

(3) the fact that the method follows well known principles, the proposed method is considered suitable for official control in the field of application that is sought.

CHECKLIST FOR THE METHODS FOR DETERMINATION OF THE ACTIVE AGENT (α-D-GALACTOSIDASE)

		Y	N	N/A	Comments
1.1	Is/are the method(s) mentioned on Premixtures accompanied by information on:				
	- Sampling method used		**x**		
	- Percentage recovery		**x**		Not directly
	- Specificity		**x**		
	- Accuracy	**x**			
	- Precision	**x**			Only repeatability
	- Limit of detection	**x**			Identical to additive
	- Limit of quantification	**x**			Identical to additive
	- Validation procedure used	**x**			Partially, not for LOD, LOQ
1.2	Is/are the method(s) mentioned on Feedingstuffs accompanied by information on:				
	- Sampling method used		**x**		
	- Percentage mecovery		**x**		Not directly
	- Specificity		**x**		
	- Accuracy	**x**			Raw data provided
	- Precision	**x**			Only repeatability
	- Limit of detection	**x**			Raw data provided
	- Limit of quantification	**x**			Raw data provided
	- Validation procedure used	**x**			

N/A: not applicable

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant submitted the same control method for the determination of the enzyme activity of α-D-galactosidase in the *feed additive* and in *premixtures* and a modified version of the method to allow for detection of lower activity levels for the analysis of the *feedingstuffs*. These two spectrophotometric methods are very similar and based on the same well known fact that α-D-galactosidase catalyses the hydrolysis of pNPG to yield D-galactose and pNP. For both methods acceptable performance characteristics were provided and the methods are therefore considered suitable for official controls in target feedingstuffs at the target activity level. However, for the measurements of the activity of α-D-galactosidase in the feedingstuffs

the applicant proposed extraction of 5 g of the feed sample. It is recommended to increase the sample amount to 20 g taking into account potential heterogeneity of the test material.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, three samples of each products - Biogalactosidase® (liquid form) and Biogalactosidase 1000P® (solid form) have been sent to the Community Reference Laboratory for Feed Additives Authorisation.

The dossier has been made available to the CRL by EFSA.

Further information provided by applicant:

- Supplementary data submitted by e-mail dated of 03/02/2006, 03/03/2006 and 10/03/2006.

6. REFERENCES

[1] Anonymous. 2004. α-galactosidase activity. Food Chemicals Codex. Fifth Edition. The National Academies Press Washington, D.C., pp. 905-906.

[2] Anonymous. 2004. Lactase (neutral) (β-galactosidase) activity. Food Chemicals Codex. Fifth Edition. The National Academies Press Washington, D.C., pp. 911-913.

[3] Anonymous. 2004. Lactase (acid) (β-galactosidase) activity. Food Chemicals Codex. Fifth Edition. The National Academies Press Washington, D.C., pp. 913-914.

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was Laboratory of the General Directorate of Fair Trading, Consumer Affairs and Fraud Control (DGCCRF), Rennes, France.