Chapter 17

Simultaneous Measurement of *Bacillus thuringiensis* Cry1Ab and Cry3B Proteins in Corn Extracts

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Transgenic corn hybrids have been developed which contain synthetic genes for the production of *Bacillus thuringiensis* insecticidal proteins. Two of these proteins are Cry1Ab and Cry3B. Enzyme-linked immunosorbent assays (ELISA) and immunochromatographic lateral flow assays, which are sensitive, specific and easy to perform, have been developed for Cry1Ab and Cry3B proteins in corn extracts. In the present work, we describe a sandwich fluorescent covalent microsphere immunoassay (FCMIA) to simultaneously measure Cry1Ab and Cry3B proteins in corn extracts. Mixtures of genetically modified organism (GMO) and non-GMO corn were prepared and extracted. The extracts were diluted and Cry1Ab and Cry3B were measured by FCMIA. Measurement of pure Cry1Ab protein by FCMIA gave a minimum detectable concentration of 0.1 ng/mL. There were no significant differences between the assays when performed singly vs. multiplexed ($P>0.588$). When the observed dilutions from the four parameter logistic fits were compared to the known dilutions, highly linear relationships were observed ($r = 0.984$ and 0.983, $P<0.001$) over a dilution range

of 1 to 100. These data give proof of principle that FCMIA can be used to simultaneously measure multiple GMO proteins in corn and may be a valuable adjunct to existing assays when the number of GMO pesticidal proteins to be measured becomes large.

Introduction

Bacillus thuringiensis (Bt, named for the Thuringia region of Germany) is a naturally occurring bacterial organism that has been known to man since the 19th century (*1*). Bt is a spore-forming bacteria (related to *Bacillus anthracis)* that, during spore formation, produces a crystalline structure. Within these crystalline inclusions are pesticidal toxins (cry(stal); Cry) named Cry proteins which were numbered in the order in which they were discovered. Genes encoding these δ-endotoxins have been transferred to major crops (such as corn, rice, cotton, potato, tomato, tobacco, soybean, etc.) which then produce Bt toxins (*2*). When ingested by insects, these toxins attack the midgut (*2*), resulting in the interruption of feeding by the insect, as well as gut paralysis, destruction of midgut epithelium and disruption of cell membrane permeability (*2*).

Plants which have been genetically modified with Bt genes are regulated in the United States by three agencies: U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS), the U.S. Environmental Protection Agency (EPA), and the Department of Health and Human Services' Food and Drug Administration (FDA). For example, USDA-APHIS under the Plant Protection Act (PPA, 7 USC 7701-7772) regulates corn as a "regulated article", based on the procedures used to introduce or express the Bt gene. EPA is responsible for setting the amounts or levels of pesticide residue that may safely be in food or feed. Developers of Bt crops also consult with FDA about possible other, unintended, changes to the food or feed, for example possible changes in nutritional composition or levels of native toxicants. Although this consultation is voluntary, all of the food/feed products commercialized to date have gone through the consultation process (*3*). Therefore efficient methods are needed to estimate the quantities of these toxic proteins.

In the present work, we evaluated the simultaneous measurement of Cry1Ab (e.g., Mon810 and Bt11 - Yieldgard™, Monsanto, St. Louis, MO) and Cry3B (e.g., MON863 YieldGard™ Rootworm) in corn extracts using a newly developed fluorescent covalent microsphere immunoassay (FCMIA) (*4, 5, 6, 7, 8*).

Methods and Materials

Antibodies and Pure Protein

Polyclonal rabbit anti-Cry1Ab, 2.7 mg/mL, polyclonal goat anti-Cry1Ab, 2.7 mg/mL, polyclonal rabbit anti-Cry3B, 5.7 mg/mL and mouse monoclonal anti-Cry3B, 5.1 mg/mL, purified Cry1Ab, as well as ground genetically modified organisms (GMO) (Cry1Ab and Cry 3B) and non-GMO corn were obtained from EnviroLogix (Portland, ME). All antibody solutions were dialyzed (Slide-A-Lyser 30 kD cassette, [Pierce Biotechnology, Rockford, IL] at 4°C against 50% diluted PBS [PBS pH 7.4, Sigma Chemical Co., St. Louis, MO]) to remove the sodium azide preservative in the commercial preparations.

