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Community Reference Laboratory For Feed Additives

Annual Report

Authorisation Activities 2007

C. von Holst, P. Robouch, G. Simone, R. Leuschner,
S. Yasar, D. Garalevičienė, S. Staes, M. De Smet

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CRL Feed Additives

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

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Contact information
Address: Retieseweg 111, B – 2440, Geel, Belgium
E-mail: christoph.von-holst@ec.europa.eu
Tel.: +32 (0) 14 571 221
Fax: +32 (0) 14 571 787

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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FOREWORD

The team of the Community Reference Laboratory for Feed Additives (CRL-FA) is very glad to present its annual report 2007. Like in the previous years the CRL-FA was very busy in quite different fields, ranging from the evaluation of dossiers to the support of the development of the new dossier guidelines. In this report we would like to explain to the reader our activities and achievements gained in the last year. In addition, we will include some background information, so it will be easier to the reader to understand the work we are conducting in the context of the authorisation of feed additives.

In 2007 the CRL-FA - along with the National Reference Laboratories (NRLs) - evaluated analytical methods related to 28 dossiers. The evaluation covered analytical methodologies on a wide variety, such as microbiological enumeration techniques for the determination of probiotics, liquid chromatography for coccidiostats and atomic absorption spectroscopy for trace elements. Based on discussions during the CRL-FA workshop in 2006 we established working groups for the individual analytical techniques with experts from NRLs in order to harmonise the evaluation of the dossiers and various meetings were organised on this topic.

An extremely important milestone for the future preparation of dossiers was reached in December 2007, when the draft Commission Regulation on the new dossier guidelines received a favourable opinion of the Standing Committee on the Food Chain and Animal Health. These guidelines also contain new requirements in respect to the analytical methods that the applicant has to present in the dossier. Following the CRL-FA's recommendations two aspects have been included, namely the requirement for the methods to fulfil the criteria laid down in official food and feed Regulation (EC) No 882/2004, and the requirement for a verification study especially designed for the single-laboratory validated methods.

Last but not least – we already launched different activities to get prepared for the deadline of November 2010 by when the industry has to submit a full application for each product that has been notified according to article 10 of the European Regulation (EC) No 1831/2003 and is currently authorised and on the market.

Christoph von Holst
Operating Manager
CRL – FA

IRMM Excellence Award 2007

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Excellence Awards 2007

SUPPORT TO EU POLICY

awarded to

**Christoph von Holst (operating manager),
Machteld De Smet, Dalia Garalevičienė,
Renata Leuschner, Giuseppe Simone,
Seppe Staes and Sulhattin Yasar**
Community Reference Laboratory for Feed Additives

on the 8th of October 2007

for their contribution to the authorisation of feed additives

Geel, February 2008

Alejandro Herrero
Director

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CRL – FA / Authorisation

Objectives

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 premixtures or 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-FA.

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

CRL
Feed Additives

Tasks

The main task of the CRL-FA 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-FA 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 the Annex.

Analytical methods are evaluated in a stepwise manner in which the CRL-FA and a rapporteur laboratory, which belongs to the consortium of NRLs and which the CRL-FA 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-FA 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, as amended by Regulation (EC) No 850/2007, the CRL-FA charges the applicant 6000€ for each application. More details on the evaluation procedure are given later on in this report. If necessary, the CRL-FA 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-FA 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-FA responsibilities also include other tasks, namely

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

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

Official Food and Feed Control Regulation

Since 1st January 2006 and in accordance to Article 32 of Regulation (EC) No 882/2004, the CRL-FA 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.

CRL-FA website

The CRL-FA website, available since early 2005, is regularly updated.

Under section "public pages" information is presented on the activities of the CRL-FA and the composition of the consortium of NRLs. 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-FA is also available for the consortium via the network pages.

www.irmm.jrc.be/html/CRLs/crl_feed_additives/index.htm

CRL-FA Dossiers Tracking System

A browsable interface to search the database was made available to the NRLs, EFSA and DG for Health and Consumer Protection (DG SANCO) via the Network pages of the CRL-FA website.Two different kinds of search can be performed: (1) samples, (2) dossiers.

https://irmm.jrc.ec.europa.eu/crldossiercat/dossier/search.do
https://irmm.jrc.ec.europa.eu/crlsamplecat/sample/search.do

CIRCA

The Communication and Information Resource Centre Administrator (CIRCA) enabled the CRL-FA and the consortium of NRLs to maintain a secure space on the Internet where they can share documents information, and they can participate in discussion fora. More than 50 users (consortium members, EFSA officers, DG for Health and Consumer Protection administrators and the CR-FA team itself) share information and documents on a daily basis, allowing for effective and fast communication. The system is managed by the CRL-FA. In the near future, CIRCA will be replaced in the near future by a more powerful system to handle efficiently the increasing number of incoming documents.

https://circa.europa.eu/

FEEDACAM, the method database

With the experience gained by the CRL–FA during this year, the FEEDACAM database revealed a need for a revision of the methods and the information it contains, in order to match the requirements and the needs of all stakeholders. This revision process started in 2007 and will be implemented in 2008.

http://www.irmm.jrc.be/crlfaacat/searchCdp.do

Main Activities of the CRL-FA Authorisation in 2007

In addition to the scientific evaluations of analytical methods the CRL-FA organised in 2007 a workshop and various expert meeting on specific analytical methodologies, 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.

Evaluations of Dossiers

In 2007, a total of 28 dossiers were finalised and submitted on due time to EFSA, as shown in Table 1. Executive summaries of the CRL-FA reports are presented in Annex 2.

Table 1: Overview of dossiers evaluated in 2007

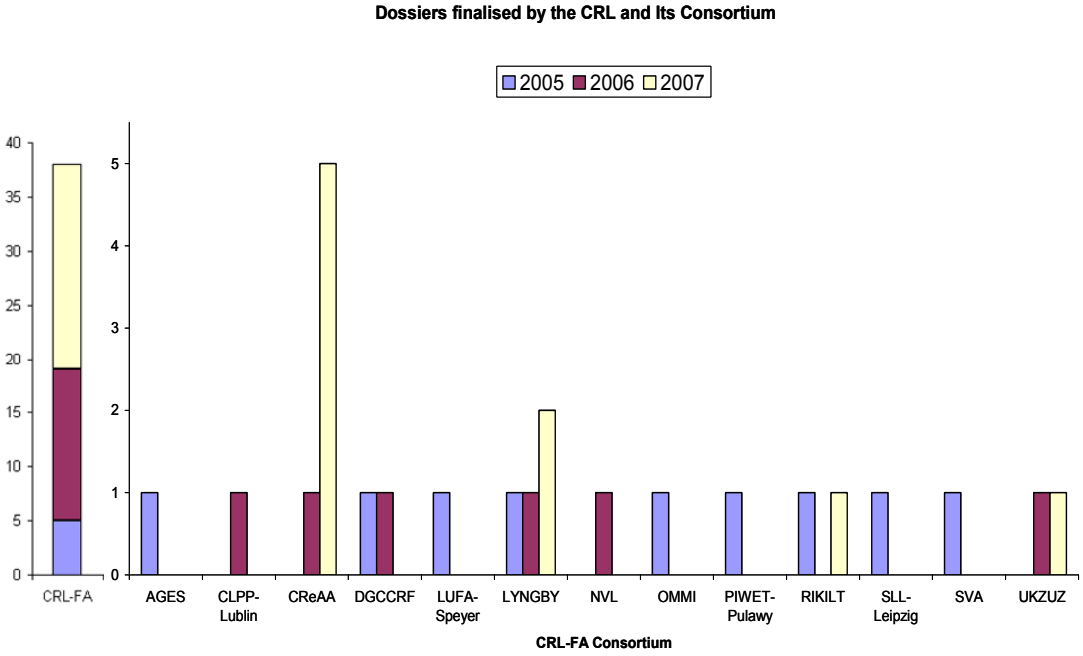
FAD number	Product/Additive Name	Active Substance	Rapporteur	Date of report
FAD-2005-0028	Quantum Phytase 5000L - 2500D	6-phytase	CRL-FA	02/02/2007
FAD-2006-0002	Toyocerin (sows)	Bacillus cereus var. toyoi	CRL-FA	28/02/2007
FAD-2006-0005	Ronozyme_Biofeed	6-phytase	CRL-FA	10/07/2007
FAD-2006-0009	L-Arginine technically pure	L-arginine	CRL-FA	31/01/2007
FAD-2006-0013	Availa Cr	Chromium-methionine chloride	CReAA	23/02/2007
FAD-2006-0014	Sorbiflore (piglets)	L. Rhamnosus, L. Farciminis	CRL-FA	15/05/2007
FAD-2006-0017	Bioplus 2B (sows)	B. subtilis, B. licheniformis	CRL-FA	16/05/2007
FAD-2006-0019	Natugrain Wheat TS and TS L	Endo-1,4-beta-xylanase	LYNGBY	21/03/2007
FAD-2006-0021	Panaferd-AX	Astaxanthin	CRL-FA	08/05/2007
FAD-2006-0023	CLINACOX 0.5%	Diclazuril	RIKILT	21/04/2007
FAD-2006-0024	Danisco Xylanase G and L	Endo-1,4 beta-xylanase	CRL-FA	27/07/2007
FAD-2006-0028	BiominC5 (chicken)	P. acidilactici, E. Faecium, B. animalis ssp. Animalis, L. reuteri, L. salivarius ssp. Salivarius	CRL-FA	25/04/2007
FAD-2006-0031	Lantharenol	Lanthanum carbonate	CReAA	06/08/2007
FAD-2006-0032	Carophyl® Stay-Pink	Astaxanthin dimethyldisuccinate	CRL-FA	16/08/2007
FAD-2006-0033	Calsporin (chicken)	Bacillus subtilis	CRL-FA	18/07/2007
FAD-2006-0037	Safizym_X (ducks)	Endo-1,4 beta-xylanase	CRL-FA	02/06/2007
FAD-2006-0038	Bonvital (sows)	E. faecium	CRL-FA	05/06/2007
FAD-2006-0039	Avizyme 1505	Endo-1,4-beta-xylanase, subtilisin, alpha-amylase	CRL-FA	11/10/2007
FAD-2007-0002	Natuphos® (ducks)	Endo-1,4-beta-xylanase	LYNGBY	31/08/2007
FAD-2007-0003	CreAminoTM	Guanideinoacetic acid	UKZUZ	29/08/2007
FAD-2007-0008	Coxidin 25%	Monensin sodium	CRL-FA	02/08/2007
FAD-2007-0009	Toyocerin (Turkeys)	Bacillus cereus var. toyoi	CRL-FA	15/09/2007
FAD-2007-0010	Mintrex-Zn	Zn chelate of hydroxy analogue of methionine	CReAA	03/12/2007
FAD-2007-0011	Mintrex-Mn	Mn chelate of hydroxy analogue of methionine	CReAA	03/12/2007
FAD-2007-0012	Mintrex-Cu	Cu chelate of hydroxy analogue of methionine	CReAA	03/12/2007
FAD-2007-0013	Biosaf Sc47 (pigs)	S. cerevisiae	CRL-FA	10/10/2007
FAD-2007-0017	Avizyme 1505 (turkeys)	Endo-1,4-beta-xylanase, subtilisin, alpha-amylase	CRL-FA	11/10/2007
FAD-2007-0018	Danisco Xylanase L & G	Endo-1,4 beta-xylanase	CRL-FA	11/10/2007

The dossier distribution - related to the different active substances - covers a variety of categories/functional groups, as presented hereafter:

Category	Functional group	
zootechnical	enzymes	9
zootechnical	micro-organisms	8
zootechnical	other	1
nutritional	trace elements	4
nutritional	amino acids	2
sensory	colourant	2
coccidiostats	coccidio	2
	Total:	**28**

As shown in the Figure bellow, nine dossiers were prepared in 2007 by the following four "Rapporteur" NRLs:

- Ústřední kontrolní a zkušební ústav zemědělský, Praha (CZ);
- Plantedirektoratets Laboratorium, Lyngby (DK);
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali, (IT); and
- RIKILT- Instituut voor Voedselveiligheid, Wageningen (NL)



Furthermore, all reports were thoroughly reviewed and commented by the above mentioned NRLs and/or the ones listed hereafter (depending on their field of expertise), thus demonstrating the enthusiastic and professional involvement of the CRL-FA network which is gratefully acknowledged. NRLs are sorted by "*country codes"*.

- Vlaamse Instelling voor Technologisch Onderzoek, Mol (BE)
- Põllumajandusuuringute Keskus, Jääkide ja saasteainete labor, Saku, Harjumaa (EE)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya,Cabrils (ES)
- Laboratoire de Rennes, Direction générale de la concurrence, de la consommation et de la répression des fraudes, Rennes (FR)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, Speyer (DE)
- Thüringer Landesanstalt für Landwirtschaft, Abteilung Untersuchungswesen. Jena (DE)
- Sächsische Landesanstalt für Landwirtschaft, Landwirtschaftliches Untersuchun-gswesen, Leipzig (DE)
- Instytut Zootechniki w Krakowie, Krajowe Laboratoriumz, Lublin (PL)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala (SE)
- Kmetijski inštitut Slovenije, Ljubljana (SI)
- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- The Laboratory of the Government Chemist, Teddington (UK)

Sample bank

Applicants seeking authorisation for feed additives have to provide the CRL-FA with samples of the product belonging to the application as outlined in Article 7 of Regulation (EC) No 1931/2003. In 2007 the CRL-FA received and registered 54 (x 3) reference samples.

