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

Dossier related to:	EFSA-Q-2005-021
Name of Additive:	Toyocerin®
Active Agent(s):	*Bacillus cereus* var. *toyoi* (NCIMB 40112 / CNCM I-1012)
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Date:	10 November 2005
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Date:	10 November 2005

EXECUTIVE SUMMARY

In the current application authorisation is sought for the use of Toyocerin® which is a preparation of viable spores of a strain of *Bacillus cereus* var. *toyoi* (NCIMB 40112 / CNCM I-1012), to be used in feed for chickens for fattening containing the permitted coccidiostats diclazuril, narasin-nicabazin and maduramicin ammonium.

Concerning the determination of the active agent of Toyocerin® (*Bacillus cereus* var. *toyoi*) in the additive, in premixtures and feedingstuffs, the applicant proposed a pour plate count method to enumerate viable counts of the preparation. The method is quantitative and uses a nutrient agar medium (NAM) for analysis of the additive and premixture, and NAM with the addition of antibiotics for feedingstuffs.

Within-laboratory validation data were submitted by the applicant regarding the method for determination of the active substance in the additive, in premixtures and in feedingstuffs. Relative standard deviations of repeatability (RSD_r) and within-laboratory reproducibility (RSD_R) for the performance of the methods in the additive, the premixture and feedingstuffs were appropriate. The limit of quantification (LOQ) for the enumeration method is 5×10^3 colony forming units (c.f.u.) per gram of sample. Taking into account the target level of application which ranges between 10^6 c.f.u./g and 10^{10} c.f.u./g of sample (additive, premixture or feedingstuffs) and the acceptable performance characteristics of RSD values, in the opinion of the CRL the proposed methods are fit for the purpose of routine control of the presence of the active agent in the feed additive Toyocerin®, in premixtures and in feedingstuffs.

The applicant provided data confirming equivalent performance of the method of analysis when enumerating viable spores of the active agent in Toyocerin® with and without the presence of three coccidiostats (diclazuril, narasin/nicarbacin and maduramicin ammonium) in broiler feed.

In summary, it is considered that the proposed methods for enumeration of viable *Bacillus cereus* var. *toyoi* spores as colony forming units in the additive, in premixtures and in feedingstuffs fulfil the requirements for routine control also in the presence of the coccidiostats (diclazuril, narasin/nicarbacin and maduramicin ammonium) at levels typically applied in broiler feed. However, it is recommended to use a larger quantity of feed samples than the proposed 2 g, such as for example 50 g of feed samples to take account of potential sample heterogeneity.

For official control purposes, the CRL recommends a fully ring-trial validated method for enumeration of bacilli spores in premixtures and feedingstuffs which was published in a peer reviewed journal (Journal of AOAC International (2003) 86, 568-575). The study

demonstrated acceptable methods performance characteristics, since the RSD_r was about 1 % and the RSD_R was about 6%.

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

KEYWORDS

Toyocerin®, *Bacillus cereus* var. *toyoi*, feed additive, zootechnical, chicken for fattening.

TABLE OF CONTENTS

1. BACKGROUND

The additive Toyocerin® is a preparation of *Bacillus cereus* var. *toyoi* (NCIMB 40112/CNCM I-1012). This product is already permanently authorised for its use in piglets up to 4 months, pigs for fattening and sows (E 1701), and was provisionally authorised (No 1) for chickens for fattening, laying hens, calves, cattle for fattening, breeding does and rabbits for fattening until 7 October 2004, including the authorisation for its use in combination with monensin sodium, lasalocid sodium, salinomycin sodium, decoquinate, robenidine, narasin and halofuginone. It is currently undergoing assessment for permanent authorisation for cattle for fattening, chickens for fattening and rabbits for fattening.

The current application seeks authorisation for use of Toyocerin® in feed products for chickens for fattening containing the permitted coccidiostats diclazuril, narasin-nicarbazin and maduramicin ammonium.

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and tasks of the Community Reference Laboratory concerning applications (No 1) for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the suitability of the control methods submitted in connection with EFSA-Q– 2005 – 021 was evaluated.

3. EVALUATION

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

Control Methods

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

Qualitative and quantitative composition of the additive

The final product contains spores of *B. cereus* var. *toyoi* and the remainder consists of maize flour and calcium carbonate.

