# Community Reference Laboratory for Feed Additives Authorisation

## Annual Report 2006

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CRL
Feed Additives Authorisation

The mission of IRMM is to promote a common and reliable European measurement system in support of EU policies.

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***FOREWORD***

We are very pleased to present the annual report on the activities of the Community Reference Laboratory (CRL) for Feed Additives Authorisation of 2006, which is the second year after the CRL started its activities in 2005. Besides the evaluation of dossiers, which is indeed the main objective of the CRL, we have still been working on several topics that are important for a smooth operation of all CRL activities such as further improvement of web tools, the method data bank and internal administration tools.

Also in 2006 we were still involved in specifying the concept of the dossier evaluation, focusing on the requirements of the applicant's analytical methods when applied to official control as outlined in Commission Regulation (EC) No 378/2005. Based on discussions with the National Reference Laboratories (NRLs), Unit D2 - Animal Welfare and Nutrition - of Directorate General (DG) for Health and Consumer Protection and the Panel on Additives and Products or substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA), we were able to come up with a general interpretation of the requirements which will definitely facilitate the evaluation of dossiers. We have also been involved in the drafting of the new Commission guidelines for the preparation of dossiers, contributing to the part regarding the analytical methods and their suitability for official control. Due to its central role in the evaluation procedure of dossiers, this report will elaborate a little bit more on this topic.

When reading the report you will recognise the challenge of the evaluation procedure, which is mainly related to the diversity of the feed additives involved and in consequence also of the analytical methods to detect the feed additives in feed. In fact, the variety of these methods covers a wide range of different techniques such as liquid chromatography to detect coccidiostats or enumeration methods for the determination of probiotics.

Moreover, Regulation (EC) No 882/2004 on official food and feed control assigned additional tasks to the CRL. Therefore we decided to change the name and logo from the CRL for Feed Additives Authorisation (CRL-FAA) into CRL for Feed Additives (CRL-FA) as from January 2007.

We would like to thank our colleagues from the NRLs for supporting us with their expert knowledge, thus ensuring that our reports are based on sound scientific evaluation. Likewise we are grateful to our colleagues from DG for Health and Consumer Protection and EFSA for the excellent cooperation. Therefore we are looking forward to achieve our tasks for 2007 in close co-operation with our partners.

*The CRL Team*

*The CRL Team*

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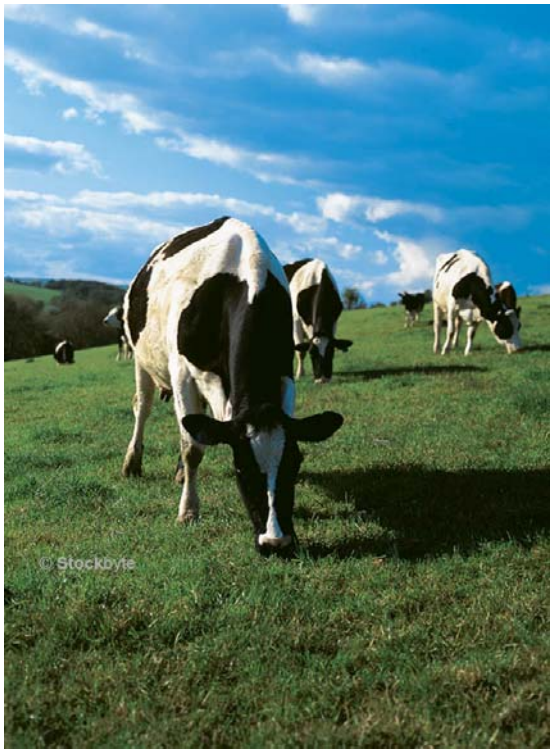



## THE OBJECTIVE AND THE TEAM OF THE CRL-FAA

Since November 2004 feed additives have to be authorised according to the procedure laid down in Regulation (EC) No 1831/2003. The procedure is based on a strict separation between scientific assessment of the feed additive - which falls under the responsibility of the European Food Safety Authority (EFSA), having the role of *risk assessor* - and the actual authorisation for placing the product on the market which is granted by the European Commission in its role as *risk manager*.

The assessment of the feed additive also includes a close evaluation of the analytical methods that are proposed by the applicant in order to determine the active substance in various matrices such as animal feed. This evaluation and some other tasks that are described later on in this report are entrusted to the Community Reference Laboratory which - according to Regulation (EC) No 1831/2003 - is the European Commission's Joint Research Centre (JRC). Within the JRC the Food Safety and Quality Unit of the Institute for Reference Materials and Measurements (IRMM) has taken up the task to establish the CRL.

Moreover, since 1st January 2006, Regulation (EC) No 882/2004 on official food and feed controls assigned additional tasks to the CRL.

In 2006 the CRL team consisted of seven people (situation: December 2006). The team members come from four Member States and one candidate country.

*Christoph von Holst*

*Anne-Mette Jensen*
*(until June 2006)*

*Giuseppe Simone*

*Dalia Garalevičienė*

*Renata Leuschner*

*Sulhattin Yasar*

*Seppe Staes*

*Machteld De Smet*


## TASKS OF THE CRL-FAA

### *The CRL-FAA and the authorisation process*

The main task of the CRL is the evaluation of the analytical methods submitted by the applicant, in order to establish whether these methods are suitable for the intended purpose. Analytical methods are for instance required to determine the active substance of the feed additive in animal feed and – if applicable – residues in animal tissue. As specified in Article 6.1 of Regulation (EC) No 378/2005 the CRL is assisted by a consortium of National Reference Laboratories (NRLs) which contribute to the evaluation procedure with their expertise on specific analytical methodology. The appointed laboratories are listed in Annex 1.

Analytical methods are evaluated in a stepwise manner in which the CRL and a rapporteur laboratory, which belongs to the consortium of NRLs and which the CRL selects individually for each dossier, conduct a documentary evaluation of the protocol of the methods and the corresponding validation report. Based on this evaluation the rapporteur laboratory and the CRL write a report, which is afterwards sent to EFSA. In the case that the submitted methods are considered suitable for official control a favourable opinion is given to EFSA, without performing experiments. In agreement with Regulation (EC) No 378/2005 the CRL charges the applicant 3000 € for each application. More details on the evaluation procedure are given later on in this report. If necessary, the CRL may also test the method in its own or a NRL laboratory, or it may organise an inter-laboratory comparison study to validate it.

In addition, the CRL maintains a bank of reference samples of all authorised additives.

In agreement with Regulation (EC) No 1831/2003 and Regulation (EC) No 378/2005 the CRL-FAA responsibilities also include other tasks, namely

- disseminating analytical methods;
- providing scientific and technical assistance to the Commission, especially in cases of dispute; and
- overall coordination of the consortium of National Reference Laboratories.

Last but not least, the CRL also aims to contribute to the mission of IRMM which is to promote a common and reliable European measurement system in support of EU policies.


***The CRL-FAA and the Official food and feed control Regulation***

Since 1st January 2006 and in accordance to Article 32 of Regulation (EC) No 882/2004, the CRL is also responsible for:

- Providing national reference laboratories (NRLs) with details of analytical methods, including reference methods;
- Coordinating the application of the above mentioned methods by the NRLs, in particular by organising comparative testing and by ensuring an appropriate follow-up of such comparative testing in accordance with internationally accepted protocols, when available;
- Coordinating practical arrangements needed to apply new analytical methods and informing the NRLs of advances in this field;
- Conducting initial and further training courses for the benefit of staff from NRLs and of experts from developing countries;
- Providing scientific and technical assistance to the Commission, especially in cases where Member States contest the result of analysis
- Collaborating with laboratories responsible for analysing feed and food in third countries.

In accordance with Article 12, sampling and analysis in the context of official control are carried out by official laboratories designated by competent authorities in each Member State.

## MAIN ACTIVITIES OF THE CRL-FAA IN 2006

In addition to the scientific evaluations of analytical methods the CRL organised in 2006 workshops, established a number of web tools, maintained a database on methods of analysis, and a sample bank of feed additives and prepared several strategic documents, especially related to the evaluation procedure of the dossiers.

### *CRL-FAA Evaluation Reports*

In 2006, final reports on the assessment of analytical methods for 20 dossiers were submitted to EFSA. All evaluations were completed within the given timeframe (see Table 1). You will find the executive summary of the CRL reports in Annex 2.

**Table 1: Overview of dossiers handled in 2006**

| Additive Name | Active Substance | Rapporteur |
|---|---|---|
| ELANCOBAN® | *Monensin sodium* | CISTA (J. Petrova), CZ |
| ALKOSEL®/SELSAF | *Selenium* | C.Re.A.A (M. C. Abete, D. Marchis), IT |
| NATUPHOS® | *3-Phytase* | Plant Directorate (Annette Plöger), DK |
| LEVUCELL®SC (for dairy goats and dairy ewes) | *Saccharomyces cerevisiae CNCM I-1077* | CRL-FAA (R. Leuschner) |
| LEVUCELL®SC (for horses) | *Saccharomyces cerevisiae CNCM I-1077* | CRL-FAA (R. Leuschner) |
| O35 | *Bacillus subtilis DSM 17299* | NVL (R. Bubulienė), LT |
| VITALYS® liquid & dry | *L-Lysine* | CRL-FAA (G. Simone) |
| BIOSAF® Sc47 (for dairy small ruminants) | *Saccharomyces cerevisiae NCYC Sc47* | CRL-FAA (R. Leuschner) |
| L-HISTIDINE monohydrochloride monohydrate | *L-Histidine* | CRL-FAA (G. Simone) |
| BIOGALACTOSIDASE® | *α-D-Galactosidase* | RENNES (Roger Ziebal), FR |
| HEMICELL® Feed Enzyme | *β-D-Mannanase* | LUBLIN (W Korol), PL |
| BELFEED B1100 MP/ML | *Endo-1,4-beta-xylanase* | CRL-FAA (D.Garalevičienė) |
| BIOPLUS 2B (for turkeys for fattening) | *B. subtilis DSM 5750, B. licheniformis DSM 5749* | CRL-FAA (R. Leuschner) |
| SAFIZYM X® | *Endo-1,4-β-xylanase* | CRL-FAA (D. Garalevičienė) |
| ROVABIO™ PHY AP/LC | *3-Phytase* | CRL-FAA (A.M. Jensen) |
| COLICURE® (for horses) | *Escherichia coli E-101-88, LMG S-1714* | CRL-FAA (R. Leuschner) |
| VEVOVITALL® (for pigs fattening) | *Benzoic Acid* | CRL-FAA (G. Simone) |
| BONVITAL (for dogs) | *Enterococcus faecium DSM 7134* | CRL-FAA (R. Leuschner) |
| BONVITAL (for piglets & pigs) | *Enterococcus faecium DSM 7134* | CRL-FAA (R. Leuschner) |
| BIOSAF® Sc47 (for calves for rearing) | *Saccharomyces cerevisiae NCYC Sc47* | CRL-FAA (R. Leuschner) |

***How does the CRL evaluate the suitability of the analytical methods?***

The core activity of the CRL is related to the evaluation of the analytical methods submitted by the applicant in order to determine the active substance in various matrices, namely the feed additive, premixtures, and feedingstuffs. As outlined in Commission Regulation (EC) No 378/2005 specifying the duties and the tasks of the CRL, the evaluation has to focus on the determination of the active substance in feedingstuffs and - where appropriate - of its residue or metabolite in food. However, also other analytical methods such as the determination of traces of contaminants in the feed additive or the identification of the feed additive may be included in the CRL's evaluation on a case by case basis.

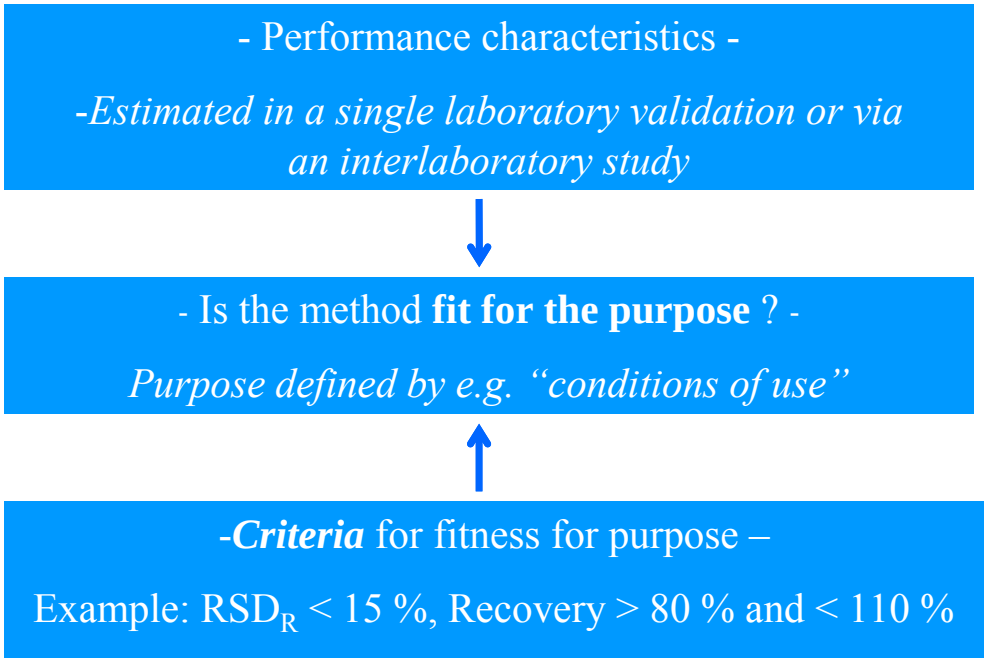
Primary objective of this evaluation is to establish whether the proposed analytical methods are fit for intended purpose. Therefore the applicant is obliged to submit the protocol of the methods along with a report of the corresponding validation studies. Given the diversity of the submitted analytical methods ranging from enumeration techniques for the determination of probiotics, to liquid chromatography for the determination of coccidistats, the expertise from all National Reference Laboratories is a key factor for a scientifically sound evaluation of the analytical methods.

