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Development and validation of a sensitive enzyme immunoassay for surveillance of Cry1Ab toxin in bovine blood plasma of cows fed Bt-maize (MON810)

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ABSTRACT

The increasing global adoption of genetically modified (GM) plant derivatives in animal feed has provoked a strong demand for an appropriate detection method to evaluate the existence of transgenic protein in animal tissues and animal by-products derived from GM plant fed animals. A highly specific and sensitive sandwich enzyme immunoassay for the surveillance of transgenic Cry1Ab protein from Bt-maize in the blood plasma of cows fed on Bt-maize was developed and validated according to the criteria of EU-Decision 2002/657/EC. The sandwich assay is based on immuno-affinity purified polyclonal antibody raised against Cry1Ab protein in rabbits. Native and biotinylated forms of this antibody served as capture antibody and detection antibody for the ELISA, respectively. Streptavidin-horseradish peroxidase conjugate and TMB substrate provided the means for enzymatic colour development.

The immunoassay allowed Cry1Ab protein determination in bovine blood plasma in an analytical range of 0.4–100 ng mL^{-1} with a decision limit (CCα) of 1.5 ng mL^{-1} and detection capability (CCβ) of 2.3 ng mL^{-1}. Recoveries ranged from 89 to 106% (mean value of 98%) in spiked plasma.

In total, 20 plasma samples from cows ($n = 7$) fed non-transgenic maize and 24 samples from cows ($n = 8$) fed transgenic maize (collected before and, after 1 and 2 months of feeding) were investigated for the presence of the Cry1Ab protein. There was no difference amongst both groups (all the samples were below 1.5 ng mL^{-1}; CCα). No plasma sample was positive for the presence of the Cry1Ab protein at CCα and CCβ of the assay.

1. Introduction

The Cry1Ab toxin is an insecticidal protein produced by the naturally occurring soil bacterium *Bacillus thuringiensis* [1,2]. The gene (truncated *cry1Ab* gene) encoding this insecticidal protein was genetically transformed into maize genome to produce a transgenic insect-resistance plant (Bt-maize; MON810) and, thereby, provide specific protection against Lepidoptera infestation [3,4]. Since past one decade such genetically modified (GM) crops have been commercialized

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and approved as an animal feed in several countries worldwide including the European Union (EU). The Cry toxins (protoxins) produced by GM crops are solubilized and activated to Cry toxins by gut proteases of susceptible insect larvae. Activated toxin binds to specific receptors localized in the midgut epithelial cells [5,6], invading the cell membrane and forming cation-selective ion channels that lead to the disruption of the epithelial barrier and larval death by osmotic cell lysis [7,8].

Cry toxins are considered harmless or non-toxic to human and farm animals [9,10], probably due to both the lack of Cry protein activation process in acidified gut [11] and of Cry toxin receptors in the mammalian small intestine [12–14]. Investigations on the effects of the Cry1Ab on mammalian cells have revealed no significant effect on bovine hepatocytes morphology or albumin secretion in vitro [15]. Furthermore, no negative effects of feeding Bt11 corn to calves, on development of any discernible clinical signs, hematological, biochemical, and rumen functions have been reported [16]. In situ studies in bovine rumen showed the time-dependent degradation of transgenic recombinant protein (Cry1Ab protein) from Bt176 corn [17]. Nevertheless, trace amounts of the Cry1Ab toxin were detected in the gastrointestinal contents of the livestock's fed on GM corns [18–20]. However, there are still safety concerns among consumers about the possible transfer and accumulation of transgenic protein in food for human consumption derived from animals receiving GM feed. A specific and sensitive method for detecting the Cry1Ab protein would facilitate monitoring of this transgenic protein in animal products.