The active constituent of the additive is a preparation of viable spores of a strain of *Bacillus cereus* var. *toyoi* originally isolated from soil. The strain has been deposited under the accession numbers NCIMB 40112 and CNCM 1-1012. Methods to identify the strain were not provided. The numbers of viable microorganisms is given in colony forming units (c.f.u.) per unit weight. The method used to enumerate the active agent in the additive was a quantitative pour plate method to quantify bacterial spores of *Bacillus cereus* var. *toyoi*. The samples of the additive are initially diluted with 0.01% Tween solution and homogenised. Decimal dilutions are prepared from these homogenates and 1 ml portions of appropriate dilutions are added to molten Nutrient agar which is poured into Petri-dishes. The method for enumeration of *Bacillus cereus* var. *toyoi* in the additive would include viable vegetative cells and damaged spores which may be present in the additive. However, this is not considered problematic as potentially occurring vegetative cells or damaged spores are expected to represent a minority.

The applicant provided protocols concerning the validation of the method of analysis for determining *Bacillus cereus* var. *toyoi* in the additive Toyocerin® using appropriate samples with 10^{10} c.f.u./g additive. Within-laboratory validation was carried out using a limited number of samples which represented different batches of the additive product. Analyses in these validation studies were performed by two laboratory analysts as well as at different dates. Relative standard deviations of repeatability (RSD_r) and within-laboratory reproducibility (RSD_R) were 4.2 % and 4.9 %, respectively. The proposed method is appropriate for routine control purposes.

Other methods recommended for routine control would represent a published method by the "Verband Deutscher Landwirtschaft Untersuchungs- und Forschungsanstalten (VDLUFA)" (1) or a fully ring-trial validated method published in Journal of AOAC International (3).

(Cf. the requirements listed in point 2.1.3 of the Guidelines.)

Qualitative and quantitative composition of any impurities

The protocol used for the routine screening of production batches for contaminants was not provided for the purpose of this extension.

(Cf. the requirements listed in point 2.1.4 of the Guidelines).

Physical state of each form of the product

Methods on how to reveal data on particle size, dusting potential and the use of processes such as encapsulation which affect the physical properties have not been provided for the purpose of this extension.

(Cf. the requirements listed in point 2.1.5 of the Guidelines).

Toxins and virulence factors

Methods to test for evidence that toxins and virulence factors are absent from the strain used in the active agents were not provided for the purpose of this extension.

(Cf. the requirements listed in point 2.2.5 of the Guidelines).

Antibiotic production and antibiotic resistance

Methods to test the active agents for the capability to produce antimicrobial substances relevant to the use of antibiotics in humans or animals have not been provided for the purpose of this extension.

(Cf. the requirements listed in point 2.2.6 of the Guidelines).

Stability of the additive

For the stability studies the methods suggested for routine analysis would be appropriate.

(Cf. the requirements listed in point 2.3.1 of the Guidelines).

Other physico-chemical or biological properties

Methods have not been provided for the purpose of this extension.

(Cf. the requirements listed in point 2.3.2 of the Guidelines).

Incompatibilities with other feed ingredients

Methods have not been provided for the purpose of this extension.

(Cf. the requirements listed in point 2.3.3 of the Guidelines).

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

A quantitative pour plate method is used to quantify bacterial spores of *Bacillus cereus* var. *toyoi* present in premixtures and feedingstuffs. Bacterial spores capable of germinating are enumerated and differentiated. The samples are initially diluted (samples of the premixture with 0.01% Tween solution, feed samples with 0.2% NaOH to inactivate vegetative cells) and homogenised. Decimal dilutions are prepared from these homogenates and 1 ml portions of appropriate dilutions are added to molten Nutrient agar which is poured into Petri-dishes. Nutrient agar supplemented with antibiotics to inhibit the background flora is used for the analysis of feedingstuffs. The method for enumeration of *Bacillus cereus* var. *toyoi* in the premixture would include potentially present viable vegetative cells and damaged spores which would not be the case for the method used for feedingstuffs. This is not considered problematic as potentially occurring vegetative cells or damaged spores are expected to represent a minority in premixtures. The apparatus, glassware, diluents and media used are specified in the method protocols supplied.

The applicant provided within-laboratory validation studies concerning the method performance for analysis of *Bacillus cereus* var. *toyoi* spores in the premixture and in feedingstuffs. Within-laboratory validation was carried out using a limited number of samples which represented different batches of premixtures or feedingstuffs. Analyses in these validation studies were performed by two laboratory analysts and were performed at different dates. In this report the results are summarised separately for the analysis of premixtures and feedingstuffs.