When evaluating the analytical methods submitted by the applicants, the CRL and the rapporteur laboratories take the following aspects into account:

- *Conditions of use as specified in the proposed register entry*: The registry entry is part of the authorisation of the feed additive and specifies important factors such as the range of concentration of the active substance in animal feed and of residues in animal tissue, if applicable. These factors together define the *purpose of the method*.
- *Method performance characteristics obtained in validation studies*. The corresponding reports submitted by the applicant contain the results of the validation experiments and the protocol of the method specifying the execution of the method. The current dossier guidelines given in Commission Directive 2001/79/EC contain a list of method performance characteristics that have to be measured in the validation experiments. Separate dossier guidelines exist for microorganisms and enzymes.
- *Criteria for fitness for purpose* are used as benchmark against which the performance characteristics of the proposed methods are compared. This comparison allows establishing whether the proposed methods are fit for the intended purpose. For specific analyte/matrix combinations such as veterinary drugs in animal tissue, Commission Decision 2002/657/EC sets a list of specific fitness for purpose criteria (e.g target values for precision and

trueness). However, for most analytes in the field of feed analysis such a list of criteria is not available which makes the evaluation of the applicant's methods more difficult. It will be the tasks of the expert groups which the CRL together with the NRL very recently established, to set appropriate evaluation criteria.

The following scheme shows, how the measured performance characteristics, the "conditions of use", and the fitness for purpose criteria are related to each other.




*Relation between method performance characteristics, fitness for purpose and criteria*

An important fitness for purpose criterion is also the suitability of the proposed methods for *official* control as specified in Regulation (EC) No 378/2005. When discussing this additional requirement we need to refer to Regulation (EC) No 882/2004 which specifies the frame in which official food and feed control has to be organised. In particular, this regulation specifies which methods should be selected when analysing samples for official control purposes. Two aspects are relevant in this context, namely the selection criteria for official methods and the characteristics of single-laboratory methods:

In the case that various methods are available for the purpose of analysis, Regulation (EC) No 882/2004 sets selection criteria, placing highest priority on Community methods. In addition, the following hierarchical structure of the analytical methods has to be applied:

(1) Community methods of analysis

(2) Internationally recognised rules or protocols, for example those that the European Committee for Standardisation (CEN) has accepted or those agreed in national legislation

(3) Methods that are fit for the intended purpose, developed in accordance with scientific protocols and validated in a ring test in accordance with an internationally recognised protocol on collaborative trials (e.g. ISO 5725 or IUPAC)

(4) Methods that are validated in-house according to international harmonised guidelines for the in-house validation of methods of analysis with respect to the characterising parameters as mentioned in Annex III of Regulation (EC) No 882/2004.

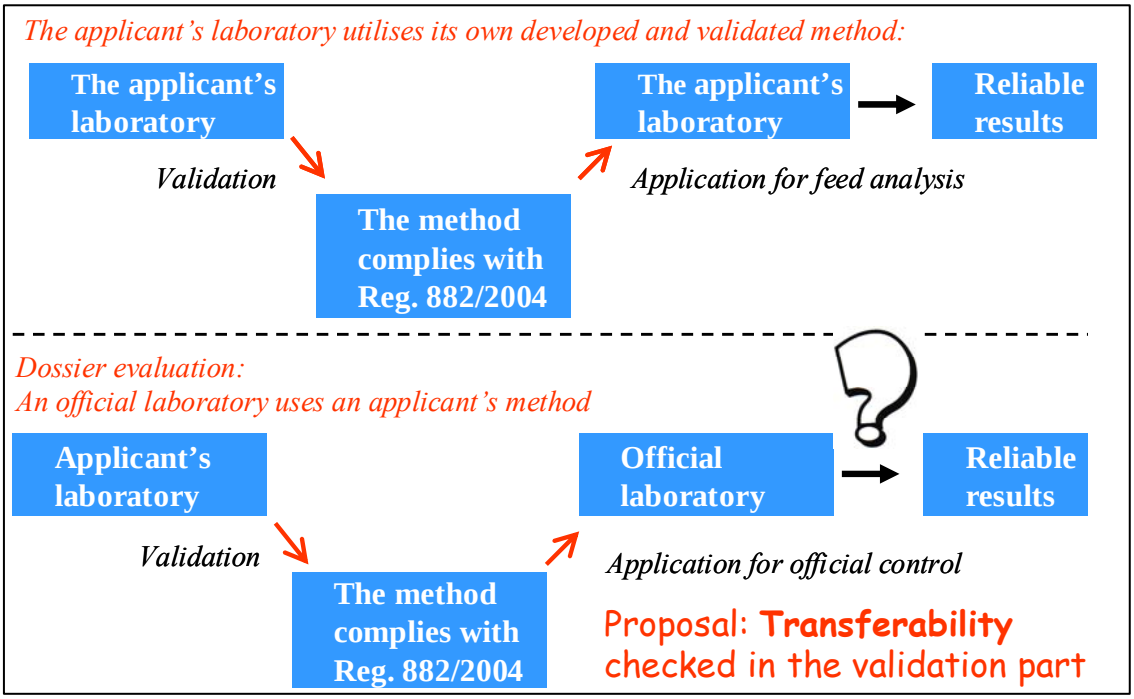
Applying this structure allows the use of single laboratory methods for official control, but only if methods belonging to a higher level such as CEN methods or Community methods are *not* available ("cascade approach").

Looking at the applicants' methods - which have been evaluated until now - reveals that the majority of these methods belong to group 3 or 4. Especially, when evaluating methods for the determination of enzyme activity, most of the proposed methods have been exclusively single-laboratory validated and therefore belong to the group of the "lowest" level. In this context it is important to emphasise that in the case that the CRL considers such a method as *"suitable for official control"* it does *not* mean that the method has been "upgraded" to the status of an "official method". The main purpose of the CRL's evaluation in fact is that at least *one* method is available that can be used for official control purposes, since the characteristics of this method fit the criteria of the Regulation (EC) No 882/2004. Therefore, official control laboratories can use other methods, provided that these methods show a comparable performance profile as the method positively evaluated by the CRL.

An important requirement for single-laboratory validated methods within the frame of the evaluation procedure of the feed additives is the proof of transferability of the analytical method. The CRL evaluation is based on the results obtained in the applicant's laboratory showing that this specific laboratory is able to use the method for the intended purpose, obtaining acceptable method performance characteristics. However, experience from interlaboratory studies has shown that this does not necessarily mean that the *same* method but applied by *another* expert laboratory (e.g. an official control laboratory) would show the same performance profile.

As shown in the following figure, the CRL therefore proposes that additional experiments are carried out by a second and independent laboratory to show that a single-laboratory method can be successfully applied in another laboratory, thus

demonstrating transferability of the method. The results of such experiments should be included in the applicants' dossier. In consequence the CRL's evaluation of the method will be based on the three types of documentations, namely (1) the method protocol, (2) the validation report and (3) the results of the transferability experiments. In this context it is worthwhile to mention that such a proposal does not modify the duties and tasks of the CRL as specified in annex III of Regulation (EC) No 378/2005, especially in terms of testing or ring-trial validating the method if this is considered necessary.




*Importance of proven transferability of the method from the applicant's laboratory to another laboratory*

### *Characteristics of the analytical methods: Examples*

Looking at the analytical methods evaluated in 2006 demonstrates again the enormous diversity of methodologies the CRL and the NRLs are confronted with. Many applications are in the field of probiotics for which a number of fully ring trial validated methods are available. Other applications cover the group of enzymes that are in most cases analysed using methods that have been exclusively single-laboratory validated. Therefore both types of analytical methods are elaborated in more detail:

*Methods for the determination of enzymatic activity*

The determination of enzymatic activity shows special features that differentiate them from other feed additives. The most important difference is related to the fact, that the

target in enzyme analysis is *not* a concentration of an active substance but the *activity* of the enzyme under specific experimental conditions. Consequently, the measurand (i.e. the enzyme activity) is defined by the specific method protocol as proposed by the applicant and is almost always linked to a specific product. Additional limitations arise from the fact that in certain cases the commercial availability of reference enzyme standards required to carry out the analysis is not clearly indicated in the submitted dossier. The CRL also has to evaluate methods in which the correct application of the method protocol requires the availability of identical feed samples *without* the feed additive (blank samples) in order to prepare matrix matched calibration standards. This might be possible when performing the method in a feed mill where such blank samples are available. However, these specific blank samples are most likely not available, when the method is applied by official control laboratories. Alternatively, the "standard addition method" can be utilised, in which various sub-samples are taken from the feed sample and a part of these sub-samples are fortified with different amounts of the target enzyme. Based on the analytical results of the sub-samples, a calibration curve is constructed from which the enzyme activity of the feed sample is calculated. A major drawback of this approach is the multiplication of the work, namely the analysis of the feed samples as such and after fortification with the target enzyme. In addition, the validity of this approach needs to be demonstrated on each enzyme product.

There are two main types of methods utilised for the determination of the enzymatic activity, namely colorimetric and viscosimetric techniques. However, colorimetric methods are by far more applied in feed analysis compared to viscosimetric methods.

When applying colorimetric methods, the enzymatic activity of a sample can be measured against the product of the enzymatic reaction – so called *absolute* methods – or against enzyme standards which are *relative* methods.

*Absolute* colorimetric methods for glycosidases (endo-1,4-β-xylanases, α- and β-glucosidases, galactosidases, amylases) are mainly based on reducing sugars assays, where the enzyme is incubated with its substrate under defined conditions. Reaction products are reducing sugars, which are quantified spectrophotometrically against reducing sugars standards. These assays are not specific enough to be used in feed analyses – mainly due to the presence of competing substrates in the feedingstuffs. In fact, they are generally used for the determination of the enzymatic activity of pure enzyme preparations. Reference enzyme standards with *known activity* are required for calibration purposes, when applying *relative* methods for the determination of the enzyme activity of *feed* samples containing the target enzyme.

*Relative* colorimetric assays are often applied for the determination of the enzyme activity in *feed* samples. When measuring xylanases, the specificity of some assays is improved by using *chromogenic* substrates which, in the presence of the enzyme release

a coloured compound. The latter compound is quantified spectrophotometrically against a reference enzyme standard.

Viscosimetric methods, mainly used for the determination of the activity of endo-1,4-β-xylanases, are based on the ability of the enzyme to reduce the viscosity of a standard substrate solution. Quantification is performed against a reference enzyme standard. Viscosimetric techniques are sensitive enough, but very tedious and only allow a limited number of assays to be performed simultaneously. In addition, the laboratory cannot apply this technique without having very specific equipment that is rarely used in analyses of food and feed. All these reasons may limit the applicability of a method, although the evaluation of its performance profile demonstrates that the method is fit for the intended purpose.

In order to facilitate the evaluation of the analytical methods and their possible application by official control laboratories, the CRL always prefers harmonised approaches. In particular, it would be beneficial of using well-defined and commercially available enzyme standards and applying harmonised analytical conditions such as the pH value, the incubation time, the temperature and the substrate concentration in the execution of the protocol. In some cases the same enzyme is produced by different bacteria or fungi and therefore has different optimal conditions for its activity depending on the producer microorganism, which makes the selection of harmonised methods even more difficult.

Another important enzyme in animal nutrition is phytase, which releases digestible phosphorus from indigestible phytate present in animal feed. For the determination of the phytase activity in premixtures and feedingstuffs, various methods have been validated in *inter-laboratory* studies. The methods follow the same principle which is the enzymatic release of phosphate from the substrate sodium phytate, but differ regarding the analytical protocol.

Two *relative* phytase methods have been validated by *inter-laboratory* studies and have been included in the method collection of two organisations, namely AOAC International (AOAC method 2000.12) and the Association of German Agricultural Analytical and Research Institutes (VDLUFA method 27.1.2). Both methods require the use of the corresponding phytase standard for calibration purposes. On behalf of the European Association of Feed Additive Manufacturers (FEFANA), an *absolute* method for the determination of the phytase activity in animal feed has been recently developed and collaboratively validated, obtaining acceptable values for the relative standard deviation for reproducibility, ranging from 5 to 14%. In the protocol the measured enzyme activity is calibrated against phosphate and therefore the assay does not require the enzyme for calibration purposes. This method, being under evaluation to become a standard of the European Committee for Standardisation (CEN), is applicable to official control of the

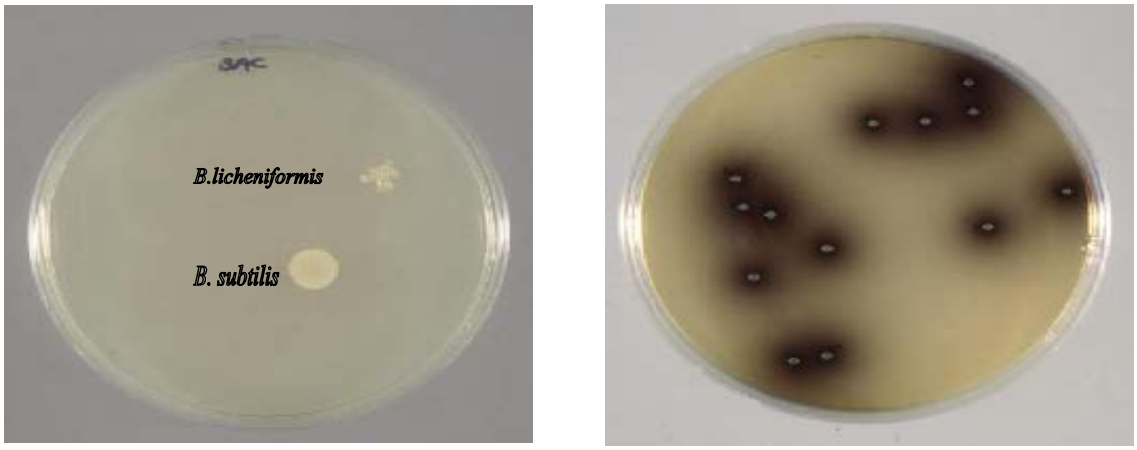
phytase activity in feedingstuffs, containing any of the phytase products (E 1600, E 1614, and E 1640) currently authorised within the European Union. It is likely that the activity of *other* phytase products can be measured with the harmonised protocol as well. However, this must be experimentally confirmed in each case, before the CRL may be able to consider the harmonised method suitable for official control of the specific product.