Working instructions and procedures for the handling, storage and registration of samples were optimized to meet the requirements for accreditation (ISO 17025).

A sample browsable catalogue has been made available online (section "Network pages"). This tool enables the stakeholders of the project (NRL's, EFSA and DG SANCO) to keep track of the submitted samples.

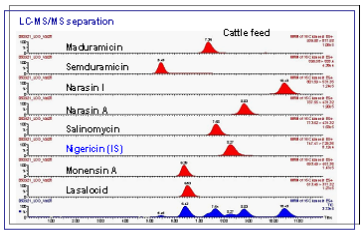
http://irmm.jrc.ec.europa.eu/html/CRLs/crl_feed_additives/index.htm

The IRMM's Informatics & Electronic Unit started to make a business analysis in cooperation with the CRL-FA concerning the handling of samples. An online interface scheduled for 2008 will allow applicants to monitor the validity of their samples and facilitate the submission of new samples.

Report activity from the Expert Groups

One of the major challenges of the evaluation procedure is the quite large variation of the analytical methodology involved. Unfortunately 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 (EG) were established according to the nature of the analytical methodology, namely (1) methods for coccidiostats, (2) methods for micro-organisms, (3) methods for trace elements, (4) methods for enzymatic activity and (5) chromatographical methods. 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 NRLs, the CRL-FA and in some case also external experts.

1. EG –Coccidiostats (G. Simone)



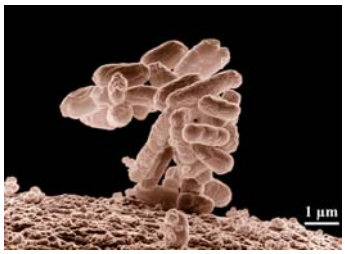
In June 2007 an enquiry within the group was launched in order to identify (1) which of the performance characteristics listed in Annex III of Regulation (EC) No 882/2004 are applicable to methods for coccidiostats in feed and food and for which criteria could be established; (2) which methods for residues of coccidiostats - in food of animal origin - are available; (3) which expert from the group member's lab could contribute to the work of the expert group.

In September 2007 a meeting between the CRL for Feed Additives and the CRL for Residues of Veterinary Drugs (Berlin) was held to identify overlapping fields of competence and to setup a co-operation in the field.

The expert group convened on 5th November 2007 in order to discuss and agree on a draft list of performance criteria, based on the results of the enquiry and also to identify which of the available methods could be used as basis to fix such criteria.

Based on the results of the discussion, a draft guidance document will be prepared and will be discussed during the next group meeting.

2. EG –Micro organisms (R. Leuschner)



The CRL-FA Expert WG dealing with microorganisms used as feed additives is chaired by Mr. Kwiatek (National Veterinary Research Institute (NVRI), Poland) and coordinated by Mrs. Leuschner (EC-JRC-IRMM, CRL-FA, Geel, Belgium). The group had meetings on 30 May and 15 November 2007 that were concerned with method performance criteria and a guidance document on method in-house validation.

The first meeting had the objective to discuss the application of performance criteria set out in Annex III Regulation (EC) 882/2004 for microbiological enumeration methods. In a prior Expert WG meeting on 12 October 2006 three criteria were identified and addressed: limit of quantification, repeatability and reproducibility. Furthermore, measurement uncertainty was included upon request of some experts.

Enumeration techniques for microorganisms are laid down in ISO 7218:2007. Microorganism feed additives are enumerated by pour plate or surface spread technique. It is generally considered necessary to count colonies on at least one dish containing at least 10 colonies for a valid result. The limits of quantification for the pour and spread plate techniques are 100 colony forming units (c.f.u.)/g and 1000 c.f.u./g, respectively.

EN ISO 4833 (2003) and EN ISO 7932 (2004) laid down limits for repeatability and

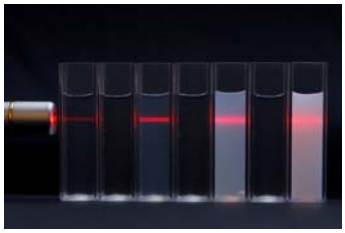
reproducibility derived from collaborative studies for the enumeration of microorganisms. Limits of repeatability and reproducibility are defined as the absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time (repeatability conditions) or in different laboratories with different operators using different equipment (reproducibility conditions). The calculations in both ISO methods were carried out on $\log_{10}$ transformed viable counts and these calculations and their interpretation were discussed in depth at the meeting and are laid down in the corresponding Minutes of the meeting. Concerning performance criteria for enumeration methods of probiotic feed additives collaborative published studies are referenced in Draft CEN Standards (prEN 15784-9) that are currently under approval by the European Committee for Standardisation (CEN) for adoption as Standard methods. These performance criteria would be considered applicable in the frame of CEN/ISO Standardisation.

MU is recommended for the interpretation of results whereby ISO/TS 19036 (2006) provides guidance on the estimation and expression of MU with regards to quantitative microbiological enumeration methods. The expression of MU in terms of $\log_{10}$ values and their conversion into percentages with regards to the final results were discussed in depth and are laid down in the corresponding Minutes of the meeting.

In the second meeting of the Expert WG, a draft guidance document for applicants regarding method in-house validation in relation to the new draft Commission Regulation (SANCO/426/2005Rev.2) on detailed rules for the implementation of Regulation (EC) No 1831/2003 was discussed in detail. A revised version was prepared following the meeting which. The guidance document on method in-house validation refers to relevant legislation, Community Rules and internationally accepted standards. The guidance document explains that modified methods, non-standard and laboratory-developed methods need to be validated. It provides guidance on preparation of a method protocol, design of in-house validation and the selection of method performance criteria. The guidance document takes account of the fact that in the new draft Commission Regulation, single-laboratory validated methods need to undergo a verification procedure applying a guidance that is currently in preparation by another working group. The Expert WG 'Microorganisms' recommended to select for the verification study repeatability and within-laboratory reproducibility criteria obtained from in-house validation of a method.

Precision: Repeatability & Within-laboratory Reproducibility

3. EG - Trace elements *(Ch. von Holst)*



The objective of the first meeting was to get an overview of the activities of CEN Technical Committee (TC) 327, focusing on the harmonisation of methods for the determination of elements in feedingstuffs. A draft standard for the determination of various elements including the feed additives "iron, zinc, copper, manganese, cobalt, molybdenum" was presented, which has been ring trial validated on various matrices including premixtures and feedingstuffs. The method has recently become a European standard (EN 15510). Another topic of the meeting was related to the determination of selenium in feedingstuffs, for which a European standard is not yet available. However, an appropriate method is currently validated by organising an inter-laboratory study. The last topic was on the question whether organically bound selenium could be analytically separated from inorganic selenium, applying instrumentation which is available in European official control laboratories. This aspect may be important in cases when selenium is added to feedingstuffs as selenised yeast containing high amounts of seleno-methionine. The evaluation of literature revealed that currently there is no method for such a separation available.

4. EG - Enzymatic activity *(Ch. von Holst, D. Garalevičienė)*

Methods for the determination of the activity of enzymes such as phytase or xylanase constitute a particular challenge, since the analytical results are expressed in terms of *activity units* that are defined by the applicants and are valid exclusively for specific products. Given the fact that the corresponding analytical protocols are adjusted to these unit definitions, harmonisation amongst the protocols for different products is impossible if the unit definitions of these products are not identical. Currently, only for the phytase activity an analytical method could be harmonised that is applicable to various European phytase products. This method is currently being reviewed to become a CEN standard. In consequence, most of the analytical methods for the determination of the enzyme activity that are submitted by applicants have been exclusively single-laboratory validated methods. Therefore, the working group discussed mainly two topics, namely (1) the setting of performance criteria to evaluate single-laboratory validated methods and (2) the feasibility of the development of harmonised methods in other fields of the enzyme activity such as xylanase.

Regarding method performance criteria, the precision data obtained in the ring trial validation of the phytase method could be utilised as benchmark for other analytical methods in this

field, but the applicability of this concept to *all* enzyme products still needs to be evaluated.

Many colorimetric methods for determination of enzymatic activity are single-laboratory validated methods, which can be considered suitable for official controls, based on acceptable performance characteristics. Since these methods do not measure a concentration but an enzyme activity, the Horwitz equation establishing fitness for purpose criteria for the precision is not applicable for this kind of measurements. Therefore the option was to use results from the ring trial validation of methods aimed at the determination of enzymes in feedingstuffs. However several weaknesses remain: - absence of commercially available standards and of matrix matched blank feed samples (used for relative analytical methods); - lack of published ring-trial studies on the methods for determination of enzymatic activity. Related problems of the implementation of a single-laboratory validated method for official controls, feasibility of the development of harmonised methods for enzymes other than phytase and the potential use of viscosimetric methods were also outlined.

CRL-FA Workshop 2007 - Executive summary

The 7th workshop of the CRL-FA was held at the Institute of Reference Materials and measurements, Geel (Belgium) on the 7-8 June 2007. 42 participants from 23 countries attended the event, including representatives from National Reference Laboratories, FEFANA and EFSA. The main topics discussed are briefly summarised hereafter.

- C. von Holst reviewed the 2006/2007 activities of the CRL-FA
- G. Simone presented the draft guidelines SANCO 426/2005 rev.3 for the "Assessment of Additives in Feedingstuffs" under revision, focussing specifically at Section 2 related to the analytical methods. Furthermore he outlined the new requirements related to the "verification/transferability" concept.
- A. Chesson presented the history and activities of the EFSA Feed Additives Panel (FEEDAP). The role and duties of EFSA were explained together with the FEEDAP mandate according to Regulation (EC) 1831/2003.
- C. von Holst, R. Leuschner and D. Garalevičienė presented the activities of the "Trace Element", "Microbiology" and "Enzymes" working groups, respectively.
- U. Vincent presented the in-house method validation of the LC-MS/MS method developed at the IRMM for the simultaneous determination of six coccidiostats in feed matrices.
- P. Robouch presented the proficiency test IMEP-101 (Cd, Pb and Hg in brown bred) organised by the Community Reference Laboratory for Heavy Metals (CRL-HM). The assessment of the performance was discussed.

The various presentations are compiled in the CRL-FA Workshop 2007 Proceedings published as the IRMM report GE/R/FSQ/03/2007 (available upon request).

The new "guidelines" are out

During 2007 the CRL-FA participated to the discussions on the new rules for the implementation of Regulation (EC) No 1831/2003 describing the requirements for the preparation of the dossiers for authorisation. The CRL-FA contributed to the draft Commission Regulation that has been adopted by the Standing Committee on the Food Chain and Animal Health in December 2007[1] that replaces Annex I of the Commission Directive 2001/79/EC. Chapter 2.6 of the new implementing rules details the requirements for the methods of analysis and reference samples that must be submitted by applicants.

The amount of the fee charged to the applicant by the CRL-FA has been revised by Regulation (EC) No 850/2007, which amended Regulation (EC) No 378/2005[2].

CEN standards

Three CEN standards related to the analysis of feeding stuffs have been published in 2007:

- **EN 15510:2007** – Animal feeding stuffs - Determination of calcium, sodium, phosphorus, magnesium, potassium, iron, zinc, copper, manganese, cobalt, molybdenum, arsenic, lead and cadmium by (ICP-AES).
- **EN 15550:2007** - Animal feeding stuffs - Determination of cadmium and lead by graphite furnace atomic absorption spectrometry (GF-AAS) after pressure digestion
- **CEN/TS 15621:2007** - Animal feeding stuffs - Determination of calcium, sodium, phosphorus, magnesium, potassium, sulphur, iron, zinc, copper, manganese, cobalt and molybdenum after pressure digestion by ICP-AES

Note: ICP-AES = Inductively coupled plasma atomic emission spectroscopy

[1] OJ L 133, 22.05.2008, p. 1, Commission Regulation (EC) No 429/2008 of 25 April 2008
[2] OJ L 188, 20.07.2007, p. 3, Commission Regulation (EC) No 850/2007 of 19 July 2007

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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. The List of NRLs is provided in Table 2.

