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Laboratory and field validation of a Cry1Ab protein quantitation method for water

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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 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.

1. Introduction

Genetically-modified crops currently dominate the agricultural landscape throughout much of the world. The primary insecticidal form of genetic modification to crops is the insertion of genetic code from the soil bacterium, *Bacillus thuringiensis* (Bt), so that the plant produces insecticidal crystalline (Cry) proteins. In 2013, approximately two-thirds of corn and cotton produced in the United States (approximately 76 million acres) contained one or more Bt genes [1]. Genetically-modified plants express the Cry proteins throughout the growing season, decreasing the need for conventional insecticide applications and reducing crop loss due to pest damage.

The Cry protein investigated in the current study was Cry1Ab, which specifically targets stalk borers, such as *Ostrinia nubilalis* (European corn borer). When the target species ingest transgenic plant material, the Cry1Ab proteins are cleaved by midgut proteases, resulting in a 60 kDa protein. The cleaved protein binds to specific membrane receptors [2], leading to pore formation and lysis of midgut epithelial cells leading to death [3]. Overall, the Cry proteins are highly specific and lethal only after ingestion, which limits the scope of insecticidal activity to target organisms.

Still, the wide-spread planting of Bt products has raised questions regarding the fate and potential toxicity of Cry protein residues in the environment. The ability to accurately monitor the concentration and movement of Cry proteins is especially crucial in the aquatic environment where dispersion potential is high [4]. Several studies have documented the presence of agricultural products, including Bt plant debris and contaminated sediments in surface waters adjacent to agricultural fields [4–7], which may be introduced via surface runoff, erosion, tissue debris, or aerial deposition of pollen or crop dust [8,9]. Jensen et al. [10] observed extended deposition of corn debris into nearby streams over several months after fall harvest until spring planting. The authors also investigated the degradation of the Cry1Ab protein in senescent corn tissue after conditioning in water. The bioactivity of the Cry1Ab protein was lost after exposure to an aquatic environment for two weeks [10]. The conditioning water was not tested for the Cry1Ab protein. Therefore, without accurate quantitation of the Cry proteins, it is difficult to ascertain whether the proteins in the leaf tissue had degraded or had leached into the surrounding aquatic environment. Prihoda and Coats [8] also showed a rapid

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decline of a Cry protein, Cry3Bb1, in senescent corn tissue after being submerged in water. No Cry3Bb1 protein was detected in the water samples using ELISA. However, water samples were not concentrated prior to analysis, making it challenging to establish the presence or absence of the Cry3Bb1 protein.

Measurable Cry protein concentrations have been documented in surface waters surrounding Bt corn fields [5] and surface waters sprayed with a Bt *kurstaki* bioinsecticide [3]. Once introduced into an aquatic setting, the Cry proteins may adsorb readily and rapidly to sediment particles due to a high affinity to clay and organic matter within soils and sediments [12–17]. The Cry proteins bound to sediment particles have been shown to retain their insecticidal properties, and are better protected from microbial degradation. While the methods used for Cry protein quantification have not been validated in water matrices, there is undeniable evidence of a widespread distribution of Cry proteins in surface waters. Aquatic systems represent a significant mode of translocation for the Cry proteins, warranting a need for a validated quantitation method. The data obtained by utilizing a validated procedure to determine Cry1Ab concentrations in water could enhance the impact of fate and effect studies regarding this protein toxicant.

Due to controversy within the public and scientific communities surrounding the production of genetically-modified crops, proven and reliable methods are needed in order for data published in environmental risk assessment studies to be considered in decision making efforts [19,20]. Researchers have developed and validated methods to quantify Cry proteins including Cry34Ab1, Cry1F, and Cry1Ab in soil matrices using ELISA [21–24], but currently there are no published studies that have a standardized and validated method for the evaluation of Cry proteins in water.

This manuscript 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.

2. Material and methods

2.1. ELISA

All field and laboratory samples were quantified using a commercially available direct double antibody sandwich ELISA kit (Agdia, Elkhart, IN, USA). The ELISA plates chosen for the current study have been confirmed by the manufacturer against other commonly used transgenic crop proteins including Cry1F, Cry2A, Cry9C, and herbicide tolerant phosphinothricin acetyltransferase (PAT) proteins. Antigen-containing samples (100 µL) were loaded in triplicate onto a qualitative 96-well microplate precoated with antibodies specific to Cry1Ab/Ac. The plates were left to incubate for 2 h and washed eight times with 300 µL assay buffer using an ELx50 microplate strip washer (BioTek, Winooski, VT, USA). The assay buffer, phosphate buffered saline plus tween (PBST), consisted of 0.14 M sodium chloride, 8.1 mM sodium phosphate, 1.5 mM potassium phosphate, 2.7 mM potassium chloride and 0.05% Tween-20, pH=7.4 [24]. A second Cry1Ab/Ac antibody linked to peroxidase enzyme was added to each well, and washed after a second incubation as before. The TMB (3,3′,5,5′-tetramethylbenzidine) substrate was then added, resulting in the formation of a blue color produced by the hydrolysis of hydrogen peroxide by peroxidase. After 20 min, the optical densities (ODs) were read at 450 and 650 nm using an Epoch microplate spectrophotometer (BioTek). The difference in absorbance was used for quantitation of the Cry1Ab protein based on a seven-point standard curve ranging from 0.1 to 10 µg/L. See example in Table 1. The Cry1Ab protein standard was purchased from Abraxis (Warminster, PA, USA). The lyophilized protein was resuspended in PBST at a concentration of 1000 µg/L and frozen at −20 °C until use. It is important to note that while the antibodies could recognize the Cry1Ac protein, the quantitative data only pertain to Cry1Ab as Cry1Ac was not expressed by the corn used in the current study.