*Methods for the determination of microorganisms used as feed additives*

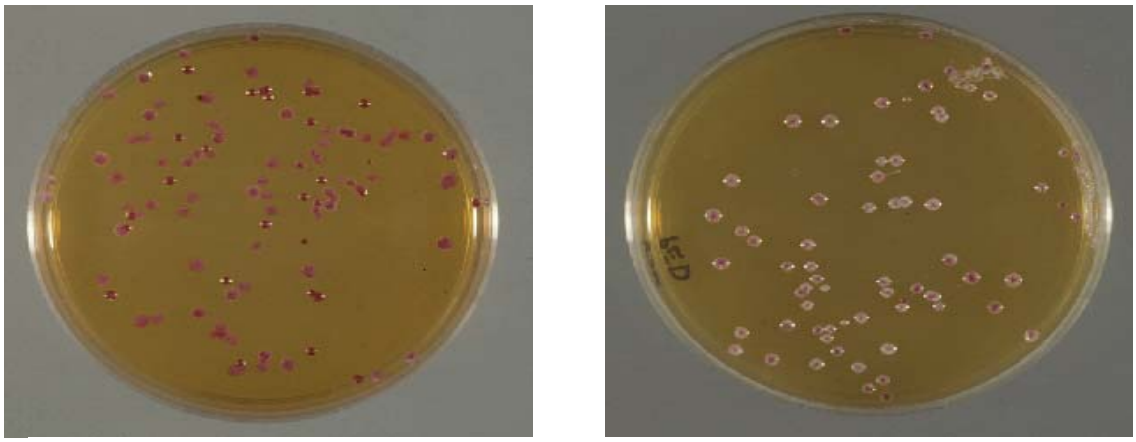
Microorganisms used as feed additives include a wide range of bacteria and yeasts. For official control in the regulatory framework, robust, reliable and standardised methods are necessary for the determination of the concentration of viable microorganisms and for strain identification.

In an effort to get standardised methodologies for selected genera of microorganisms used as feed additives, a number of laboratories participated within a European Community Standards, Measurements and Testing (SMT) program in collaboration with FEFANA. Within the project (SMT4-CT98-2235) existing methods for the enumeration and identification of probiotic bacteria and *S. cerevisiae* used as feed additives were reviewed and evaluated, with modifications as appropriate, to be used as prospective control methods.

The performance of enumeration methods with regards to repeatability and reproducibility and the potential to selectively enumerate specific microorganisms when present in various concentrations i.e. as majority, equal, or minority components, in mixtures within probiotic feed preparations were established.




*Colony forming units of Bacillus subtilis and B. licheniformis on tryptone soya agar and of enterococci on bile esculin azide agar*



*Lactobacilli and pediococci colonies on MRS agar supplemented with 0.01 % TTC*

A number of techniques are available for the identification of probiotic microorganisms at strain level. These include biochemical or molecular techniques based on restriction endonuclease analysis (REA), rRNA genes, polymerase chain reaction (PCR), amplified fragment length polymorphism (AFLP), and pulse field gel electrophoresis (PFGE). Within the project (SMT4 CT98-2235), PFGE was reviewed and recommended by the project partners for lactic acid bacteria including the bacterial genera *Lactobacillus*, *Pediococcus*, *Enterococcus* and for *Bacillus* strains as a technique that was robust, reliable, user-friendly and providing the required unambiguous fingerprints for authorised probiotic strains. For the yeast *S. cerevisiae*, a PCR-based method which had a history of use in the feed industry was tested in an inter-laboratory comparison and revealed appropriate performance data.

***Workshops***

The CRL held two workshops in 2006, which took place at the Institute for Reference Materials and Measurements in Geel, Belgium. Participants came from the NRLs, European Food Safety Authority (EFSA), DG for Health and Consumer Protection, industry (EU Feed additives & Premixtures association (FEFANA)) and academia.

In the workshop held in April 2006 the CRL presented the outcome from discussions with the NRLs, EFSA and DG for Health and Consumer Protection regarding the 'fitness for purpose' criteria for the analytical methods submitted by the applicant. In particular it was emphasised that the applicants' methods need to be suitable for the determination of the active substance at the target concentration and in the target matrix as specified in the proposed register entry. In addition, the CRL presented the concept of the *transferability* of those analytical methods that have been exclusively single-laboratory validated. Scientific presentations covered various topics, namely the concept of measurement uncertainty, proficiency testing in microbiology and alternative methods for the determination of vitamins A and E in animal feed. A representative from FEFANA reported on the industry initiatives on the harmonisation of analytical methods in the area of enzymes and probiotics. The current activities of the Technical Committee 327 (Animal feeding stuffs - Methods of sampling and analysis) of the European Committee for Standardization (CEN) regarding the harmonisation of analytical methods - especially in the field of the determination of feed additives - were also presented.

An important topic of the second workshop in October 2006 was the presentation of the Regulation (EC) No 882/2004 which sets criteria for analytical method suitable for *official* food and feed control. This is an important aspect, since the CRL's evaluation reports on the applicant's methods need to contain a statement regarding their suitability for official control. A presentation from the representative of DG for Health and Consumer Protection showed how this aspect will also be reflected in the new dossiers guidelines which are going to replace the current guidelines (Commission Directive 2001/79/EC). When analysing feed samples for specific analytes - such as the determination of the enzyme activity - additional information (e.g. the exact origin of the enzyme) that can only be retrieved from the documentation in connection with the animal feed are needed. These aspects are related to the implementation of the feed hygiene Regulation (EC) No 183/2005 which was presented by an industry representative. Scientific presentation included a presentation on enzyme analysis, clarifying that for some products such as phytase, harmonised methods are possible, whereas for other enzymes such as xylanase, most likely the applicant's exclusively in-house validated methods for each xylanase product, need to be applied in the frame of official control. In addition, an expert from the NRLs gave an update of an interlaboratory validation

exercise on a method for the determination of specific marker substances present in essential oils that are intended to be used as feed additives.

*5th CRL-FA Workshop (Geel, 25-26 April 2006)*

***Expert Groups***

One of the major challenges of the evaluation procedure is the quite large variation of the analytical methodology involved. As pointed out earlier in this report harmonised methods, that would facilitate the evaluation, are only available in some fields. Likewise, well accepted and achievable performance criteria (e.g. target value for the precision) for many analytical methods used in the feed sector are missing. Discussing this issue with the NRLs revealed a strong need for establishing specific criteria for the various methods, in order to assist the rapporteur laboratories in the evaluation of the dossiers. This aspect is also extremely important when considering the large number of feed additives that need to be re-authorised by 2010. In order to address all questions related to this topic, five expert groups were established according to the nature of the analytical methodology, namely (1) methods for micro-organisms, (2) methods for enzymatic activity, (3) methods for coccidiostats, (4) chromatographical methods and (5) methods for trace elements. In addition, the expert groups will contribute to the maintenance of the methods data base and will give recommendations regarding the suitability of methods for official control. The expert groups consist of experts from the NRL's, the CRL and in some case also external experts.

*Tasks of the expert groups*

*Microbiological methods* (Chair: K. Kwiatek, National Veterinary Research Institute (NVRI), Poland; CRL Contact person: R. Leuschner). The group will deal with microorganisms in the "technological" and "zootechnical" functional feed additives categories. The relevant methods applicable to the work in the frame of the CRL were classified in quantitative enumeration and qualitative identification methods. It was agreed between the experts to prepare an overview of microoganisms involved and to review available information related to method performance criteria and analytical results.

*Methods for enzymatic activity* (Interim Chair: K.-W. Wagner, Austrian Agency for Health and Food Safety (AGES), Austria; CRL Contact person: D. Garalevičienė). The group identified various aspects of enzyme analysis where further information is required, especially when applying viscosimetric methods. Except for phytase, no harmonised methods are available for the determination of enzyme activity in feed. The experts will collect all available information on performance criteria for enzymatic methods (including present standardised and published methods) to draft up the guidelines for their evaluation. In addition, literature and legislation will be screened for ring-trial validated methods regarding the determination of enzymatic activity in food, which could provide valuable information when applied to feed.

*Methods for coccidiostats* (Chair: J. de Jong, Institute of Food Safety (RIKILT), The Netherlands; CRL Contact person: G. Simone). All members of the expert group have the required experience in the field, e.g. liquid chromatography coupled to various detectors including mass spectrometry. Relevant performance characteristics from the list in Annex III of Regulation (EC) No 882/2004 need to be discussed, taking into account the method performance criteria as specified in Commission Decision (EC) No 657/2002 which are for methods related to food analysis, but not feed. Contacts with another Commission Directorate General (Enterprise and Industry DG) and the European Agency for the Evaluation of medical products (EMEA) have to be established, since they also deal with analytical methods for the enforcement of MRLs when coccidiostats are used as veterinary drugs. In this context another CRL dealing with the determination of coccidiostats in food of animal origin may be contacted.

*Chromatographical methods* (Chair: E. Nordkvist, National Veterinary Institute (SVA), Sweden; CRL Contact person: U. Vincent). The Group has a broad scope due to the wide application of chromatographical methods. The first goal of the expert group will be to prepare a practical document (e.g. a checklist) to have a guide for method's evaluation. The present legislation such as Annex III of Regulation No (EC) 882/2004 and Commission Decision (EC) No 657/2002 will be used as a starting tool for discussion.

*Methods for trace elements* (Chair: M. C. Abete, Centro di Referenza Nazionale per la Sorveglianza ed il Controllo degli Alimenti per Animali (Cre.A.A.), Italy; CRL Contact persons: C. von Holst, S. Yasar). It is planned to establish criteria for the method performance characteristics, taking into account the requirements from Commission Decision (EC) No 657/2002 which specified corresponding criteria for the food area. The Technical Committee 327 of CEN needs to be contacted to establish for which trace elements appropriate methods in animal feed are currently validated. In addition, a list of trace elements currently authorised as feed additive as published in the Community Register for Feed Additives will be established.

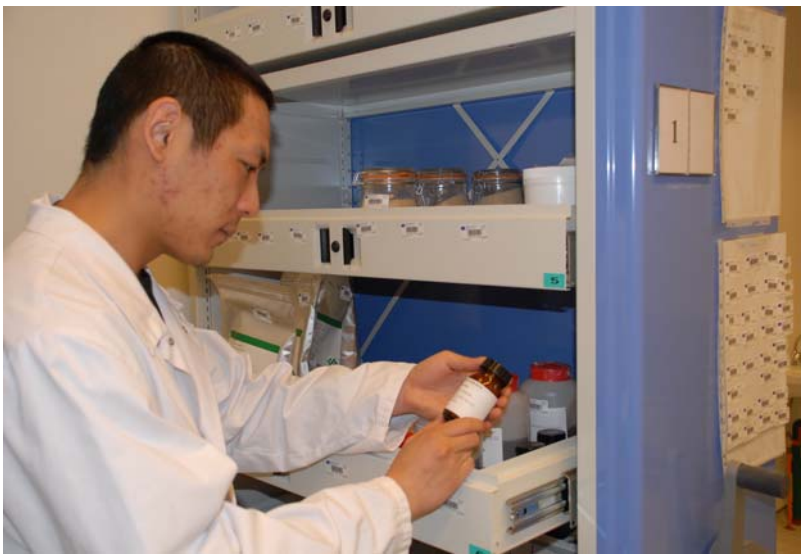
### *The CRL-FAA Sample Bank for Feed Additives*

The CRL sample bank consists of 8 cells of -30°C freezers, 8 cells of +5°C fridges and 15 mobile cupboards at ambient temperature.

In 2006 we received 138 reference samples in total. We predict about 7500 new samples by 2010, based on information available from the Community Register for Feed Additives.

All relevant information related to the samples (arrival date, weight, validity checklist, expiry date, etc…) and accessory dossiers are introduced in a database, which has been specifically designed for this purpose (CRL-FAA samples database).

Due to growing needs and in order to meet the requirements for accreditation, the working instructions, procedures for handling, storage, registration of the samples and the CRL-FAA samples database have been reviewed and adapted in 2006. A browsable interface to search the database will be available to the NRLs, EFSA and DG for Health and Consumer Protection via the Network pages of the CRL website by the end of 2007.



*Samples in the CRL –FAA Samples Bank*

### *Accreditation*

An important step forward to demonstrate the CRL's expertise in feed analysis is the successful accreditation according to ISO 17025 granted by the Belgian authorities. This important accreditation was focussed on the needs of the CRL and its scope covered (1) the determination of Vitamin A applying a Community method (Commission Directive 2000/45/EC) and (2) the determination of monensin, narasin and salinomycin in feed based on the ISO/DIS standard 14183.