The CRL-FA Authorisation group

Table 2: The list of NRLs of the CRL-FA network

	Acronomy	Institute
AT	AGES	- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien
BE	AFSCA-FAVV	- Federaal Voedingslabo Tervuren (AFSCA-FAVV), Tervuren
BE	VITO	- Vlaamse Instelling voor Technologisch Onderzoek (VITO), Mol
CY	FAL	- Feedingstuffs Analytical Laboratory, Department of Agriculture
CZ	UKZUZ	- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha
DE	LGL-Obers.	- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim
DE	LUFA-Speyer	- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer
DE	SLL-Leipzig	- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig
DE	TLL-Jena	- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena
DK	LYNGBY	- Plantedirektoratets Laboratorium, Lyngby
EE	PMK-JSL	- Põllumajandusuuringute Keskus (PMK), Jääkide ja saasteainete labor, Saku, Harjumaa
EE	PMK-TMAL	- Põllumajandusuuringute Keskus (PMK), Taimse materjali analüüsi labor, Saku, Harjumaa
ES	GENCAT	- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya,Cabrils
ES	MAPYA	- Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid
FI	KTTK	- Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors
FR	DGCCRF	- Laboratoire de Rennes, Direction générale de la concurrence, de la consommation et de la répression des fraudes (DGCCRF), Rennes
HU	OMNI	- Mezőgazdasági Szakigazgatási Hivatal (MgSzH) Élelmiszer- és Takarmánybiztonsági Igazgatóság, Központi Takarmányvizsgáló Laboratórium – Nemzeti Referencia Laboratórium, Budapest
IE	State Lab	- The State Laboratory, Kildare
IT	CReAA	- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino
IT	ISS	- Istituto Superiore di Sanita' - Dipartimento di Sanita' alimentare ed animale, Roma
LT	NVL	- Nacionalinė veterinarijos laboratorija, Vilnius
LT	VMVT	- Klaipėdos apskrities VMVT laboratorija, Klaipėda
LU	ASTA	- Laboratoire de Controle et d'essais - ASTA, Ettelbruck
LV	VVMDC	- Valsts veterinārmedicīnas diagnostikas centrs (VVMDC), Rīga
NL	RIKILT	- Instituut voor Voedselveiligheid, Wageningen
NL	RIVM	- Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven
NO	LabNett	- LabNett AS, Agricultural Chemistry Laboratory, Stjørdal
PL	CLPP-Lublin	- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin
PL	PIWET-Pulawy	- Państwowy Instytut Weterynaryjny, Puławy
PT	LNIV	- Laboratorio Nacional de Investigação Veterinária, Lisboa
SE	SVA	- Foderavdelningen, Statens Veterinärmedicinska Anstalt (SVA), Uppsala
SI	KIS	- Kmetijski inštitut Slovenije, Ljubljana
SI	VF-UNI-LJ	- Univerza v Ljubljani, Veterinarska fakulteta. Nacionalni veterinarski inštitut, Enota za patologijo prehrane in higieno okolja, Ljubljana
SK	UKSUP	- Skúšobné laboratórium – Oddelenie analýzy krmív, Ústredný kontrolný a skúšobný ústav poľnohospodársky, Bratislava
UK	LGC	- The Laboratory of the Government Chemist, Teddington

Annex
CRL-FA Evaluation Reports
Executive Summaries

FAD number	Product/Additive Name
FAD-2005-0028	Quantum Phytase 5000L - 2500D
FAD-2006-0002	Toyocerin (sows)
FAD-2006-0005	Ronozyme_Biofeed
FAD-2006-0009	L-Arginine technically pure
FAD-2006-0013	Availa Cr
FAD-2006-0014	Sorbiflore (piglets)
FAD-2006-0017	Bioplus 2B (sows)
FAD-2006-0019	Natugrain Wheat TS and TS L
FAD-2006-0021	Panaferd-AX
FAD-2006-0023	CLINACOX 0.5%
FAD-2006-0024	Danisco Xylanase G and L
FAD-2006-0028	BiominC5 (chicken)
FAD-2006-0031	Lantharenol
FAD-2006-0032	Carophyl® Stay-Pink
FAD-2006-0033	Calsporin (chicken)
FAD-2006-0037	Safizym_X (ducks)
FAD-2006-0038	Bonvital (sows)
FAD-2006-0039	Avizyme 1505
FAD-2007-0002	Natuphos® (ducks)
FAD-2007-0003	CreAminoTM
FAD-2007-0008	Coxidin 25%
FAD-2007-0009	Toyocerin (Turkeys)
FAD-2007-0010	Mintrex-Zn
FAD-2007-0011	Mintrex-Mn
FAD-2007-0012	Mintrex-Cu
FAD-2007-0013	Biosaf Sc47 (pigs)
FAD-2007-0017	Avizyme 1505 (turkeys)
FAD-2007-0018	Danisco Xylanase L & G

Full reports available on the CRL-FA website
http://irmm.jrc.ec.europa.eu/html/CRLs/crl_feed_additives/authorisation/evaluation_reports/index.htm

FAD-2005-0028	EFSA-Q-2006-025
Product Name *Active substance*	Quantum Phytase 5000L - 2500D 6-phytase
Rapporteur	Dalia Garalevičienė CRL-FA, European Commission

In the current application authorisation is sought for Quantum™ Phytase under the category 'zootechnical additives', functional groups 4(a) 'digestibility enhancers' and 4(c) 'substances, which favourably affect the environment', according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Quantum™ Phytase as a digestibility enhancer and a substance, which favourably affects the environment, for chickens, ducks and turkeys for fattening, laying hens and weaned piglets. The additive is intended to be marketed as a powder (Quantum™ Phytase 2500 D) and as liquid formulation (Quantum™ Phytase 5000 L).

The active agent of Quantum™ Phytase is 6-phytase, produced by a strain of *Pichia pastoris* (DSM 15927). The enzymatic activity is expressed in phytase units (FTU). One FTU is the amount of enzyme which liberates one □mole of inorganic phosphate per minute from sodium phytate at pH 5.5 and 37°C. Quantum™ Phytase 2500 D and Quantum™ Phytase 5000 L have a target activity of 2500 FTU/g and 5000 FTU/ml of product. Quantum™ Phytase 2500 D is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas Quantum Phytase™ 5000 L is sprayed directly onto feed. Both formulations are used to obtain an enzyme activity level of 100 to 2700 FTU/kg in *feedingstuffs*.

For the determination of the activity of 6-phytase in the *feed additive,* the applicant proposes a method which measures the enzyme-catalysed formation of inorganic phosphate released from sodium phytate. The phosphate forms with molybdate and vanadate ions a coloured complex, which is measured on a spectrophotometer and quantified via a phosphate standard curve. The measurements are carried out at pH 5.5 and 37°C and therefore the activity is expressed in terms of FTU. Method performance characteristics include a relative standard deviation for reproducibility (RSD_R) of 3.4 % calculated from the results of two laboratories and recovery rates ranging from 80 to 101 %.

For the determination of the activity of 6-phytase in *premixtures,* the applicant proposes the same method as for the *feed additive*, but using a modified extraction buffer. The measurements are carried out at pH 5.5 and 37°C and the activity is expressed in terms of FTU/g. Method performance characteristics include a RSD_R of 5.5 % calculated from the results of two laboratories and recovery rates ranging from 80 to 94 %.

For the quantification of the activity of 6-phytase in *feedingstuffs,* the applicant proposes a different in-house developed method, performing the activity measurements at *pH 4.5* and *60°C* - and not at FTU conditions - , thereby introducing a new activity Unit: Quantum™ Phytase unit (QPU). One QPU is the amount of enzyme liberating one μmole of inorganic phosphate from sodium phytate per minute measured at 60°C and at a pH of 4.5. In the final step of the analysis the measured QPUs are converted into FTUs by using an experimentally obtained conversion factor. However, the method protocol does not explicitly explain the determination of this conversion factor. The limit of detection of the method is 25 FTU/kg, the limit of quantification is 75 FTU/kg, the intermediate relative standard deviation for reproducibility (RSD_R) varies from 5.6 to 11.9 % and the recovery rate varies from 93 to 126 %. The obtained method performance characteristics are considered acceptable.

Since the CRL favours the use of inter-laboratory validated methods, the applicant applied, upon request of the CRL, a recently collaboratively validated method which has been developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) and which measures the enzyme activity at FTU conditions. This harmonised method is suitable for the determination of the activity of *various* phytase products in *feedingstuffs*. However, the experimental results revealed that the FTU values obtained with the FEFANA method were up to 15 % *lower* than the corresponding values of the applicant's method. Therefore the applicant proposed to use its in-house validated method for the determination of the Quantum™ Phytase activity in *feedingstuffs* and not the FEFANA method. The CRL agrees with this proposal but is concerned that the suggested approach of measuring the enzyme activity in *feedingstuffs* at QPU conditions (pH 4.5 and 60°C) followed by conversion from QPU units to FTU units, introduces additional uncertainty into the measurements. In addition the CRL considers that, for consistent analytical results, the enzyme activity in the *feed additive*, in *premixtures* and in *feedingstuffs* should be determined at the same conditions and expressed in the same units.

Therefore, the CRL recommends:

- to express the enzyme activity of 6-phytase, regardless of the matrix, in QPU;
- to *modify* the proposed register entry by expressing the target activity values in *feedingstuffs* as given in the "conditions of use" in terms of QPUs and not in terms of FTUs. In addition, the *definition* of the QPU needs to be included in the register entry instead of the definition of FTU;
- to employ the applicant's method in the frame of official controls for the determination of the enzyme activity of 6-phytase, in *feed additive, premixtures* and complete *feedingstuffs,* expressed in terms of QPUs, thereby excluding the employment of a conversion factor from QPU to FTU.

In the case that the "conditions of use" in the final register entry will be expressed in terms of FTUs and not – as proposed by the CRL – in terms of QPUs, the CRL recommends the FEFANA method to be used for official controls.

Further testing or validation is not considered necessary.

FAD-2006-0002	**EFSA-Q-2006-037**
Product Name	Toyocerin (sows)
Active substance	Bacillus cereus var. toyoi
Rapporteur	Renata Leuschner CRL-FA, European Commission

In the current application authorisation is sought for the microbial feed additive Toyocerin® 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 are viable spores of the microorganism Bacillus cereus var. *toyoi* NCIMB 40112/CNCM I-1012. The additive is an odourless, white greyish-brown dry powder containing a minimum concentration of 1 x 10^{10} colony forming units (c.f.u.) per gram additive. Specifically, authorisation is sought to use Toyocerin® for sows from service until weaning. The conditions of use are proposed with a recommended dosage of 0.5 to 2 x 10^{9} c.f.u./kg complete feedingstuffs.

For the quantification of the active agent (*Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012) of Toyocerin® in the *feed additive*, *premixtures* and *feedingstuffs* appropriate enumeration methods using a selective agar were proposed by the applicant. Analysis data confirmed appropriate method performance in a second laboratory. It is however recommended to use a larger amount of feed sample for analysis than the proposed 2 g, such as for example 50 g to take account of potential sample heterogeneity.

For official controls regarding the quantitative determination of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, another surface plate count enumeration method is recommended which has been fully ring-trial validated (J.AOAC Int. 2003, 86, 568-575). The method performance characteristics include a relative standard deviation for repeatability (RSD_r) of around 1 % and a relative standard deviation for reproducibility (RSD_R) of around 6 %. 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, *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012, was analysed by a range of techniques including biochemistry, phage-typing, molecular methods and pyrolysis mass spectrometry. Pulsed field gel electrophoresis (PFGE) is a generally recognised standard methodology for microbial identification and is considered suitable for official controls in the frame of the authorisation.

Further testing or validation is not considered necessary.

FAD-2006-0005	**EFSA-Q-2006-060**
Product Name	Ronozyme_Biofeed
Active substance	6-phytase
Rapporteur	Dalia Garalevičienė CRL-FA, European Commission

In the current application authorisation is sought for *Ronozyme® P/Bio-Feed® Phytase* under the category 'zootechnical additives' and the functional groups 4(a) and 4(c), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Ronozyme® P/Bio-Feed® Phytase* as a digestibility enhancer for ducks and as a substance, which favourably affects the environment.

The active agent of *Ronozyme® P/Bio-Feed® Phytase* is 6-phytase, produced by a strain of *Aspergillus oryzae* (DSM 14223). Enzymatic activity is expressed in FYT (phytase) units. One FYT unit is defined as the amount of enzyme that liberates one μmol of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37°C. The additive is intended to be marketed as a solid formulation (*Ronozyme® P5000 (CT)/Bio-Feed® Phytase CT 2X)* containing 5000 FYT/g and as liquid formulation (*Ronozyme® P20000 (L)/Bio-Feed® Phytase L 4X*) containing 20000 FYT/g. The products are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain a recommended enzyme activity level of 500 to 1000 FYT/kg in *feedingstuffs*.

For the determination of the activity of 6-phytase in *feed additive*, *premixtures* and *feedingstuffs,* the applicant proposes a colorimetric method, based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37°C by the enzyme phytase. Released phosphate forms with molybdate and vanadate ions a coloured complex that is measured on a spectrophotometer at 415 nm and quantified against the phosphate standard curve.

The applicant submitted method's validation data on *feed additive* and *premixtures*, obtained in a single laboratory. For the *feed additive*, method performance characteristics include a limit of quantification (LOQ) of 0.1 FYT/ml, a relative standard deviation for repeatability (RSD_r) of 0.5 to 1.4% and a relative within-laboratory standard deviation for reproducibility (RSD_R) of 1.4 to 2.6%. For the *premixtures*, an RSD_r of 1.2 to 5.1%, a within-laboratory RSD_R of 2.4 to 4.1 % and recovery rates of 95% and 99% were obtained.