Preparation of Capture Microspheres

The polyclonal rabbit anti-Cry1Ab and mouse monoclonal anti-Cry3B were used as capture antibodies. They were conjugated to two sets of microspheres (2.5×10^6, Luminex Corp., Austin, TX). The microspheres were 5.6 µm in diameter and composed of polystyrene, divinyl benzene and methacrylic acid, which provided surface carboxylate functionality for covalent attachment of biomolecules. Internally, the microspheres were dyed with red and infrared emitting fluorochromes. By proportioning the concentrations of each fluorochrome, spectrally addressable microsphere sets were obtained. Prior to conjugation, the microspheres were activated. Briefly, the microspheres were pelleted (5,000 x g for 2 min) in 1.5 mL centrifuge tubes using a microcentrifuge (Eppendorf, Hamburg, Germany). They were then resuspended by sonication [mini sonicator, Cole Parmer, Vernon Hills, IL], and gentle vortexing [VWR, Intl., West Chester, PA]) in 80 µL activation buffer (0.1M NaH_2PO_4, pH 6.2) to which 10 µL of 50 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, Pierce Chemical Company, Rockford, IL), and 10 µL of 50 mg/mL N-hydroxysulfosuccinimide, sodium salt (sulfo-NHS, Pierce Chemical Company) were added. The mixture was allowed to incubate for 20 minutes at room temperature. The microspheres were then washed twice in 500 µL coupling buffer (0.05 M 2-[N-morpholino]ethanesulfonic acid, MES, [Sigma Chemical Co.], pH 5.0) and solutions of anti-Cry1Ab, or anti-Cry3B (50 µg/mL) in 500 µl coupling buffer was added and incubated for 2 hrs at room temperature. The coupled microspheres were then washed twice in 1 mL wash buffer (PBS containing 0 .05 % Tween 20 [Sigma #3563]) and stored in 0.5 mL PBS-BSA (PBS, 1% BSA [Sigma #3688], 0.05% Na Azide [EM Sciences, Cherry Hill, NJ], pH 7.4). Microsphere concentrations were determined using a hemacytometer (Bright Line, VWR, Intl.).

Biotinylation of Detector Antibodies

Goat anti-Cry1Ab and rabbit anti-Cry3B were biotinylated using a commercially available biotinylation kit (BiotinTag Micro Biotinylation Kit, Sigma Chemical Co., St. Louis, MO) following the manufacturer's directions.

GMO and Non-GMO Corn Mixtures and Extraction

Mixtures of GMO and non-GMO corn were prepared to yield 10% and 50% by dry weight two component mixtures (e.g. 10% Cry 1Ab corn, 90% non-GMO corn). In addition, 50% Cry1Ab and 50% Cry3B mixtures were made. Three component mixtures of corn were also prepared (e.g., 10% Cry1Ab corn +10% Cry3B corn +80% non-GMO corn). Two grams of each of these mixtures, as well as 100% Cry1Ab and 100% Cry3B, were extracted in 5 mL extraction buffer (PBS-0.55% Tween 20). The mixture was then vortexed until all solids were suspended and then left undisturbed for 1 hour at room temperature. The mixture was re-vortexed and the solids allowed to settle until the extraction liquid became visibly clear. An aliquot of the extraction liquid was pressure filtered (0.45 μ Acrodisc) and placed in a clean vial. The primary corn extracts were further diluted (PBS-BSA) 1:3 and 1:10. This effectively yielded 1, 3.3, 5, 10, 16.7 and 50% mixtures which were extracted from a mixture of GMO corn and either Cry1Ab or Cry3B corn or non-GMO corn and Cry1Ab and Cry3B corn. Blanks were prepared by diluting extraction buffer in the same manner as the corn extracts.

Monoplex and Diplex Suspension Array Measurements

The Bio-Plex suspension array used to measure fluorescence (Bio-Rad Laboratories, Hercules, CA) was systematically calibrated and validated before each run using a maintenance, calibration, and validation plate, and standards supplied by the manufacturer. Conjugated microspheres (50 μL), at a working concentration of $1x10^5$ microspheres/mL for each microsphere type in PBS-BSA, were added to the wells of a 1.2 μm filter membrane microtiter plate (Millipore Corp., Part #MABVN1250, Bedford, MA) and the liquid aspirated by use of a vacuum manifold filtration system (Millipore, Part #MAVM09601). For each experiment, 50 μL (in duplicate) of pure Cry1Ab protein, corn extract, diluted extract or control buffer were added to either one (monoplex) or a mixture (diplex) of microspheres in the wells of the filter membrane microtiter plate and incubated for 30 min at 37° C with shaking. The contents of the wells were aspirated and the wells were washed three times with 200 μL wash buffer.

