European Commission

Draft Renewal Assessment Report prepared according to the Commission Regulation (EU) N° 1107/2009

Microbial Pest Control Agent (MPCA) Bacillus thuringiensis subsp. *kurstaki* SA-12

Volume 3 B.5 (MPCA)

Analytical method

Rapporteur Member State: Denmark

Co- Rapporteur Member State: The Netherlands

Version history

When	What
2008	DAR
2011	Addendum to the DAR
2019	Initial RAR

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INTRODUCTION

Bacillus thuringiensis subsp. *kurstaki* SA-12 (in the following abbreviated as Btk SA-12) was one of the existing active substances covered by the Regulation (EC) No 2229/2004 on the implementation of the fourth stage of the program of work referred to in Article 8(2) of Council Directive 91/414/EEC. In Annex I to Regulation (EC) No 2229/2004 the Commission designated Denmark as rapporteur Member State to carry out the assessment of Btk SA-12 on the basis of a joint dossier submitted for the Btk strains SA-11, SA-12 and EG 2348. The notifier for Btk SA-11 and SA-12 was Mitsui AgriScience International SA/NV while EG 2348 was notified by Mitsui AgriScience International SA/NV and Intrachem Bio Italia S.p.A. (now CBC (Europe) S.r.l.). In accordance with the provisions of Article 22(1) of Regulation (EC) No 2229/2004, Denmark submitted in January and February 2008 to the EFSA the draft assessment report, including, as required, a recommendation concerning the possible inclusion of Btk SA-12 in Annex I to the Directive. The Commission examined the draft assessment report, the recommendations by the rapporteur Member State and the comments received from other Member States in consultation with experts from a certain number of Member States. The Commission referred on 12 July 2008 a draft review report to the Standing Committee on the Food Chain and Animal Health, for final examination. The draft review report was finalized in the meeting of the Standing Committee on 12 July 2008. Subsequently Regulation (EC) No 1107/2009 repealed and replaced Directive 91/414/EEC and the active substance Btk SA-12, was deemed to be approved under that Regulation and included in the Annex to Regulation (EC) No 540/2011. EFSA delivered its conclusions on *Bacillus thuringiensis* ssp. *kurstaki* (strains ABTS-351, PB-54, SA-11, SA-12, EG2348) on the 16 December 2011 (published 23 February 2012). Based on this new information available, no need to change the conditions of approval of Btk SA-12 was identified. The Commission filed on 13 December 2013 an updated review report for Btk strains SA-11, SA-12 and EG 2348 to the Standing Committee on the Food Chain and Animal Health for examination.

The approval of Btk SA-12 under the Regulation (EC) No 1107/2009 expires 30 April 2019. In accordance with the same Regulation the original notifier Mitsui AgriScience International SA/NV has filed to the Commission an application for the renewal of the approval of the active substance Btk SA-12 on 30 April 2016. In accordance with Regulation (EU) 2016/183 the notifier submitted to the designated RMS Denmark, the co-RMS The Netherlands as well as to EFSA and Commission a dossier for renewal of Btk SA-12 considering the deadline stated in SANTE-2016-10616–rev. 3.

Btk SA-12 is a wild type strain originating from infested insects. Btk 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 Btk is mainly attributed to spore bound insecticidal pro-proteins (*Cry* toxins) which are ingested by the target pests and activated under alkaline conditions in the midgut of the larvae. The first assessment of the strain proved that it does not have any harmful effects on human or animal health or on groundwater or any unacceptable influence on the environment. The overall conclusion from EFSA (2012) confirms that no critical areas of concern are identified within the framework of the use which was supported.

As the manufacturing process of Btk SA-12 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.

For the renewal of the Btk strains SA-11, SA-12 and EG 2348 under Regulation (EC) 1107/2009, a separate dossier was submitted for each strain only including data, which have previously not been submitted or evaluated. Nevertheless, there is some information which is applicable to all three Btk strains, e.g. published information for Btk in general obtained during searches for peer reviewed literature according to EFSA Guidance (2011)[1] carried out for relevant sections.

In the following for ease of information, full study summaries/sections taken from the DAR (2008) or its Final Addendum (2011) are included if they are considered relevant for renewal of Btk SA-12. In order to facilitate discrimination between new data and data already evaluated during the first approval process, the headline "New Data" begins the section with data, which have previously not been submitted or evaluated. Data and their evaluations from the original DAR and addenda to the DAR are highlighted by grey background.

1 Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092

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

Methods for the identification of the micro-organism

Relevant information for identification and characterisation of the strain has already been submitted for the first approval of the strain. Please refer to Volume 3 MA, B.1.3.3.