This method has been developed on behalf of the European Association of Feed Additive Manufacturers (FEFANA) for the measurement of phytase activity in *feedingstuffs* and validated in an inter-laboratory study on various phytase products including *Ronozyme® P/Bio-Feed® Phytase*. The obtained values for the RSD_R, ranging from 5 to 14%, are considered acceptable and the method is therefore suitable for official control purposes. This method is currently under evaluation to become a standard of the European Committee for Standardisation (CEN).

Based on acceptable performance characteristics, the proposed methods are considered suitable for determination of phytase's activity in *feed additive*, *premixtures* and *feedingstuffs* for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

FAD-2006-0009	**EFSA-Q-2006-031**
Product Name *Active substance*	L-Arginine technically pure L-arginine
Rapporteur	Giueseppe Simone CRL-FA, European Commission

In the current application authorisation is sought for L-arginine 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-arginine for supplementing feed for all animal species. The product is a crystalline powder with a minimum content of 80 % L-arginine. The feed additive is intended to be included into feedingstuffs at a final concentration up to 5-6 % of total L-arginine, depending on the concentration of L-arginine already present in the feed components.

For the determination of the active substance (L-arginine) in the *feed additive* the applicant proposes a titrimetric method using perchloric acid. Since the feed additive contains L-arginine in its base form, the CRL considers the more appropriate titrimetric method using hydrochloric acid as prescribed by the Ph.Eur.. The applicant also provides a High Performance Liquid Chromatography (HPLC) method which is specific for the analyte, but without specifying related validation data. For official control purposes, the CRL recommends validated methods based on the same technique, such as the method 4.11.6 of the Association of German Agricultural Analytical and Research Institutes (VDLUFA) [Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt] and the similar AOAC Method 999.13 [Fontaine and Eudaimon, J. of AOAC Int., Vol. 83, No. 4, 2000]. These methods have been validated for the quantitative determination of three free (non protein bound) amino acids (lysine, methionine and threonine) in feed grade amino acid commercial products and premixtures with more than 10 % individual amino acid content, and can be applied also for the determination of L-arginine.

The applicant does not describe whether the additive is intended to be directly incorporated into feedingstuffs or through *premixtures*. However, for the determination of L-arginine in *premixtures* for official controls, the same above mentioned Official or validated methods are recommended by the CRL.

For the determination of the active substance (L-arginine) in *feedingstuffs* the applicant proposes 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 analyser or 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 performed on different premixtures and feeds [Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994]. Performance characteristics for the target analyte (L-arginine) include the relative repeatability standard deviation (RSD_r) ranging from 2.34 to 3.31 % and relative reproducibility standard deviation (RSD_R) ranging from 7.18 to 9.66 %, 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 by the CRL is not considered necessary.

FAD-2006-0013	**EFSA-Q-2006-066**
Product Name	Availa Cr
Active substance	Chromium-methionine chloride
Rapporteur	Maria Cesarina Abete C.Re.A.A, Torino, Italy

Availa®Cr is a feed additive for which authorisation is sought under the category "*Nutritional additives*" functional group 3(b) "compounds of trace elements", according to the classification system of Annex I of Regulation (EC) No 1831/2003. *Availa®Cr* contains 3% trivalent chromium (Cr) in the complexed form chromium-methionine chloride, which is the active substance. The concentration of the active substance in the feed additive is 31 %.

In the current application authorisation is sought for the use of *Availa®Cr* for all animal species. The feed additive is intended to be added to the compound feed through premixtures. According to the proposed register entry, the dosage of the feed additive is defined in terms of the concentration of *Availa®Cr* in feedingstuffs, ranging from 13.3 to 52.8 mg/kg depending on the target animal. These limits correspond to a concentration of the active substance (chromium-methionine chloride) in feedingstuffs varying from 4.1 to 16.4 mg/kg. The *theoretical* concentration of chromium introduced via the addition of *Availa®Cr* to feedingstuffs ranges from 0.4 to 1.6 mg/kg. However, the *actual* chromium concentration in feedingstuffs is most likely very different from these values due to the contributions from other chromium sources to the overall chromium concentration.

For the determination of the complexed chromium (i.e. chromium-methionine chloride) in the *feed additive* the applicant proposed a method in which *Availa®Cr* is dissolved in water and the complexed chromium is directly measured from the solution. Prior to the measurement free Cr^{3+} is precipitated and thereby separated from

complexed Cr^{3+} by using sodium carbonate as a Cr^{3+} specific precipitating agent. Complexed chromium is assayed in the supernatant filtrate using Inductively Coupled Plasma (ICP), measuring the chromium emission at 357.869 nm. The chromium content is calculated from the standard curve. This method is considered suitable for the intended purposes.

The quantitative analysis of the active substance in *premixtures* and *feed* requires an analytical technique that can distinguish between chromium introduced into the feed via the additive and chromium from other sources already present in the premixture or feedingstuffs sample. Theoretically, the same method employed for the determination of chromium-methionine chloride in the additive could be considered to detect the active substance also in feed. However, according to the applicant's opinion the determination of the active substance in feedingstuffs is not possible, due to the high and rather varying concentration of non specific chromium in this matrix, which could be up to a factor 1000 higher than the chromium content originating from the added feed additive. Therefore the applicant did not propose a method suitable for the detection of the active substance in premixtures and feedingstuffs.

Based on the submitted documentation, the CRL is therefore unable to recommend an analytical method suitable for the determination of the active substance in *premixtures* and *feedingstuffs* for official control purposes.

For official controls regarding the determination of *total* chromium in *feedingstuffs* the CRL recommends in-house validated methods such as "Determination of chromium in feeds by automated microwave digestion and atomic absorption spectrometry" [Gallo et all., J. AOAC Int. 1997 80 (5): 956-960] or draft methods from the European Committee for Standardisation (CEN).

According to the applicant, chromium residues in animal tissues that are related to feeding the animal with *Availa®Cr* are not anymore linked to methionine. In consequence, chromium measurements in animal tissues do not allow for distinguishing between Cr derived from *Availa®Cr* and Cr naturally present in animal tissues. In addition, no maximum residue limit (MRL) has been proposed by the applicant. For these reasons, the applicant did not submit a method suitable for the analysis of animal tissues.

For quantitative analysis to determine *total* Cr in *animal tissues* the standard method EN 14083 could be applied. This method was developed to detect total Chromium in food by graphite furnace absorption spectrometry (GFAAS) after pressure digestion. According to this European standard the achievable limit of quantification for chromium in foodstuffs is in the range of about 0.04 to 0.16 mg/kg, mainly depending on the specific experimental conditions of the measurements.

FAD-2006-0014	**EFSA-Q-2006-062**
Product Name	Sorbiflore (piglets)
Active substance	L. Rhamnosus, L. Farciminis
Rapporteur	Renata Leuschner CRL-FA, European Commission

In the current application authorisation is sought for the microbial feed additive Sorbiflore® 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 are viable cells of two microorganism strains, *Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R. The additive is a light brown free-flowing powder containing equivalent numbers of both strains at concentrations of 0.5 x 10^8 colony forming units (c.f.u.) per gram additive. Specifically, authorisation is sought to use Sorbiflore® for piglets from weaning up to two months. The conditions of use are proposed with a recommended dosage of 1 to 5 x 10^8 c.f.u./kg complete feedingstuffs.

For the quantification of the active agents (*Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R) of Sorbiflore® in the *feed additive*, *premixtures* and *feedingstuffs* the applicant uses a microbiological plate count enumeration method and epifuorescence microscopy. The methods are appropriate for the purpose.

For official controls in the frame of the authorisation concerning the quantitative determination of the colony forming units of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, a spread plate enumeration method is recommended which has been fully ring-trial validated (Food Microbiol. 2003, 20, 57-66). The method performance characteristics include a relative standard deviation for repeatability (RSD_r) of around 1 to 3 % and a relative standard deviation for between-laboratory reproducibility (RSD_R) of around 2 to 5 %. 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 strains, *Lactobacillus rhamnosus* MA27/6B and *Lactobacillus farciminis* MA27/6R, was analysed by pulsed-field gel electrophoresis (PFGE) which showed a sufficient degree of differentiation. 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.

FAD-2006-0017	**EFSA-Q-2006-136**
Product Name	Bioplus 2B (sows)
Active substance	B. subtilis, B. licheniformis
Rapporteur	Renata Leuschner CRL-FA, European Commission

In the current application authorisation is sought for the microbial feed additive BioPlus® 2B under the category 'zootechnical additives', functional group 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive are viable cells of two microorganism strains, *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749. The additive is a free-flowing powder product with a yellowish colour containing equivalent numbers of both strains and a minimum total concentration of 3.2 x 10^9 colony forming units (c.f.u.) per gram additive. Specifically, authorisation is sought to use BioPlus® 2B for sows. The conditions of use are proposed with a recommended total dosage of 1.28 x 10^9 c.f.u./kg complete feedingstuffs including both strains.

For the quantification of the active agents (*Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749) of BioPlus® 2B in the *feed additive*, *premixtures* and *feedingstuffs* the applicant applies plate count enumeration methods using tryptose blood agar. The methods are appropriate for the intended purpose.

For the quantitative determination of the colony forming units of the active agents for official controls in the *feed additive*, *premixtures* and *feedingstuffs*, a spread plate enumeration method is recommended which has been ring-trial validated using premixtures and feedingstuff samples (J. AOAC Int. 2003. 86, 568-575). The method performance characteristics include a relative standard deviation for repeatability (RSD_r) of around 1 % and a relative standard deviation for between-laboratory reproducibility (RSD_R) of around 6 %. 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 strains, *Bacillus subtilis* DSM 5750 and *Bacillus licheniformis* DSM 5749, was analysed by pulsed-field gel electrophoresis (PFGE) which showed a sufficient degree of differentiation. 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.

FAD-2006-0019	**EFSA-Q-2006-119**
Product Name	Natugrain Wheat TS and TS L
Active substance	Endo-1,4-beta-xylanase
Rapporteur	Annette Ploeger Plantedirektoratet, Lyngby, Denmark

In the current application authorisation is sought for *Natugrain Wheat® TS* and *TS L*, under the category 'zootechnical additives' and the functional group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, *cf*. EFSA-Q-2006-119, authorisation is sought to use *Natugrain Wheat® TS* and *TS L* as a digestibility enhancer for turkeys for fattening.

The active agent of *Natugrain Wheat® TS* and *TS L* is a thermostable endo-1,4-β-xylanase, produced by a strain of *Aspergillus niger (*CBS 109.713*).* Enzymatic activity is expressed in thermostable xylanase units (TXU). One TXU is defined as the amount of enzyme that liberates 5 µmol of reducing sugars (xylose equivalents) from wheat arabinoxylan per minute at pH 3.5 and 55° C. The additive is intended to be marketed as a powder (*Natugrain Wheat® TS*) and as liquid formulation (*Natugrain Wheat® TS L*). *Natugrain Wheat® TS* and *Natugrain Wheat® TS L* contain 5600 TXU/g of product and are intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of 400 to 800 TXU/kg in *feedingstuffs*.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes an *in-house* developed and validated viscosimetric method based on the fact that endo-1,4-β-xylanase catalyses the hydrolysis of glycosidic bonds in the substrate wheat arabinoxylan. The decrease in sample viscosity, expressed in terms of a drop time, is a measure for the endo-1,4-β-xylanase's activity and is determined using a falling ball viscosimeter at pH 3.5 and 55°C. The quantification is performed via an endo-1,4-β-xylanase's standard curve based on reference enzyme obtainable from the applicant. Method performance characteristics, when measured on various matrices (*feed additive, premixtures* and *feedingstuffs*), include relative standard deviation for repeatability (RSD_r) of 2.4 to 5.7%, within-laboratory relative standard

deviation for reproducibility (RSD_R) of 2.4 to 11.1% and recovery rates ranging from 85 to 115%. The limit of detection (LOD) is 11 TXU/kg and the limit of quantification (LOQ) is 36 TXU/kg *feedingstuffs*. Based on acceptable performance characteristics, the method is considered to be suitable for official control purposes in the frame of authorisation.

Further testing or validation is not considered necessary.

FAD-2006-0021	**EFSA-Q-2006-173**
Product Name	Panaferd-AX
Active substance	Astaxanthin
Rapporteur	María José González de la Huebra CRL-FA, European Commission

Panaferd-AX® is a feed additive for which authorisation is sought under the category "sensory additives", functional group "colorants: substances which, when fed to animals, add colours to food of animal origin", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Panaferd-AX® contains astaxanthin as active substance.

Panaferd-AX® is composed of sterilised dried cells of astaxanthin-rich *Paracoccus carotinifaciens* containing at least 20 g/kg of astaxanthin and is intended to be added to salmonid fish feed at a rate providing up to 85 mg/kg as astaxanthin in the final feedingstuff. Panaferd-AX® will be added directly to the final feedingstuffs and not prepared in pre-mixtures.