2.2. Method comparison

Lyophilization [18] and filter centrifugation [5] 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 (Corning #430304, Corning, NY, USA) for each method and each tube was spiked with purified Cry1Ab protein at 167.5 ng/L. This concentration was used in several experiments throughout the validation process as it falls in the middle of the quantitative range of the standard curve. Samples processed via lyophilization were frozen at −20 °C overnight and freeze-dried at −50 °C and 0.120 mBar until dry. Each sample was resuspended in 0.5 mL of PBST assay buffer, vortexed for 30 s and transferred to a 1.5 mL microcentrifuge tube. Another 0.5 mL of PBST was used to wash the 50 mL tube and added to the sample so that the final volume of each sample was 1 mL. Samples were also processed using a filter centrifugation method (Fig. 1). A 15 mL sample was added to a 30,000 molecular weight cut-off (MWCO) Amicon® Ultra centrifugation tube (Millipore, Billerica, MA, USA) and centrifuged at 870 × *g* for 30 min at room temperature (5810 centrifuge, Eppendorf, Hauppauge, NY, USA). The eluted component was

Table 1
Standard curve with acceptable prediction.

Standard	OD values	Mean OD	Expected Bt Cry1Ab concentration (µg/L)	Mean observed Bt Cry1Ab concentration (µg/L)	Mean RPD*
Std 1 (Blank)	0.026 0.020 0.018	0.021	0.00	−0.010	19.1
Std 2	0.919 0.917 0.967	0.934	10.0	9.974	0.26
Std 3	0.515 0.488 0.520	0.508	5.00	4.981	0.38
Std 4	0.281 0.274 0.265	0.273	2.50	2.499	0.03
Std 5	0.128 0.125 0.131	0.128	1.00	1.033	3.31
Std 6	0.079 0.071 0.071	0.074	0.50	0.499	0.26
Std 7	0.049 0.047 0.046	0.047	0.25	0.242	3.33
Std 8	0.040 0.030 0.029	0.033	0.10	0.102	2.60

Quadratic formula $y=-0.0012x^2+0.1031x+0.0231$, $R^2=1$.
OD=optical density.

* Relative percent difference (RPD) calculated using the following equation

$$\mathrm{RPD}=\frac{(\mathrm{abs}(\mathrm{Observed\ \ concentration}\ (O)-\mathrm{expected\ \ concentration}\ (E))}{\mathrm{Average}\ (O,E)}\times 100\%$$