Système Belge d'Accréditation
Essais et Inspection

Belgisch Accreditatiesysteem
Testen en Keuring

BELTEST

Member of EA and the EA-MLA (testing)
Member of ILAC

**ACCREDITATION CERTIFICATE**

**Nr. 357-T**

*In compliance with the provisions of the Royal Decree of December 22nd 1992 concerning the setting up of BELTEST, the Minister of Economy, hereby confirms, on advice of the Accreditation Bureau, that the test laboratory*

**Institute for Reference Materials and Measurements IRMM**
**Food Safety and Quality Unit (FSQ)**
**Retieseweg, 111**
**2440 GEEL**

*has the competence to perform the tests, mentioned in the attached enclosure, in accordance with the requirements of the standard NBN EN ISO/IEC 17025. The present accreditation certificate is granted for a period of 3 years starting from the date of issue and is submitted to a yearly surveillance.*

*The chair of the Accreditation Bureau,*

*Nicole MEURÉE-VANLAETHEM*

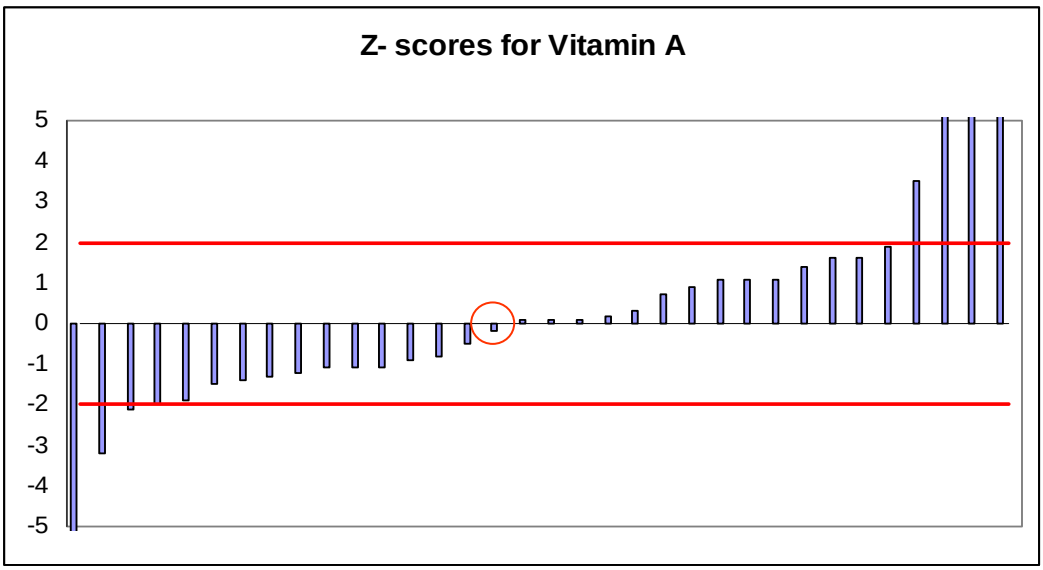
*The Minister of Economy,*

*Marc VERWILGHEN*

*Date of issue :* 15 -06- 2006

A2-0034

Furthermore the CRL participated in proficiency tests on analytical methods included in the accreditation to demonstrate its competence in producing reliable results as shown in the following figure:




*Result from the CRL's participation - indicated by the red circle - in a proficiency test on the determination of Vitamin A in a premixture. The bars represent the laboratories' performance expressed in terms of the z-score, which is the deviation of the laboratories' results from the target Vitamin A concentration divided by the target value for the standard deviation. Z-scores between ± 2 are considered acceptable. The CRL z-score is – 0.2 and therefore very close to the ideal result of 0, which would mean complete agreement with the target Vitamin A concentration.*

### *CRL-FAA Informatics tools*

*Website*

The CRL website, available since early 2005, has been regularly updated during 2006.

In the public pages information on the CRL's activities and the composition of the consortium of NRLs are presented. In addition support is given to applicants seeking feed additive authorisations.

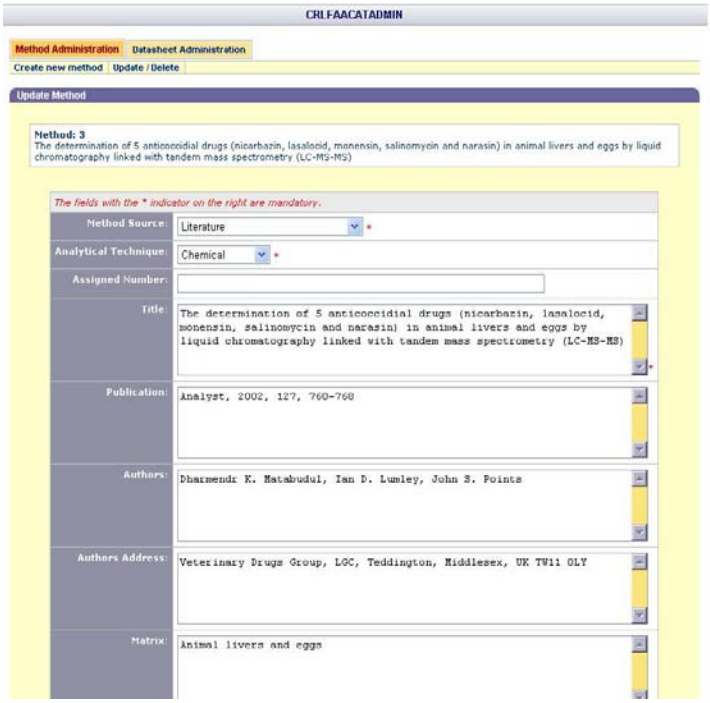
In the network pages assistance is given to the NRLs on the procedures and activities in which the consortium is involved. A list of the reference samples stored at the CRL is also available for the consortium via the network pages.

*CIRCA*

CIRCA (Communication and Information Resource Centre Administrator) enabled the CRL and the consortium of NRLs to maintain a secure space on the Internet where they can share documents and information, also participating in a discussion forum. More than 50 users (consortium members, EFSA staff members, DG for Health and Consumer Protection administrators and the CRL team itself) share information and documents on a daily basis, allowing for effective and fast communication. The system is managed by the CRL. Due to the rapidly increasing quantity of documents to be handled, the Informatics & Electronic Unit of the IRMM, together with the CRL, has performed a business analysis in order to identify more powerful systems (collaborative tools) to replace CIRCA in the near future. Some pilots will be set up and tested presumably during the first half of 2007.

*Methods Database (FEEDACAM Database)*

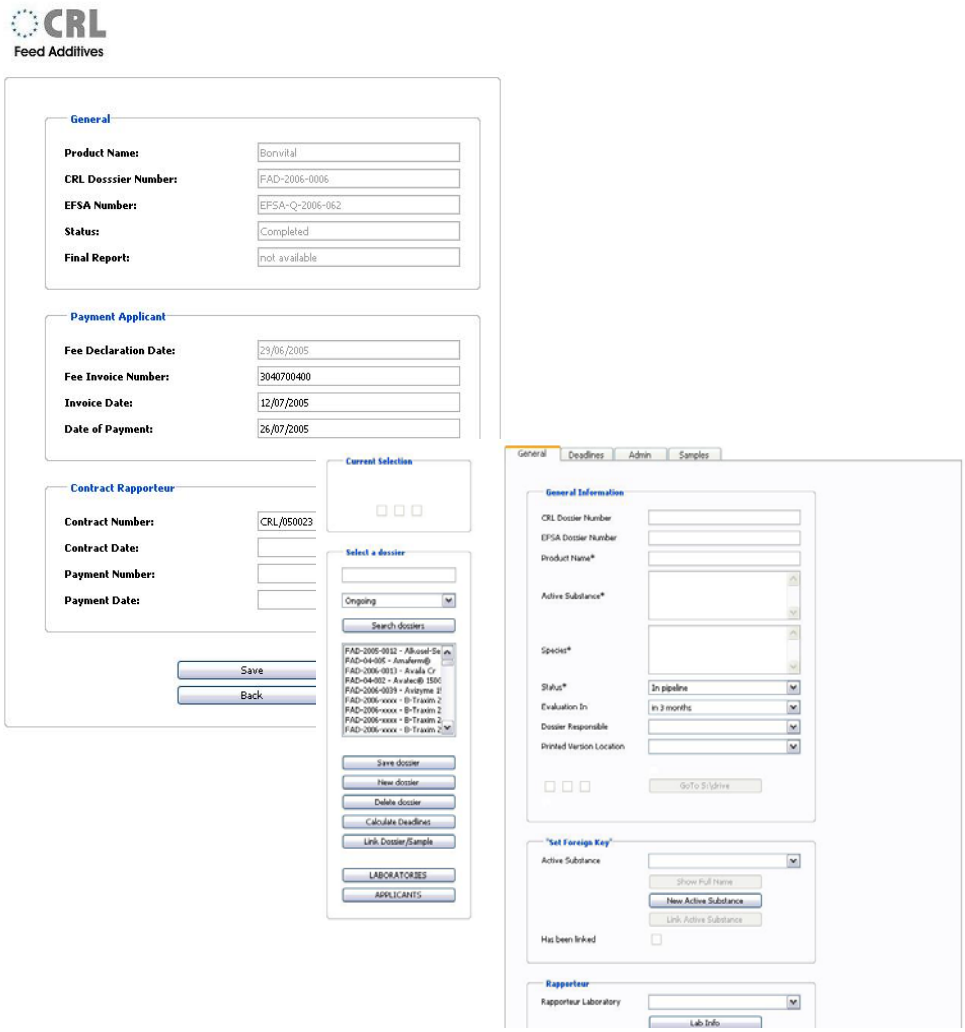
The last phase of the development of the database on Methods of Analysis for Feed Additives has been completed in June 2006, containing an "administration interface" which is used by the CRL not only to input new methods but also to update the contents of the database. Further minor modifications have been done and the database has been updated, especially by inputting methods related to additives for which authorisations have been published in the Official Journal of the European Union in 2005 and 2006.




*CRL-FEEDACAM administration interface*

*CRL Dossiers Tracking System*

The original MS Access databases established in 2005 with the aim of keeping track of every single step in the evaluation process (dossiers, samples, laboratories) have been further developed. In cooperation with the Informatics & Electronics Unit of the IRMM a fully consistent and robust system (so called normalised datamodel) has been established. The existing databases have been integrated into one single database and transferred into Oracle resulting in the CRL Dossiers Tracking System (CRL-DTS) database. A web based interface has been developed internally in order to allow the CRL team to input and update data into the database. The CRL-DTS database is a crucial tool for the smooth operating of the CRL, for managing the strict deadlines laid down by the EU legislation and for keeping all the information related to each dossier handled by the CRL. The final aim will be the establishment of an integrated CRL application which will be used by the CRL staff for the management of the whole workflow of the evaluation process. A browsable interface to search the database will be available to the NRLs, EFSA and DG for Health and Consumer Protection via the Network pages of the CRL website by the end of 2007.




*CRL-FA Dossiers Tracking System*

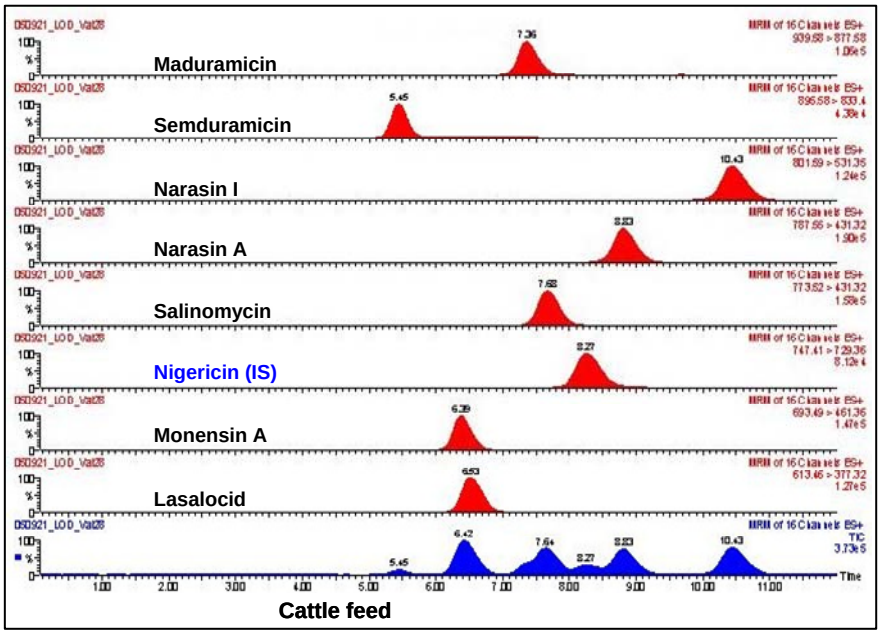
### *Additional activities*

The CRL contributed to the discussion on the implementing rules and guidelines concerning applications for authorisation of additives held by DG for Health and Consumer Protection and the Standing Committee on the Food Chain and Animal Health – Section Animal Nutrition. The discussion resulted in the draft document SANCO/426/2005. The CRL contribution is mainly related to the section concerning the methods of analysis and it is based on the experience gained during the first two years of operation of the CRL. Most of the proposals of the CRL have been "taken on board" in the document. These proposals aim to establish appropriate quality standards of the dossiers submitted by the applicants in order to enable the CRL for an assessment, which is in line with scientific and legal requirements.

The CRL also asked the Commission to amend the Regulation (EC) No 378/2005 in order to increase the fee paid by the applicant. The main reason of this request is to cover to a greater extent the expenses related to the evaluation procedures as well as to

guarantee a fair remuneration of the NRLs acting as rapporteurs. The Regulation will presumably be amended during 2007.

Furthermore, the activities of the CRL are closely linked to other projects of the IRMM, especially those on the validation and harmonisation of analytical methods in the field of food and feed analysis. Several members of the IRMM are working in the TC 327 'Animal feeding stuffs – Methods of sampling and analysis' and in particular in working group 3 'Feed additives and drugs' of the European Committee for Standardisation (CEN). In this context the Residues and Contaminants group of the Food Safety and Quality Unit supporting the CRL developed and validated an analytical method for the simultaneous determination of six ionophore coccidiostats by LC-MS/MS.




