Renewal Assessment Report

Bacillus thuringiensis subsp. *aizawai strain GC-91*

- Agree 50 WG -

Volume 3 – B.5 Analytical methods

July 2018

Rapporteur Member State: The Netherlands

Co-Rapporteur Member State: Germany

Version history

When	What
July 2018	Initial RAR

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B Summary, evaluation and assessment of the data and information

Introduction

Bacillus thuringiensis subsp. *aizawai* GC-91 (in the following abbreviated as Bta GC-91) is a transconjugant strain originating from a Bta and a Bt subsp. *kurstaki* strain. Bta in general occurs ubiquitous in soils on plants as well as in infested insects. Bta acts highly specific against insect species of the order Lepidoptera and is not expected to have any harmful effects on beneficials and other non-target species of other insect orders. The insecticidal activity of Bta is mainly attributed to spore bound insecticidal pro-toxins (Cry toxins) which are ingested by the target pests and activated under alkaline conditions in the midgut of the larvae.

As the manufacturing process of Bta GC-91 has not been changed since original approval, all data submitted for the original approval of the strain are considered fully applicable for the current evaluation.

Besides new information, the submitted dossier includes all data, which have been presented in the DAR (Jan 2008) and DAR addendum (Nov 2012). This information is marked grey with a clear indication where the information is originating from.

B.5 Analytical methods

B.5.1 Methods for the analysis of the preparation

B.5.1.1 Methods for the identification and the determination of the content of the micro-organism(s) in the preparation

New data

Methods for identification of the strain are described in B.1.3.3.

An analytical method has been developed and validated for the determination of the content of active ingredient Bta GC-91 in the formulated product Agree 50 WG and in aqueous dilutions obtained after suspensibility and dispersibility tests.

The following analytical method for the determination of the active substance has not previously been reviewed and is provided in support of this assessment.

Report:	KMP 5.1/01, Coranelli, S. (2011)
Title:	Analytical method for the determination of the active ingredient content in the formulated product Agree WG and in aqueous dilutions
Document No:	Study BT064/11
Guidelines:	SANCO/3030/99 rev.4.
GLP	Yes

Material	Agree WG, Batch No. 4093650, containing *Bacillus thuringiensis aizawai* strain GC-91, content: 11 BIU/lb, 2.75×10^{10} CFU/g Reference item: Agree technical powder, Batch No. 041211, containing *Bacillus thuringiensis aizawai* strain GC-91, content: 22 BIU/lb, 5.6×10^{10} CFU/g Blank Formulation: Agree WG inert, Batch No. 106-48 Demineralized water and Standard Water D.
Principle of the method:	The active ingredient content was determined by colonies counting and reported as spore concentration. After a first dilution of the sample (100 times) the suspension was heat shocked for 45 min. at 65°C for killing of vegetative cells. Then sufficient dilutions were prepared to obtain 30 - 300 colonies. Namely, 0.1 mL of the already diluted dispersions were plated on nutrient agar (15 - 20 mL of agar were added). Finally, the Petri dishes were incubated at 30 ± 1°C for 24 hours and the colonies were counted. The calculation of spore/g was performed according to the following formula: [spore] = (dilution) × (mean number of colonies counted)/(weight or volume of sample) The method was validated with regard to specificity, linearity, precision and accuracy. For the validation of the method in aqueous dilutions (dispersions), the samples were prepared using Standard Water D.
Conclusion:	The analytical method is suitable and reliable for the determination of concentration of the number of spores of *Bacillus thuringiensis aizawai* strain GC-91 in the product Agree WG and in aqueous dilutions. The method was validated by definition of the linearity, the precision and the accuracy according to the criteria set by SANCO/3030/99 rev. 4.

Validation

The following validation of the analytical method for the determination of the active substances in the product and in aqueous dilutions has not previously been reviewed and is provided in support of this assessment.

Linearity over an appropriate range:

The linearity of the response was determined by analysing five solutions of reference item (technical powder) at different concentrations in demineralized water, for the formulated product-base matrix, and in Standard Water D, for the matrix consisting of aqueous dilutions.

Results validation in the formulated product:

The results were linear within the range between 0.1 and 4 g/L. The equation of the calibration line (n = 5) was found to be $y = 6 \times 10^{10} - 4 \times 10^{9}$ (correlation coefficient 0.9945). Where x is the concentration in g/L.

Results validation in the aqueous dilution:

The results were linear within the range between 0.1 and 4 g/L. The equation of the calibration line (n = 5) was found to be $y = 6 \times 10^{10} - 4 \times 10^{8}$ (correlation coefficient 0.9965). Where x is the concentration in g/L.