An HPLC (high performance liquid chromatography) method with spectrophotometric detection is proposed for the quantification of the active substance (astaxanthin) in the *feed additive*, *feedingstuffs* and *fish tissues*. The validation of the proposed method has been performed according to the requirements laid down by Commission Directive 2001/79/EC in fish pellet feed and fish tissues. For the determination of astaxanthin in *feedingstuffs* the following performance characteristics were obtained. The percentage of the recovery rate was estimated through blank feed samples fortified with the feed additive at different concentrations and ranged between 80 and 93 %. The obtained precision values, expressed as relative standard deviation were below 3.8 %. The limit of detection (LOD) and limit of quantification (LOQ) were 0.124 and 0.412 mg/kg, respectively. These performance characteristics are considered acceptable and the method is therefore considered suitable for official control purposes in *feedingstuffs*. Performance characteristics have also been provided for the method for the determination of the target analyte in *fish tissue*. However, since there are no Maximum Residue Limits (MRLs) for astaxanthin, the CRL cannot evaluate the suitability of the proposed method for official control of astaxathin in *fish tissue*.

Different control methods are proposed for the identification and quantification of impurities. Most of the proposed methods are classical methods that are often part of the relevant legislation, therefore the proposed methodologies can be considered suitable for the intended purposes.

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

FAD-2006-0023	EFSA-Q-2006-134
Product Name	Clinacox 0.5%
Active substance	Diclazuril
Rapporteur	Wim M.J. Beek; Jacob de Jong RIKILT – Institute of Food Safety, The Netherlands

Clinacox® 0.5% is a feed additive authorised as coccidiostat according to Directive 70/527/EEC. Clinacox® 0.5% contains 0.5% w/w diclazuril as active substance. The proposed inclusion rate of the active substance in feedingstuff for chickens and turkeys for fattening is 1 mg/kg, corresponding to 200 mg Clinacox® 0.5% per kg feed. The recommended withdrawal period is 5 days.

In the current application a modification of the authorisation is sought for establishing Maximum Residue Levels (MRLs) of diclazuril in tissues of all poultry species after application of Clinacox® 0.5%. The proposed MRLs are: 3000 µg/kg in liver, 2000 µg/kg in kidney, 1000 µg/kg in skin with fat and 500 µg/kg in muscle.

Diclazuril is the established marker residue, after application of Clinacox® 0.5%.

For determination of the marker residue diclazuril in *animal tissues* several methods have been proposed by the applicant. The procedures are based on high performance liquid chromatography (HPLC), gas chromatography (GC) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In our opinion the LC-MS/MS procedure is satisfactory validated for chicken (broiler) tissues, but in principle also applicable to turkey tissues. The obtained validation results carried out at 100 µg/kg of diclazuril in tissue show a relative within-laboratory standard deviation for reproducibility ranging from 3.7 % to 8.3 % depending on the specific tissue (muscle, liver, kidney, fat/skin). The limit of quantification (LOQ) is 5 µg/kg and therefore well below the target MRLs. According to the validation results the LC-MS/MS method can be considered suitable as a quantitative method. The suitability as a confirmatory method for official control is not fully established, since the applied mass spectrometry parameters do not fulfil the identification criteria as established by Commission Decision 2002/657/EC. However, most probably the LC-MS/MS method can fulfil the required confirmation criteria for diclazuril in the frame of official control when using the confirmatory mass spectrometry conditions proposed by another but similar LC-MS/MS method (Mortier, L et al. (2005) Analytica Chimica Acta 529, 229-234), although this method was only described for poultry meat and not for liver, kidney and fat/skin.

Besides, in order to establish the suitability for official control, it is strongly recommended that the method will be checked by a second independent laboratory.

FAD-2006-0024	EFSA-Q-2006-137
Product Name	Danisco Xylanase G and L
Active substance	Endo-1,4 beta-xylanase
Rapporteur	Dalia Garalevičienė CRL-FA, European Commission

In the current application authorisation is sought for *Danisco Xylanase* 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 *Danisco Xylanase* as a digestibility enhancer for chickens for fattening, ducks for fattening and laying hens. The product is intended to be marketed as a powder (*Danisco Xylanase G*) and as liquid formulation (*Danisco Xylanase L*).

The active agent of *Danisco Xylanase* is endo-1,4-β-xylanase produced by a strain of *Trichoderma reesei* (ATCC PTA 5588). The enzymatic activity is expressed in units (U). One U is the amount of endo-1,4-β-xylanase that liberates 0.5 μmol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50°C. The product has a target activity of 40000 U/g. *Danisco Xylanase G* is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas *Danisco Xylanase L* is sprayed directly onto feed to obtain an enzyme activity level of 250 to 2500 U/kg in *feedingstuffs*.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a colorimetric method based on the quantification of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on commercially available cross-linked xylan substrates. Enzymatic activity of the sample is calculated using a reference enzyme standard. The applicant introduced some adaptations to the protocol. The modified methods have been single laboratory validated.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive,* the applicant proposes a method which measures the enzyme-catalysed formation of water soluble dyed fragments released from cross-linked wheat arabinoxylan. The rate of release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard, available from the applicant upon request. The analysis – however – is carried out at *different* conditions (pH 4.0 and 40°C on a cross-linked wheat arabinoxylan) compared to those given in the proposed register entry (pH 5.3 and 50°C on a cross-linked oat spelt xylan) and the enzymatic activity is calibrated against a reference enzyme of which the activity is obtained applying the conditions of the proposed register entry. Method performance characteristics include a limit of detection (LOD) of 1.2 U/g, limit of quantification (LOQ) of 1.5 U/g products and a relative standard deviation for repeatability (RSD_r) of 4.4%.

For the determination of the activity of endo-1,4-β-xylanase in *premixtures,* the applicant proposes a method based on the same principle as described above, but employing a different extraction procedure. The measurements are carried out at pH 5.3 and 40°C on a cross-linked wheat arabinoxylan. Method performance characteristics include a LOD of 13.0 U/g, LOQ of 19.3 U/g, an RSD_r of 3.5 % and recovery rates of 96.4 %.

For the quantification of the activity of endo-1,4-β-xylanase in *feedingstuffs,* the applicant proposes a method, based on the same principle as described above, measuring enzymatic activity on a cross-linked wheat arabinoxylan at pH 4.2 and 50°C. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzyme, available from the applicant. Method

performance characteristics include a LOD of 285 U/kg, a LOQ of 530 U/kg, a RSD_r of 7.5% and a recovery rate of 97%. In the case that identical blank feed samples are *not* available, a standard addition technique is employed.

Though the methods proposed by the applicant are based on well known principles and show acceptable performance characteristics, the CRL is concerned that the suggested approach of measuring the enzymatic activity at *different* conditions compared to the conditions of the proposed register entry and to the conditions of the determination of the activity of a reference enzyme, introduces additional uncertainty into the measurements. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions specified in the register entry;
- and that the minimum activity specified in the register entry (250 U/kg) is replaced by the limit of quantification of the method, which is 500 U/kg.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as proposed in the Register entry, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

FAD-2006-0028	**EFSA-Q-2006-169**
Product Name	BiominC5 (chicken)
Active substance	P. acidilactici, E. Faecium, B. animalis ssp. Animalis, L. reuteri, L. salivarius ssp. Salivarius
Rapporteur	Renata Leuschner CRL-FA, European Commission

In the current application authorisation is sought for the microbial feed additive Biomin® C5 under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agents in the additive are viable cells of five microorganism strains, *Pediococcus acidilactici* DSM 16210, *Enterococcus faecium* DSM 16211, *Bifidobacterium animalis* ssp. *animalis* DSM 16284, *Lactobacillus reuteri* DSM 16350, *Lactobacillus salivarius* ssp. *salivarius* DSM 16351. The additive is a whitish powder containing 5×10^{10} colony forming units (cfu)/g microorganisms, whereby each strain is represented in a different concentration: *P. acidilactici* 1.3×10^{10} cfu/g, *E. faecium* 3.0×10^{10} cfu/g, *B. animalis* 5.0×10^{9} cfu/g, *L. reuteri* 1.0×10^{9} cfu/g, *L. salivarius* 1.0×10^{9} cfu/g. Specifically, authorisation is sought to add Biomin® C5 to drinking water for chickens for fattening until the slaughter age of about 42 days. The conditions of use are proposed with a recommended dosage of the additive resulting in 1.0×10^{9} to 1.0×10^{10} c.f.u. per liter drinking water.

For the quantification of the total viable counts in the *feed additive* Biomin® C5 and in its 'drinking water suspension', the applicant provides a validated pour plate method which is appropriate for the purpose. Validated plate count methods are further provided for an enumeration of each strain prior to addition to the final product which are appropriate.

For the quantification of the c.f.u. of each strain in the feed additive and in its 'drinking water suspensions' for official controls in the frame of the authorisation ring-trial validated methods are recommended, whereby it may be difficult to differentiate c.f.u. of the two *Lactobacillus* strains. (J. AOAC Int. 2003. 86, 4, 791-801, J. Appl. Microbiol. 2002, 93, 781-786, Food Microbiol. 2003, 20, 57-66, Int. J. Microbiol. 2003, 83, 161-170). The performance characteristics of the published methods were relative standard deviations for repeatability (RSD_r) between 0.5 to 6 % and relative standard deviations for between-laboratory reproducibility (RSD_R) of 1 to 9 %. The limit of quantification (LOQ) for the method is around 2 to 3 x 10^6 c.f.u./kg sample or liter suspension which is well below the minimum anticipated target level of application.

The identity of the bacterial strains was demonstrated by a range of morphological, physiological and genotypic methods. Pulsed-field gel electrophoresis is a generally recognised standard methodology for microbial identification and is considered as a suitable technique 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.

FAD-2006-0031	**EFSA-Q-2006-317**
Product Name	Lantharenol
Active substance	Lanthanum carbonate
Rapporteur	Maria Cesarina Abete C.Re.A.A, Torino, Italy

Lantharenol® is a product for which authorisation is sought under the category "zootechnical additives", functional group "others zootechnical additives", according to the classification system of Annex I, of Regulation (EC) No 1831/2003. According to the applicant, *Lantharenol®* contains at least 85% lanthanum carbonate octahydrate as active substance.

In the current application authorisation is sought for use of *Lantharenol®* for cat feed. *Lantharenol®* is intended to be added to complete feed in concentration of 1500 to 7500 mg/kg expressed as concentration of lanthanum carbonate octahydrate and is not intended to be used in premixtures.

For the determination of the active substance in the *additive* the applicant proposed an inductively coupled plasma optical emission spectrometry (ICP-OES) method. The carbonate content of *Lantharenol®* was determined by electrochemical volumetric analysis. Both methods have been in-house validated. The performance characteristics are considered acceptable, therefore the methods are considered suitable for official control.

For *feed* analysis the applicant proposed the same ICP-OES method used to quantify lanthanum in the additive, but with a different extraction procedure. The method has been in house validated and a recovery study has been carried out on wet and dry cat feed, obtaining percentage recovery rate between 90.7% and 103%. The

concentration of lanthanum carbonate octahydrate is calculated on the basis of the measured concentration of lanthanum. This is possible, since the applicant performed analyses to evaluate the background content of lanthanum in regular cat feed showing that the concentration of lanthanum in blank feed samples was less than 1/1000 of lanthanum present in the lowest recommended dose of lanthanum carbonate octahydrate. The results show that the method is robust and suitable for official control of lanthanum in cat feed.

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

FAD-2006-0032	**EFSA-Q-2007-018**
Product Name	Carophyl® Stay-Pink
Active substance	Astaxanthin dimethyldisuccinate
Rapporteur	Christoph von Holst CRL-FA, European Commission

Astaxanthin dimethyldisuccinate (AXN-DMDS) is a product for which authorisation is sought as feed additive under the category "sensory additives", functional group "colorants: substances which, when fed to animals, adds colours to food of animal origin", according to the classification system of Annex I of Regulation (EC) No 1831/2003. The active substance is all-trans astaxanthin dimethyldisuccinate which is a specific geometrical isomer of AXN-DMDS. The applicant intends to place the product on the market in the specific beadlets formulation Carophyll® Stay Pink.

The product contains in addition to all-trans AXN-DMDS about 1.5 % of two cis isomers of AXN-DMDS and less than 4 % of carotenoids other than AXN-DMDS. It is intended to add the feed additive to fish feed for salmon and trout at a recommended rate ranging from 55 to 97 mg/kg of the active substance in complete feedingstuff. The recommended maximum concentration of the active substance is 138 mg/kg of complete feedingstuff. The concentration of the active substance in all matrices is measured as the sum of the concentration of all-trans and two cis isomers of AXN-DMDS.

The applicant proposes analytical methods for the determination of the active substance that are specifically designed for this particular product formulation (beadlets). The analytical procedures for the analysis of AXN-DMDS in the formulated product, premixtures and feedingstuffs are similar and are comprised of the following steps: (1) The enzymatic release of AXN-DMDS from the beadlets formulation, (2) the extraction of AXN-DMDS with dichloromethane and ethanol, (3) the purification of the extract with solid phase extraction columns when analysing feedingstuffs and (4) the determination of AXN-DMDS by normal phase high performance liquid chromatography (HPLC) coupled to ultraviolet detection (UV detection) measuring at about 470 nm. The method allows for the simultaneous determination of the all-trans and cis isomers of AXN-DMDS and the other carotenoids present in the matrix, since these substances are well separated in the HPLC chromatogram.