Validation experiments for the analysis of premixtures: Samples of the premixtures containing viable counts of the active substances of 10^9 c.f.u./g were used in the validation study. The RSD_r and within-laboratory RSD_R of 4.2 % and 4.9 % were determined in an appropriate testing scheme (in respect of categories of different products, number of replications, number of analysts). The performance characteristics are within the range of other studies reported in the scientific literature (2, 3). The method proposed for analysis of the premixture is considered appropriate for routine control.

Validation experiments for the analysis of feedingstuffs: The procedure for determination of *Bacillus cereus* var. *toyoi* spores described in the method for feedingstuffs ("Analysis method for viable *Bacillus toyoi* in feed") differs from the previous method (additive and premixtures) in respect of the extraction solution and the selective medium: Feed samples are extracted and diluted with 0.2% NaOH to inactivate vegetative cells and a nutrient agar supplemented with antibiotics (chloramphenicol and polymyxin B) is used. In the validation study for feedingstuffs samples of piglet and rabbit feed containing viable counts of the active substance at a concentration of 10^6 spores/g were used. The RSD_r and within-laboratory RSD_R of 7.4 % and 8.6 % were determined in an appropriate testing scheme and the performance characteristics are within the range of other studies reported in the scientific literature (2, 3). From the studies it can be concluded that the method performs adequately in the presence of the three tested coccidiostats (diclazuril, narasin/nicarbacin and maduramicin ammonium) in feedingstuffs. The method is therefore considered appropriate for routine control. This also applies if the samples contain the three coccidiostats diclazuril, narasin/nicarbacin and maduramicin ammonium in an amount that is typical in broiler feed.

The applicant's method for the enumeration of viable *Bacillus cereus* var. *toyoi* spores in feedingstuffs is similar to a recommended method published by the VDLUFA (1) but differs in two points: The sample amount for analysis and the agar base:

- According to the applicant's method a sample amount of 2 g feedstuff is subjected to analysis, whereas the ring trial validated method (3) uses 50 g of sample and the VDLUFA-Method (1) uses two portions of 20 g sample for analysis. Though the applicant demonstrated that a sample amount of 2 g is appropriate for types of pelleted feed for which Toyocerin® is intended, the CRL considers this sample amount as too small given the potential heterogeneity of feed samples. In fact, typical amounts of feed samples subjected to analyses in this field are at least 20 g.
- The agar base proposed by the applicant seems not to be commercially available in form of a dehydrated medium, which appears unfavourable with reference to measures of quality management in a laboratory. Nevertheless, this agar medium seems appropriate for the intended purpose.

The limit of quantification of the enumeration methods for the active agent provided by the applicant is 5×10^3 c.f.u./g sample (feedingstuffs or premixtures) which is well below expected concentrations of around 10^6 c.f.u./g feedingstuff or concentrations of around 10^9 c.f.u./g premixture.

The CRL recommends for official control purposes a fully ring-trial validated method applicable for the enumeration of bacilli spores in premixtures and feedingstuffs (3). Methods performance characteristics for the method using samples of premixture and feedingstuffs include values of RSD_r and RSD_R of around 1 % and 6 %, respectively.

(Cf. the requirements listed in point 2.5.2 of the Guidelines).

CHECKLIST

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned in Part I (1.- A. Premixtures) accompanied by information on:				
	- Sampling Method used		X		
	- Percentage Recovery			X	
	- Specificity		X		
	- Accuracy		X		
	- Precision	X			
	- Limits of detection			X	
	- Limits of quantification	X			
	- Validation procedure used	X			
1.2	Is/Are the method(s) mentioned in Part I (1.- A. Feedingstuffs) accompanied by information on:				
	- Sampling Method used		X		
	- Percentage Recovery			X	
	- Specificity		X		
	- Accuracy		X		
	- Precision	X			
	- Limits of detection			X	
	- Limits of quantification	X			
	- Validation procedure used	X			

4. CONCLUSIONS AND RECOMMENDATIONS

Concerning the determination of the active agent of Toyocerin® (*Bacillus cereus* var. *toyoi*) in the additive, in premixtures and feedingstuffs, a pour plate count method was proposed by the applicant to enumerate viable counts of the preparation. The method is quantitative and uses a nutrient agar medium (NAM) for analysis of the additive and premixture, and NAM with the addition of antibiotics for feedingstuffs.