The results showed that the analytical method is linear in range of interest. The correlation coefficients meet the criterion $R > 0.99$.

Representative labelled documentation e.g. chromatograms

Tables containing all the data are submitted in the corresponding report.

Accuracy

The accuracy of the method was evaluated by a recovery assessment.

Results validation in the formulated product:

The mean recovery rate of samples fortified with the active ingredient (technical powder) at two concentration levels was 100.11%.

Results validation in the aqueous dilution:

The mean recovery rate of samples fortified with the active ingredient (technical powder) at two concentration levels was 101.76%.

The results showed that the analytical method is accurate according to the criteria set by SANCO/3030/99 rev. 4, for which the mean recovery accepted should be within 98.0 and 102.0% in case the nominal concentration of active ingredient is > 10%.

Repeatability (at least 5 determinations)

5 replicates independent sample determinations were prepared and measured to establish the precision of the analytical method.

Results validation of formulated product:

Mean Active ingredient content: 2.80×10^{10} spores/g ± RSD 2.28%, n = 5.

Results validation of aqueous dilution:

Mean Active ingredient content: 3.05×10^{8} spores/mL ± RSD 2.13%, n = 5.

Since the concentration of the test item is 1%, the value of RSD (coefficient of variation) shall be ≤ 2.68% according to the criteria set by SANCO/3030/99 rev. 4. The method is therefore considered precise to determine the active substance both in the formulated product Agree WG and in the aqueous dilutions.

Indication as to whether outliers identified have been discarded

Outliers did not occur.

Reasons for the occurrence of outliers

Not applicable: Outliers did not occur.

Table 5.1-1: Summary of validation of the method for the determination of Bta CG-91.

Reference	Linearity	Accuracy	Repeatability

		(Recoveries)	(Precision)
KMP 5.1/01	Calculated between 0.1 and 4 g/L, where x is the concentration in g/L: In the formulated product: $y = 6 \times 10^{10} - 4 \times 10^{9}$ (correlation coefficient 0.9945) In aqueous dilutions: $y = 6 \times 10^{10} - 4 \times 10^{8}$ (correlation coefficient 0.9965)	The accuracy of the method was evaluated by a recovery assessment on two fortification levels. High recovery values were obtained: In the formulated product: 100.11% In aqueous dilutions: 101.76%	The precision was evaluated upon measuring five samples from independent weightings. A high precision, expressed as coefficient of variation was found: In the formulated product: RSD 2.28% In aqueous dilutions: 2.13%

In conclusion, the analytical method is suitable and reliable for the determination of the concentration of the numbers of spores of *Bacillus thuringiensis* strain GC-91 in the product Agree WG and in aqueous dilutions (dispersions). Its validation, provided in support of this application, satisfies all requirements given by SANCO/3030/99 rev.4 guideline, concerning linearity, precision and accuracy.

B.5.1.2 Methods to establish regular control of the preparation to show that it does not contain other organisms than the indicated ones and to establish uniform

Relevant data on microbial contaminants have been provided for the technical material. See volume 3, B.5 MA.

B.5.1.3 Methods to identify any contaminating micro-organisms of the preparation

If any of the analyses performed during production of the technical material indicates a contamination, the batch is isolated and investigated. Depending on the results, the batch is either released (non-pathogenic and low titer), or inactivated and disposed off. As during the manufacture of Bta Tech products, human pathogens have never been associated with its manufacture (see further volume 4) and during storage of Agree WG at 20°C for 24 months, the biopotency of the product is maintained at a level meeting the specification of the product (see further B.2) no contaminating micro-organisms are expected and determination of contaminating micro-organism is considered not necessary in the preparation.

The relevant data on microbial contaminants have been provided for the technical material. See volume 3, B.5 MA.

B.5.1.4 Methods for the determination of relevant impurities or metabolites in the manufactured material

See volume 3, B.5 MA.

B.5.1.5 Methods used to determine the storage stability and shelf life of the preparation

See B.2.7 MP

B.5.2 Methods to determine and quantify residues (viable or non-viable)

Information from DAR and DAR addendum The active micro-organism(s) on and/or in crop, in foodstuffs and feeding stuffs, in animal and human body tissues and fluids, in soil, in water (including drinking water, ground water and surface water) and in air where relevant

Post-registration monitoring methods are different only in the isolation method that depends on the substrate that is being analysed. The method(s) used for subsequent determination and quantification are independent from the source of the isolates. Therefore, a determination method for *B. thuringiensis* ssp. *aizawai* strain GC-91 that can be used in all cases and that can as well be applied to any *Bacillus* isolate independent from its origin is presented hereafter.