Method performance characteristics were determined on *feedingstuffs* containing AXN-DMDS of about 50 mg/kg, obtaining a percentage recovery rate of 98 % and a relative within laboratory reproducibility standard deviation of about 2%. The limit of quantification was 0.2 mg/kg. Performance characteristics for other carotenoids have not been provided. Based on the results from the validation study the method is considered

suitable for official control purposes to determine the active substance in *feedingstuffs* within the frame of this application.

The applicant proposed a Maximum Residue Limit (MRL) for astaxanthin in the target *fish tissue* of 25 mg/kg, for which an analytical method has been proposed. The astaxanthin content is expressed in terms of the concentration of the sum of the measured geometrical isomers of this compound. The method is comprised of two steps, which are the extraction of the sample with acetone and the determination of the target analytes with HPLC coupled to UV detection. The parameters of the HPLC detection of the target analytes in all matrices (i.e fish tissue, formulations, premixtures and feedingstuffs) are identical.

Method performance characteristics were determined on *fish flesh* fortified with astaxanthin. The percentage recovery rate was 98 % obtained on samples containing 10 mg/kg of the target analyte and the relative within laboratory reproducibility standard deviation was about 3 %, obtained on samples containing about 6.5 mg/kg of the target analyte. The limit of quantification was 0.2 mg/kg. Based on the results from the validation study the method is considered suitable for official control purposes.

The applicant proposed limits of various impurities in the feed additive including some heavy metals for which appropriate methods are available.

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

FAD-2006-0033	**EFSA-Q-2007-040**
Product Name	Calsporin (chicken)
Active substance	Bacillus subtilis
Rapporteur	Renata Leuschner CRL-FA, European Commission

In the current application authorisation is sought for the microbial feed additive Calsporin® under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. Specifically, the use of Calsporin® as a gut flora stabiliser for chickens for fattening (broilers) is requested. Calsporin® consists of a minimum of 1 x 10^{10} of viable spores (c.f.u., colony-forming units) of *Bacillus subtilis* C-3102 (as active agent) per gram and calcium carbonate as carrier. The feed additive is intended to be mixed into complete feedingstuffs at a final concentration of 5 x 10^{8} to 1 x 10^{9} c.f.u./kg.

For the determination of the active agent in the *feed additive, premixtures and feedingstuffs*, a surface plate count method is proposed by the applicant to enumerate viable spores of *Bacillus subtilis* C-3102. Vegetative cells are inactivated by a heat treatment and not taken into account. The method is quantitative using trypticase soy agar (TSA) as medium. The method's performance characteristics for enumerating the active agent in the *feed additive, premixtures and feedingstuffs* revealed acceptable method's performance characteristics. However, for official controls a fully ring-trial validated, peer reviewed and published spread plate method for enumeration of bacilli spores including those of *B. subtilis,* is recommended [J. AOAC Int. 2003. 86, 568-575]. In the method a heat treatment of the initial sample suspension and tryptone soya agar is used. Methods performance characteristics for samples of premixtures and feedingstuffs were determined after logarithmic transformation of the measured colony forming units. A standard deviation for repeatability (s_r) of 0.09 $\log_{10}$ and a standard deviation for between-laboratory reproducibility (s_R) of 0.32 $\log_{10}$ for premixtures were concluded. For

feedingstuffs a s_r of 0.07 log_{10} and a s_R of 0.35 log_{10} were found. The limit of quantification (LOQ) for the method for feedingstuffs is around 1 x 10^7 c.f.u./kg sample which is well below the minimum anticipated target level of application.

For identification of the active agent molecular methods suitable for the purpose of analysis were used by the applicant. For official controls pulsed-field gel electrophoresis (PFGE) is recommended.

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

FAD-2006-0037	**EFSA-Q-2006-320**
Product Name	Safizym_X (ducks)
Active substance	Endo-1,4 beta-xylanase
Rapporteur	Dalia Garalevičienė CRL-FA, European Commission

In the current application authorisation is sought for *Safizym X* under the category 'zootechnical additives' and the functional group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, *cf*. EFSA-Q-2006-320, authorisation is sought to use *Safizym X* as a digestibility enhancer for ducks.

The active agent of *Safizym X* is endo-1,4-β-xylanase, produced by a strain of *Trichoderma longibranchiatum* CNCM MA 6-10W. Enzymatic activity is expressed in IFP (Institut Français du Pétrole) units. One IFP unit is defined as the amount of enzyme that liberates one □mol of reducing sugars (xylose equivalents) from oat xylan per minute at pH 4.8 and 50°C. The additive is intended to be marketed in two forms, namely as a powder (*Safizym XP20*) containing 70000 IFP/g and as liquid formulation (*Safizym XL200*) containing 7000 IFP/ml of the product. *Safizym X* is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of minimum 700 IFP/kg in *feedingstuffs.* The recommended enzyme activity in feed is 2800 IFP/kg. *Safizym X* also contains a residual activity of endo-1,3-(4)-β̃-glucanase.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive,* the applicant proposes an *absolute* colorimetric method based on the fact that endo-1,4-β-xylanase releases xylose from the substrate oat spelt xylan. Released sugar reduces the added 3,5-dinitro-salicylic acid to a coloured compound that is measured spectrophotometrically and quantified against the xylose standard curve. Transferability of the method on *Safizym XP20* has been checked among three laboratories [*Anim. Feed Sci. Techn*. 1999, 77:345-353] obtaining similar results.

For the determination of the endo-1,4-β-xylanase activity in *premixtures,* the applicant proposes a *relative* colorimetric method, based on the principle that xylanase releases water soluble dyed fragments from the substrate oat azo-xylan. The formed dyed fragments are then measured with a spectrophotometer and quantification is performed via a standard line based on the reference enzyme *Safizym XP20,* available from the applicant upon request. Method performance characteristics, when checked between two laboratories, include a limit of detection (LOD) of 0.14 IFP/g, a limit of quantification (LOQ) of 0.35 IFP/g, a relative standard deviation for repeatability (RSD_r) of 6.5% and an intermediate relative standard deviation for reproducibility (RSD_R) of 16.6%.

For the quantification of the endo-1,4-β-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 which are blank feed samples fortified with a known dose of the reference enzyme *Safizym XP20*. In the case that a matrix matched blank feed is not available the applicant proposes the use of the standard addition technique for quantifying the enzyme activity in feed.

Transferability of the method on *Safizym XP20*, using matrix matched standards, has been checked between two laboratories [*Anim. Feed Sci. Techn*. 1999, 77:345-353] obtaining similar results. The applicant also reported on another study with independent measurements between two laboratories. The LOD and LOQ correspond to 140 IFP/kg and 350 IFP/kg of *feedingstuffs*, respectively. The average RSD_r is 8.3% for powder form and 9.2% for liquid form. The average intermediate RSD_R is 15.8% for the liquid form and 24% for the powder form. Since the method shows acceptable performance characteristics, it is considered fit for official controls in the frame of the authorisation.

The CRL recommends that for the quantification of the enzyme activity in *feedingstuffs* the declared activity of endo-1,4-β-xylanase in *Safizym XP20* is confirmed by applying the method proposed for the pure additive or – in the case of major deviations - substituted by the actual measured activity of the enzyme.

Further testing or validation is not considered necessary.

FAD-2006-0038	**EFSA-Q-2007-033**
Product Name	Bonvital (sows)
Active substance	E. faecium
Rapporteur	Renata Leuschner CRL-FA, European Commission

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 1×10^{10} colony forming units (c.f.u.) per gram. Specifically, authorisation is sought to use Bonvital for sows. The conditions of use are proposed with a recommended dosage of 0.5 to 1×10^9 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 pour plate method using a selective enterococci agar 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 *premixtures* and *feedingstuffs*, a spread plate enumeration method is recommended which has been fully ring-trial validated (J. Appl. Microbiol. 2002, 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.

FAD-2006-0039	**EFSA-Q-2007-020**
Product Name	Avizyme 1505
Active substance	Endo-1,4-beta-xylanase, subtilisin, alpha-amylase
Rapporteur	Dalia Garalevičienė CRL-FA, European Commission

In the current application authorisation is sought for *Avizyme 1505* 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 *Avizyme 1505* as a digestibility enhancer for chickens for fattening and ducks for fattening. The product is intended to be marketed as a granular powder formulation.

The active agents of Avizyme 1505 are 1) endo-1,4-β-xylanase, produced by a strain of *Trichoderma reesei* (ATCC PTA 5588), 2) α-amylase, produced by a strain of *Bacillus amyloliquefaciens* (ATCC 3978) and 3) subtilisin, produced by a strain of *Bacillus subtilis* (ATCC 2107). Enzymatic activity of the active agents is expressed in units (U):

- One U of endo-1,4-β-xylanase is the amount of enzyme that liberates 0.5 µmol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50°C;
- One U of α-amylase is the amount of enzyme that liberates 1 µmol of glucosidic linkages per minute from a water insoluble cross-linked starch polymer substrate at pH 6.5 and 37°C;
- One U of subtilisin is the amount of enzyme that liberates 1 µmol of phenolic compound (tyrosine equivalents) per minute from a casein substrate at pH 7.5 and 40°C.

The product has a target activity of 1500 U endo-1,4-β-xylanase/g, 2000 U α-amylase/g and 20000 U subtilisin/g. *Avizyme 1505* is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of 75 to 300 U endo-1,4-β-xylanase/kg, 100 to 400 U α-amylase/kg and 1000 to 4000 U subtilisin/kg in *feedingstuffs.*

In general, the methods proposed for the determination of the activity of the active agents in different matrices are based on quantification of dyed compounds produced by enzymatic action of commercially available substrates. Enzymatic activity of the samples is calculated using reference enzyme standards, available from the applicant upon request, of which the activity is obtained applying the conditions described by the definitions

of units. When analysing *feedingstuffs,* calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzymes. In the case that identical blank feed samples are *not* available, a standard addition technique is employed. The applicant introduced some adaptations to the protocols provided by the suppliers of substrates. All modified methods have been single-laboratory validated and showed acceptable performance characteristics such as limit of detection, limit of quantification and relative standard deviation for repeatability.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a method based on the quantification (λ = 590 nm) of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on cross-linked wheat xylan substrates. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 5.3 and 50°C on a cross-linked oat spelt xylan. Analyses are carried out at pH 4.0 and 40°C (*feed additive*), at pH 5.3 and 40°C (*premixtures*) and at pH 4.2 and 50°C (*feedingstuffs*).

For the determination of the activity of α-amylase in the *feed additive,* the applicant proposes a method based on the quantification (λ = 405 nm) of free *p*-nitrophenol produced by the action of α-amylase on blocked *p*-nitrophenyl maltoheptaoside at pH 5.6 and 37°C. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 6.5 and 37°C. For the analysis of the activity of α-amylase in *premixtures* and *feedingstuffs,* quantification (λ = 620 nm) of dyed oligomers produced by the action of α-amylase on azurine-crosslinked starch at pH 6.4 and 37°C is proposed.

For the determination of the activity of subtilisin in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes a method based on the quantification (λ = 590 nm) of *dyed oligomers* produced by the action of subtilisin on azurine-cross linked casein. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured by quantification of *phenolic compounds* released from casein at pH 7.5 and 40°C. Analyses are carried out at pH 10 and 50°C (*feed additive* and *feedingstuffs*) and at pH 8.0 and 40°C (*premixtures*).

Though the methods proposed by the applicant are based on well known principles and show acceptable performance characteristics, the CRL is concerned that the suggested approach of measuring the enzymatic activity at *different* conditions in various matrices compared to the conditions described by the definitions of units and to the conditions of the determination of the activity of reference enzymes, introduces additional uncertainty into the measurements. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions described by the definitions of units;
- that the minimum activity of endo-1,4-β-xylanase, specified in the register entry (75 U/kg) is replaced by the limit of quantification of the method, which is 500 U/kg;
- that the minimum activity of α-amylase, specified in the register entry (100 U/kg) is replaced by the limit of quantification of the method, which is 160 U/kg;
- and that the minimum activity of subtilisin, specified in the register entry (1000 U/kg) is replaced by the limit of quantification of the method, which is 2000 U/kg.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as described by the definitions of units, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

FAD-2007-0002	**FAD-2007-0002**
Product Name *Active substance*	Natuphos® (ducks) Endo-1,4-beta-xylanase
Rapporteur	Annette Ploeger Plantedirektoratet, Lyngby, Denmark

In the current application authorisation is sought for *Natuphos*P$^{®P}$ under the category 'zootechnical additives' and the functional group 4(a), according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use *Natuphos*P$^{®P}$ as a digestibility enhancer for ducks.

The active agent of *Natuphos*P$^{®P}$ is 3-phytase, produced by a strain of *Aspergillus niger* (CBS 101.672). Enzymatic activity is expressed in FTU (phytase) units. One FTU is defined as the amount of enzyme that liberates one µmol of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37P^{oP}C. The product is intended to be marketed in three forms, as a powder *(Natuphos*P$^{®P}$*5000),* as a granulate *(Natuphos*P$^{®P}$ *5000 G* and *10000 G)* and as liquid formulation *(Natuphos*P$^{®P}$ *5000 L* and *10000 L),* containing, as indicated in the trade names, either 5000 or 10000 FTU/g. The products are intended to be incorporated into *premixtures* and/or complete *feedingstuffs* to obtain enzyme activity level of minimum 300 FTU/kg to recommended 750 FTU/kg in complete *feedingstuffs*.