Fifty µL of the biotinylated detector antibody mixture in PBS/BSA was added to the wells and incubated for 30 min at 37° C. The contents of the wells were aspirated and the wells were washed three times with 200 µL wash buffer. An aliquot (50 µL) of a 4 µg/mL solution of streptavidin R-phycoerythrin (PE, Molecular Probes, Eugene, OR) in dilution buffer (PBS/BSA) was added to the wells of the plate and incubated for 15 min at 37° C. The wells were again washed three times with 200 µL wash buffer and resuspended in 100 µL wash buffer. The plate was shaken vigorously for approximately one min to disperse the microspheres and was placed into the autosampler platform of the BioRad Bioplex Suspension Array instrument using software, calibration microspheres, and sheath fluid supplied by the manufacturer. The instrument was programmed to collect data from 100 microspheres (classified by their internal fluorescence ratio) and acquire the median fluorescence intensity (MFI) of the microsphere-antibody-toxin-antibody-biotin-R-PE complex(es). The MFI response to Cry1Ab protein at 0, 0.1, 0.3, 1, 3, 10, 30 and 100 ng/mL was also evaluated in a sandwich FCMIA.

Data Analyses

For Cry1Ab, a standard curve was constructed from 4-parameter logistic log fits (4-PL) of MFI vs. the logarithm of ng/mL (*5, 7, 8*) (SigmaPlot, Systat, Point Richmond, CA) using the following relationship:

$$Y = \{(A - D) / [1 + (X/C^B)]\}$$

where A is the maximum MFI response, B is the slope at the inflection point, C the concentration of analyte at 50% maximal MFI, and D is maximal MFI. The Cry1Ab minimum detectable concentration (MDC) was calculated from the intersection of the asymptote of the regression's 95% confidence interval (CI) with the regression line (*6, 8, 9*). Assessment of the "goodness of fit" and the dynamic ranges of the assays were investigated by evaluating the fit of the standards data to the 4-PL model by "standards recovery", calculated by evaluating interpolated results from each 4-PL fit and comparing it to the actual concentration of Cry1Ab added to the system (*10*) using the following relationship:

$$\text{Percent recovery} = 100 \times \left(\frac{\text{Observed Concentration from 4PL fit}}{\text{Expected Concentration from 4PL fit}} \right)$$

The resultant data were analyzed for linearity by regression analysis (*7*). Extracts from GMO corn:non-GMO corn two and three component mixtures were either analyzed or diluted and analyzed as monoplex or diplex FCMIAs (1.0, 3.3, 5.0, 10, 16.7 and 50% GMO:non-GMO mixtures) and linear regressions of percentage GMO corn:non-GMO corn vs. MFI evaluated. Differences in MFI when the assays were run as monoplex or duplex were investigated using one-way repeated measures analysis of variance (SPSS, Chicago, IL).

Results

The FCMIA for Cry1Ab had an excellent fit to the 4-PL model (Figure 1A, r=0.998, P <0.0001). When the observed vs. expected concentration returned from the 4-PL model was evaluated, highly linear responses (r=0.996, P<0.001) were observed (Figure 1B). The MDC for Cry1Ab was 0.1 ng/mL. When extracts and dilutions from increasing ratios of Cry1Ab corn:non-GMO corn were evaluated in a monoplex format FCMIA (Figure 2 A), a linear relationship (MFI = [3217.7*Log(percent Cry1Ab) - 100.33]) was observed (r=0.983, P<0.001). Similarly, extracts and dilutions from increasing ratios of Cry3B corn:non-GMO corn were evaluated (Figure 2 B) in a monoplex format FCMIA, a linear relationship (MFI = [3221.2*Log(percent Cry3B)+7470.4]) was observed (r=0.984, P<0.001). When extracts and extract dilutions of mixtures of Cry1Ab corn, Cry3B corn, and non-GMO corn were evaluated (Figure 3) in a diplex FCMIA, the resulting linear relationships [(MFI = [3007.3*Log(percent Cry1Ab) + 173.6]), (r=0.965, P<0.002) and (MFI = [4177.4*Log(percent Cry3B) + 6807.3]), (r=.940, P=0.005)] were observed. MFI responses for the FCMIA assays executed in monoplex vs. diplex modes were are shown in Figures 4 A and B. One way repeated measures ANOVAs of these data demonstrate that no statistically significant differences were observed related to performance of diplex vs. monoplex FCMIA for either Cry3B non-GMO corn or Cry1Ab non-GMO corn mixtures (P=0.602 and 0.588, respectively). These results strongly suggest that there is no cross-reactivity between Cry1Ab and Cry3B.