New data

During original approval of SA-12, a set of methods was used for identification purpose. However, for renewal of the strain, an attempt was made to develop strain specific markers based on the whole genome and plasmid sequences of strain SA-12 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 other Bt subspecies and pathogenic *B. cereus* strains

- Reproducibility by running independent experiments (triplicate).

Based on these tests, two primer pairs are considered to be highly specific for Btk SA-12. For the two other ones it cannot be excluded that they also target other Btk strains.

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, as for PCR, 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, C.1.4.1.

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

Details are confidential information. Please refer to Vol. 4, C.1.1.3.

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

Btk SA-12 is a wild strain and not a mutant or GMO. If mutations should occur, spontaneous changes can be detected by a range of methods applied in the quality control. See Volume 4 for further information.

B.5.1.4 Methods for the establishment of purity of seed stock from which batches are produced and methods to control that purity

CONFIDENTIAL information. Please refer to Vol. 4, C.1.2.2.

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

CONFIDENTIAL information. Please refer to Vol. 4, C.1.2.1 and C.1.2.2. In addition, a validated method is submitted for the quantification of Btk SA-12 in the end-use product CoStar WG and aqueous suspensions. Please refer to Vol. 3MP, B.5.1.1.

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

CONFIDENTIAL information. Please refer to Volume 4, Point C.1.2.2

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

New data

A screening for microbial contaminants has been carried out in the technical material of Btk SA-12. The results are in compliance with SANCO/12116/2012. Please refer to Volume 4, Point C.1.2.2.

The below mentioned methods were used to screen the technical material of Btk SA-12 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 are 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 confirmation if required.

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°C to 35°C for 18 to 24 hours. Afterwards 1 mL of the pre-culture is added to 100 mL of MacConkey Broth, and incubated at 42°C to 44°C for 24 to 48 hours. When subcultures are plated on MacConkey Agar they need to be incubated at 30°C to 35°C for 18 to 72 hours. Growth of colonies indicates the possible presence of E. coli. This is confirmed by identification tests.

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.

Salmonella **(USP 38 Method 62):**

10 g of the sample is inoculated with a suitable amount of Soybean–Casein Digest Broth, mixed and incubated at 30°C to 35°C for 18 to 24 hours. 0.1 mL of the pre-culture is added to 10 mL of Rappaport Vassiliadis *Salmonella* Enrichment Broth and incubated at 30°C to 35°C for 18 to 24 hours. Subcultures on plates of Xylose Lysine Deoxycholate Agar are incubated at 30°C to 35°C 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.

Shigella **(BAM Chapter 6):**

Twenty-five g of the sample is 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 then streaked to MacConkey agar and incubated for additional 20 hours at 35°C. *Shigella* colonies are slightly pink and translucent with or without round edges. Suspicious colonies are subjected isolation and confirmation procedures if required.

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°C to 35°C for 18 to 24 hours. The subculture is then plated on Mannitol Salt Agar and incubated at 30°C to 35°C 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.

Vibrio cholerae **(BAM Chapter 9):**

Twenty-five g of the sample is 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.

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 then 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.

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

The technical material as such is not stored or shipped elsewhere for the final formulation process. Therefore, no information is required here.

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

Btk SA-12, such as all Btk 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 infor-

mation, please refer to information provided for the Vol. 3 MA, Section B.7. Strain specific markers are available for monitoring of the strain on treated plants. Please refer to Vol. 3 MA, B.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 criterion 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 5.2-01) 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.

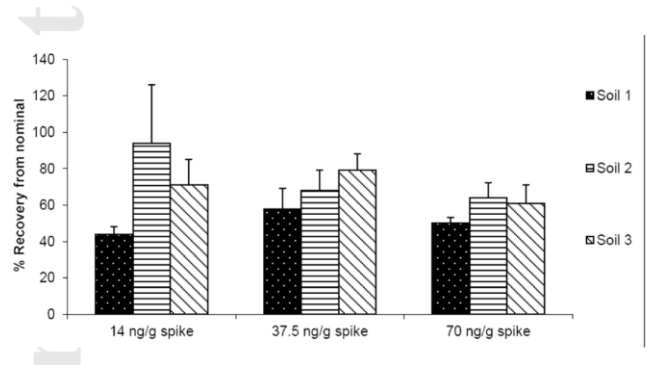

Figure 5.2-01 Results from fortification recovery tests in which purified Cry1Ab protein standard was added to the extraction buffer and the spiked buffer was then added to soils and the extraction procedure was conducted. Mean recoveries across the three spiking levels were 51, 75, and 70% for Soils 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 determined 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 5.2-02). 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 5.2-02).