For the determination of the activity of 3-phytase in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes a colorimetric method, based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37P^{oP}C by the enzyme 3-phytase. Released phosphate forms with molybdate and vanadate ions a coloured complex that is measured with a spectrophotometer at 415 nm and quantified against a standard curve based on the reference enzyme *Natuphos*P$^{®P}$*,* available from the applicant upon request.

The applicant reported precision data of the methods for the determination of the phytase activity in all three matrices that were obtained via inter-laboratory studies performed by the German Agricultural and Research Institutes (VDLUFA, Germany).

For the determination of the 3-phytase activity in the feed *additive,* method performance characteristics include a limit of detection (LOD) of 45 FTU/kg, a limit of quantification (LOQ) of 90 FTU/kg, a relative standard deviation for repeatability ($RSDB_{rB}$) of 2.5% and a relative standard deviation for reproducibility ($RSDB_{RB}$). The values for the recovery rate ranged from 98 to 102%. The inter-laboratory study of the method for the determination of 3-phytase activity in *premixtures* revealed an $RSDB_{rB}$ of 4.9% and an $RSDB_{RB}$ of 8.4%. Since the obtained method performance characteristics are acceptable, the methods are considered suitable for the official control purposes in the frame of authorisation.

The validation of the method for the determination of the phytase activity in *feedingstuffs* showed values for the LOD, LOQ and the recovery rate that were comparable to those of the methods for the determination of the

phytase activity in the *feed additive* and in *premixtures*. The $RSDB_{rB}$ was 6.9%, and the $RSDB_{RB}$ was 11.1%, obtained in an inter-laboratory study organised by VDLUFA. Therefore, the method is considered suitable for official control purposes.

Several other methods for the determination of phytase activity in *feedingstuffs* exist and have also been ring trial validated. These include a colorimetric method developed by FEFANA (European Association of Feed Additive Manufacturers) which has been validated according to the IUPAC guidelines on various phytase products and obtaining values for the $RSDB_{R\,B}$of 5 to 14%. In contrast to the method proposed by the applicant, the quantification is based on phosphate standard curve and not on the reference enzyme. Based on acceptable performance characteristics, this method, which is currently under evaluation to become a standard of the European Committee for Standardisation (CEN), is considered suitable for official control purposes and therefore recommended by the CRL for determination of enzymatic activity in *feedingstuffs*.

FAD-2007-0003	**EFSA-Q-2007-050**
Product Name *Active substance*	CreAminoTM Guanideinoacetic acid
Rapporteur	Jaroslava Petrová ÚKZÚZ, NRL RO Praha, The Czech Republic

In the current application authorisation is sought for guanidinoacetic acid (GAA) 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.

The applicant intends to place GAA on the market as a granular product containing at least 96% of GAA and at maximum 1% of starch as granulation agent under the trade name *CreAmino*™. The product is intended being mixed into *feedingstuffs* for broilers and turkeys at a minimum concentration of 300 mg/kg and at a recommended concentration of 600 mg/kg of GAA in complete *feedingstuffs*.

For the determination of the active substance in the *product* and in *feedingstuffs,* the same single-laboratory validated method is proposed, which is based on ion chromatography (IC) method coupled to ultraviolet-visible (UV-VIS) detection operated at a wavelength of 200 nm. Method performance characteristics were determined by conducting replicate analyses of *CreAmino*™ and two types of feed samples containing the active substance at different concentrations. The limit of detection (LOD) and limit of quantification (LOQ) was 20 mg/kg and 55 mg/kg, respectively. The relative standard deviation for repeatability (RSD_r) was 0.1% for the feed additive and varied from 0.77% to 2.86% for the feed samples depending on the concentration of the active substance. The values for the recovery rate were in all cases above 97%. Also the ruggedness of the method was confirmed by analysing feed samples of different composition on different instruments and days. The performance characteristics are considered acceptable and therefore the method is suitable for official control purposes within the frame of the authorisation.

For the determination of the active substance in *premixtures,* the applicant proposed a different method which has also been single-laboratory validated and which is based on high performance liquid chromatography (HPLC) coupled to ultraviolet-visible (UV-VIS) detection operated at a wavelength of 210 nm. The validation of

the method was performed on typical premixtures with supplementation levels of the active substance at 1, 5, 25 and 50%, respectively. The obtained values for the recovery rate varied between 96 and 108% and the obtained values for the RSD_r ranged from 0.6 to 1.4%. All methods have also been successfully tested by a second independent analytical laboratory. The performance characteristics are considered acceptable and therefore the method is considered suitable for the intended purpose.

For the determination of GAA in *muscle tissues*, *liver* and *kidney* an IC method is proposed which is based on the same principle as the method for the determination of GAA in the *product* and in the *feedingstuffs*. The method was single-laboratory validated on the target tissues, obtaining acceptable values for the recovery rate and the precision. The limit of quantification (LOQ) was 0,8 mg/kg in *muscle tissues* and 1,6 mg/kg in *liver* and *kidney*. Since the applicant did not propose Maximum Residue Levels of GAA in the target tissues, the CRL is unable to comment on the suitability of the proposed method for official control purposes.

Further testing or validation is not considered necessary.

FAD-2007-0008	**EFSA-Q-2007-067**
Product Name *Active substance*	Coxidin 25% Monensin sodium
Rapporteur	Giuseppe Simone CRL-FA, European Commission

Coxidin®25% is a feed additive authorised [Regulation (EC) No 109/2007] in EU (Registration number E 1701) under the category "zootechnical additives", functional group "coccidiostats and histomonostats" according to Annex I of Regulation (EC) No 1831/2003. The additive is authorised for use in compound feedingstuffs for chickens for fattening and for turkeys at concentrations of 100 to 125 mg/kg and 90 to 100 mg/kg, respectively.

In the current application a modification of the authorisation is sought for reducing the authorised minimum dose, within the conditions of use for turkeys, from current 90 to 60 mg/kg complete feedingstuffs. In accordance with Article 13 (3) of Regulation (EC) No. 1831/2003, only the technical documentation supporting this request has been submitted by the applicant. Therefore, the same methods already evaluated by the CRL in relation to the Dossier No. FAD-2005-003 (*Coxidin®*) [Evaluation Report of the Community Reference Laboratory Feed Additives Authorisation on the Method(s) of Analysis for Coxidin®, 20 July 2005[3]] have been considered in this report.

The conclusions drawn in the report issued by the CRL on 20 July 2005 as regards the analytical methods for the determination of the active substance (monensin) in the *additive*, *premixtures* and *feedingstuffs* are valid and applicable for the purpose of this application. Therefore, the methods are considered suitable for official control.

For the determination of *residues* in chicken and turkey tissues in the frame of the implementation of the provisional Maximum Residue Limits (MRLs) for monensin sodium, the FEEDAP Panel proposed a method [Opinion on the Maximum Residue Limits for monensin sodium for chickens and turkey for fattening, The EFSA Journal (2006) 413, 1-13] available in literature [Chéneau *et al.*, J. Chromatography B, 850 (2007) 15-23]. This

method is based on liquid chromatography coupled to low resolution tandem mass spectrometry (LC-MS/MS) and uses the authorised feed additive narasin as internal standard. Furthermore, the mass spectrometry detection of monensin is based on one precursor ion and *one* ion transition (688.4 >635.3). The method has been validated for liver, muscle and fat. Since monensin belongs to group B of Annex I of Council Directive 96/23/EC[4], analytical methods for the determination of this substance in the target matrices for official control purposes have to comply with the criteria specified in Commission Decision 2002/657/EC[5]. Applying these criteria indicates acceptable validation results in terms of sensitivity, precision and trueness. However, the application of the method in the frame of official control is limited due to the following two aspects. The proposed method can *only* be considered suitable as a *quantitative* method to determine residues of monensin in target tissue samples at or around the provisional MRL values in cases where the presence of narasin can be *excluded*. In addition, the proposed protocol does not allow for the *unequivocal* identification of monensin in the case of a suspected non-compliant result, i.e. when the analytical results indicate exceeding the provisional MRLs. This is due the fact that the protocol uses one precursor ion and *one* transition thereby obtaining 2.5 identification points whereas at least *three* identification points would be required for identification of these substances according to Commission Decision 2002/657/EC. The CRL assumes that this method could slightly be modified by measuring a second transition in order to fulfil the criteria of the Commission Decision. However, validation data for this modification have not been provided.

Therefore, the CRL considers the proposed method only suitable for official control if (1) its application is limited to the target tissues in the frame of this authorisation, (2) the sample does not contain narasin, *and* (3) the measured concentration of monensin is below the provisional MRLs. In all other cases the proposed method is not suitable for official purposes.

As an alternative, another LC-MS/MS method using an internal standard which is different than the currently authorised coccidiostats has been identified and is available at the Community Reference Laboratory for Residues of Veterinary Drugs at the German Federal Office of Consumer Protection and Food Safety[6]. The detection mode used grants *four* identification points obtained by one precursor ion and *two* transitions and the method is routinely used by official control laboratories in the EU. The method was successfully in-house validated in accordance with the requirements of Commission Decision 2002/657/EC in liver and muscle of calf, lamb, chicken, and turkey with acceptable performance characteristics. The CRL therefore recommends this method for official control purposes in the frame of this authorisation.

[3] Available at http://www.irmm.jrc.be/crl-feed-additives
[4] Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues there of in live animals and animal products
[5] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC)
[6] Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Berlin, Germany

FAD-2007-0009	EFSA-Q-2007-090
Product Name	Toyocerin (Turkeys)
Active substance	Bacillus cereus var. toyoi
Rapporteur	Renata Leuschner CRL-FA, European Commission

In the current application authorisation is sought for the microbial feed additive Toyocerin® under the category 'zootechnical additives', functional group 'gut flora stabiliser' according to Annex I of Regulation (EC) No 1831/2003. Specifically, the use of Toyocerin® for turkeys for fattening from day one until slaughtering is requested. Toyocerin® contains a minimum of 1 x 10^{10} colony forming units (c.f.u.) of viable spores of *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012 per gram (g). The feed additive is intended to be mixed into complete feedingstuffs at a final concentration of 0.2 to 1 x 10^{9} c.f.u./kg.

For the quantification of the active agent (*Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012) of Toyocerin® in the *feed additive*, *premixtures* and *feedingstuffs* appropriate enumeration methods were proposed by the applicant. Analysis data confirmed an appropriate method performance in a second laboratory.

For official controls regarding the quantitative determination of the active agent in the *feed additive*, *premixtures* and *feedingstuffs*, another surface plate count enumeration method is recommended which includes a heat-treatment of the initial sample suspension to inactivate vegetative cells and uses subsequently a non-selective agar. This method has been fully ring-trial validated (J.AOAC Int. 2003, 86, 568-575). The method's performance characteristics revealed standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.07 – 0.09 $\log_{10}$ and 0.35 – 0.32 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g premixture or feedingstuff, respectively. The limits of quantification (LOQ) of this method are 100 c.f.u./g feed additive or premixture and 10^{7} c.f.u./kg feedingstuff which is well below the minimum anticipated target level of application.

The identity of the bacterial strain, *Bacillus cereus* var. *toyoi* NCIMB 40112/CNCM I-1012, was analysed by a range of techniques including biochemistry, phage-typing, molecular methods and pyrolysis mass spectrometry. Pulsed field gel electrophoresis (PFGE) is a generally recognised standard methodology for microbial identification and is therefore 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.

FAD-2007-0010	EFSA-Q-2007-098
Product Name *Active substance*	Mintrex-Zn Zn chelate of hydroxy analogue of methionine
Rapporteur	Maria Cesarina Abete C.Re.A.A, Torino, Italy

Mintrex® Zn is a product for which authorisation is sought under the category "nutritional additives", functional group 3b "compounds of trace elements", according to the classification system of Annex I, of Regulation (EC) No 1831/2003. According to the applicant, *Mintrex® Zn* contains 16% of Zinc as chelate of hydroxyl analogue of methionine, 2-hydroxy-4-methylthiobutanoic acid (HMTBa) as active substance. *Mintrex®Zn* is also a source of methionine activity as HMTBa.

In the current application authorisation is sought for use of *Mintrex®Zn* for all animal species. *Mintrex®Zn* is intended to be added to complete feed to supplement Zn within legal limits for each species which are: pet animals 250 mg /kg, fish 200 mg/kg, milk replacers 200 mg/kg and other species 150 mg/kg.

For the determination of Zn in the feed additive, premixtures and feedingstuffs for official control the CEN standard method EN 15510:2007, as proposed by the applicant, is recommended by the CRL.

The proposed methods for the determination of HMTBa are considered suitable for the intended purpose.

FAD-2007-0011	EFSA-Q-2007-094
Product Name *Active substance*	Mintrex-Mn Mn chelate of hydroxy analogue of methionine
Rapporteur	Maria Cesarina Abete C.Re.A.A, Torino, Italy

Mintrex® Mn is a product for which authorisation is sought under the category "nutritional additives", functional group 3b "compounds of trace elements", according to the classification system of Annex I, of Regulation (EC) No 1831/2003. According to the applicant, *Mintrex® Mn* contains 13% of Manganese as chelate of hydroxyl analogue of methionine, 2-hydroxy-4-methylthiobutanoic acid (HMTBa) as active substance. *Mintrex®Mn* is also a source of methionine activity as HMTBa.