Discussion

Genetic modifications are made to plants generally for two reasons, either to impart pesticidal properties to the plant itself, usually by adding Bt genes such that the plant can make pesticidal proteins (Cry1Ab, Cry1Ac, Cry1C, Cry1F, Cry2A, Cry3B and Cry9C) (*11, 12*) or to make the plant resistant to herbicides

such as glyphosate (*13*). This enables the grower to apply herbicides in a more targeted and efficient manner without harming the crop. Presently, real-time PCR is the most widely applied technique for the quantification of genetically modified organisms in foods and feeds (*14*). Traditional PCR for detecting genetically modified crops (*15*) has high sensitivity and specificity, but only one target gene can be detected in most cases. Multiplex PCR methods can be used for detecting more than one target sequence, such as CaMV 35S-P, nos-P, nos-t, npt II, and epsps in soybean and tobacco (*16*). Enzyme-linked immunosorbent assays (ELISAs) have also been used to detect GMO proteins (*17, 18*), however, ELISA's can only detect one analyte at a time. Commercially available lateral flow immunochromatographic dipsticks have been developed for both single and multianalyte detection of GMOs (e.g., QuickStix™ Kit for Roundup Ready® Grain - AS 010 BG and QuickStix™ Combo Comb Kit for Cry1Ac/Cry2A/Roundup Ready® - AS 046 STC, EnviroLogix). Single and multianalyte dipsticks, while convenient, still yield mostly qualitative yes/no results.

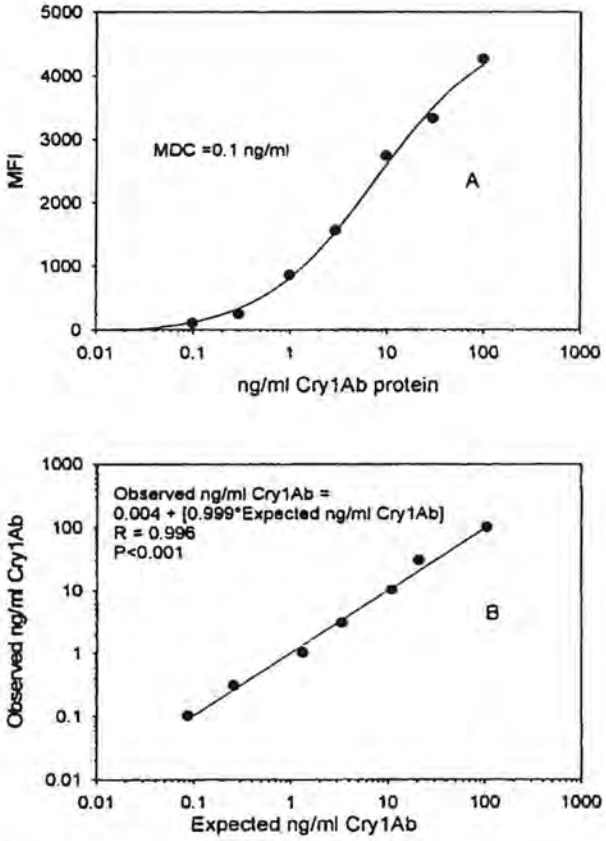

Figure 1. Panel A- Fluorescent covalent microsphere immunoassay 4-PL plot of ng/mL Cry1Ab vs. median fluorescence intensity. Panel B- Linear regression of the results of observed vs. expected MFIs from 4-PL fit.