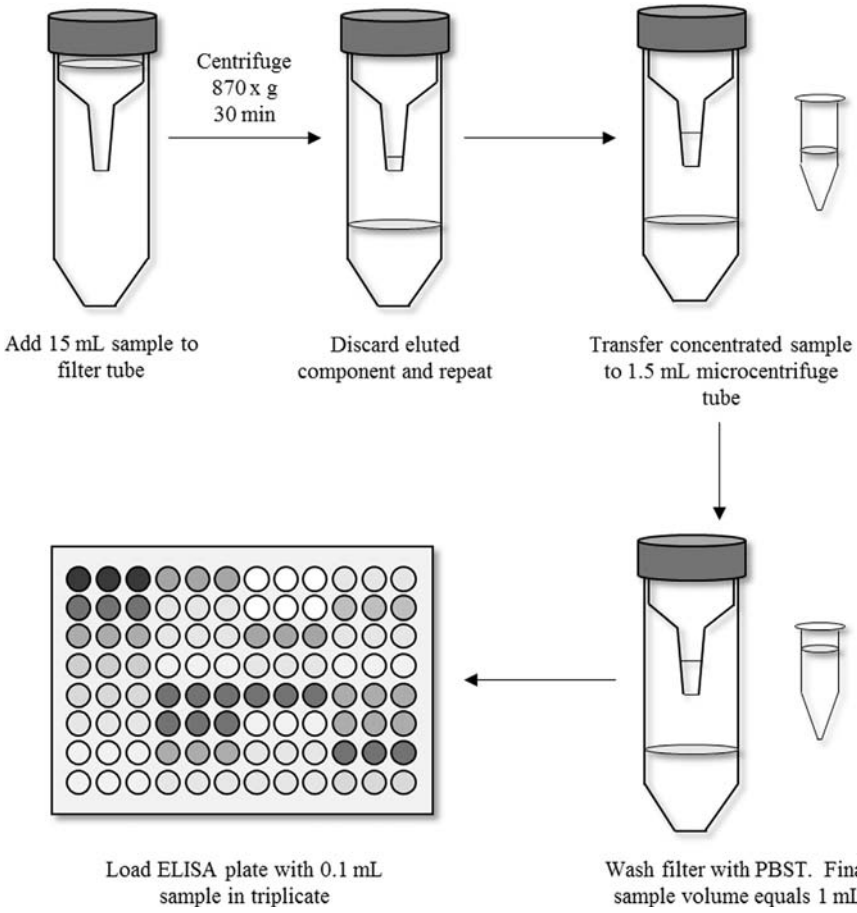

Fig. 1. Flow chart depiction of the filter centrifugation extraction and quantification method.

discarded and the remaining 15 mL sample was added to the Amicon® tube for a second centrifugation cycle. The volume of the liquid retained by the membrane was measured to the nearest 5 μL and transferred to a 1.5 mL microcentrifuge tube. The Amicon® membrane was rinsed with PBST buffer so that the final sample volume was 1 mL. All samples were stored at 4 °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 percent recoveries were converted to proportions and transformed using the arc sin square root function. A one-way analysis of variance (ANOVA) was conducted to compare recoveries between extraction methods using SAS software [25].

2.3. Specificity

The sample matrix may contain components that could interfere with the ability of the ELISA to accurately determine protein concentrations [23]. In order to assess the effect of the environmental matrices and specificity of the ELISA method used in the current study, a matrix testing experiment was conducted. Matrices (100%) were prepared by concentrating 30 mL of reference water (groundwater, river water, or runoff water) following the filter centrifugation method explained in the previous section. 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 or 50% matrix. The relative percent difference (RPD) between the matrix and standard curve ODs were calculated, with a RPD > 15% being indicative of matrix effects [23,26].

2.4. Precision

Two matrix dilution agreement experiments were conducted for each matrix. A 30 mL aliquot of reference water was spiked with the Cry1Ab protein standard at 2 μg/L and concentrated using filter centrifugation. The concentrated sample was then serially diluted from 1:1 to 1:512 for a total of 10 dilutions ranging from above the upper limit of quantitation (ULOQ) to below the lower limit of quantitation (LLOQ). Samples were quantified as described in Section 2.1 and concentrations were back-calculated by applying a dilution factor. Final adjusted concentrations for each extract were required to have several dilutions with a coefficient of variation (CV) less than 20% [23].

2.5. Accuracy

The ability of the Amicon® membrane to retain Cry1Ab protein was investigated through qualitatively assessing the extraction process using ELISA and dot blot. A 30 mL aliquot of each reference water was spiked near the ULOQ at 800 ng/L and concentrated using the filter centrifugation method. The eluted components from both centrifugation steps were pooled and concentrated again using a 3000 MWCO Amicon® Ultra centrifugation tube (Millipore) in order to detect Cry1Ab protein fragments that had passed through the larger filter. A 100 μL aliquot of the volume retained by the filter was added to the ELISA plate in triplicate and quantified as previously described. For dot blot confirmation, a

200 μL aliquot from each sample was vacuum-fixed in triplicate to a 0.4 μm nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using the Bio-Dot Microfiltration Apparatus (Bio-Rad). Following transfer, the membrane was placed into a 20 mL blocking solution (1% bovine serum albumin, BSA in TBST (tris-buffered saline +0.05% Tween-20)) for 90 min, followed by a 90 min incubation in anti-Bt-Cry1Ab rabbit polyclonal primary antibody (Abraxis, Warminster, PA, USA) at 1:2000 dilution in TBST. The membrane was washed three times with 20 mL TBST then incubated with alkaline phosphatase goat anti-rabbit secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) at 1:1000 dilution in TBS (tris-buffered saline) for 30 min. Following final incubation, the membrane was rinsed three times with 20 mL TBS followed by a 5 min wash and developed with alkaline-phosphatase developer (Sigma-Aldrich). Once antigen was visible, the membrane was washed with de-ionized water and dried before analysis using Gel Pro Analyzer Densitometry software (Media Cybernetics, Bethesda, MD, USA). The OD values were transferred to a Microsoft Excel spreadsheet for quantitation based on a seven point standard curve ranging from 1 to 100 μg/L.

Two extraction efficiency experiments were conducted for each water matrix in which three 30 mL replicates of reference water were spiked with Cry1Ab protein at concentrations encompassing the entire range of the standard curve (8, 80, or 800 ng/L) and extracted using the filter centrifugation method. The percent recovery of Cry1Ab protein was determined for each matrix at each concentration in order to determine the accuracy of the extraction method. The CV between two extractions performed on different days was required to be below 20% in order to validate the precision of the extraction and quantitation method [23].