Quantification of *B. thuringiensis* ssp. *aizawai* is accomplished by plating of bacteria suspensions as described in Ohba & Aizawa (1986) and Travers et al. (1987). Enrichment of spore-forming bacteria during heat treatment is followed by dilution plating on non-selective nutrient agar (Ohba & Aizawa 1986) or selective sodium acetate medium (Travers et al. 1987). Enumeration is accomplished by plating of different dilutions on appropriate media and counting of resulting colonies.

Identification at strain level is according to Hill et al (2004).

Report: Hill, K.K., Ticknor, L.O., Okinaka, R.T., Asay, M., Blair, H., Bliss, K.A., Laker, M., Pardington, P.E., Richardson, A.P., Tonks, M., Beecher, D.J., Kemp, J.D., Kolsto, A.B., Wong, A.C., Keim, P., Jackson, P.J. (2004). Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* isolates. Applied and Environmental Microbiology, 70, 1068-80.

Guideline: Not applicable

GLP: Not applicable

Principle: Bacteria are grown in liquid cultures and genomic DNA is isolated. Amplified fragment length polymorphism (AFLP) analysis is carried out by restriction of DNA with the restriction endonucleases EcoRI and MseI. Resulting fragments are ligated to double-stranded adapters. Primers binding to the adapter sites are then used to amplify the DNA by PCR. In a second PCR, fragments are amplified with specific 6'-carboxyfluorescein-labelled primers as described. Detection of amplified DNA is accomplished with fluorescence detection after gel electrophoresis. Every strain then gives a typical profile of amplified DNA fragments that is characteristic for this strain.

Comment: the method does not allow to discriminate GC-91 from other Bta strains unequivocally.

New data:

For a new identification method at strain level it is referred to Vol 3 MA B.5.1.1.

Bacillus thuringiensis subsp. *aizawai* GC-91, such as all Bta strains currently registered at EU level, was proposed for inclusion into Annex IV of Regulation (EC) No 396/2005. This means that no residue definition applies to the microorganism and no MRL is set for any of the existing or intended uses. This issue, however, is still under discussion. For more information, please refer to information provided for the MA in Section M-MA,

Section 1, Point MA 2.8. In principle, strain specific methods are available for monitoring of the strain on treated plants. Please refer to Volume 4 and volume 3 B.5 MA.

B.5.3 References relied on

Data point CADDY (ongoing numbering)	Author(s)	Year	Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previously submitted Y/N* If Y => old data point
KMP 5.1/01	Coranelli, S.	2011	ANALYTICAL METHOD FOR THE DETERMINATION OF THE ACTIVE INGREDIENT CONTENT IN THE FORMULATED PRODUCT AGREE WG AND IN AQUEOUS DILUTIONS Certis Europe B.V., NL, BT064/11 Biotecnologie BT Srl, Fraz. Pantalla, Italy GLP: yes Published: no	no	yes	New data for existing formulation, not previously submitted nor evaluated	CER	N
KMP 5.2/01	Ohba, M., Aizawa, K.	1986	DISTRIBUTION OF BACILLUS THURINGIENSIS IN SOILS OF JAPAN not available, not applicable Journal of invertebrate Pathology, 47, 277-282 GLP/GEP: no Published: yes	no	no	not protected	-	Y
KMP 5.2/02	Travers, R.S., Martin, P.A.W., Reichelderfer, C.F.	1987	SELECTIVE PROCESS FOR EFFICIENT ISOLATION OF SOIL BACILLUS SPP. not available, not applicable Applied and Environmental Microbiology, 53, 1263-1266 GLP/GEP: no Published: yes	no	no	not protected	-	Y

Data point CADDY (ongoing numbering)	**Author(s)**	**Year**	**Title Owner, Report No. Source (where different from owner) GLP or GEP status Published or not**	**Vertebrate study Y/N**	**Data protection claimed Y/N**	**Justification if data protection is claimed**	**Owner**	**Previously submitted Y/N* If Y => old data point**
KMP 5.2/03	Hill, K.K., Ticknor, L.O., Okinaka, R.T., Asay, M., Blair, H., Bliss, K.A., Laker, M., Pardington, P.E., Richardson, A.P., Tonks, M., Beecher, D.J., Kemp, J.D., Kolsto, A.B., Wong, A.C., Keim, P., Jackson, P.J.	2004	FLUORESCENT AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF BACILLUS ANTHRACIS, BACILLUS CEREUS, AND BACILLUS THURINGIENSIS ISOLATES not available, not applicable Applied and Environmental Microbiology, 70, 1068-1080 GLP/GEP: no Published: yes	no	no	not protected	-	Y