*Chromatogram of the LC-MS/MS method for the simultaneous determination of six ionophore coccidiostats in animal feed using nigericin as internal standard. The LC-MS/MS was operated in the Multiple Reaction Mode*

## ACKNOWLEDGEMENTS

We sincerely thank our colleagues within the Institute for their strong support and interest in the CRL activities, both with regards to secretarial support, review of reports and development of tailor made systems. Special thanks to Anne-Mette Jensen, Bibi Kortsen, Franz Ulberth, María José González de la Huebra, Annegret Erkelenz, Michael Bickel, Gaida Lapitajs, Federica Serano, Stefano Bellorini, Stephane Marcon, Sandra Pauwels, Peter Lambert, Ursula Vincent, Leen Peetermans, Ivan Celen, Elizabeth Garlick, Sari Lehto, Sigrid Beutels, Bartel Meersman, Marc Wellens, and last but not least: Elke Anklam (former Deputy Director of the IRMM and Head of Food Safety & Quality Unit, now Director of JRC-IHCP) and Alejandro Herrero (Director of JRC-IRMM) for their constant support in all CRL related activities.

We are also very grateful to all experts from the NRLs for contributing to the evaluation of the dossiers and to the discussions in the workshops and working groups which was indispensable for the successful operation of the evaluation procedure.

# ANNEX 1: NATIONAL REFERENCE LABORATORIES

| EU Member States | Name of laboratory |
|---|---|
| Belgique/België | • Federaal Voedingslabo Tervuren (FAVV), Tervuren<br>• Vlaamse Instelling voor Technologisch Onderzoek (VITO), Mol |
| Česká republika | • Central Inst. Superv. Test. Agriculture, Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha |
| Danmark | • Plantedirektoratets Laboratorium, Lyngby |
| Deutschland | • Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim<br>• Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Speyer<br>• Sächsische Landesanstalt für Landwirtschaft Fachbereich 8 Landwirtschaftliches Untersuchungswesen, Leipzig<br>• Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen Jena |
| Eesti | • Põllumajandusuuringute Keskus (PMK), Jääkide ja saasteainete labor, Saku, Harjumaa<br>• Põllumajandusuuringute Keskus (PMK), Taimse materjali analüüsi labor, Saku, Harjumaa |
| España | • Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid<br>• Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Cabrils |
| France | • Laboratoire de Rennes, Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (DGCCRF), Rennes |
| Ireland | • The State Laboratory, Dublin |
| Italia | • Istituto Superiore di Sanità. Dipartimento di Sanità alimentare ed animale, Roma<br>• Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino |
| Κύπρος | • Feedingstuffs Analytical Laboratory, Department of Agriculture, Nicosia |
| Latvija | • Valsts veterinārmedicīnas diagnostikas centrs (VVMDC), Rīga |
| Lietuva | • Nacionalinė veterinarijos laboratorija, Vilnius<br>• Klaipėdos apskrities VMVT laboratorija, Klaipėda |
| Luxembourg | • Laboratoire de contrôle et d'essais — ASTA, Ettelbrück |
| Magyarország | • Országos Mezőgazdasági Minősítő Intézet (OMMI) Központi Laboratórium, Budapest |
| Nederland | • RIKILT- Instituut voor Voedselveiligheid, Wageningen<br>• Rijkinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven |
| Österreich | • Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien |
| Polska | • Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin,<br>• Państwowy Instytut Weterynaryjny, Puławy |
| Portugal | • Laboratório Nacional de Investigação Veterinária, Lisboa. |
| Slovenija | • Univerza v Ljubljani. Veterinarska fakulteta, Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higienookolja, Ljubljana<br>• Kmetijski inštitut Slovenije, Ljubljana |
| Slovensko | • Skúšobné laboratórium – oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava |
| Suomi/Finland | • Kasvintuotannon tarkastuskeskus/ Kontrollcentralen för växtproduktion (KTTK). Vantaa/Vanda |
| Sverige | • Foderavdelningen, Statens veterinärmedicinska anstalt (SVA), Uppsala |
| United Kingdom | • The Laboratory of the Government Chemist, Teddington |
| **EFTA Countries** | |
| Norway | • LabNett AS, Agricultural Chemistry Laboratory, Stjørdal |

# ANNEX 2: CRL EVALUATION REPORTS - EXECUTIVE SUMMARIES

**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**

| | |
|---|---|
| Name of the additive | *Elancoban®* |
| Active substance | Monensin sodium |
| | EFSA-Q-2005-168 |
| Author: | Jaroslava Petrova (CISTA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 05/01/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for Elancoban® under the category 5. coccidiostats and histomonostats, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Elancoban® for the control of coccidiosis in calves for rearing and cattle for fattening.

The active substance in Elancoban® is monensin as sodium salt which contains the two major components monensin A contributing 97.6 % to the overall activity and monensin B. The monensin concentration in the feed additive, in premixtures and feedingstuffs is expressed in terms of monensin activity which is calculated from the measured concentration of both components. The target monensin activity in Elancoban® G 100 is 100 g/kg and the monensin activity in Elancoban G 200 is 200 g/kg. For both products the applicant proposed purity criteria expressed as range of monensin activity of 92.5 g/kg to 107.5 g/kg for Elancoban® 100 and of 185 to 215 g/kg for Elancoban® 200. The appearance is a dark brown, speckled with straw-coloured particles, free flowing meal, which contains rice hull or limestone granular, antidusting oil and monensin. The proposed dosage ranges from 40 to 120 mg of monensin /kg in the feedingstuff for calves and for cattle.

For the determination of the active substance (monensin) in the feed additive (Elancoban®)an isocratic High Performance Liquid Chromatography (HPLC) method with post-column derivatisation and Ultraviolet (UV) detection is proposed by the applicant. The method is also used to demonstrate the purity of monensin, which mainly consists of monensin A. The performance characteristics are considered acceptable, thus the method is considered suitable for official control purposes.

For the determination of the monensin in premixtures and feedingstuffs the applicant proposes a HPLC method, which is based on the same principle as mentioned above. The limit of quantification (LOQ) of the method for the determination of monensin is 4 mg /kg.

The method has also been validated by conducting an interlaboratory study (J. of AOAC International 1997 80 693) performed on various feed matrices including cattle feedingstuff. Acceptable precision data were obtained for the feedingstuffs, since the relative repeatability

standard deviation (RSDr) ranged from 6.1 to 15 % and the relative reproducibility standard deviation (RSDR) ranged from 8.6 to 15 %.

The applicant's method has been adopted as AOAC Official method (AOAC Official Method 997.04). It is therefore considered suitable for official control purposes for the field of application that is sought. For official control purposes the CRL also recommends the ISO standard 14183:2005 which is a multi-analyte method, since it allows for the simultaneous determination of monensin, narasin and salinomycin in feedingstuffs. This method is based on the same principle as the method proposed by the applicant.

For determination of monensin in edible bovine tissues and milk a HPLC method based on the same principle as the method for the detection of the active substances in the other matrices has been submitted. The LOQ for monensin is 25 μg/kg in bovine muscle, liver, kidney and fat and 5 μg/kg in milk. The recovery rate of the target analyte in the four tissues types and milk ranged from 80 to 88% and the relative within-laboratory standard deviation for reproducibility varied from 3.6 to 9.1%. The obtained method performance characteristics are considered acceptable for the intended purpose. However, since there are no maximum residue levels (MRLs) for monensin fixed by European legislation, the suitability of the method for official control purposes cannot be evaluated.

Further testing or validation of the submitted methods is not considered necessary.

**Additional conclusion regarding the suitability of the analytical method for official control purposes:**

The analytical method for the determination of the active substance in feedingstuff is, however, considered suitable for official control purposes, if the analysis aims at the quantification of the target analyte in feedingstuffs samples in the frame of the sought authorisation, i.e. in target feed samples (feedingstuffs for weaned piglets) at the target concentration level of the active substance (5000 mg/kg).

**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**

| | |
|---|---|
| Name of the additive | *Alkosel®/Selsaf* |
| Active substance | Selenium enriched yeast |
| | EFSA-Q-2005-117 |
| Authors: | Maria Cesarina Abete, Daniela Marchis (C.Re.A.A) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 10/01/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for Alkosel®/Selsaf under the category/group 3(b), nutritional additives/compounds of trace elements, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Alkosel®/Selsaf as a source of selenium for all animal species. Alkosel®/Selsaf is an inactivated Selenium enriched yeast (Saccharomyces cerevisiae) product, containing high levels of the essential trace element selenium. The inactivated and dried Selenium enriched yeast product is blended with non viable dehydrated yeast (Saccharomyces cerevisiae) to adjust the selenium content. The final product is an inactivated whole cell yeast containing minimum 2000 mg/kg of total selenium of which 2% are residual inorganic selenium. At least 60% of the total organic selenium is in the form of selenomethionine. The active substance in Alkosel®/Selsaf is selenium (Se). Alkosel®/Selsaf is added to the feedingstuffs obtaining a concentration of the feed additive in the feed of 250 mg/kg which corresponds to a concentration of selenium in the feedingstuffs of 0.5 mg/kg.

The active substance is measured as total selenium regardless of its chemical form, i.e. independently of whether it is present as organically bound Se or as inorganic Se.

For the determination of the active substance in Alkosel®/Selsaf either flame atomic absorption spectrometry (FAAS) or inductively coupled plasma atomic emission spectrometry (ICP-AES) methods are proposed by the applicant. Since both methods are based on well known principles, they are considered suitable for the determination of selenium in the feed additive.

For determination of the active substance (total selenium) in premixtures and feedingstuffs also two methods based on FAAS or ICP-AES are proposed. Since information on a complete validation study performed on the target feed was not available, the suitability of this method for official control purposes cannot be evaluated.

For official control regarding the determination of the active substance in premixtures and feedingstuffs, the CRL recommends an analytical method that has been fully ring trial validated

at relevant concentrations of the active substance in relevant matrices. The method and the results from the related interlaboratory study are presented in the method collection of the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany). The obtained method performance characteristics for this method are considered acceptable, since the relative between-laboratory reproducibility standard deviation (RSDR) for a premixture containing 112 mg/kg of Se was 7.3 % and the between-laboratory RSDR for a feedingstuff matrix containing 0.48 mg/kg of Se was 7.4 %. However, the validated method includes different options for the mineralisation procedure and also for the type of instrumentation, since either Zeeman graphit furnace Atomic Absorption Spectrometry (AAS) or Hydrid AAS can be used for the final measurement of Se. Therefore, the laboratory has to select a specific analytical procedure based on these options and must demonstrate that the method performance criteria as obtained in the ring trial can be met. The VDLUFA method shows different limits of quantification depending on the specific analytical procedure selected, but they are all sufficiently below the legal limit of 0.5 mg Se /kg feed and therefore acceptable for the purpose of analysis.

For the determination of selenium in target tissues and animal products the applicant proposed the same methods as described for the quantification of selenium in premixtures and feeds, but without submitting corresponding validated data. Validated methods are available in the literature such as a recently published method aiming at the determination of selenium in chicken meat and using inductively coupled plasma mass spectrometry (ICP-MS) for analysis. The obtained method performance characteristics included a limit of detection (LOD) of 83 μg/kg, a relative recovery rate ranging from 97 to 100 % and a relative standard deviation of about 3 %. (J. Anat. At. Spectrom., 2004, 19, 1361-1369). However, since there are no legal limits for the active substance in animal products fixed by the European legislation, the suitability for official control purposes cannot be evaluated.

Further testing or validation is not considered necessary.

**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**

| | |
|---|---|
| Name of the additive | *Natuphos®* |
| Active substance | 3-phytase |
| | EFSA-Q-2005-116 |
| Author: | Annette Plöger (Plantedirektoratet) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 17/01/2006 |

## EXECUTIVE SUMMARY

In the current application, authorisation is sought for Natuphos®.

The active agent is the enzyme 3-phytase produced by *Aspergillus niger*. The additive is already authorised in EU for the animal categories chickens for fattening, laying hens, piglets, pigs for fattening, sows and turkeys for fattening. The content of the active substance is expressed in terms of the enzyme activity FTU, where 1 FTU is the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate at pH 5.5 and 37°C in one minute. The current application is for the use of two products which are Natuphos® 5000 G (granulate) and Natuphos® 5000 L (liquid) with a minimum activity of 5000 FTU/g and 5000 FTU/ml, respectively.

The additive improves the utilisation of phosphorous in the feedingstuff by the target animal. The proposed dosages for the six categories of animals range from 250 to 700 FTU / kg feedingstuff, depending on the target animal.

For the determination of the phytase activity in the *feed additive,* in *premixes* and in *feedingstuff* the applicant proposes very similar analytical methods as published in a peer reviewed journal. The method is based on the principle that phytase releases inorganic phosphate from a substrate, which in the presence of molybdate/vanadate reagent forms a yellow complex. The yellow complex is measured with a spectrophotometer. The phytase activity of the sample is quantified against a phytase standard with defined activity. The applicant reported precision data of the methods for the determination of the phytase activity in all three matrices that were obtained via interlaboratory studies performed by the German Agricultural Analytical and Research Institutes (VDLUFA,Germany).

For the determination of the phytase activity in the *feed additive* the method proposed by the applicant has a limit of detection (LOD) of 45 FTU/kg and a limit of quantification (LOQ) of 90 FTU/kg. The same precision data were reported for the liquid and the granulated product, which were 2.5 % for the relative standard deviation for repeatability ($RSD_r$) and 4.9 % for the relative standard deviation for reproducibility ($RSD_R$). Acceptable values for the accuracy were reported, since the relative recovery rate for the solid formulation varied between 98 and 102 % and for the

liquid formulation the relative recovery rate was 94 %. The method is considered suitable for the intended purpose.