Additional extraction efficiency experiments were conducted with seven water matrices including groundwater, runoff water, river water, soil pore water, de-ionized water, tap water, and reconstituted moderately-hard water (RMHW), to determine the influence of physiochemical water properties on Cry1Ab protein recoveries. Conductivity and temperature were measured using a YSI 30 salinity, conductivity and temperature meter (YSI Inc., Yellow Springs, Ohio, USA). Dissolved oxygen (DO) was measured with a YSI 55 dissolved oxygen probe. Ammonia nitrogen was estimated using a Hach surface waters kit (Loveland, CO, USA). The water pH was measured with an Orion 4 Star pH meter after calibration following manufacturer's instructions (Thermo Scientific, Chelmsford, MA, USA). The values for each metric from triplicate water samples were averaged. Total suspended solids (TSS) and total organic carbon (TOC) were measured by Midwest Laboratories (Omaha, NE) using standard methods 2540D and 5310B, respectively [27]. Each water matrix was spiked with purified Cry1Ab protein at 80 ng/L and processed using the filter centrifugation method. Recoveries of Cry1Ab protein were determined and physiochemical properties (Table 2) were compared using a Pearson rank correlation and multiple regression analysis using SAS software [25].

2.6. Stability

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 [25].

2.7. Sensitivity

The method detection limit (MDL) for the quantitation of Cry1Ab protein from water matrices was determined according to U.S. Environmental Protection Agency (EPA) guidelines [28]. Briefly, seven samples from each reference water were spiked near the LLOQ at 8 ng/L, extracted using filter centrifugation, and quantified using ELISA. The standard deviation was calculated and multiplied by Student's t-value at the $\alpha=0.01$ level with six degrees of freedom to determine the MDL [28]. The highest MDL from the three matrices was deemed the method MDL and used to determine the reporting limit (RL), which was equal to three times the method MDL. The RL was used as the lower quantitation limit for field and laboratory samples.

2.8. Field sample validation

Soil pore water, groundwater, and runoff waters were collected throughout the 2013 corn growing season from a farm located in Christian County, Illinois. Samples were taken before planting, and monthly throughout the growing season from a 40-acre field planted with transgenic corn expressing Cry1Ab protein (MON810), and an adjacent 40-acre field planted with a non-Bt isoline. Soil pore water was vacuumed from the unsaturated zone of the soil into lysimeters buried at 1 m soil depth. Groundwater was taken from wells 4 m deep. Overland samplers made from halved 30.48 cm diameter capped polyvinyl chloride (PVC) pipe, 3.6 m long were installed in the ground to collect runoff water. Runoff water was collected following a rain event of 1.2 cm or greater. Samples were collected into acetone-rinsed clear glass mason jars and stored at 4 °C for up to one week before being processed for Cry1Ab protein using filter centrifugation as previously described.

Table 2
Physical and chemical characteristics of waters used for the correlation analysis.

Matrix	Conductivity (μS/cm^2)	pH	Dissolved oxygen (mg/L)	Total suspended solids (mg/L)	Total organic carbon (mg/L)	Recovery (%)
Groundwater	578 ± 42	7.8 ± 0.2	7.8 ± 1.4	n.d.	1.1	42 ± 5
Runoff water	61 ± 10	6.7 ± 0.7	5.8 ± 0.9	25	46	65 ± 2
River water	249 ± 11	7.5 ± 0.4	8.5 ± 1.5	10	5.9	66 ± 13
DI water	0.8 ± 0.8	6.2 ± 0.4	6.5 ± 0.8	n.d.	n.d.	59 ± 11
RMHW	354 ± 25	7.5 ± 0.2	7.4 ± 0.4	n.d.	n.d.	73 ± 5
Pore water	351 ± 25	7.4 ± 0.1	8.4 ± 1.5	4.0	2.6	107 ± 10
Tap water	171 ± 3	8.4 ± 0.3	8.0 ± 0.9	n.d.	3.0	41 ± 6

DI=de-ionized; RMHW=reconstituted moderately-hard water; n.d.=non-detect. Water quality parameters are given as mean ± standard deviation of three replicates, except for total suspended solids and total organic carbon, which were processed by Midwest Laboratories, Omaha, NE. Detection limits for TSS and TOC were 4.0 and 1.0 mg/L, respectively. Percent recoveries of Cry1Ab protein are given as mean ± standard deviation of six replicates collected over two separate extraction procedures.

2.9. Laboratory sample validation

Water was removed from aquatic bioassays in order to demonstrate the ability of the method to quantify Cry1Ab protein concentrations in the water column. Senescent leaves from Bt and non-Bt corn were stamped into discs using a 1.8 cm diameter punch, with each disc weighing approximately 7.4 mg. The leaves were conditioned in a tank of water for one week, then added to a jar with 250 mL of RMHW and 37.5 g of sediment. Ten *Hyallela azteca*, an epibenthic amphipod, were added to each beaker and kept at 23 °C for 10 days. Daily water changes were performed throughout the bioassays. A 30 mL aliquot of water was taken from the conditioning water, and from bioassay microcosms upon initiation and conclusion of the bioassays. Samples were concentrated and quantified as previously described using filter centrifugation and ELISA.

