Renewal Assessment Report

Bacillus thuringiensis subsp. *aizawai strain GC-91*

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 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.

As agreed with the RMS, 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 micro-organism as manufactured

B.5.1.1 Methods for the identification of the micro-organism

Information from DAR

Bacillus thuringiensis ssp. *aizawai* strain GC-91 can be isolated from different substrates using one of two standard methods for *Bacillus thuringiensis*. Both are based on enrichment of spore-forming bacteria during heat treatment 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 of the strains can be accomplished by AFLP analysis as described by Hill et al., (2004) and comparison of the fingerprint with previous results.

Morphological and biochemical characterization

The strain GC-91 was analysed due to its morphological and biochemical properties (Verma, 1991). The results of the various test methods provide information about growth conditions and properties, enzymatic activity and morphological characteristics, like size and shape of cells and spores and the presence of parasporal crystal proteins. The strain is a spore forming and gram+ bacilli, generating bipyrimidal and cuboidal parasporal crystal proteins.

Serotyping

According to the results of a flagella antigen serotyping of *B. thuringiensis* aizawai GC-91 indicating the kind of subspecies using analytical methods described by de Barjac (1981), strain GC-91 can be classified as serotype H:7 (Verma, 1991).

Fatty Acid Analysis

An analysis of the fatty acid composition of Bta strain GC-91, and Btk strains SA-11, SA-12 was conducted by FAME-GC using the identification software Sherlock Version 4.5. The three strains were compared to the fatty acid profile of *B. thuringiensis kurstaki* strain HD-1.

DNA fingerprinting

DNA fingerprinting for each single strain was generated by AFLP and PCR analysis (Jackson et al., 1999; Hill et al., 2004). The data generated by AFLP analysis contain fragment sizes with each fragment having a peak height. The chromatograph shows a high similarity of the strains regarding the average of the DNA fragments sizes and their intensity (Chen, 2005a).

A phylogenetic tree was generated by Jackson et al. (2005) at the Lawrence National Laboratory, Livermore CA and Los Alamos National Laboratory, Los Almos, NM, USA, based on the analytical methods of Hill et al. (2004) and including the commercially important serovars of *B. thuringiensis* and strains of *B. cereus* and *B. anthracis* isolates.

Plasmid analysis

Another method to distinguish different strains is the analysis of the plasmid profile. The plasmid profile of Bta strain GC-91 was analysed in comparison to several other strains using agarose gel electrophoresis in 0.5 % agarose with a horizontal submarine apparatus in electrophoresis buffer. The analyses were conducted in accordance with the methods of Gonzalez & Carlton (1980).

New data

During original approval of Bta GC-91, a set of methods was used for identification purposes. However, for renewal of the strain, an attempt was made to develop strain specific markers based on the whole genome and plasmids sequencing of Bta GC-91 and comparison to available Bt genomes in open data bases. The study was carried out using next generation sequencing representing most recent developments in the area of molecular biology. In the study, strain specific primers were developed and tested for:

- Specificity by testing it in 16 reference strains, covering other commercial Bt strains, as well as type strains of different Bt subspecies and pathogenic *B. cereus* strains

- Reproducibility by running independent experiments (triplicate).

Based on the results it can be concluded that four highly specific primer pairs are available allowing an unequivocal identification of Bta GC-91.

Considering that the analysis was done according to standard molecular biological methods, which are described in the published literature and used by researchers, including the lab where the study was carried out, since years or even decades it is considered that they are sufficient to cover the data requirement and a validation according to SANCO/3030/99 is not required. A full description of the study is provided in Volume 4.

B.5.1.2 Methods for providing information on possible variability of seed stock/active micro-organism

Information from DAR

Alteration of Bta through conjugation is highly unlikely, as conjugation requires close contact between two bacteria cells. Due to agitation and aeration, shearing between cells is too high for such contact. If they should occur, spontaneous changes can be detected by the methods. Bacteriophage sensitivity profile, fermentation analysis, SDS-PAGE analysis of the toxin, confirmation of serotype and subspecies, PCR analysis of the toxin genes, insecticidal host range confirmation, and plasmid analysis. Three slants from each working culture line are grown under simulated production conditions and analysed microscopically, for pH, spore yield and bioactivity against *Trichoplusia ni*. Working cultures are only released for production if all values are within Statistical Process limits. For further details refer to Chen & Hargrove (2003) in C.1.1.3 in Volume 4.