For the determination of the phytase activity in *mineral premixtures*, the reported values for the LOD and LOQ were 45 FTU/kg and 90 FTU/kg, respectively. The reported precision data were 4.9% for the $RSD_r$ and 8.4% for the $RSD_R$. Accuracy data were derived from the analysis of four samples, revealing a relative recovery rate ranging from 93 to 101 %. The method performance characteristics are considered acceptable and the method is therefore considered suitable for the intended purpose.

For the determination of the phytase activity in *feedingstuff*, the obtained LOD of 45 FTU/kg and LOQ of 90 FTU/kg are acceptable, considering the lowest target level of the enzyme activity in feedingstuffs for laying hens and turkeys for fattening, which is 250 FTU/kg. The reported precision was 6.7 % for $RSD_r$ and 11.1 % for $RSD_R$. Accuracy data were derived from the analysis of four chicken feedingstuff samples, revealing a relative recovery rate ranging from 97 to 103 %. Furthermore, the method has been adopted as AOAC Official Method (2000.12) and has been fully ring trial validated obtaining values for the $RSD_r$ ranging from 2.5 to 8.6% and values for the $RSD_R$ ranging from 14.0 to 27.6%. Therefore, the method is considered suitable for the intended purpose.

Several other very similar analytical methods for the determination of phytase activity in *feedingstuffs* exist and have also been ring trial validated. These include a method developed by FEFANA (European Association of Feed Additive Manufacturers) which has been validated according to IUPAC guidelines. It is an *absolute* method in contrast to the *relative* method proposed in the dossier, because it quantifies the samples against a phosphate standard and not an enzyme standard. In addition, the validation showed that the FEFANA method can be applied to the analysis of samples regardless of the specific phytase product present in the feedingstuffs. The $RSD_r$ varied between 3.1 and 13.0% and the $RSD_R$ varied between 5.2 and 14.2%. Both method performance characteristics are considered acceptable. This method, which is currently under evaluation to become a standard of the European Committee for Standardisation (CEN) is therefore recommended by the CRL for official control purposes.

No further testing or validation are required.

**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**

| | |
|---|---|
| Name of the additive | *Levucell® SC (for dairy goats and dairy ewes)* |
| Active substance | Saccharomyces cerevisiae CNCM I-1077 |
| | EFSA-Q-2005-176 |
| Author: | Renata Leuschner (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 17/01/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for LEVUCELL® SC under the category 'zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use LEVUCELL® SC in its two forms LEVUCELL® SC 20 and LEVUCELL® SC 10ME for dairy goats and dairy ewes. LEVUCELL® SC is a light brown powdery product whereby LEVUCELL® SC 20 is uncoated and LEVUCELL® SC 10ME micro-encapsulated. The feed additive is proposed for use in premixtures and feedingstuffs for dairy goats at a minimum concentration of 4.8 x 108 and maximum concentration of 2.9 x 109 colony forming units (c.f.u.) per kg complete feedingstuffs and for dairy ewes at a concentration of 1.2 x 109 c.f.u./kg complete feedingstuffs.

For the determination of the active agent (Saccharomyces cerevisiae CNCM I-1077) in the feed additive LEVUCELL® SC, a pour plate method and a molecular identification method (polymerase chain reaction (PCR)) are proposed, which are considered appropriate for the intended purpose.

For the determination of the active agent S. cerevisiae CNCM I-1077 in premixtures and feedingstuffs, the same methods as for the additives are proposed. The method's performance characteristics of the enumeration method include relative standard deviations for repeatability (RSDr) and between-laboratory reproducibility (RSDR) of around 5 % and 8 %, respectively [System. Appl. Microbiol. 2003, 26, 147-153]. The limit of quantification (LOQ) of the method is 100 c.f.u./g. These performance characteristics are considered acceptable. The PCR method for strain identification was ring trial validated and performed appropriately. Both methods are considered suitable for official control for the field of application that is sought [System. Appl. Microbiol. 2004, 27, 492-500].

Official and/or standard methods are proposed by the applicant for the determination of impurities (heavy metals, mycotoxins, microbiological quality) in the feed additive. The methods are therefore suitable for official control purposes.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required by the CRL.

**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**

| | |
|---|---|
| Name of the additive | *Levucell® SC (for horses)* |
| Active substance | Saccharomyces cerevisiae CNCM I-1077 |
| | EFSA-Q-2005-234 |
| Authors: | Renata Leuschner (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 03/03/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for LEVUCELL® SC under the category 'zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use LEVUCELL® SC in its two forms LEVUCELL® SC 20 and LEVUCELL® SC 10ME for horses. LEVUCELL® SC 20 is a light brown powdery uncoated product whereby LEVUCELL® SC 10ME is micro-encapsulated. The feed additive is proposed for use in premixtures and feedingstuffs for horses at a concentration of 3 x 109 colony forming units (c.f.u.) per kg complete feedingstuff.

For the determination of the active agent (Saccharomyces cerevisiae CNCM I-1077) in the feed additive LEVUCELL® SC, a pour plate method for enumeration and a polymerase chain reaction (PCR) method for identification are proposed which are considered appropriate for the intended purpose.

For the determination of the active agent S. cerevisiae CNCM I-1077 in premixtures and feedingstuffs, the same methods as for the feed additive are proposed. The method's performance characteristics of the enumeration method include relative standard deviations for repeatability (RSDr) and between-laboratory reproducibility (RSDR) of around 5 % and 8 %, respectively [System. Appl. Microbiol. 2003, 26, 147-153]. The limit of quantification (LOQ) of the method is 105 c.f.u./kg. These performance characteristics are considered acceptable. The PCR method for strain identification was ring trial validated and performed appropriately [System. Appl. Microbiol. 2004, 27, 492-500]. Both methods are considered suitable for official control for the field of application that is sought.

Official and/or standard methods are proposed by the applicant for the determination of impurities (heavy metals, mycotoxins, microbiological quality) in the feed additive. The methods are therefore considered suitable for official control purposes.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required by the CRL.

**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**

| | |
|---|---|
| Name of the additive | *O35* |
| Active substance | Bacillus subtilis DSM 17299 |
| | EFSA-Q-2005-237 |
| Author: | Rūta Bubulienė (National Veterinary Laboratory, LT) |
| Checked by: | Dalia Garalevičienė (CRL-FAA)<br>Renata Leuschner (CRL-FAA) |
| Approved by: | Anne-Mette Jensen (CRL-FAA) |
| Date: | 07/04/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the feed additive '035' under the category 'zootechnical additives 4(b) – gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use '035' for chickens for fattening. The feed additive '035' is a yellowish free-flowing powdery product containing at least 1.6 x $10^9$ colony forming units (c.f.u.) per gram of the additive and it is proposed for use in premixtures and feedingstuffs obtaining a concentration ranging from 0.8-1.6 x $10^9$ c.f.u. of active agent per kg complete feedingstuffs.

For the determination of the active agent (*Bacillus subtilis* DSM 17299) in the *feed additive*, *premixtures* and *feedingstuffs*, a surface plate count method is proposed. The method uses Tryptose Blood Agar (TBA) base with inclusion of 5% defibrinated calf or sheep blood. The limit of quantification (LOQ) of this method is 1.0 x $10^3$ c.f.u./g (1.0 x $10^6$ c.f.u./kg) which is well below the anticipated minimum target level of application in the animal feedingstuffs. This method is very similar to another method that was ring-trial validated [J.AOAC Int. 2003, 86, 568-575] and which uses Tryptose Soya Agar (TSA) as medium. The applicant provides data which confirm that the use of TBA as medium results in comparable performance as when using TSA, and that there are no statistically significant differences between the two methods. The method performance characteristics for the ring trial validated method include relative standard deviations for repeatability ($RSD_r$) and between-laboratory reproducibility ($RSD_R$) of around 1% and 6%, respectively. These performance characteristics are considered acceptable. Therefore, in the opinion of the CRL, the ring trial validated method is suitable for official control purposes.

The purity and correct identity of the *Bacillus subtilis* strain (DSM 17299) is examined by molecular DNA fingerprinting methodology, which is considered to be appropriate for the intended purpose. Pulsed field gel electrophoresis would be considered a suitable technique for official control purposes.

Standard and/or official methods are proposed by the applicant for the determination of impurities (heavy metals, microbiological agents) in the feed additive. The methods are considered suitable for the intended purposes.

On the basis of supplied documentation, no supplementary experimental work (testing or method validation) is required.

**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**

| | |
|---|---|
| Name of the additive | *VitaLys®* |
| Active substance | l-lysine |
| | EFSA-Q-2005-230 |
| Author: | Giuseppe Simone (CRL-FAA) |
| Checked by: | Anne-Mette Jensen(CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 05/05/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for VitaLys® under the category "nutritional additives", functional group "amino acids, their salts and analogues", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use VitaLys® in its two forms, VitaLys® Liquid and VitaLys® Dry, for all animal species. VitaLys® Liquid is a brown liquid lysine concentrate with a minimum content of 25 % L-lysine and a maximum water content of 40 %. VitaLys® Dry is a light brown, powder lysine concentrate with a minimum content of 44 % L-lysine and a maximum content of 5 % moisture. The feed additive is intended to be mixed into premixtures, mineral mixed feedingstuffs, complementary or complete feedingstuffs at a final concentration range depending on both the nutritional requirements of the animals and the concentration of L-lysine in the feed materials contained in the complete feedingstuffs.

For the determination of the active substance (L-lysine) in the *feed additive,* in *premixtures* and in *feedingstuffs* the official Community and fully ring-trial validated method for determination of amino acids [Commission Directive 98/64/EC] is proposed by the applicant. The method is applicable for both the determination of free (synthetic and natural) and the determination of total (peptide-bound and free) amino acids, using an amino acid analyzer or an High Performance Liquid Chromatography (HPLC) equipment with post column derivatisation with ninhydrin and photometric detection at 570 nm. The method's performance characteristics related to the target analyte include a relative repeatability standard deviation ($RSD_r$) ranging between 2.1 and 2.8 % and a relative reproducibility standard deviation ($RSD_R$) ranging between 3.0 and 6.7 %, depending on the matrix.

The same method is described in the ISO standard 13903:2005 [Animal feedingstuffs – determination of amino acids content - ISO 13903:2005], which reports also the results from a second intercomparison study [Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994]. Performance characteristics for the target analyte (L-lysine) included the relative repeatability standard deviation ($RSD_r$) ranging between 2.37 and 3.46 % and relative reproducibility standard deviation ($RSD_R$) ranging between 7.94 and 13.08 %, depending on the matrix. The reported

limits of quantification (LOQ) are 0.3 g/kg for total lysine and 0.035 g/kg for free lysine. The method is considered suitable for official control purposes for the determination of total and free L-lysine in *feedingstuffs* and in *premixtures*.

However, for official control purposes regarding the determination of free L-lysine in the *feed additive* the CRL recommends a fully ring trial validated method which is very similar to the above mentioned Community method and which is explicitly designed for the determination of *free* amino acids in feed additives. The method has been published in the method collection of the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany) [VDLUFA Methodenbuch III, 1993 2. Erg., Aminosaeuren 4.11.1]. The reported $RSD_R$ of the method for the determination of free L-lysine in a feed additive matrix was 1.95 %.

Neither method distinguishes between the salts of amino acids, nor differentiates between D and L forms of amino acids.

For the identification/characterisation of the producer micro-organism the applicant proposed a molecular method for the detection of *Corynebacterium glutamicum* (DSM 14764) based on colony hybridization developed by the Flemming Jørgensen Biotechnological Institute (Denmark). The method is considered suitable for the intended purpose.

For the determination of heavy metals (As, Pb and Cd), mycotoxins (aflatoxin B1, B2, G1 and G2) and dioxins (PCDD/PCDF) the applicant proposed standard methods which are considered suitable for official control purposes. Internal methods are proposed by the applicant for other mycotoxins (ochratoxin, zearalenone, T2, deoxynivalenol (DON)). For official control purposes fully ring trial validated methods that are available at the CRL are recommended.

Methods issued by the Nordic Committee on Food Analysis are proposed by the applicant for microbiological quality of the final products, which the CRL considers suitable for the intended purpose. However, for official control purposes the CRL recommends corresponding ISO/CEN standard methods.

Further testing or validation is not considered necessary.

**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**

| | |
|---|---|
| Name of the additive | *BIOSAF® Sc47 (for dairy small ruminants)* |
| Active substance | *Saccharomyces cerevisiae* NCYC Sc47 |
| | EFSA-Q-2006-003 |
| Author: | Renata Leuschner (CRL-FAA) |
| Checked by: | Anne-Mette Jensen (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 19/05/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for BIOSAF® Sc47 under the category 'zootechnical additives', functional group 'other zootechnical additives', according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use BIOSAF® Sc47 for dairy small ruminants. BIOSAF® Sc47 contains as active agent *Saccharomyces cerevisiae* NCYC Sc47 at a minimum concentration of 5 x $10^9$ colony forming units (c.f.u.) per gram of additive. It is proposed for use in complete feedingstuffs for dairy small ruminants at a concentration between 7 x $10^8$ and 7.5 x $10^9$ c.f.u./kg.

For quantifying the active agent *Saccharomyces cerevisiae* (NCYC Sc47) in the *additive* the ISO 7954 method is provided by the applicant. For official control purposes a similar pour plate method which was validated by an interlaboratory study is recommended [System. Appl. Microbiol. 2003, 26, 147-153].

This same method [System. Appl. Microbiol. 2003, 26, 147-153] is used by the applicant for enumeration of the active agent in *premixtures* and *feedingstuffs*. The method's performance characteristics obtained in the validation study included relative standard deviations for within-laboratory repeatability ($RSD_r$) and for between-laboratory reproducibility ($RSD_R$) of around 5 % and 8 %, respectively. The limit of quantification (LOQ) of the method is $10^5$ c.f.u./kg sample, corresponding to 100 c.f.u./g, which is well below anticipated concentrations in feedingstuffs. The method is recommended for official control purposes.