In the current application authorisation is sought for use of *Mintrex®Mn* for all animal species. *Mintrex®Mn* is intended to be added to complete feed to supplement Mn within legal limits for each species which are: fish 100 mg/kg, other species 150 mg/kg.

For the determination of Mn in the feed additive, premixtures and feedingstuffs for official control the CEN standard method EN 15510:2007, as proposed by the applicant, is recommended by the CRL.

The proposed methods for the determination of HMTBa are considered suitable for the intended purpose.

FAD-2007-0012	EFSA-Q-2007-097
Product Name	Mintrex-Cu
Active substance	Cu chelate of hydroxy analogue of methionine
Rapporteur	Maria Cesarina Abete C.Re.A.A, Torino, Italy

Mintrex®Cu is a product for which authorisation is sought under the category "nutritional additives", functional group 3b "compounds of trace elements", according to the classification system of Annex I, of Regulation (EC) No 1831/2003. According to the applicant, *Mintrex® Cu* contains 15% of Copper as chelate of hydroxyl analogue of methionine, 2-hydroxy-4-methylthiobutanoic acid (HMTBa) as active substance. *Mintrex®Cu* is also a source of methionine activity as HMTBa.

In the current application authorisation is sought for use of *Mintrex®Cu* for all animal species. *Mintrex®Cu* is intended to be added to complete feed to supplement Cu within legal limits for each species which are: piglets up to 12 weeks 170 mg/kg, other pigs 25 mg/kg, milk replacers 15 mg/kg, other complete feed for bovine before the start of rumination 15 mg/kg, other bovine 35 mg/kg, ovine 15 mg/kg, fish 25 mg/kg, crustaceans 50 mg/kg and other species 25 mg/kg.

For the determination of Cu in the feed additive, premixtures and feedingstuffs for official control the CEN standard method EN 15510:2007, as proposed by the applicant, is recommended by the CRL.

The proposed method for the determination of HMTBa in the feed additive is considered suitable for the intended purpose.

The suitability of the proposed method for the determination of HMTBa in feedingstuffs cannot be evaluated by the CRL.

FAD-2007-0013	EFSA-Q-2007-104
Product Name	Biosaf Sc47 (pigs)
Active substance	S. cerevisiae
Rapporteur	Renata Leuschner CRL-FA, European Commission

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. Specifically, the use of BIOSAF® Sc47 for pigs for fattening is requested. BIOSAF® Sc47 contains a minimum of 5×10^9 of viable cells (c.f.u., colony-forming units) of *Saccharomyces cerevisiae* NCYC Sc47 (as active agent) per gram. The feed additive is intended to be mixed into complete feedingstuffs at a final concentration of 1.25×10^9 to 1.00×10^{10} c.f.u./kg.

For the determination of the active agent, a strain of *Saccharomyces cerevisiae* NCYC Sc47, in the *feed additive*, a pour plate method and a molecular identification method (polymerase chain reaction (PCR)) are proposed by the applicant, which are considered appropriate. For the determination of the active agent *S. cerevisiae* NCYC Sc47 in *feedingstuffs* a similar pour plate method for enumeration and the same molecular

PCR method for identification of the strain are proposed. The enumeration method was validated in a collaborative study [System. Appl. Microbiol. 2003. 26, 147-153]. The method's performance characteristics of the enumeration method are standard deviations for repeatability (s_r) and reproducibility (s_R) of around 0.17 – 0.36 $\log_{10}$ and 0.55 – 0.60 $\log_{10}$ calculated from the base 10 logarithms of the measured c.f.u./g in feedingstuffs, respectively. The limits of quantification (LOQ) of this method are 100 colony forming units (c.f.u) per gram (g) feed additive or premixture and 10^7 c.f.u./kg feedingstuff to take account of natural background flora. These performance characteristics are considered acceptable. This method is recommended for official controls of the active agent in the feed additive, premixtures and feedingstuffs.

The PCR method for strain identification was ring trial validated and demonstrated a high level of correct identification between laboratories [System. Appl. Microbiol. 2004. 27, 492-500]. It is therefore considered appropriate for official controls.

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

FAD-2007-0017	**EFSA-Q-2007-112**
Product Name	Avizyme 1505 (turkeys)
Active substance	Endo-1,4-beta-xylanase, subtilisin, alpha-amylase
Rapporteur	Dalia Garalevičienė CRL-FA, European Commission

In the current application authorisation is sought for *Avizyme 1505* 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 *Avizyme 1505* as a digestibility enhancer for turkeys for fattening. The product is intended to be marketed as a granular powder formulation.

The active agents of *Avizyme 1505* are 1) endo-1,4-β-xylanase, produced by a strain of *Trichoderma reesei* (ATCC PTA 5588), 2) α-amylase, produced by a strain of *Bacillus amyloliquefaciens* (ATCC 3978) and 3) subtilisin, produced by a strain of *Bacillus subtilis* (ATCC 2107). Enzymatic activity of the active agents is expressed in units (U):

- One U of endo-1,4-β-xylanase is the amount of enzyme that liberates 0.5 □mol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50°C;
- One U of α-amylase is the amount of enzyme that liberates 1 □mol of glucosidic linkages per minute from a water insoluble cross-linked starch polymer substrate at pH 6.5 and 37°C;
- One U of subtilisin is the amount of enzyme that liberates 1 □mol of phenolic compound (tyrosine equivalents) per minute from a casein substrate at pH 7.5 and 40°C.

The product has a target activity of 1500 U endo-1,4-β-xylanase/g, 2000 U α-amylase/g and 20000 U subtilisin/g. *Avizyme 1505* is intended to be mixed into *premixtures* and/or *feedingstuffs* to obtain an enzyme activity level of 300 U endo-1,4-β-xylanase/kg, 400 U α-amylase/kg and 4000 U subtilisin/kg in *feedingstuffs.*

In general, the methods proposed for the determination of the activity of the active agents in different matrices are based on quantification of dyed compounds produced by enzymatic action of commercially available substrates. Enzymatic activity of the samples is calculated using reference enzyme standards, available from the applicant upon request, of which the activity is obtained applying the conditions described by the definitions of units. When analysing *feedingstuffs,* calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzymes. In the case that identical blank feed samples are *not* available, a standard addition technique is employed. The applicant introduced some adaptations to the protocols provided by the suppliers of substrates. All modified methods have been single-laboratory validated and showed acceptable performance characteristics such as limit of detection, limit of quantification and relative standard deviation for repeatability.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a method based on the quantification (λ = 590 nm) of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on cross-linked wheat xylan substrates. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 5.3 and 50°C on a cross-linked oat spelt xylan. Analyses are carried out at pH 4.0 and 40°C (*feed additive*), at pH 5.3 and 40°C (*premixtures*) and at pH 4.2 and 50°C (*feedingstuffs*).

For the determination of the activity of α-amylase in the *feed additive,* the applicant proposes a method based on the quantification (λ = 405 nm) of free *p*-nitrophenol produced by the action of α-amylase on blocked *p*-nitrophenyl maltoheptaoside at pH 5.6 and 37°C. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured at pH 6.5 and 37°C. For the analysis of the activity of α-amylase in *premixtures* and *feedingstuffs,* quantification (λ = 620 nm) of dyed oligomers produced by the action of α-amylase on azurine-crosslinked starch at pH 6.4 and 37°C is proposed.

For the determination of the activity of subtilisin in the *feed additive, premixtures* and *feedingstuffs,* the applicant proposes a method based on the quantification (λ = 590 nm) of *dyed oligomers* produced by the action of subtilisin on azurine-cross linked casein. Enzymatic activity is calculated using a reference enzyme standard, of which the activity is measured by quantification of *phenolic compounds* released from casein at pH 7.5 and 40°C. Analyses are carried out at pH 10 and 50°C (*feed additive* and *feedingstuffs*) and at pH 8.0 and 40°C (*premixtures*).

Though the methods proposed by the applicant are based on well known principles and show acceptable performance characteristics, the CRL is concerned that the suggested approach of measuring the enzymatic activity at *different* conditions in various matrices compared to the conditions described by the definitions of units and to the conditions of the determination of the activity of reference enzymes, introduces additional uncertainty into the measurements. Therefore, for consistent analytical results, the CRL recommends:

- that the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- that the harmonised analytical conditions are identical with conditions described by the definitions of units;
- that the minimum activity of endo-1,4-β-xylanase, specified in the register entry (300 U/kg) is replaced by the limit of quantification of the method, which is 500 U/kg.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as described by the definitions of units, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

FAD-2007-0018	**EFSA-Q-2007-109**
Product Name	Danisco Xylanase L & G
Active substance	Endo-1,4 beta-xylanase
Rapporteur	Dalia Garalevičienė CRL-FA, European Commission

In the current application authorisation is sought for *Danisco Xylanase* 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 *Danisco Xylanase* as a digestibility enhancer for turkeys for fattening. The product is intended to be marketed as a powder (*Danisco Xylanase G*) and as liquid formulation (*Danisco Xylanase L*).

The active agent of *Danisco Xylanase* is endo-1,4-β-xylanase produced by a strain of *Trichoderma reesei* (ATCC PTA 5588). The enzymatic activity is expressed in units (U). One U is the amount of endo-1,4-β-xylanase that liberates 0.5 □mol of reducing sugar (xylose equivalents) per minute from a cross-linked oat spelt xylan at pH 5.3 and 50°C. The product has a target activity of 40000 U/g. *Danisco Xylanase G* is intended to be mixed into *premixtures* and/or *feedingstuffs*, whereas *Danisco Xylanase L* is sprayed directly onto feed to obtain an enzyme activity level of 1250 to 2500 U/kg in *feedingstuffs*.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive*, *premixtures* and *feedingstuffs*, the applicant proposes a colorimetric method based on the quantification of water soluble dyed fragments produced by the action of endo-1,4-β-xylanase on commercially available cross-linked xylan substrates. Enzymatic activity of the sample is calculated using a reference enzyme standard. The applicant introduced some adaptations to the protocol. The modified methods have been single laboratory validated.

For the determination of the activity of endo-1,4-β-xylanase in the *feed additive,* the applicant proposes a method which measures the enzyme-catalysed formation of water soluble dyed fragments released from cross-linked wheat arabinoxylan. The rate of release is measured on a spectrophotometer at 590 nm and quantified against a reference enzyme standard, available from the applicant upon request. The analysis – however – is carried out at *different* conditions (pH 4.0 and 40°C on a cross-linked wheat arabinoxylan) compared to those given in the proposed register entry (pH 5.3 and 50°C on a cross-linked oat spelt xylan) and the enzymatic activity is calibrated against a reference enzyme of which the activity is obtained applying the conditions of the proposed register entry. Method performance characteristics include a limit of detection (LOD) of 1.2 U/g, limit of quantification (LOQ) of 1.5 U/g products and a relative standard deviation for repeatability (RSD_r) of 4.4%.

For the determination of the activity of endo-1,4-β-xylanase in *premixtures,* the applicant proposes a method based on the same principle as described above, but employing a different extraction procedure. The

measurements are carried out at pH 5.3 and 40°C on a cross-linked wheat arabinoxylan. Method performance characteristics include a LOD of 13.0 U/g, LOQ of 19.3 U/g, an RSD_r of 3.5 % and recovery rates of 96.4 %.

For the quantification of the activity of endo-1,4-β-xylanase in *feedingstuffs,* the applicant proposes a method, based on the same principle as described above, measuring enzymatic activity on a cross-linked wheat arabinoxylan at pH 4.2 and 50°C. Calibration is performed on standards prepared from identical blank feed samples fortified with exact amounts of the reference enzyme, available from the applicant. Method performance characteristics include a LOD of 285 U/kg, a LOQ of 530 U/kg, a RSD_r of 7.5% and a recovery rate of 97%. In the case that identical blank feed samples are *not* available, a standard addition technique is employed.

Though the methods proposed by the applicant are based on well known principles and show acceptable performance characteristics, the CRL is concerned that the suggested approach of measuring the enzymatic activity at *different* conditions compared to the conditions of the proposed register entry and to the conditions of the determination of the activity of a reference enzyme, introduces additional uncertainty into the measurements. Therefore, for consistent analytical results, the CRL recommends that:

- the enzymatic activity in the *feed additive*, in *premixtures* and in *feedingstuffs* is determined at identical conditions;
- the harmonised analytical conditions are identical with conditions specified in the register entry.

In the case that the analytical conditions remain *different* for determination of enzymatic activity in various matrices and *different* from those as proposed in the Register entry, the CRL cannot evaluate the proposed methods for their suitability for official controls.

Further testing or validation is not considered necessary.

European Commission

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Abstract
In 2007 the CRL-FA Authorisation submitted a total of 28 evaluation reports to EFSA and organised the annual workshop attended by 23 National Reference Laboratories. This report provides a detailed overview of the yearly activity, thus including the activity report of the four expert groups: coccidiostats, micro-organisms, enzymes and trace elements.

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