B.5.1.3 Methods to differentiate a mutant of the micro-organism from the parent wild strain

Information from DAR

GC-91 is not a mutant but a transconjugant strain. The strain GC-91 is a product of or conjugation between two parental strains that differ in their flagella serotype as well as in their δ-endotoxin genes. The actual event was the transfer of two plasmids carrying a Cry1Ac and a Cry2A δ-endotoxin gene, respectively, from the strain HD-191-A2 (flagella serotype kurstaki) into the strain HD-135-S4 (flagella serotype *aizawai*), which already carried δ-endotoxin genes of the Cry1C and Cry1D types. So the strain GC-91 has the *aizawai* flagella serotype (H 7) and carries the Cry1Ac, Cry1C, Cry1D and Cry2A genes. The resulting strain is called a transconjugant, a strain incorporating genes from two different Bt strains. This strain has flagella serotype *aizawai* and δ-endotoxin genes mixture *kurstaki-aizawai*. Methods for the analysis of the flagella serotype, the plasmid content, and identification of the strain are reported in Volume 3 B.1 and above in B.5.1.1.

B.5.1.4 Methods for the establishment of purity of seed stock from which

batches are produced and methods to control that purity

See C.1.1.3 in Volume 4.

B.5.1.5 Methods to determine the content of the micro-organism in the manufactured material used for the production of formulated products and methods to show that contaminating micro-organisms are controlled to an acceptable level

Information from DAR

Content of MPCA

The insecticidal activity (Potency in IU / mg) is not correlated with the amount of CFU/g MPCA. Potency in a batch is determined by a bioassay with the target organisms *Spodoptera exigua* and *Trichoplusia ni*. Determination of the number of viable spores is done by heating of spore suspensions for 45 min at 65 °C, plating of different dilutions of and counting of colonies. See also Iqbal & Chen (2005) in C.1.2.1 in Volume 4.

Biological Activity determination - Potency

Two samples are selected from each lot group for biotests and biochemical analyses. Potency is determined in bioassays against *Trichoplusia ni*, methods [redacted] respectively (Chen & Hargrove, 2003).

The content of δ-endotoxins is determined by SDS-PAGE. Proteins are denatured and separated in a polyacrylamide gel. After staining, gels are scanned and quantities are calculated from the intensity of the bands. For further details, please refer to Chen (2005 b). Values must fall within the manufacturing specifications and [redacted] If either the physical or biological parameters are not met, product is either reworked or disposed off.

Content of additives (preservatives, stabilisers, diluents)

Not applicable, because the MPCA does not contain any additives (refer to Doc J-IIM, Point IIM 1.4.1).

Content of microbial impurities, classified/identified to a taxonomic level required by quality criteria to support the hygienic state of the production process

Contamination tests are performed on all stages of fermentation starting from seed flask up to production fermenters (Chen & Hargrove, 2003). Each batch of the dried technical powder is subjected to the following quality control tests:

• Physical tests (moisture, bulk density, appearance, pH)

• Microbial contamination test

• Microscopic examination

• Absence of human/animal pathogens

• Absence of B. anthracis by subcutaneous injection of at least 1 million spores per mouse

• Absence of ß-exotoxins by fly larval assay and HPLC with concentrations of technical material at 20,000 ppm (detection limit of ß-exotoxin by fly larvae assay is 0.028 µg/mL)

• Potency / Biological activity determination by bioassay or protein assay (bioassay on Trichoplusia ni, SDS-PAGE)

The identification of a cross-contaminated fermentation is done utilizing the Fermentation Monitoring Program (FMP) testing procedures (refer to Doc J-IIM, Point 1.4.3.1). If contamination occurs at exponential phase, the

resultant pH, and dO2 profiles and FMP results will indicate an atypical fermentation and will be handled accordingly (fermentation broth will be disposed). For detection of contamination in the stationary phase, the maximum allowed titre is less than 1.2 x 10^3 CFU / mL or 0.12 ppm. Contamination below this level is considered insignificant.

Contamination during the fermentation cycle and / or down-stream processing is prevented or minimized and detected by the following procedures and precautions:

- Sterile fermentation media and tanks
- Use of pre-sanitized equipment during down-stream processing (tanks equipped with a CIP system)
- FMP testing (Fermentation Monitoring Program)
- Low processing pH to inhibit bacterial growth
- Use of cooled holding/recovery tanks to inhibit bacterial growth
- Monitoring of production batches for certain human pathogens, i.e. *Salmonella, Shigella, Vibrio*.

New data

Additives

The technical material of Bta GC-91 does not contain any additives.