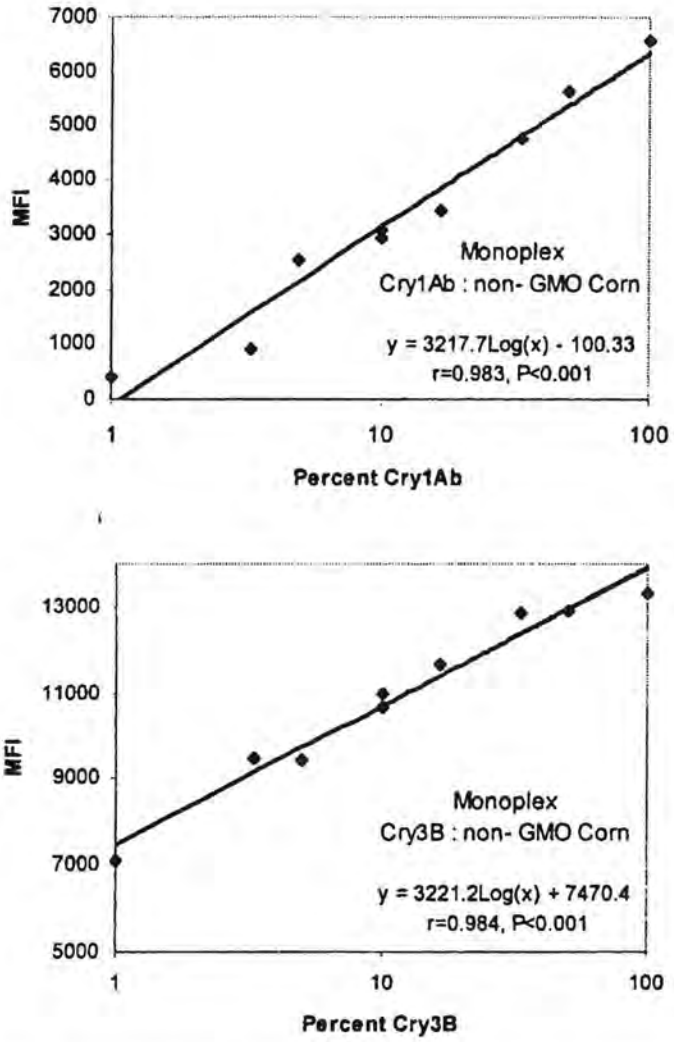

Figure 2. Linear regression of monoplexed fluorescent covalent microsphere immunoassay vs. percent Cry1Ab (Panel A) or Cry3B (Panel B) extracted from non-GMO:GMO corn mixtures.

The FCMIA could easily detect 1% GMO:non-GMO mixtures (the equivalent of 1 GMO kernel of Cry1Ab or Cry3B in 100 non-GMO kernels). One percent was the lowest ratio of GMO:non-GMO corn we used, however, the assay still had 1000s of MFI units before it would be expected to level off. This is supported by the demonstration of an MDC of 0.1 ng/mL for pure Cry1Ab.

Bt is now the most widely used biologically produced pest control agent. In 1995, worldwide sales of Bt were projected at $90 million, representing about 2% of the total global insecticide market with a worldwide distribution of 2.3 x 10^6 kg (*19*). As of early 1998, there were nearly 200 registered Bt products in

the United States (*19*). While the use of biological pesticides in agriculture remains significantly behind that of synthetic chemical pesticides, several environmental and safety considerations favor the future development of Bt for the control of pests in forestry, and the control of mosquitoes and blackflies (*19*). Therefore rapid analytical methods are needed.

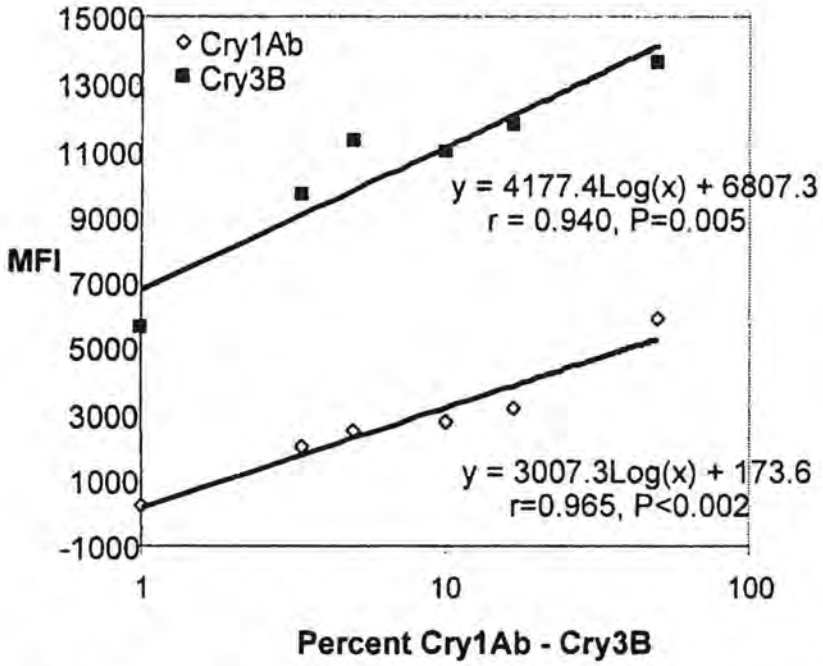

Figure 3. Linear regression of diplexed fluorescent covalent microsphere immunoassays vs. percent Cry1Ab and Cry3B extracted from non-GMO:GMO corn mixtures.