2.10. Quality assurance and quality control

The acceptance criteria for a standard curve with good prediction are outlined in detail by Schmidt and Alarcon [23] with an example provided in Table 1. 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. Samples with ODs reading above the average OD for the 10 μg/L standard were considered to be above the upper limit of quantitation (> ULOQ) and were subsequently diluted and re-evaluated. The ODs falling below the average OD for the lowest standard with a relative percent difference (RPD) < 20% were considered to be below the lower limit of quantitation (< LLOQ). In the example shown in Table 1, a sample with an average OD below 0.033 would have been considered < LLOQ.

A matrix spike (MS) and matrix spike duplicate (MSD) were included for each plate consisting of field or laboratory samples. The MS and MSD samples consisted of a non-Bt sample from the same batch that was spiked near the ULOQ with purified Cry1Ab protein at 500 ng/L and extracted simultaneously with the samples. The percent recovery and RPD was determined for the MS and MSD samples, with an RPD < 20% indicating good precision throughout the extraction process.

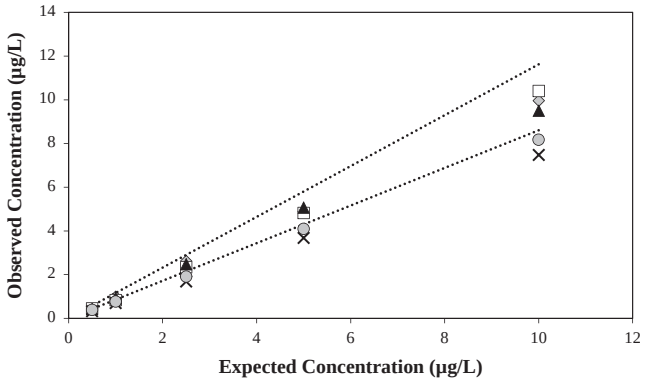

Fig. 2. Results of matrix testing of standard curves in phosphate buffered saline plus tween (PBST) buffer control (◇), 100% groundwater (□), 100% runoff water (▲), 100% river water (×), and 50% river water (●) matrices. The dotted lines represent the range of acceptance indicating no matrix effects.

Table 3
Results from river water matrix dilution agreement. Values within range of the standard curve were used to calculate the coefficient of variation (CV).

Dilution factor	Mean Bt Cry1Ab result (ng/L)	Adjusted Bt Cry1Ab result (ng/L)	Quantitative range 10 to 0.1 ng/L (±10%)
4	*Out of range*	*Out of range*	*> ULOQ*
8	7.14	**57.1**	Within range
16	3.80	**60.7**	Within range
32	1.94	**62.1**	Within range
64	0.91	**58.6**	Within range
128	0.46	**58.8**	Within range
256	0.18	**47.0**	Within range
512	*Out of range*	*Out of range*	*< LLOQ*
Mean adjusted result		57.4	
Standard deviation		5.39	
CV		**9.4%**	

> ULOQ=above the upper limit of quantitation; < LLOQ=below the lower limit of quantitation.

3. Results and discussion

3.1. Method comparison

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%. The results from the one-way ANOVA indicated a highly-significant effect of extraction method on percent recovery ($F_{1,16}=30.8$, $P<0.01$). These findings indicate superior extraction efficiencies of Cry1Ab protein using filter centrifugation over freeze-drying, and therefore this method was chosen for further validation.

3.2. Specificity

The results of the matrix testing experiments showed no matrix effects in groundwater or runoff water (Fig. 2). Minor matrix effects were observed in 100% river water, with an average RPD 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 (Fig. 2). Dilutions beyond 50% were not investigated, because excessive dilution may not have removed matrix effects and would have decreased the sensitivity of the method. Acceptable recoveries of Cry1Ab proteins were observed in river water and ranged from 50 to 90%, despite minor matrix hindrance, suggesting the effect of matrix using the current method was negligible in quantitation of Cry1Ab proteins.

3.3. Precision

The results from the matrix dilution agreement experiments support a high level of precision using the ELISA quantitation method. A single sample from each matrix was spiked at a level above the ULOQ and serially-diluted over a wide range surpassing the LLOQ. The CV's for groundwater, runoff water and river water were 5.0, 9.7, and 9.4%, respectively, with a minimum of six consecutive dilutions for each matrix. A detailed description of the dilution agreement ELISA experiment for river water is shown in Table 3.