Microbial contaminants

As the production process of the technical material, including fermentation of the strain, has not changed since the first evaluation (please see the certificate of the manufacturer, Chen 2016a), all information submitted previously is still considered valid. However, when the strain was evaluated first, there was no specific guidance for the determination of microbial contaminants available. To meet current requirements for allowed densities of microbial contaminants in microbial plant protection products as laid down in SANCO/12116/2012-rev. 0 (September 2012), new five batch data are provided for microbial contaminants in the technical material (Rodriguez, 2016). For the complete profile of five recent production batches please refer to Volume 4.

In the table below the indicator organisms which were screened for, detection limits and indication if the data are compliant with SANCO/12116/2012-rev. 0 (September 2012) are provided.

Table 5.1.5-1 Microbial contaminant screening

Indicator species	Method	Detection limit	SANCO/12116/2012-rev. 0 limit (compliant/not compliant)	Comment
Coliforms	BAM Chapter 4	< 10 CFU/g	< 10 CFU/g	Compliant
E. coli	USP 38 Method 62	Absence in 10 g	Absence in 1 g	Compliant
Listeria	AOAC 2004.06	Absence in 25 g	Absence in 25 g	Compliant
Salmonella	USP 38 Method 62	Absence in 10 g	Absence in 25 g	Lower amount of tested substance
Shigella	BAM Chapter 6	Absence in 25 g	Absence in 25 g	Compliant
Staphylococcus aureus	USP 38 Method 62	Absence in 10 g	Absence in 1 g	Compliant
Vibrio cholera	BAM Chapter 9	Absence in 10 g	Absence in 25 g	Lower amount of tested substance
Mold	BAM Chapter 18	Determination CFU/g	< 1000 CFU/g	Compliant
Yeast	BAM Chapter 18	Determination CFU/g	< 1000 CFU/g	Compliant

Indicator species	Method	Detection limit	SANCO/12116/2012-rev. 0 limit (compliant/not compliant)	Comment
Mouse IP/SC	Internal method	10^6 CFU/mouse	-	Absence of *B. anthrax*

The tested indicator species are in compliance with those indicated in SANCO/12116/2012-rev. 0 (September 2012). Although there are some trigger values which were not fully met (*Salmonella, Vibrio*) available data are considered acceptable as they demonstrate absence of a broad range of critical contaminating microorganisms. Total aerobic and anaerobic counts have been also performed but are not presented as they would count the active ingredient Bta GC-91 also and are thus not required for spore forming bacteria.

A screening for microbial contaminants has been carried out in the technical material of Bta GC-91. The results are in compliance with SANCO/12116/2012 (see Volume 4).
The below mentioned methods were used to screen the technical material of Bta GC-91 for the presence of microbial contaminants. The methods used are standard microbiological methods comparable to EN ISO methods and are as such considered validated.

Coliforms (BAM Chapter 4):
Solid medium method coliforms: Add 450 mL of Butterfield´s Phosphate Buffered Water (BPBW) to 50 g of the sample (or adequate portions to obtain a 1:10 dilution of the test item). Serial dilutions in BPBW and prepared and. 1 mL of each dilution is transferred to a sterile petri dish. Afterwards 10 mL of Violet Red Bile Agar (VRBA) are than poured into the plates and incubated for 18-24 hours at 35°C after solidification. Purple-red colonies are counted and subjected to conformation if required. All details are provided in "BAM Chapter 4", submitted in KMA 4.1/01).

E. coli **(USP 38 Method 62):**
A sample is prepared using a 1:10 dilution of not less than 1 g of the product and 10 mL or the quantity corresponding to 1 g or 1 mL, to inoculate a suitable amount of Soybean–Casein Digest Broth. The suspension is mixed and incubated at 30° to 35° for 18 to 24 hours. Afterwards 1 mL of the pre-culture is added to 100 mL of MacConkey Broth, and incubated at 42° to 44° for 24 to 48 hours. When subcultures are plated on MacConkey Agar they need to be incubated at 30° to 35° for 18 to 72 hours. Growth of colonies indicates the possible presence of E. coli. This is confirmed by identification tests. All details are provided in "USP 38 Method 62", submitted in KMA 4.1/02).