For identification of the yeast strain a molecular pulsed field gel electrophoresis (PFGE) and a polymerase chain reaction (PCR) method are proposed by the applicant. For official control purposes the PCR method which performed appropriately in an interlaboratory study is recommended [System. Appl. Microbiol. 2004, 27, 492-500].

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required by the CRL.

**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**

| | |
|---|---|
| Name of the additive | *L-histidine monohydrochloride monohydrate* |
| Active substance | L-histidine |
| | EFSA-Q-2004-030 |
| Author: | Giuseppe Simone (CRL-FAA) |
| Checked by: | Renata Leuschner (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 07/06/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for L-histidine monohydrochloride monohydrate under the category "nutritional additives", functional group "amino acids, their salts and analogues", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use L-histidine monohydrochloride monohydrate for supplementing salmon feed. The product is a white crystalline powder with a minimum content of 98 % L-histidine monohydrochloride monohydrate, corresponding to 74 % L-histidine. The feed additive is intended to be mixed directly in feedingstuffs at a final concentration up to 1.7 % of total L-histidine, depending on the concentration of L-histidine already present in the feed components.

For the determination of the active substance (L-histidine) in the *feed additive* and in *feedingstuffs* the applicant proposed the official Community and fully ring-trial validated method for determination of amino acids [Commission Directive 98/64/EC]. The method is applicable for both the determination of free (synthetic and natural) and the determination of total (peptide-bound and free) amino acids, using an amino acid analyzer or an High Performance Liquid Chromatography (HPLC) equipment with post column derivatisation with ninhydrin and photometric detection at 570 nm. The same method is adopted by ISO and described in the ISO standard 13903:2005 [Animal feedingstuffs – determination of amino acids content - ISO 13903:2005], which additionally reports the results from a second intercomparison study [Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994]. Performance characteristics for the target analyte (L-histidine) included the relative repeatability standard deviation ($RSD_r$) ranging between 2.41 and 7.04 % and relative reproducibility standard deviation ($RSD_R$) ranging between 12.85 and 23.33 %, depending on the matrix. The method does not distinguish between the salts of amino acids, nor differentiates between D and L forms of amino acids. The method is considered suitable for official controls.

Further testing or validation is not considered necessary.

**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**

| | |
|---|---|
| Name of the additive | *Biogalactosidase®* |
| Active substance | α-D-galactosidase |
| | EFSA-Q-2005-224 |
| Author: | Roger Ziebal (DGCCRF) |
| Checked by: | Christoph von Holst (CRL-FAA)<br>Dalia Garalevičienė (CRL-FAA) |
| Approved by: | Anne-Mette Jensen (CRL-FAA) |
| 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.

# 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

| | |
|---|---|
| Name of the additive | *Hemicell® Feed Enzyme ®* |
| Active substance | *β-D-mannanase* |
| | EFSA-Q-2006-004 |
| Author: | Waldemar Korol (National Feed Laboratory ,PL) |
| Checked by: | Giuseppe Simone(CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 19/06/2006 |

## 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.

**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**

| | |
|---|---|
| Name of the additive | *Belfeed B1100 MP/ML®* |
| Active substance | Endo-1,4-beta-xylanase |
| | EFSA-Q-2005-115 |
| Author: | Dalia Garaleviciene (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 19/01/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for Belfeed B1100M® 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 Belfeed B1100M® as a digestibility enhancer for ducks for fattening. The active substance of Belfeed B1100M® is endo-1,4-®-xylanase (produced by the genetically modified microorganism *Bacillus subtilis* LMG S-15136), which degrades the polysaccharide ®-1,4-xylan. The additive is intended to be marketed in two forms, as a granulated powder (Belfeed B1100MP®) and as liquid formulation (Belfeed B1100ML®).

The activity of endo-1,4-®-xylanase is expressed as international units (IU). According to the applicant, one IU is the amount of enzyme which liberates 1 mole of reducing sugars (xylose equivalents) from birchwood xylan per minute at pH 4,5 and 30°C. Belfeed B1100MP® and Belfeed B1100ML® have a minimum specific activity of 100 IU endo-1,4-®-xylanase /g of product. The product also has other limited enzymatic activities (®-glucanase, α-amylase and pectinase). Belfeed B1100M® is intended to be mixed into feedingstuffs obtaining a minimum enzyme activity of 10 IU/kg.

For the determination of the xylanase activity in the *feed additive* the applicant proposes an in-house developed test which is based on the principle that xylanase releases xylose from the substrate xylane, which in the presence of copper neocuproin forms a yellow coloured complex. The yellow complex is measured with a spectrophotometer using an enzyme standard curve. Based on the obtained method performance characteristics, that include a limit of detection of 5 IU/g and a relative standard deviation for within-laboratory reproducibility of 3.2 %, the method is considered suitable for the intended purpose.

For the determination of the xylanase activity in *premixtures* the applicant proposes the same method obtaining relative standard deviations for within-laboratory reproducibility ranging from 0.8 to 2.3 % for the solid form and from 1.7-6.5% for the liquid form of the feed additive. The method is considered suitable for the intended purpose.

For the determination of the xylanase activity in *feedingstuffs* a modified method of a commercially available test kit is proposed. The method is based on the principle that xylanase

releases water soluble dyed fragments from a substrate, which are directly related to the enzyme activity. The formed dyed fragments are then measured with a spectrophotometer and the enzyme activity is quantified against matrix matched standards. The linear range of the test, in which the activity of the enzyme can be quantified, is from 5 to 75 IU/kg. The relative standard deviations for within-laboratory reproducibility range from 2.8-5.9% for the solid form of the feed additive and 3.1-7.4% for the liquid form of the product. Taking into account the target level of application which is equal to 10 IU /kg of feedingstuffs and the acceptable values of method performance characteristics, in the opinion of the CRL the proposed method is fit for official control purposes to determine the activity of the xylanase in target feedingstuffs at the target activity level.

Further testing or validation is not considered necessary.

**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**

| | |
|---|---|
| Name of the additive | *BioPlus 2B (for turkeys for fattening)* |
| Active substance | *Bacillus subtilis* DSM 5750<br>*Bacillus licheniformis* DSM 5749 |
| | EFSA-Q-2005-275 |
| Author: | Renata Leuschner (CRL-FAA) |
| Checked by: | Anne-Mette Jensen (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 06/06/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive BioPlus 2B under the category 'zootechnical additives', functional group 'others (microorganism)', according to Annex I of Regulation (EC) No 1831/2003. The active agents of the additive are *Bacillus licheniformis* DSM 5749 and *Bacillus subtilis* DSM 5750 at a ratio of 1:1. Specifically, authorisation is sought to use BioPlus 2B for turkeys for fattening. It is proposed for use in feedingstuffs for turkeys at a concentration of $1.3 \times 10^9$ colony forming units (c.f.u.) of active agents *Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749 per kilogram (kg) complete feedingstuffs.

For the quantification of the two active agents (*Bacillus subtilis* DSM 5750 and *B. licheniformis* DSM 5749) of BioPlus 2B in the *feed additive, premixtures and feedingstuffs*, a surface plate count method was proposed by the applicant. The method was shown to perform equivalently for compound feedingstuffs containing the permitted coccidiostat maduramicin ammonium. The method is very similar to a method which uses tryptone soya agar (TSA) and which was validated by an interlaboratory study. This method is characterised by method performance data including a relative within-laboratory repeatability standard deviation ($RSD_r$) and relative between-laboratory reproducibility standard deviation ($RSD_R$) of around 1% and 6%, respectively (J. AOAC Int. 2003. 86, 568-575). The limit of quantification (LOQ) for the method is $1.0 \times 10^6$ c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs. For official controls the validated method is recommended.

The molecular identity of the two bacilli strains was determined by using pulsed field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and would be considered suitable for official controls.

Standard and/or official methods are proposed by the applicant for the determination of impurities (heavy metals, microbiological quality) in the feed additive. The methods are therefore considered suitable for official controls.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

**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**

| | |
|---|---|
| Name of the additive | *Safizym X®* |
| Active substance | Endo-1,4-β-xylanase |
| | EFSA-Q-2005-276 |
| Author: | Dalia Garalevičienė (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 29/06/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for Safizym X® under the category zootechnical additives, group 4(d), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Safizym X® as a zootechnical feed additive for piglets, according to EFSA-Q-2005-276. The additive is intended to be marketed in two forms, as a powder (Safizym XP20®) and as liquid formulation (Safizym XL200®).

The active agent of Safizym X® is endo-1,4-®-xylanase, produced by a microorganism *Trichoderma longibranchiatum* (CNCM MA 6-10W). According to the nomenclature of the International Union of Biochemistry and Molecular Biology (IUBMB), endo-1,4-®-xylanase has the number EC 3.2.1.8.

The activity of endo-1,4-®-xylanase is expressed as IFP (Institut Français du Pétrole) units. According to the applicant, one IFP unit is the quantity of enzyme which liberates one mole of reducing sugars in equivalent xylose per minute from oat xylan under specific conditions (pH 4.8 and 50°C). According to the applicant, Safizym XP20® and Safizym XL200® have a guaranteed minimum activity of 70000 IFP/g and 7000 IFP/ml of product, respectively. The additive also contains a residual activity of endo-1,3-(4)-®-glucanase. Safizym XP20® is intended to be incorporated into premixtures or complete feedingstuffs, whereas the liquid formulation Safizym XL200® is sprayed onto the feedingstuffs to obtain enzyme activity levels of minimum 840 to recommended 1680 IFP/kg in complete feedingstuffs.

For the determination of the activity of endo-1,4-®-xylanase in the *feed additive,* the applicant proposes an absolute colorimetric method based on reducing sugar properties. Xylose is released from the substrate oat spelt xylan when incubated with endo-1,4-®-xylanase. Method's transferability on Safizym XP20® has been checked among three laboratories [*Anim. Feed Sci. Techn*. 1999, 77:345-353] and similar results were obtained. Therefore, the method is considered suitable for the intended purpose.

For the determination of the endo-1,4-®-xylanase activity in *premixtures,* the applicant proposes a relative colorimetric method, based on the principle that xylanase releases water soluble dyed

fragments, when incubated with oat azo-xylan. Method's transferability on Safizym XP20® has been checked among two laboratories, obtaining similar results. Based on the method performance characteristics, that include a limit of detection (LOD) of 0.14 IFP/g, limit of quantification (LOQ) of 0.35 IFP/g and the within-laboratory relative standard deviation for repeatability ($RSD_r$) of 6.5 %, the method is considered suitable for the intended purpose.

For the quantification of the endo-1,4-®-xylanase activity in *feedingstuffs,* the applicant proposes the same method as for *premixtures*, just the sample extraction is modified and the incubation time is prolonged. The enzyme activity in *feedingstuffs* is quantified against matrix matched standards (blank feed samples supplemented by a known dose of Safizym XP20® with declared activity). The transferability of the method has been checked among two laboratories [*Anim. Feed Sci. Techn*. 1999, 77:345-353] obtaining similar results. A separate check among two laboratories has been performed on the liquid formulation Safizym XL200®. The LOD and LOQ correspond to 140 IFP/kg and 350 IFP/kg of feedingstuffs, respectively, and the average within-laboratory $RSD_r$ is 8.3 % for the powder and 9.2% for the liquid formulation. Taking into account the target enzyme activity level of 840 to 1680 IFP/kg of complete feedingstuffs and the acceptable values of method performance characteristics, the proposed method is considered fit for official controls to determine the activity of the endo-1,4-®-xylanase in target feedingstuffs at the target activity level, when the standard feed (blank feed supplemented by a known dose of Safizym XP20® with declared activity) is available.

It is recommended by the CRL that for the preparation of matrix matched standards for the quantification of the enzyme activity in feedingstuffs, the declared activity of endo-1,4-®-xylanase in Safizym XP20® is confirmed by applying the method proposed for the pure additive and the actual measured activity is taken into account for the calculation of the final enzyme activity in feedingstuffs.

In the case, that the standard feed (blank feed supplemented by a known dose of Safizym XP20® with declared activity) is not available, a standard addition method is recommended.

Further testing or validation is not considered necessary.

**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**

| | |
|---|---|
| Name of the additive | *Rovabio™ PHY AP/LC* |
| Active substance | 3-phytase |
| | EFSA-Q-2005-281 |
| Author: | Anne-Mette Jensen (CRL-FAA) |
| Checked by: | Dalia Garaleviciene (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 16/08/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for ROVABIO™ PHY under the category zootechnical additives, digestibility enhancers, group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use ROVABIO™ PHY for chickens for fattening, laying hens, weaned piglets and fattening pigs, according to EFSA-Q-2005-281. ROVABIO™ PHY is an enzyme preparation, available in powder form (ROVABIO™ PHY AP) and in liquid form (ROVABIO™ PHY LC). The enzyme is produced by *Penicillium funiculosum* 4.05 b (CBS 111443). The feed additive has a target activity of minimum 2500 RPU/g for ROVABIO™ PHY AP and of minimum 1000 RPU/ml for ROVABIO™ PHY LC, where 1 RPU is the amount of enzyme that releases 1 µM inorganic ortho-phosphate per minute from sodium phytate as substrate at pH 5.5 and 37° C. It is intended to be mixed into compound feedingstuffs to a level of 250 RPU/kg.

For the determination of the enzyme activity of 3-phytase, in the *feed additive,* ROVABIO™ PHY*,* a colorimetric method is proposed by the applicant. The principle of the method is that after diluting the feed additive and performing an enzyme kinetics test in the presence of sodium phytate, the amount of phosphate released is measured via the formation of a phosphomolybdate complex by reduction of iron (II). The amount of phosphate released is read off a calibration curve constructed using inorganic phosphate. The method is considered suitable for official control purposes.