Multiplexed FCMIA technology has been shown to be useful in the measurement of diverse analytes such as cytokines (*20, 21*), cancer markers (*22*) gene expression (*23*), and markers of exposure to bioterrorism agents (*4, 6, 8*) and pesticides (*7*), etc. FCMIA has been shown to be more sensitive, faster and cheaper than alternative ELISA or chemical methodologies, and also has the capacity for remarkable throughput. Utilizing as few as 5 microplates, we have described an FCMIA, which has the capability of performing over 12,000 separate analyses in under 6 h (*5*).

In the present experiment we developed monoplex and diplex indirect sandwich FCMIAs for Cry1Ab and Cry3B extracted from GMO:non-GMO corn mixtures as well as a monoplex FCMIA for purified Cry1Ab. The monoplex FCMIA for Cry1Ab yielded a highly significant linear relationship when the observed vs. expected concentrations were calculated from the 4-PL fit. The mean recovery was 101.7 $\pm$ 19.8% (SD) over the concentration range of 0.1–100

ng/mL Cry1Ab while 6 of the 7 Cry1Ab dilutions added to construct the curve were in the 70–130% range considered acceptable for these types of assay (*10*) . The Cry1Ab MDC was 0.1 ng/mL. When extracts from pre-mixed GMO:non-GMO corn were evaluated for their Cry1Ab and Cry3B content highly linear relationships were observed on dilution, both in monoplex and diplex formats. Performing the FCMIA assay in diplex vs. a monoplex format showed no significant differences in MFI suggesting no cross-reactivity between Cry1Ab and Cry3B.

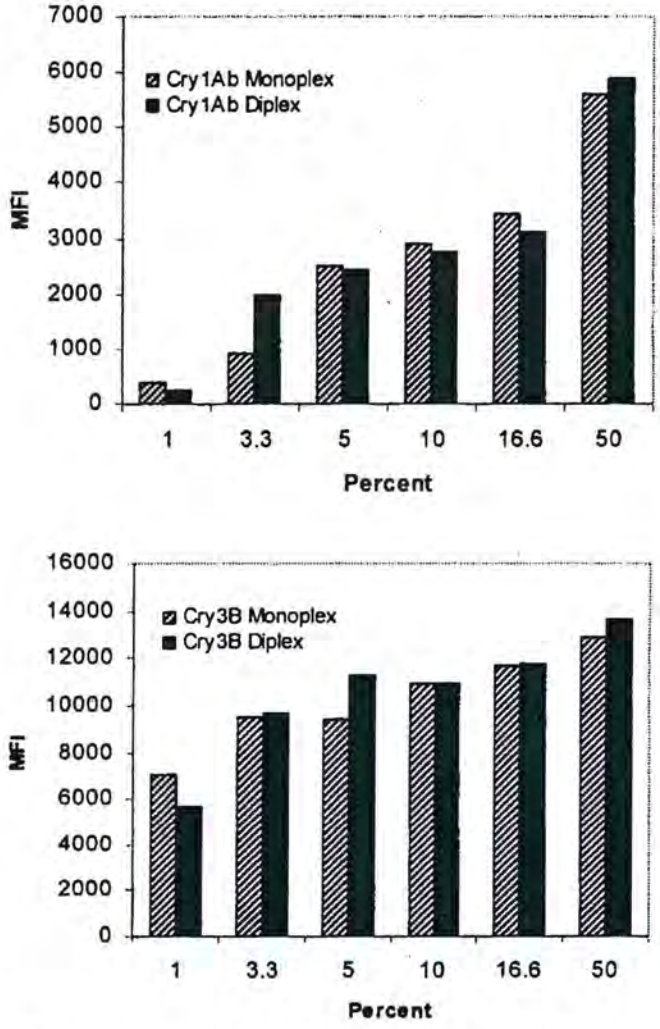

Figure 4. Comparison of monoplexed vs. diplexed fluorescent covalent microsphere immunoassays for Cry1Ab and Cry3B extracted from non-GMO:GMO corn mixtures. One way repeated measures ANOVA Cry1Ab (Panel A), P=.602. One way repeated measures ANOVA Cry3B (Panel B), P=.588.