Listeria **(AOAC 2004.06):**
Listeria is screened for using the VIDAS LIS test kit form Biomerieux. Therefore, 25 g of the test item are suspended in 225 mL Fraser broth supplied with the kit, pre-warmed at room temperature and incubated at 30°C for 25 hours. Afterwards 1 mL of the culture is transferred to Fraser broth without ferric ammonium citrate and incubated another 25 hours at 30°C. Afterwards 1 mL aliquot is heat treated for 15 min at 95-100°C and subjected to a test with the VIDAS kit according to the package insert. If positive results are obtained the presence of *Listeria* is confirmed by enrichment in selective media, e.g. *Listeria* chromogenic agars. All details are provided in "AOAC 2004.06", submitted in KMA 4.1/03).

Salmonella **(USP 38 Method 62):**
10 g of the sample are inoculated with a suitable amount of Soybean–Casein Digest Broth, mixed and incubated at 30° to 35° for 18 to 24 hours. 0.1 mL of the pre-culture are added to 10 mL of Rappaport Vassiliadis *Salmonella* Enrichment Broth, and incubate at 30° to 35° for 18 to 24 hours. Subcultures on plates of Xylose Lysine Deoxycholate Agar are incubated at 30° to 35° for 18 to 48 hours. The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centres. This is confirmed by identification tests. All details are provided in "USP 38 Method 62", submitted in KMA 4.1/02).

Shigella **(BAM Chapter 6):**
Twenty five g of the sample are suspended in 225 mL *Shigella* broth (with 3.0 µg/mL novobiocin). After 10 min. shaking at room temperature the supernatant is poured into a new flask and anaerobically incubated at 42°C for 20 hours. Enrichment cultures are than streaked to MacConkey agar and incubated for additional 20 hours ate 35°C. *Shigella* colonies are slightly pink and translucent with or without round edges. Suspicious colonies are subjected isolation and confirmation procedures if required. All details are provided in "BAM Chapter 6", submitted in KMA 4.1/04).

Staphylococcus aureus **(USP 38 Method 62):**
Not less than 1 g of the product is added to 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount of Soybean–Casein Digest Broth. The suspension is homogenized and incubated at 30° to 35° for 18 to 24 hours. The subculture is than plated on Mannitol Salt Agar and incubated at 30° to 35° for 18 to 72 hours. The possible presence of *S. aureus* is indicated by the growth of yellow or white colonies surrounded by a yellow zone. This is confirmed by identification tests. All details are provided in "USP 38 Method 62", submitted in KMA 4.1/02).

Vibrio cholerae **(BAM Chapter 9):**
Twenty five g of the sample are suspended in Alkaline Peptone Water (APW) and incubated at 35°C for 6-8 hours. A 3-mm loopful from the surface pellicale of the APW culture are transferred to Thiosulfate Citrate Bile Salts Sucrose (TCBS) plates in a manner that single colonies can be obtained and incubated overnight at 35°C. typical colonies of *V. cholerae* are large, smooth, yellow and slight flattened with opaque centres and translucent peripheries. Such colonies would be submitted to biochemical identification/screening and confirmation. All details are provided in "BAM Chapter 9", submitted in KMA 4.1/05).

Mold/yeast (BAM Chapter 18):
A ten-fold dilution of the test item is prepared in 0.1% Peptone Water, homogenized and serial dilutions are than prepared in the same medium. 0.1 mL aliquots of the dilutions are pipetted to Dichloran Rose Bengal Chloramphenicol (DRBC) agar and incubated at 25 °C for 5 days. If there are no colonies present, re-incubate for another 48 hours. Individual colonies can be isolated for species identification if required. All details are provided in "BAM Chapter 18", submitted in KMA 4.1/06).

B.5.1.6 Methods for the determination of relevant impurities in the manufactured material

Information from DAR
Content of non-microbial impurities (e.g. metabolic products, impurities in starting materials, fermentation residues, extraneous host residues)

Due to the fermentation process, the technical product is composed of fermentation solids, spores and insecticidal toxins (Dively, 2005). After the fermentation process the MPCA is spray dried and subsequently processed to the formulated end product. The production batches are analysed regularly for the content of non-microbial impurities using the Fermentation Monitoring Program (FMP) testing procedures (refer to Doc J-IIM, Point 1.4.3.1).

B.5.1.7 Methods to control the absence and to quantify (with appropriate limits of determination) the possible presence of any human and mammalian pathogen

See B.5.1.5

B.5.1.8 Methods to determine storage stability, shelf-life of the micro-organism, if appropriate

See B.5.1.5.

B.5.2 Methods to determine and quantify residues (viable or non-viable) of the active micro-organism

No information was provided in the DAR.