For the determination of the enzyme activity in *premixtures* and in *feedingstuffs* the applicant proposes an adapted version of the colorimetric method applied for feed additives. In the adapted version an initial step of extraction of the active agent from the premixture or feedingstuff is included.

When tested on *premixtures* for chicken and piglet feed the method's performance characteristics include relative repeatability standard deviation ($RSD_r$) values of 1.1-4.2 %; and recovery rates between 34 and 67 %.

When tested on *feedingstuffs* (chicken and piglet feed) the method's performance characteristics include $RSD_r$ values of 1.0-7.6 %; and recovery rates between 100 and 131 %.

The applicant's method follows well known principles for the determination of phytase activity in various matrices, and transferability of the method has been verified by testing the method with a second laboratory. It should be noted though that other analytical methods for the determination of phytase activity in premixtures and feedingstuffs exist, which have been validated in inter-laboratory studies. These include a method proposed by the Association of German Agricultural Analytical and Research Institutes (*Bestimmung der Phytaseaktivität in Futtermitteln und Vormischungen (Determination of the phytase activity in feedingstuffs and premixtures)* Method book III of VDLUFA „The chemical analysis of feedingstuffs"; Method Number 27.1.2; 4th Auxiliary supply 1997 ; VDLUFA ISBN 3-922712-66-7, in German] which resulted in a relative between-laboratory standard deviation for reproducibility ($RSD_R$) of about 12 % for feedingstuffs and 8.4 % for a mineral premixture. Another method [Engelen et al. (2001) J. AOAC Int., 84, 629-633] obtained $RSD_R$ values ranging from 14.0 to 27.6 % for feedingstuffs. However, data regarding ROVABIO™ pertaining to these two methods was not submitted by the applicant.

The European Association of Feed Additive Manufacturers (FEFANA) developed a method, suitable for the analysis of all phytase products currently authorised within the EU, which follows the same principle as the applicant's method, in order to allow for the measurement of phytase activity in feedingstuffs, regardless of the specific phytase product used. The FEFANA method has been validated in an inter-laboratory study which was performed on feedingstuffs containing different 3- or 6- phytase products. The obtained values for the $RSD_R$, ranging from 5 to 14 %, are considered acceptable for the intended use. This method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN). For these reasons, the CRL asked the applicant to compare the proposed in-house method with the inter-laboratory validated FEFANA method for chicken and piglet feed. The applicant provided results, showing a comparison between the proposed in-house method and a, to some extent modified, FEFANA method (different sample weight, different extraction solutions, buffer used during detection stage instead of water). With the modified FEFANA method, performance characteristics comparable to the method proposed by the applicant were obtained. While it is likely that the FEFANA method would be suitable for official control purposes for determining the enzyme activity of ROVABIO™ PHY in feedingstuffs, the data provided by the applicant concerns a *modified* version of the FEFANA method. For this reason, the CRL has no evidence of the suitability of the FEFANA method for official control purposes for this particular feed additive. Taking into account these facts, for determination of the enzyme activity of ROVABIO™ PHY in feedingstuffs, the CRL recommends the applicant's own method for official control purposes.

Regarding identification and characterisation of the additive, methods pertaining to the enumeration and detection of micro-organisms, identification of the strain including DNA profile for *Penicillium funiculosum* 4.05 b (CBS 111443), and determination of mycotoxins are provided.

No further testing or validation is required.

# 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

| | |
|---|---|
| Name of the additive | *ColiCure®* |
| Active substance | *Escherichia coli* E-101-88, LMG S-1714 |
| | EFSA-Q-2005-167 |
| Author: | Renata Leuschner (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 20/09/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive ColiCure® under the category ‘zootechnical additives’, functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is *Escherichia coli* E-101-88, LMG S-1746. Specifically, authorisation is sought to use ColiCure® for horses. It is proposed that one bottle of 100 ml ColiCure® which contains at least 1 x $10^9$ colony forming units (c.f.u.)/ml of the active agent *Escherichia coli* E-101-88, LMG S-1746 suspended in phosphate buffered saline is added to a small amount of feedingstuffs for horses for immediate consumption. For the quantification of the active agent (*Escherichia coli* E-101-88, LMG S-1746) of ColiCure® in the feed additive and feedingstuffs, an appropriate non-selective surface plate count method based on well-known principles using nutrient agar supplemented with 5 % bovine blood and an incubation temperature of 37 °C was proposed by the applicant. The method is considered suitable for the intended purpose. For official controls of the *feed additive* ColiCure® and if required of *feedingstuffs* supplemented with ColiCure® corresponding officially recognised standard methods such as ISO and/or CEN methods for example ISO 4832, ISO 16649-2 or ISO 21528-2 are recommended. The identity of the microbial strain was analysed by a range of techniques including microscopy, serology, biochemistry, polymerase chain reaction (PCR) and restriction enzyme analysis using three enzymes. The applicant used amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

**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**

| | |
|---|---|
| Name of the additive | *VevoVitall® (for pigs for fattening)* |
| Active substance | Benzoic acid |
| FAD-: | EFSA-Q-2006-056 |
| Author: | Giuseppe Simone (CRL-FAA) |
| Checked by: | Federica Serano(CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 23/11/2006 |

## EXECUTIVE SUMMARY

VevoVitall® is a feed additive for which authorisation is sought under the category "zootechnical additives", functional groups "other zootechnical additives" and "substances which favourably affect the environment", according to the classification system of Annex I of Regulation (EC) No 1831/2003. VevoVitall® contains high purity benzoic acid (≥ 99.9 % on anhydrous basis) as active substance.

In the current application authorisation is sought for use of VevoVitall® for pigs for fattening. The feed additive is intended to be mixed into compound feedingstuffs at a concentration of 5000 mg to 10000 mg/kg feedingstuffs.

For the determination of the benzoic acid in the *feed additive* the CRL recommends a titrimetric assay as specified by the corresponding monograph of the European Pharmacopoeia.

For the determination of the active substance in *feedingstuffs* a Reversed Phase High Performance Liquid Chromatography (RP HPLC) method with diode array detection (DAD) is submitted. The method's performance characteristics include a recovery rate between 94 % and 113 %, a relative repeatability standard deviation ($RSD_r$) of 2 % and a relative within-laboratory reproducibility standard deviation ($RSD_R$) of 5 %. The limit of detection of the method is 500 mg/kg and the limit of quantification is 2000 mg/kg. These performance characteristics are considered acceptable and the method is therefore considered suitable for official control purposes, if the analysis aims at the quantification of the target analyte in feedingstuffs samples in the frame of the sought authorisation, i.e. in target feed samples (feedingstuffs for pigs for fattening) at the target concentration range of benzoic acid (5000 to 10000 mg/kg).

Control methods are submitted for determination of possible contaminants and impurities (heavy metals, arsenic, organic impurities) in the feed additive which are considered suitable for the intended purposes. For official controls of heavy metals various standard methods, based on the same analytical technique and routinely applied by official control authorities are available and recommended by the CRL.

Further testing or validation by the CRL is not considered necessary.

**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**

| | |
|---|---|
| Name of the additive | *Bonvital (for dogs)* |
| Active substance | *Enterococcus faecium* DSM 7134 |
| FAD-: | EFSA-Q-2006-061 |
| Author: | Renata Leuschner (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 13/12/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Bonvital under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is *Enterococcus faecium* DSM 7134. The additive is available in two forms (powder or granules (micro-encapsulated)) both of which contain a minimum concentration of 1 x $10^{10}$ colony forming units (c.f.u.) per gram. Specifically, authorisation is sought to use Bonvital for dogs. The conditions of use are proposed with a recommended dosage of 1 x $10^{9}$ c.f.u./kg.

For the quantification of the active agent (*Enterococcus faecium* DSM 7134) of Bonvital in the *feed additive*, *premixtures* and *feedingstuffs*, an appropriate surface plate count method was proposed by the applicant. The method was in-house validated and shown to be transferable to four external laboratories. The method precision data resulting from the in-house and four laboratory trials were acceptable for the intended purpose.

For official controls regarding the quantitative determination of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, another plate count enumeration method is recommended which has been fully ring-trial validated (Leuschner R.G.K. et al. 2002. J. Appl. Microbiol. 93, 781-786). The method performance characteristics include a relative standard deviation for repeatability ($RSD_r$) ranging between 1.5 to 3.6 % and a relative standard deviation for reproducibility ($RSD_R$) ranging between 2.9 to 7.4 %. The limit of quantification (LOQ) for the method is around 2 to 3 x $10^{6}$ c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

The identity of the bacterial strain, *Enterococcus faecium* DSM 7134, was analysed by a range of techniques including biochemistry, protein-fingerprinting and molecular methods such as polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

**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**

| | |
|---|---|
| Name of the additive | *Bonvital (for piglets and pigs)* |
| Active substance | *Enterococcus faecium* DSM 7134 |
| | EFSA-Q-2006-061 |
| Author: | Renata Leuschner (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 13/12/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive Bonvital under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is *Enterococcus faecium* DSM 7134. The additive is available in two forms (powder or granules (micro-encapsulated)) both of which contain a minimum concentration of 1 x $10^{10}$ colony forming units (c.f.u.) per gram. Specifically, authorisation is sought to use Bonvital for piglets and pigs for fattening. For piglets the conditions of use are proposed at a minimum content of 0.5 x $10^9$ c.f.u./kg and at a maximum content of 4 x $10^9$ c.f.u./ kg complete feedingstuffs. For pigs for fattening a minimum content of 0.2 x $10^9$ c.f.u./kg and a maximum content of 1 x $10^9$ c.f.u./kg complete feedingstuffs is suggested.

For the quantification of the active agent (*Enterococcus faecium* DSM 7134) of Bonvital in the *feed additive*, *premixtures* and *feedingstuffs*, an appropriate surface plate count method was proposed by the applicant. The method was in-house validated and shown to be transferable to four external laboratories. The method precision data resulting from the in-house and four laboratory trials were acceptable for the intended purpose.

For official controls regarding the quantitative determination of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, another plate count enumeration method is recommended which has been fully ring-trial validated (Leuschner R.G.K. et al. 2002. J. Appl. Microbiol. 93, 781-786). The method performance characteristics include a relative standard deviation for repeatability ($RSD_r$) ranging between 1.5 to 3.6 % and a relative standard deviation for reproducibility ($RSD_R$) ranging between 2.9 to 7.4 %. The limit of quantification (LOQ) for the method is around 2 to 3 x $10^6$ c.f.u./kg sample which is well below the minimum anticipated target level of application in feedingstuffs.

The identity of the bacterial strain, *Enterococcus faecium* DSM 7134, was analysed by a range of techniques including biochemistry, protein-fingerprinting and molecular methods such as polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

# 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

| | |
|---|---|
| Name of the additive | *BIOSAF® Sc47 (for calves for rearing)* |
| Active substance | *Saccharomyces cerevisiae* NCYC Sc47 |
| | EFSA-Q-2006-067 |
| Author: | Renata Leuschner (CRL-FAA) |
| Checked by: | Giuseppe Simone (CRL-FAA) |
| Approved by: | Christoph von Holst (CRL-FAA) |
| Date: | 13/12/2006 |

## EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive BIOSAF® Sc47 under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is a strain of *Saccharomyces cerevisiae* NCYC Sc47. The additive represents heat resistant micro-granules containing live yeast cells with a minimum concentration of 5 x $10^9$ colony forming units (c.f.u.) per gram. Specifically, authorisation is sought to use BIOSAF® Sc47 for calves for rearing. The conditions of use are proposed with a recommended dosage of 1.5 x $10^9$ to 2.0 x $10^{10}$ c.f.u./kg complete feedingstuffs.

For the quantification of the active agent (*Saccharomyces cerevisiae* NCYC Sc47) of BIOSAF® Sc47 in the *feed additive* an appropriate pour plate method (ISO 7954) using a yeast extract agar was proposed by the applicant. For an analysis of the active agent in *premixtures* and *feedingstuffs* a ring-trial validated pour plate method using chloramphenicol glucose yeast extract (CGYE) agar (Leuschner R.G.K. et al., 2003. System. Appl. Microbiol. 26, 147-153) is suggested. The method performance characteristics of the enumeration method include relative standard deviations for within-laboratory repeatability ($RSDB_{rB}$) and between-laboratory reproducibility ($RSDB_{RB}$) of 2 to 5 % and of around 8 %, respectively. For official controls of *premixtures* and *feedingstuffs* supplemented with BIOSAF® Sc47 this ring-trial validated method using CGYE agar is recommended. The limit of quantification (LOQ) for the method is around 2 to 3 x $10^5$ c.f.u./kg sample which is well below the minimum target level of application in feedingstuffs.

The identity of the bacterial strain, *Saccharomyces cerevisiae* NCYC Sc47, was analysed by polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE). The PCR method was ring-trial validated and performed appropriately (Leuschner R.G.K. et al., 2004. System. Appl. Microbiol. 27, 492-500). The PCR method is considered suitable for official controls in the frame of the authorisation.

Supplementary experimental work is not considered necessary based on the documentation provided.

European Commission

**EUR 22776 EN – DG Joint Research Centre, Institute for Reference Materials and Measurements –**
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**Abstract**

The objective of this report is to present the activities of the Community Reference Laboratory for Feed Additives Authorisation in 2006 (CRL-FAA). The report shows that besides the evaluation of dossiers, the CRL-FAA carried out with many other tasks. Important additional matters were the successful accreditation of the laboratory according to ISO 17025, the support of the European Commission regarding the drafting of the new guidelines for the applicants, further improvement of informatics tools and the organisation of two workshops to discuss these topics with the National Reference Laboratory.

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