EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory – Feed Additives Authorisation

D08-FSQ(2005)D/29129

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-2005-175**
Name of Additive:	**Amaferm®**
Active Agent(s):	**1,4-α-D-glucan glucano-hydrolase, endo-1,4-β-D-glucan glucano-hydrolase, a vitamin B complex and citric acid**
Rapporteur Laboratory:	**Community Reference Laboratory for Feed Additive Authorisation, IRMM, Geel, Belgium (CRL-FAA)**
Report prepared by:	**Renata Leuschner**
Report checked by:	**Anne-Mette Jensen (CRL-FAA)**
Date:	**15 November 2005**
Report approved by:	**Christoph von Holst (CRL-FAA)**
Date:	**16 November 2005**

EXECUTIVE SUMMARY

In the current application authorisation is sought for the feed additive Amaferm® under the category 'zootechnical additives', functional group 4 'other zootechnical additives', according to the categorisation system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Amaferm® for dairy cows and beef cattle. Amaferm® consists of a fermentation extract originating from a non-pathogenic strain of the fungus *Aspergillus oryzae* (ATCC 10063) (4 %) sprayed onto wheat bran (96 %). A daily dose between 2 and 5 g Amaferm® per animal (beef cattle, cows), mixed in concentrate feed, total mixed ratio or as a top-feeding is recommended.

Amaferm® contains four defined active agents, an alpha-amylase (1,4-α-D-glucan glucano-hydrolase), an endo-1,4-β-D-glucan glucano-hydrolase, a vitamin B complex and citric acid.

The applicant proposes colorimetric methods for the two enzymes, a fluorometric rapid method for the vitamin B complex (determined as vitamin B_1) and a gravimetric method for citric acid. Although the applicant has cited official methods of analysis from the Association Of the Analytical Community (AOAC) International and one publication for the determination of the four active agents of Amaferm® in the fermentation extract, the target matrices of these methods do not cover the additive Amaferm®, premixtures and feedingstuffs.

In summary, taking into account the proposed methods for the four active agents it is not possible to conclude on their suitability for routine analysis of the additive, premixtures or feedingstuffs due to the absence of supporting experimental data. Equally, it is not possible to comment on the suitability of the proposed methods for the use in official control.

KEYWORDS

Amaferm®, *Aspergillus oryzae*, fermentation extract, feed additive, cattle

TABLE OF CONTENTS

1. BACKGROUND

Amaferm® is a feed additive consisting of a dried extract of a non-pathogenic strain of the fungus *Aspergillus oryzae* (ATCC 10063). The additive Amaferm® is produced by spraying the fermentation extract which was secreted by *A. oryzae* in a 2-stage fermentation process onto a carrier of food grade wheat bran. This is followed by drying and grinding to produce the additive Amaferm® with a final concentration of 4 % fermentation extract and 96 % wheat bran.

The additive contains four active agents which are an alpha-amylase (1,4-α-D-glucan glucano-hydrolase), a cellulase (endo-1,4-β-D-glucan glucano-hydrolase), a vitamin B complex (determined as vitamin B_1) and citric acid. Typical concentration levels of the active agents in the supernatant obtained at the end of the fermentation process are: 1330 mIU[1]/g of 1,4-α-D-glucan glucano-hydrolase, 100 mIU/g of endo-1,4- β-D-glucan glucano-hydrolase, 237 mg/kg of thiamine and 6.7 % citric acid ($C_6H_8O_7$). After spraying the extract onto the carrier and subsequent drying, the content in the additive would correspond to 40 mIU/g for 1,4-α-D-glucan glucano-hydrolase, 3.0 mIU/g for endo-1,4-β-D-glucan glucano-hydrolase, 7.1 mg/kg thiamine and 0.2 % citric acid. The product is supplied in dry form.

The proposed dosages for Amaferm® are:

- between 2 and 5 g Amaferm® per animal per day, mixed into concentrate feed, total mixed ration or as a top-feeding for dairy cows
- between 2 and 4 g Amaferm® per animal per day, mixed into concentrate feed, total mixed ration or as a top-feeding for beef cattle in growing/finishing stage.

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

[1] IU = International Unit(s)

applications for authorisations of feed additives, the CRL is required to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the suitability of the control methods submitted in connection with EFSA-Q – 2005 -175 was evaluated.

3. EVALUATION

The numbering system under this point refers to the corresponding numbering system of the Guidelines for the assessment of additives in animal nutrition – Part II: Enzymes and Microorganisms –, as adopted by the Scientific committee on Animal Nutrition on 22 October 1999.