New data

No specific MRL was fixed for the active substance under Reg. (EC) No 396/2005, according to Art. 18(1)(b) of that Regulation. Up till now *Bacillus thuringiensis* subsp. *aizawai strain* GC-91 is not included in Annex IV due to delay at EFSA. Moreover, the default MRL of 0.01 mg/kg is not applicable because agencies are not used to follow enforcement or maintenance procedures for micro-organisms. Therefore, provided that no MRLs are set in Reg (EC) No 396/2005, no analytical methods are required for monitoring of residues in food and feed as well as in water, soil and air samples."

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 Doc J, Point MA 1.3.

Measurements of Cry proteins in the environment have gained more and more attention. Below two studies from the open literature are presented that provide a description of a validated analytical method for analysis of soil and water.

Soil

Mueting et al., (2014) provided a validation of an extraction method for soil. A selection of three soils was used for the study. Soils were air dried, homogenized, and verified for the absence of Cry1Ab before use. To extract Cry1Ab from soil, 0.5 g (wet mass) of soil was weighed into a 1.5 mL microcentrifuge tube and 1 mL of 10 × PBST (phosphate buffered saline with tween) was added to the tube. The non-interference of the 10 × PBST with the commercial ELISA kit used for quantification was tested in a preliminary experiment. Samples were extracted 3 times and the supernatant was combined. An aliquot of the pooled supernatant was added to the ELISA plate. In order to verify that complex soil matrices do not interfere with the quantification of Bt Cry1Ab, a series of matrix testing experiments was conducted. Un-spiked soil was extracted using the method described above to obtain blank soil matrix. Standard curves using purified Cry1Ab were created in blank matrix diluted with 1 × PBST assay buffer to obtain 25, 50, and 100% matrix and all 3 were compared to a standard curve in 1 × PBST assay buffer. An absorbance relative percentage of difference (RPD) of ≤ 15% from the 1 × PBST curve was indicative of no matrix effects. Purified Cry1Ab protein standard was added to the 3 soils at a concentration of 160 ng/g, extracted, and then linearly diluted and run on the ELISA plate. Dilutions were above, within, and below the standard curve to determine the quantitative range. All in-range dilutions had to be consistent with a CV of ≤ 20%. The extracts were also quantified on a dot blot for further confirmation of the ELISA results using at least 2 dilutions within the range of quantification. Fortification recovery was conducted by adding the purified Cry1Ab protein standard to the extraction buffer then adding that buffer to a pre-weighed clean soil sample and conducting the entire extraction procedure. Three concentrations of Cry1Ab were extracted including 14, 37.5, and 70 ng/g, which represented the range of the standard curve and these extractions were run in triplicate at each level with an acceptance criteria of 70 to 120% recovery of the protein and a CV of ≤ 20% across levels and days One analyst repeated the extractions on 3 separate days. Recoveries below 70% may be acceptable if results are reproducible and consistent (CV ≤ 20%) The extraction efficiency test was the final component of the method validation. Dry reference soils were spiked with purified Cry1Ab protein standard at 3 concentrations representative of the range of the standard curve and extracted using the method described above. To determine the amount of protein remaining bound to the soil after 4 extractions with 10 × PBST, a fifth extract was conducted using a high salt-high pH buffer containing 50 mM sodium borate, 0.75 M KCl, 10 mM ascorbic acid, 0.075% Tween-20 at pH = 10.5 and was quantified on a dot blot assay. The 10 × PBST extracts and the fifth extract with the high salt-high pH buffer were run separately on the dot blot and the 2 values were combined to determine overall extraction efficiency. The buffer used for the fifth extract is harsher than 10 × PBST and exhibited interference with the ELISA plates. Therefore, the high salt- high pH buffer could not be used as the primary extraction buffer. For every 18 soil samples processed on an ELISA plate, a lab blank consisting of a known blank soil, and matrix spike (MS) and matrix spike duplicate (MSD), which consisted of a sample from the batch spiked with a known amount of purified Cry1Ab protein standard were processed. A plate passed the quality assurance test if the RPD between the MS and MSD was below 20%. The lower limit of quantification (LOQ) was defined as the lowest point on the standard curve that was measured with acceptable precision and

accuracy, which was usually the absorbance below the 0.25 ng/mL protein standard. Soil samples with absorbencies below this point were designated as < LLOQ.