3.4. Accuracy

The ability of the 30,000 MWCO Amicon® membrane to sufficiently retain the Cry1Ab protein was confirmed using both ELISA and dot blot. No Cry1Ab protein was detected in the eluted

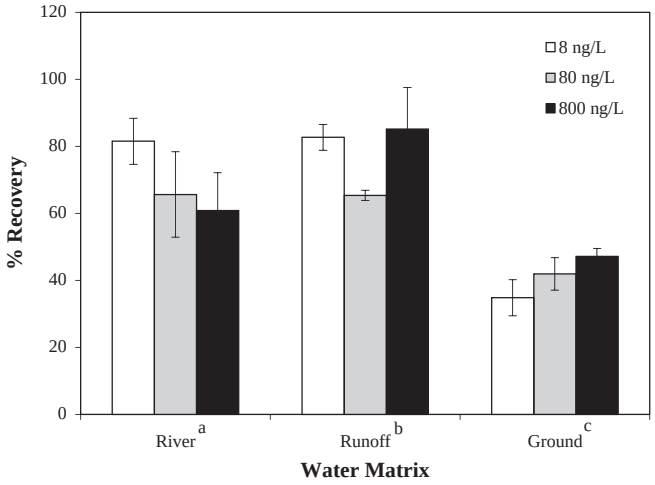

Fig. 3. Extraction efficiency of the filter centrifugation extraction method. Error bars represent the standard deviations among six replicates from two extractions. Different letters indicate a significant difference according to Tukey's HSD comparison.

fraction from any of the 30,000 MWCO ultracentrifugation tubes, thus indicating the membrane sufficiently retains Cry1Ab protein during the extraction process (data not shown). Percent recoveries for each matrix were calculated at three spiking levels over two extractions conducted on different days in order to demonstrate both the precision and accuracy of the extraction and quantitation method (Fig. 3). Groundwater had the lowest Cry1Ab protein recoveries with an overall mean near 40%, and ranged from 30 to 50% among all spiking levels. Percent recoveries in runoff water were the highest, with an average near 80% and ranged from 70 to 100%. River water had a mean recovery of 70% and ranged from 50 to 90% (Fig. 3). Excellent precision was detected as the CV for each matrix and spiking level was below 20% both within and among the extractions.

Percent recovery data was converted to proportions and transformed with the arc sin square root function. A two-way ANOVA was conducted, comparing the effects of spiking level and matrix on recoveries. There was a highly-significant effect of matrix ($F_{8,45}=80.4$, $P<0.01$) and spiking level ($F_{8,45}=5.3$, $P<0.01$) on recoveries of Cry1Ab protein. A Tukey's honestly significant difference (HSD) test revealed a significant difference in recoveries from all three matrices (Fig. 3). The interaction term was also highly-significant ($F_{4,45}=8.41$, $P<0.01$), indicating the matrix may influence the recovery of Cry1Ab protein near the limits of quantitation.

With significant variation in the recoveries among the three matrices tested, it is difficult to compare concentrations among the water sources. Diluting certain matrices may improve the recovery of the Cry1Ab protein. The river water matrix revealed a slight matrix hindrance that was slightly improved by diluting to 50% with assay buffer (Fig. 2). The groundwater matrix, on the other hand, did not have matrix interference with the ELISA, but did have increased recoveries by diluting with groundwater (see below). For these reasons, it is pertinent for investigators to fully validate their extraction and quantitation methods for each matrix to determine the method that will provide the best recoveries for the analyte of interest. In this case, the concentrations of the Cry1Ab protein are low enough that diluting the samples with de-ionized water or assay buffer could push the samples below the limits of detection. Therefore, for this study, the samples were not diluted prior to extraction.

The recovery of Cry1Ab protein was the lowest in groundwater, indicating difficulty in extracting the protein from this matrix. Groundwater had the highest conductivity values compared to the other matrices tested (Table 2). While water conductivity was not significantly correlated with recovery of the Cry1Ab protein (see below), the high conductance of groundwater could be causing the Cry1Ab protein to precipitate out of solution due to aggregation or conformational changes [29,30]. These changes might also be effecting the solubility, stability, or the capacity for recognition by the ELISA antibodies. Lowering the conductance of groundwater by diluting with de-ionized water at a 1:1 ratio does improve the recovery of the Cry1Ab protein to 64%. The inefficiency of Cry1Ab protein recovery in groundwater may be compounded at concentrations near the limit of detection, as groundwater spiked at 8 ng/L had the lowest overall recoveries.

The filter centrifugation and ELISA methods were evaluated in four additional water matrices to further investigate the effect of variation in physiochemical properties on Cry1Ab protein recoveries. A multiple regression analysis was conducted to determine relationships between percent recoveries of the seven total matrices tested and commonly observed water metrics, such as pH, DO, conductivity, TSS and TOC. Ammonia nitrogen was also measured, but was negligible in each matrix and therefore was not included in the regression analysis. Water matrices and characteristics included in the analyses are summarized in Table 2. Correlation analysis using a Pearson ranking in SAS software [25] showed significant correlations between pH and conductivity ($P=0.041$) and TSS and TOC ($P<0.01$). In order to eliminate colinearity amongst the dependent variables, conductivity or pH and TSS or TOC were experimentally dropped from the model prior to analysis. Recovery data was transformed as described above and used as the dependent variable in multiple regression analysis against the independent variables pH, DO and TSS. However, the results from all of the above combinations showed no effects on Cry1Ab protein recoveries.