Within-laboratory validation data were submitted by the applicant regarding the method for determination of the active agent in the additive, in premixtures and in feedingstuffs. In the case of additives and premixtures a RSD_r of around 4 % and a RSD_R of around 5 % were

determined. Concerning the method for feedingstuffs, values for RSD_r and within-laboratory RSD_R of around 7 % and 9 %, respectively, were determined. The limit of quantification (LOQ) for the enumeration methods is 5 x 10^3 c.f.u./g additive, premixture or feedingstuffs. The applicant provided data confirming equivalent performance of the method of analysis when enumerating viable spores of the active agent in Toyocerin® with and without the presence of three coccidiostats (diclazuril, narasin/nicarbacin and maduramicin ammonium) in feedingstuffs. Therefore, these methods are fit for purpose for routine control of the active agent in the feed additive, in premixtures and in feedingstuffs.

In summary, it is considered that the proposed methods for determination of viable *Bacillus cereus* var. *toyoi* spores in the additive, in premixtures and in feedingstuffs fulfil the requirements to quantitatively determine, as part of a routine control, the colony forming units present in Toyocerin® in the proposed concentration range also in the presence of coccidiostats (diclazuril, narasin/nicarbacin and maduramicin ammonium) at levels typically applied in broiler feed. However, it is recommended to use a larger quantity of feed samples than the proposed 2 g, e.g. 50 g of feed samples for analysis as also suggested by referenced methods in the literature (3), to take account of potential sample heterogeneity.

For official control purposes a fully ring-trial validated method for enumeration of bacilli spores which was published in a peer reviewed journal (3) is recommended. Methods performance characteristics for the method using samples of premixture and feedingstuff include a RSD_r and a RSD_R of around 1 % and 6 %, respectively.

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

Information provided by the applicant in several parts:

- Analysis method for determination of viable *Bacillus toyoi* spores, active substance of the feed additive Toyocerin® (EC No. E1701). Rubinum, Barcelona, Spain.
- Analysis method for viable *Bacillus toyoi* in feed. Asahi Vet S.A., Barcelona, Spain.
- Compatibility of Toyocerin® with coccidiostats in chicken for fattening. Asahi Vet S.A., Barcelona, Spain. January 2005.
- Complementary information to application reference no. FAD-2005-0004. Asahi Vet S.A., Barcelona, Spain. March 2005.

- Complementary information to application reference no. FAD-2005-0004. Summary for publication. Rubinum, Barcelona, Spain. April 2005

Further information provided by applicant upon request:

- A response from the applicant (Rubinum, S.A.) concerning the particle size of the product and the homogeneity of the active substance observed in the final product. Email via JRC IRMM FSQ CRL, 9 June 2005.
- A response from the applicant (Asahi Vet S.A.) concerning the 'Validation of the method of analysis for determining *Bacillus toyoi* in the final product Toyocerin® 10^{10} and in the premixture Toyocerin® 10^{9}'. Email via JRC IRMM FSQ CRL, 29 June 2005.
- A response from the applicant (Rubinum, S.A.) concerning the 'Validation of the method of analysis for determining *Bacillus toyoi* in the premixture Toyocerin® 10^{9} and in the feed'. Email via JRC IRMM FSQ CRL, 11 October 2005.

Product samples have been made available to the CRL on 04/04/2005.

6. REFERENCES

(1) VDLUFA-Method 28.2.1 "*Bacillus cereus*" (2004), VDLUFA c/o LUFA Speyer, DE

(2) Leuschner, R.G.K., Bew, J., Domig, K., & Kneifel, W. (2002) J. Appl. Microbiol. 93, 781-786

(3) Leuschner, R.G.K., Bew, J., & Cruz, A. (2003) J. AOAC Int. 86, 568-575

Additional information on the active agent may be found in the 'First report of the Scientific Committee on Animal Nutrition on question 58 by the Commission on the use of spores of *Bacillus toyoi* in feedingstuffs for Calves (milk replacers), Cattle for fattening, Chickens for fattening, Laying hens, Piglets, Pigs, Rabbits and Sows for breeding. Opinion expressed on 13 January 1995' and the 'Report of the Scientific Committee on Animal Nutrition on product Toyocerin® for use as feed additive. Adopted on 5 December 2001'.

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Austrian Agency for Health and Food Safety, Vienna, Austria.