Results

The 10 × PBST extraction buffer caused no significant interferences with the ELISA or dot blot in the matrix test or the buffer dilution agreement test. For soil 1 the RPDs comparing the assay buffer standard curve and the curve at the 100% matrix level were above 15% indicating there was interference with this matrix on the ELISA plate. All 3 soils passed the matrix dilution agreement tests by having a CV of less than 20% between dilutions for the calculated adjusted result without any indication of a required minimum dilution. Fortification recovery and extraction efficiency tests indicated that the matrix of Soil 1 affected Cry1Ab recovery (Figure 4.2-01) with only 51 and 41% recoveries being found for these tests across all spiking levels, respectively. This was considered acceptable however, due to its consistency with CVs less than 20% despite being below the 70% recovery limit. Acceptable recoveries of Cry1Ab were found for the other 2 soils and recoveries were consistent across extractions. An additional extraction with a high salt- high pH extraction buffer was used to determine the remaining amount of Cry1Ab in soil after the 10 × PBST extraction. Quantification of the fifth extraction with a dot blot assay revealed that almost 50% of the Cry1Ab remained bound to Soil 1 following the 4 extractions in 10 × PBST. Only 25% of Cry1Ab remained in Soil 2 and 4% in Soil 3 after the final extraction with 10 × PBST. However, despite higher extraction efficiencies the high salt-high pH buffer had significant interaction effects with the ELISA plate.

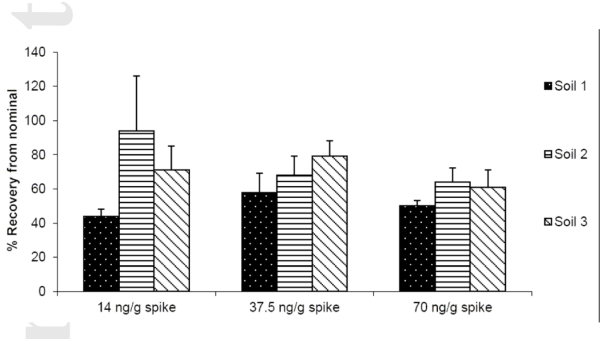

Figure 5.2-01 Results from fortification recovery tests in which purified Cry1Ab protein standard was added to the extraction buffer and the spiked buffer was then added to soils and the extraction procedure was conducted. Mean recoveries across the three spiking levels were 51, 75, and 70% for Soils 1, 2, and 3, respectively. Error bars indicate ± standard deviation.

Water

Strain et al., (2014) published a study on optimisation and validation of the commonly used ELISA method. The study describes a validated method for the extraction and quantitation of Cry1Ab proteins in water. Three water matrices were selected based on ecological relevance and a wide range of physiochemical characteristics. The ELISA method was validated for specificity, accuracy, precision, stability, and sensitivity, and then was demonstrated in field and aquatic bioassay samples. Lyophilization and filter centrifugation methods were adapted from the literature and optimized for the extraction of Cry1Ab protein from water samples. A 30 mL aliquot of each reference water was added to three 50 mL conical tubes for each method and each tube was spiked with purified Cry1Ab protein at 167.5 ng/L. Samples were processed via lyophilization and using a filter centrifugation method. All samples were stored at 4 ± 1°C and quantified using ELISA within 24 h. Triplicate sample values were averaged to determine the percent recovery per matrix for each extraction method. The specificity of the ELISA method used was determined. A five-point curve ranging from 0.5 to 10 µg/L was created by spiking 100% matrix with purified Cry1Ab protein and serially-diluting to obtain the desired concentrations. The 100% matrix was also diluted with PBST assay buffer to obtain a curve in 50% matrix at each concentration. All samples were processed using ELISA and a standard curve in PBST assay buffer was used to generate predicted concentrations for each point on the standard curves in 100 and 50% matrix. Precision was determine by spiking reference water with Cry1Ab protein and prepare a serial dilution from above the upper limit of quantification to below the lower limit of quantification. The stability of the Cry1Ab protein in the spiked water samples was determined after storage at: -80, -20, 4, or 23°C in order to determine appropriate storage conditions for a two week holding time. A 30 mL aliquot of each reference water was spiked with Cry1Ab protein at 167.5 ng/L in triplicate for each storage temperature, and maintained at that temperature for 14 days ± 1 day. Following the storage period, all samples were processed simultaneously using the filter centrifugation method with three 'control' replicates that were spiked using the same stock protein on the day of the extraction. Extracts were analyzed via ELISA as previously described and recoveries were determined and compared with one-way ANOVA using SAS software. Sensitivity was determined by spiking 7 samples of each reference water near the LLOQ, extracted and quantified. A seven-point standard curve ranging from 0.1 to 10 µg/L. was prepared. Each sample was run on the ELISA plate in triplicate along with a blank of the same matrix. The CV was calculated for each sample, with a CV < 20% deemed acceptable within a sample.