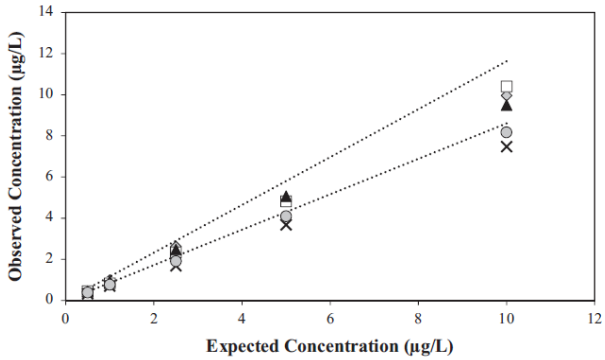

Figure 5.2-02 Results of matrix testing of standard curves in phosphate buffered saline plus tween (PBST) buffer control (◊), 100% groundwater (□), 100% runoff water (▲), 100% river water (x), and 50% river water (●) matrices. The dotted lines represent the range of acceptance indicating no matrix effects.

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 were 5.0, 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 5.2/01**
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.

Evaluation RMS	The reference is acceptable.

Reference	**KMA 5.2/02**
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 immunosorbent 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.

Evaluation RMS	The reference is acceptable.

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)

Not relevant

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)

Not relevant

B.5.3 References relied on

Several literature review reports have been provided according to the guidance of EFSA (Guidance of EFSA: Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009. EFSA Journal 2011;9(2):2092). The aim of these reports was to provide a global overview of peer-reviewed literature concerning potential side effects of *B. thuringiensis* subsp. *kurstaki* strain SA-12.

Overview of literature reports provided according to the guidance of EFSA

Data point	**Author**	**Year**	**Title**	**Section of RMS evaluation**
KMA 2.7/12 & 3.5/06	Süß, J.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Biological properties	Vol. 3MA, B.2.10
KMA 6.1.1/07	Seehase, S.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Toxicology	Vol. 3MA, B.6.3
KMA 7.1/01	Cornelese, A.	2016a	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12 and metabolites: Residues in or on treated products, food and feed	Vol. 3MA, B.7.4
KMA 8.1/10	Cornelese, A.	2016b	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Fate and behaviour in the environment	Vol. 3MA, B.8.3
KMA 9/01	Schöbinger, U.	2016	Literature review on *B. thuringiensis* subsp. *kurstaki* strain SA-12: Effects on non-target organisms	Vol. 3MA, B.9.8

Data point	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	Previous evaluation
KMA 5.1.7/01	Feng, P., Weagant, S.D., Grant, M.A., Burkhardt, W.	2002	BAM: ENUMERATION OF ESCHERICHIA COLI AND THE COLIFORM BACTERIA not available, not applicable Bacteriological Analytical Manual, Chapter 4 GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted
KMA 5.1.7/02	Anonymous	1900	USP 38 METHOD 62 not available, not applicable Microbiological Examination / Microbiological Tests, 61, 112-120 GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted
KMA 5.1.7/03	Anonymous	2012	AOAC OFFICIAL METHOD 2004.06 LISTERIA IN FOODS; MODIFIED VODAS LIS FIRST ACTION 2004, FINAL ACTION 2008 not available, not stated GLP/GEP: no Published: no	no	no	not protected	-	New data for active ingredient, not previously submitted
KMA 5.1.7/04	Andrews, J., Jacobson, A.	2001	BAM: SHIGELLA not available, not applicable Bacteriological Analytical Manual, 6 GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted
KMA 5.1.7/05	Kaysner, C.A., DePaola, A.,	2004	BAM: VIBRIO not available, not applicable Bacteriological Analytical Manual, Chapter 9 GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted
KMA 4.1/06	Tournas, V., Stack, M.E., Mislivec, P.B., Koch, H.A., Bandler, R.	2001	BAM: YEASTS, MOLDS AND MYCOTOXINS not available, not applicable Bacteriological Analytical Manual, Chapter 18 GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted

KMA 5.2 /01	Mueting, S.A., Strain, K.E., Lydy, M.J	2014	VALIDATION OF AN EXTRACTION METHOD FOR Cry1Ab PROTEIN FROM SOIL Environmental Toxicology and Chemistry, 33(1), 18-25 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated
KMA 5.2 /02	Strain, K.E., Whiting, S.A., Lydy, M.J.	2014	LABORATORY AND FIELD VALIDATION OF A Cry1Ab PROTEIN QUANTITATION METHOD FOR WATER Talanta, 128, 109-116 Report-no.: not applicable GLP/GEP: no Published: yes	no	no	not protected	-	New data for active ingredient, not previously submitted nor evaluated