Control Methods

Description of the methods used for the determination of the criteria listed under items 2.1.3, 2.1.4, 2.1.5, 2.2.5, 2.2.6, 2.3.1, 2.3.2, and 2.3.3

Qualitative and quantitative composition of the additive

There are four active agents in the additive Amaferm® which were described with their corresponding concentrations in section four (background) of this evaluation report.

The applicant cited published methods (AOAC Int. official methods of analysis and a method published in a scientific journal), and provided copies of the four methods for determining the active agents of the fermentation extract [1-4]. The enzyme concentration of the α-amylase (1,4-α-D-glucan glucano-hydrolase) is measured spectrophotometrically at 400 nm. The results are expressed in Ceralpha units whereby 1 unit is defined as the amount of enzyme required to release 1 µmol *p*-nitrophenol per minute at 40 ºC [1]. The cellulase (endo-1,4-β-D-glucan glucano-hydrolase) activity is quantified as the generation of reducing sugars from carboxymethyl cellulose (CMC). Reducing sugars are measured by colorimetry using tetrazolium blue chloride. One international unit of cellulose activity has been defined as 1 µmol of reducing sugar produced per minute [2]. Thiamine (vitamin B_1) is determined following an extraction and subsequent oxidation procedure. Fluorescence of an isobutanol extract is measured and thiamine hydrochloride content is calculated [3]. Citric acid is determined gravimetrically following an AOAC procedure suggested for analyses of citric acid in cheese [4].

The applicant has not provided method protocols for the analysis of the four active agents in the additive Amaferm® which would outline each procedure containing all necessary information to conduct the methods of analysis in the laboratory. The applicant explained that the methods are not used on a routinely basis to analyse the concentrations of the agents in the

additive Amaferm®. Method performance data are not provided. There is a limited amount of data in stability studies conducted for Amaferm® in the dossier which may indicate that those methods would perform adequately for the analysis of the four active agents in the additive Amaferm®. However, in the absence of appropriate performance or validation data these can not be considered as sufficient and in particular for the two enzymes applicability of the proposed methods appears doubtful.

The applicant outlined further that he would ensure the reproducibility of the production process and an analysis of the liquid fermentation extract prior to production of the additive Amaferm®. To ensure consistency of the final fermentation extract, the production process is controlled using methods such as pH measurements and those described above for analyses of thiamine and citric acid [3, 4]. The proposed methods of analysis for vitamin B_1 and citric acid may be appropriate to analyse the fermentation extract, however they can not necessarily be considered equally appropriate to analyse the additive Amaferm® which is a dry product containing the fermentation extract at a concentration of 4 % and wheat bran with 96 %, since naturally occurring substances in wheat bran may interfere with the analysis.

Internal quality control methods are documented (The applicant has a quality system in place to ensure consistent production (see Quality Manual ISO 9001). While AOAC and other methods are referenced in a table no protocols of analysis including methodological information or method performance data are provided.

Methods to assess the purity and identity of the production strains *Aspergillus oryzae* (ATCC 10063), deposited at an offically recognised culture collection, are considered appropriate to ensure the correct strain identity for manufacturing purposes. They are based on commonly used principles for mould identification such as light microscopy to examine characteristic morphology. Upon request, the applicant provided further methods to identify *A. oryzae* in the fermentation extract such as high performance liquid chromatography (HPLC) chromatograms showing a characteristic amino acid profile. While these methods are appropriate for internal control by the applicant of e.g. the manufacturing process, they can not be considered appropriate to identify unambiguously the additive Amaferm® .
(*Cf*. the requirements listed in point 2.1.3 of the Guidelines.)

Qualitative and quantitative composition of any impurities

Methods to analyse for impurities of the additive Amaferm® are not provided.
(*Cf*. the requirements listed in point 2.1.4 of the Guidelines).

Physical state of each form of the product

Methods for determination of particle size, dusting potential and density are briefly mentioned in the dossier. They include the use of ISO filters, Stauber-Heubach dust test and volumetric measurements and appear to be appropriate for determining product characteristics. Detailed method procedures or references are not provided.
(Cf. the requirements listed in point 2.1.5 of the Guidelines).

Toxins and virulence factors

Methods of analysis were not provided to assess toxins and virulence factors of Amaferm®.
(*Cf*. the requirements listed in point 2.2.5 of the Guidelines).

Antibiotic production and antibiotic resistance

Methods of analysis to assess antibiotic production/resistance of the production strain were not provided.
(*Cf*. the requirements listed in point 2.2.6 of the Guidelines).