Results

The mean recoveries of Cry1Ab protein from groundwater, runoff, and river waters using the freeze-drying extraction method were low at 10.9, 54.1, and 14.7%, respectively, with an overall mean of 26% among all three matrices. In contrast, the recoveries using the filter centrifugation method were much higher at 59.4, 95.5 and 79.2%, with a mean of 78%. These findings indicate superior extraction efficiencies of Cry1Ab protein using filter centrifugation over freeze-drying, and therefore this method was chosen for further validation. The results of the matrix testing experiments showed no matrix effects in groundwater or runoff water (Figure 4.2-02). Minor matrix effects were observed in 100% river water, with an average RPD (relative percentage of difference) from the control near 30%. Matrix hindrance was improved when river water was diluted to 50%, however the RPD (20%) still indicated slight matrix effects (Figure 4.2-02).

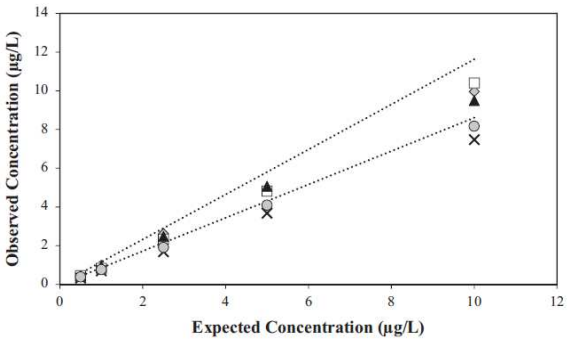

Figure 4.2-02 Results of matrix testing of standard curves in phosphate buffered saline plus tween (PBST) buffercontrol (◊), 100% groundwater (□), 100% runoff water (▲), 100% riverwater (x), and 50% riverwater (●) matrices. The dotted lines represent the range of acceptance indicating no matrix effects.

The results from the matrix dilution agreement experiments support a high level of precision using the ELISA quantitation method. The CV's for groundwater, runoff water and river water were5.0, 9.7 and 9.4%, respectively. The results of the stability experiment revealed a highly-significant effect of matrix ($F_{2,30}$ = 233, P < 0.01), temperature ($F_{4,30}$ = 199, P < 0.01) and matrix × temperature interaction ($F_{8,30}$ = 4.9, P < 0.01) on recoveries of Cry1Ab protein. There was no significant difference between water samples frozen at -80 or -20°C, which had acceptable recoveries relative to the control group. Samples stored at 4 and 23°C had low recoveries. The method detection limits (MDLs) for the detection of Cry1Ab protein in groundwater, runoff water, and river water were 1.7, 2.1 and 0.9 ng/L, respectively. The MDL was measured at 2.1 ng/L and a reporting limit (RL) of 6.3 ng/L was used for the quantitation of Cry1Ab proteins in all water samples.

Cited references:

Reference	**KMA 4.2/01**
Report	Mueting S.A., Strain K.E., and Lydy M.J., 2014 Validation of an extraction method for Cry1Ab protein from soil Published report Environmental Toxicology and Chemistry, 33(1):18-25
Guideline:	Not applicable
GLP:	No
Abstract:	Corn expressing insecticidal proteins derived from Bacillus thuringiensis (Bt corn) has increased in usage in the United States from 8% of total corn acreage in 1996 to 67% in 2012. Due to this increase, it is important to be able to monitor the fate and transport of the insecticidal Bt proteins to evaluate environmental exposure and effects. Accurate and validated methods are needed to quantify these proteins in environmental matrices. A method to extract Bt Cry1Ab proteins from 3 soil types using a 10 × phosphate buffered saline with tween (PBST) buffer and a commercially available enzyme-linked immunosorbent assay (ELISA) was validated through a series of 6 tests. The validation process for Cry1Ab extractions in soil has not yet been reported in the scientific literature. The extraction buffer and each soil matrix was tested and validated for the ELISA analysis. Extraction efficiencies were 41, 74 and 89% for the 3 soil types and were significantly correlated with the organic matter content of the soil. Despite low recoveries, consistent results with low coefficients of variation allowed for accurate measurements. Through validating this method with 3 different soils, a sensitive, specific, precise, and accurate quantification of Bt-Cry1Ab was developed. The validation process can be expanded and implemented in other environmental matrices, adding consistency to data across a wide range of samples.