In conclusion, we have shown proof of principle that Bt pesticidal proteins can be analyzed by multiplexed FCMIA.

Acknowledgements

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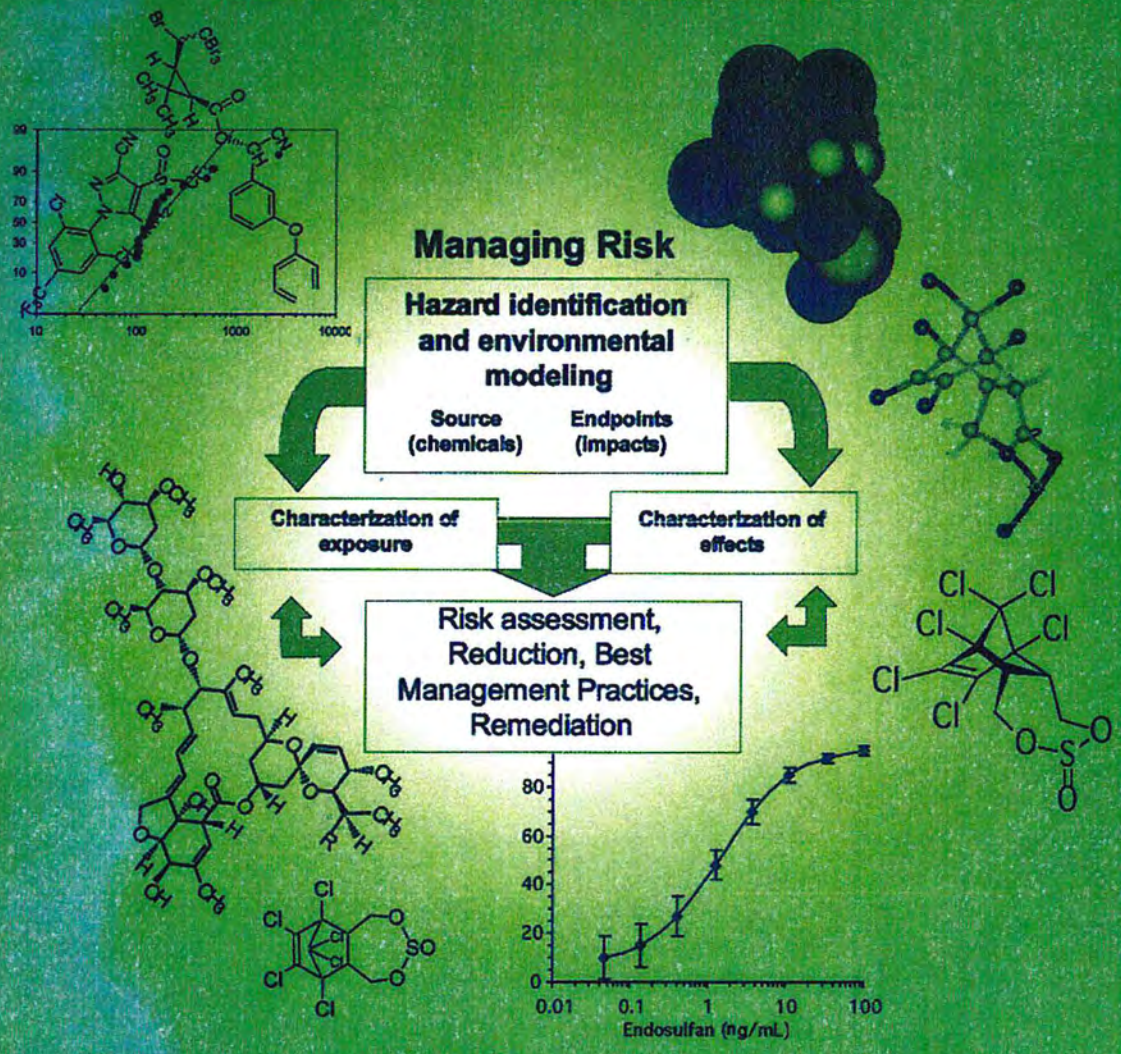

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