Stability of the additive

The four methods described above are used for the stability studies for this application [1 – 4] as well. The applicant provided no experimental data on the stability of the active agents of Amaferm® in the premixtures and feedingstuffs. The questionable suitability of the proposed methods is discussed in the corresponding sections of this evaluation report.
(*Cf*. the requirements listed in point 2.3.1 of the Guidelines).

Other physico-chemical or biological properties

Methods to investigate other physico-chemical or biological properties of the additive Amaferm® were not provided by the applicant.
(*Cf*. the requirements listed in point 2.3.2 of the Guidelines).

Incompatibilities with other feed ingredients

Information on methods to assess incompatibilities or interactions with other feed ingredients was not provided.
(*Cf*. the requirements listed in point 2.3.3 of the Guidelines).

Description of qualitative and quantitative methods for routine control of the active agents in premixtures and feedingstuffs

Literature citations concerning the four control methods for the active agents in the fermentation extract were provided as outlined in the section of the report dealing with the

feed additive [1-4]. However, no data is presented to demonstrate that those methods perform adequately for the analysis of the four active agents in the premixture or the animal feed containing Amaferm®. There is in fact doubt, whether the proposed methods of analysis of the active agents in the fermentation extract would be applicable to premixtures or feedingstuffs because of the low concentrations of the active substances in premixtures or feedingstuffs and because matrix effects are to be expected. The applicant confirmed that no routine control is carried out on the active agents in the premixtures or animal feed containing Amaferm® and consequently limits of quantification (LOQ) of the methods were not provided.

Although the applicant recommended an external cobolt marker (Microtracer™ F-Cobalt) and its incorporation in premixtures and feedingstuffs (premixtures should be formulated to contain 5 grams tracer per metric ton of finished feedingstuffs) as a way to quantify Amaferm®, this is not considered an appropriate alternative to the direct quantitative analysis of the four active agents in premixture and feedingstuffs. A cobolt microtracer may provide an indication towards the identity of the manufacturer, however such an indication would not provide unambiguous identification.
(*Cf*. the requirements listed in point 2.5.2 of the Guidelines).

4. CONCLUSIONS AND RECOMMENDATIONS

In summary, taking into account the methods provided for the analysis of the four active agents in the fermentation extract of Amaferm®, it is not possible to conclude on their suitability for the analysis of the additive Amaferm® or premixtures or feedingstuffs containing Amaferm® without supporting protocols of analysis and validation or method performance data.

As none of the proposed methods are routinely used to directly analyse the active agents in the additive Amaferm® it is doubtful, in particular concerning the two enzymes, whether the proposed methods of analysis are appropriate for the additive. This hold true, equally, for the analysis of premixtures and feedingstuffs, particularly when taking into account the low concentrations of the active agents and expected matrix effects.

No performance characteristics are provided for the proposed methods for any of the active agents in Amaferm®, premixtures or feedingstuffs. The absence of validation or performance data in the dossier makes it impossible to comment on the suitability of the proposed methods for routine control. Equally, it is also not possible to comment on the suitability of the proposed methods for use in official control.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

Information on methodologies is provided by the applicant in the following sections of the dossier:

- Introduction
- Section II: Identity, characterisations and conditions of use of the additive; methods of control
- Section IV: Studies concerning the safety of use of the additive
- Section V: Form of monograph
- Section VI: Form of identification note
- Annexes I – XV
- Testing methods (AOAC Official Control Methods of Analysis 2000 & 2003)
- Literature

Additional information provided by the applicant:

- Supplied by e-mail on 15/07/05 a letter of justification of methods to analyse four active components of Amaferm® dated 11 July 2005 and a morphological description of the production strain of Amaferm®
- Information on production strain identity e-mailed on 23/07/05
- Supplement to section II 2.5 Control methods dated 6 October 2005-10-27
- Supplement 2 to section II 2.5 Control methods dated 18 October 2005

Product samples have been made available to the CRL on 02.02.2005.

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

6. REFERENCES

[1] AOAC Int. Official Method of Analysis. 2000. Section 32.1.35A. Measurement of α-amylase activity in white wheat flour, milled malt, and microbial enzyme preparations.

[2] Barichievich E.B., Calza R.E. 1990. Supernatant protein and cellulose activities of the anaerobic ruminal fungus *Neocallimastix frontalis* EB 188. Appl. Environ. Microbiol. 56, 43.

[3] AOAC Int. Official Method of Analysis, 2000. Section 45.1.06. Thiamine (vitamine B_1) in grain products.

[4] AOAC Int. Official Method of Analysis. 2000. Section 33.7.21. Citric acid in cheese.

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additive Authorisation (CRL-FAA), IRMM, Geel, Belgium.