Reference	**KMA 4.2/02**
Report	Strain K.E., Whiting S.A. and Lydy M.J., 2014 Laboratory and field validation of a Cry1Ab protein quantitation method for water Published report Talanta, 128: 109–116
Guideline:	Not applicable
GLP:	No
Abstract:	The widespread planting of crops expressing insecticidal proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt) has given rise to concerns regarding potential exposure to non-target species. These proteins are released from the plant throughout the growing season into soil and surface runoff and may enter adjacent waterways as runoff, erosion, aerial deposition of particulates, or plant debris. It is crucial to be able to accurately quantify Bt protein concentrations in the environment to aid in risk analyses and decision making. Enzyme-linked immuno sorbent assay (ELISA) is commonly used for quantitation of Bt proteins in the environment; however, there are no published methods detailing and validating the extraction and quantitation of Bt proteins in water. The objective of the current study was to optimize the extraction of a Bt protein, Cry1Ab, from three water matrices and validate the ELISA method for specificity, precision, accuracy, stability, and sensitivity. Recovery of the Cry1Ab protein was matrix-dependent and ranged from 40 to 88% in the validated matrices, with an overall method detection limit of 2.1 ng/L. Precision among two plates and within a single plate was confirmed with a coefficient of variation (CV) less than 20%. The ELISA method was verified in field and laboratory samples, demonstrating the utility of the validated method. The implementation of a validated extraction and quantitation protocol adds consistency and reliability to field-collected data regarding transgenic products.

B.5.2.1 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 (i.e. viable residues)

See B.5.2

B.5.2.2 Relevant metabolites (especially toxins) 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 (i.e. non -viable residues)

SeeB.5.2

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
KMA 4.1/01	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 KIIM 4.3.1
KMA 4.1/02	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 KIIM 4.3.1
KMA 4.1/03	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 KIIM 4.3.1

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
KMA 1.3/03	Verma, M.	1991	CONFIDENTIAL APPENDIX TO VOLUME OF SUBMISSION TECHINCAL CGA-237218 PRODUCT CHEMISTRY Certis USA LLC, PC-91-005 Agricultural Division Ciba-Geigy Corporation, Greensboro, NC GLP/GEP: no Published: no	no	yes	protected	CEU	Y KIIM 1.3.3
KMA 4.1/05	de Barjac, H.	1981	Identification of H-serotypes of Bacillus thuringiensis not available, not applicable Microbial control of pests and plant diseases, 35-43 GLP/GEP: no Published: yes	no	no	not protected	-	Y
KMA 4.1/06	Jackson, P.J., Hill, K.K., Laker, M.T., Ticknor, L.O., Keim, P.	1999	Genetic comparison of Bacillus anthracis and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis not available, not applicable Journal of Applied Microbiology, 87, 263-269 GLP/GEP: no Published: yes	no	no	not protected	-	Y
KMA 1.3/10	Chen, C.Y.	2005a	FINGERPRINTS OF BACILLUS THURINGIENSIS Certis USA LLC, not applicable not available GLP/GEP: no Published: no	no	yes	protected	CEU	Y KIIM 1.3.3
KMA 1.3/11	Jackson et al.	2005	AFLP-BASED PHYLOGENETIC COMPARISIONS OF CERTIS SAMPLES TO OTHER B. THURINGIENSIS, B. CEREUS AND B. ANTHRACIS ISOLATES Certis USA LLC, not applicable Los Alamos National Laboratory, Los Alamos, NM, USA GLP/GEP: no Published: no **Submitted in: KMA 1.3**	no	yes	protected		

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
KMA 4.1/10	González, J.M.,Carlton, B.C.	1980	Pattern of plasmid DNA in crystalliferous and acrystalliferous strains of Bacillus thuringiensis not available, not applicable Plasmid, 3, 92-98 GLP/GEP: no Published: yes	no	no	not protected	-	Y
KMA 4.2/01	Mueting, S.A., Strain, K.E., Lydy, M.J.	2014	Validation of an Extraction method for Cry1Ab Protein from soil not available, not applicable Environmental Toxicology and Chemistry, 33, 18-25 GLP/GEP: no Published: yes	no	no	not protected	-	N
KMA 4.2/02	Strain, K.E., Whiting, S.A., Lydy, M.J.	2014	Laboratory and field validation of a Cry1Ab protein quantitation method for water not available, not applicable Talanta, 128, 109-116 GLP/GEP: no Published: yes	no	no	not protected	-	N