While the current study revealed no direct relationships between the physiochemical characteristics of the water matrix and recovery of Cry1Ab protein, possible interactions may still exist. Several studies report adsorption of Cry proteins to clay and organic matter within sediments [12–14,18,24]. Suspended solids and organic matter in the water retained by the concentration filter could therefore interact with Cry proteins spiked into the water and possibly alter extraction efficiencies. The pH of the matrix might also impact the binding of Cry1Ab protein to water particulates [18], protein aggregation, and electrostatic interactions [29]. Formation of protein aggregates or conformation changes might also be influenced by conductivity [29,30]. In many cases, conductivity can also serve as a surrogate for total dissolved solids, assuming a major composition of ionic substituents capable of conductance [31]. As slight differences in the properties of the environmental matrix can affect protein folding and stability, the effect of water source could potentially influence the longevity of Cry proteins in aquatic settings.

3.5. Stability

A stability experiment was conducted in which each matrix was spiked with Cry1Ab protein and maintained for two weeks at −80, −20, 4, or 23 °C. All samples for the same matrix were extracted together, along with triplicate 'control' samples spiked immediately before processing using protein from the same initial stock. A two-way ANOVA 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. As shown in Fig. 4, the effect of temperature had a disproportionally larger negative impact on recovery of Cry1Ab protein in groundwater, suggesting Cry1Ab protein is very unstable in this matrix at warmer temperatures.

A Tukey's HSD test was employed to determine differences among matrices and temperatures. The 'control' group had significantly higher recoveries than any of the samples stored for two weeks. 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 (Fig. 4). The results from this experiment suggest that water samples should be frozen if immediate processing is not possible.

3.6. Sensitivity

The 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 detection limits obtained in the current study were lower but comparable to those published by Tank et al. [5] and Wang et al. [18]. The MDL was measured at 2.1 ng/L and a RL of 6.3 ng/L was used for the quantitation of Cry1Ab proteins in all water samples.

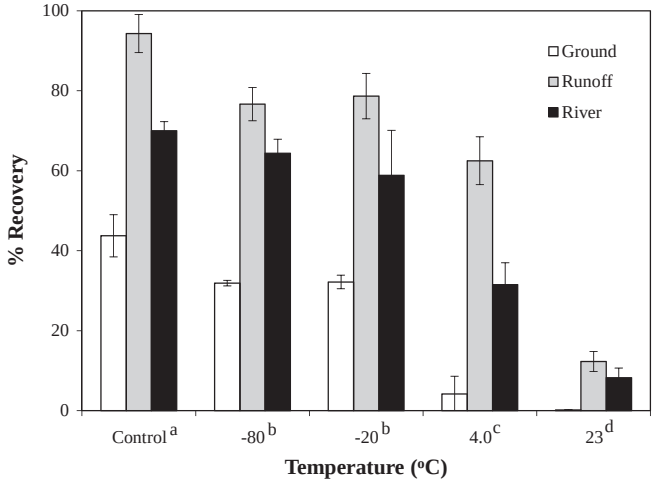

Fig. 4. Stability of Cry1Ab proteins in spiked water samples after two weeks of storage at different temperatures. Error bars represent the standard deviation between triplicate replicates from a single extraction. Different letters indicate a significant difference according to Tukey's HSD comparison for temperatures. Tukey's HSD test determined all matrices were significantly different from one another (letters not shown).

3.7. Field and laboratory validation

The use of a validated ELISA method is necessary for the appropriate interpretation of data collected in field and laboratory studies [9]. Groundwater, soil pore water, and runoff water samples were collected monthly throughout the 2013 growing season. Of the 35 groundwater and 81 soil pore water samples collected in 2013, only one groundwater sample was above the RL for the Cry1Ab protein. This is consistent with data our laboratory has analyzed from previous growing seasons (data not shown), suggesting rapid degradation or little transport of the protein through these matrices. Runoff troughs in the non-Bt field contained consistently low concentrations of Cry1Ab protein and ranged from non-detect to 42 ng/L (Fig. 5a). Runoff water collected from the Bt field had higher concentrations of Cry1Ab protein, with a maximum concentration of 130 ng/L detected in September (Fig. 5b).

The results reported in the current study are consistent with previous publications regarding the fate and transport of the Cry1Ab protein in water. Wang et al. [18] detected up to 31 ng/L of the Cry1Ab protein in the water of Bt rice fields. Additionally, the highest Cry1Ab concentrations were detected during the flowering stage. These results corroborate the findings in the current study, as Cry1Ab concentrations increased in the runoff water samples after corn pollination. In a study by Tank et al. [5], approximately 25% of samples collected from streams draining agricultural fields were positive for the Cry1Ab protein, and concentrations ranged from 6 to 32 ng/L.

Senesced leaves from Bt and non-Bt corn were conditioned for seven days prior to their use in laboratory bioassays. Initial Cry1Ab protein concentrations in the leaf were 50.4 μg/g and dropped to 3.3 μg/g after seven days of conditioning. Concentrations of Cry1Ab protein reached 25.6 ng/L in the water at the end of the conditioning period. No Cry1Ab protein was detected in any of the non-Bt samples. Conditioned leaves were added to fresh water for toxicity testing and water samples were taken before organisms were added. No Cry1Ab protein was detected in either sample before test initiation. Upon conclusion of the bioassay, water samples were taken to determine final Cry1Ab protein concentrations of 10.0 ng/L in the Bt-treatment, with no Cry1Ab protein found in the non-Bt samples. No toxicity of the Bt leaves to *H. azteca* was observed during the experiment. According to Johnson et al. [20], exposure conditions should be defined and accurately monitored throughout ecotoxicological studies. Thus, the ability to

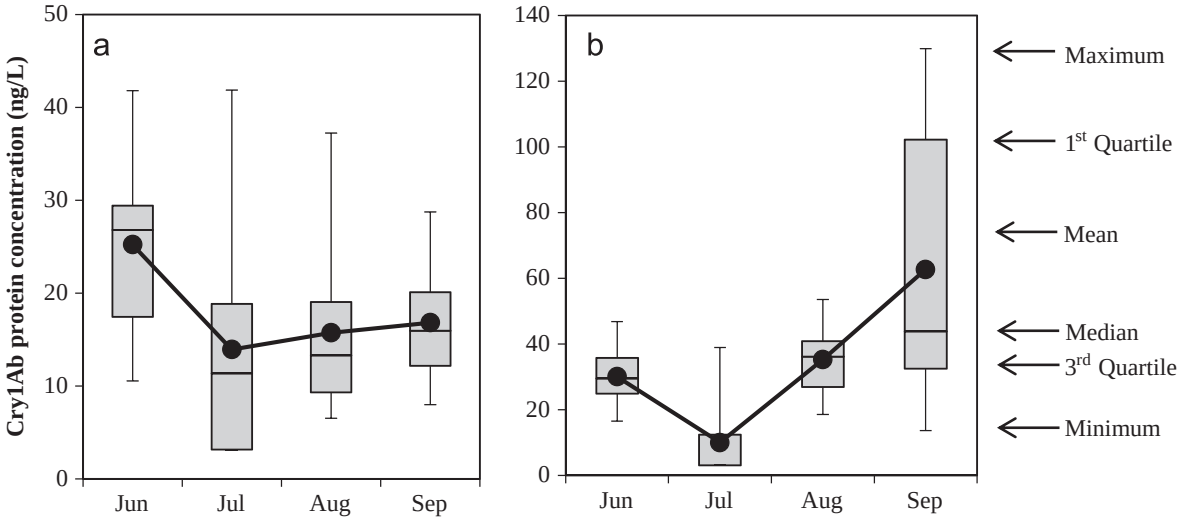

Fig. 5. Concentrations of Cry1Ab protein in runoff water collected from non-Bt (a) and Bt (b) corn fields throughout the 2013 growing season.

quantify Cry1Ab protein concentrations in the water column throughout aquatic bioassays satisfies a pertinent component for toxicity tests and relevancy for risk analyses.

4. Conclusion

The production of genetically-modified crops has sparked debates amongst the public and scientists alike. The results from a single study may have the power to influence policy makers and food activists, making certain readers especially critical of research involving genetically-modified crops [32]. In thorough risk assessments, hazard and exposure should be accurately quantified [20]. Perhaps of more value, the presently-described method may be explored for rapid diagnosis of transgenic products in aquatic systems. The reliable quantitation of Cry proteins for risk analysis is pertinent to protect the integrity of the data and to continue the un-biased pursuit of knowledge regarding the impact of these toxins.

The quantitation method outlined in the current study was validated for specificity, accuracy, precision, stability, and sensitivity based on previously-published validation criteria [23,24,26]. The development of a standardized water extraction method bolsters the integrity of research involving the quantitation of Cry proteins in aquatic settings, and provides a foundation for future ecological risk assessments. The data presented in the current study benefit the scientific community by serving as a reference for alternative method validations and quantitation of the Cry1Ab protein in water samples.

Novelty statement

This manuscript is the first to validate an analytical method for the detection of a crystalline (Cry) insecticidal protein derived from *Bacillus thuringiensis* (Bt) in water. Accurate and reproducible assessments of Cry proteins in aquatic settings is pertinent as materials from Bt agricultural fields can enter surface waters, exposing susceptible non-target organisms to the insecticidal proteins.

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