A number of ELISA [21–25] and commercial kits (QuantiPlate kit for Cry1Ab/Cry1Ac, Envirologix and DAS ELISA kit for Bt-Cry1Ab/1Ac protein, Agdia) are already existing for the detection and quantification of Cry1Ab protein expressed in GM crops and their by-products. These commercial kits have been also used in various livestock feeding studies on GMO for the surveillance of transgenic protein in the animal tissues and gastrointestinal contents [17–20,26]. Though the commercially available Cry1Ab protein ELISA kits (QuantiPlate kit for Cry1Ab/Cry1Ac, Envirologix and Agdia) were reported to detect Cry1Ab protein down to 1 ng mL^{-1} of spiked blood [26], however, the study missed the most important assay validation part. Further, in another study [19] the same ELISA kit (Envirologix) did not work for the analysis of blood plasma for the surveillance of transgenic protein. Hence, such commercial kits designed for transgenic protein (Cry1Ab or Cry1Ac) quantification in plant materials warrants for a proper assay validation before used for protein analysis in animal systems.

Therefore, an assay for the specific detection, including all validation criteria, is required, in particular for the monitoring of transgenic protein in animal products (like blood plasma, milk and meat) derived form GM plant fed animals.

The aim of the present study was: (a) to raise and purify the Cry1Ab toxin specific polyclonal antibody for the development of a sensitive, specific sandwich ELISA for the surveillance of Cry1Ab protein in blood plasma of cows receiving ration supplemented with Bt-maize (MON810) and (b) to validate of this assay as per the requirements of assay validation cited in the guidelines of EU-Decision 2002/657/EC [27].

2. Materials and methods

2.1. Materials and instrumentation

All the reagents were of analytical grade and supplied by Merck (Darmstadt, Germany) unless specified otherwise. HPLC-purified trypsin-activated Cry1Ab protein for immunization and standard preparation was generously provided by Dr. William J. Moar, Auburn University, USA. Other Cry proteins (Cry3Bb1, Cry1C, Cry9C, Cry2A and Cry3A) were purchased from Agdia Inc. (Elkhart, USA). Bovine serum albumin (BSA) and 100 kDa MWCO SERVAPOR® dialysis tubing were from SERVA (Heidelberg, Germany). 3,3′,5,5′-tetramethylbenzidine (TMB), Freund's incomplete adjuvant, Biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Biotin-x-NHS, B2643), Sepharose-4B were purchased from Sigma (Germany). Streptavidin-horseradish peroxidase conjugate was purchased from Roche Diagnostics (Mannheim, Germany).

Maxisorp™ 96-well ELISA microplates were from Nunc (Roskilde, Denmark). 96PW microplate washer was from SLT Lab Instruments (Tecan, Germany). ELISA plates were read at 450 nm on a Sunrise™ microplate reader (Tecan, Germany) with online data calculation software Magellan V6.1 (Tecan, Austria).

Antibody coating buffer (CB) was 50 mmol L^{-1}, sodium carbonate/bicarbonate buffer pH 9.6. Assay buffer (PBST) was phosphate buffered saline (PBS; 8 mmol L^{-1} sodium phosphate, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 1.5 mmol L^{-1} potassium phosphate pH 7.4) containing 0.1% Tween® 20.

Matrix-matched calibrators (0.4–100 ng mL^{-1}) and controls (2.5, 20 and 60 ng mL^{-1}) were prepared by fortifying Cry1Ab protein in analyte free pooled blood plasma collected from the cows fed non-transgenic ration.

2.2. Production and purification of polyclonal antibody

2.2.1. Production of antibodies

Polyclonal antibodies were raised in crossbred rabbits ($n = 5$) through repeated immunization with 500 μg HPLC purified trypsin-resistant Cry1Ab toxin protein (65 kDa). Routinely, 500 μg of immunogen (Cry1Ab toxin) dissolved in PBS (150 μL) emulsified with Freund's incomplete adjuvant (1:1 volume ratio) was injected intra-cutaneously at multiple sites on the back of each rabbit. Booster injections (500 μg) were administered every 4 weeks for first 3 months, and later after each 2 months. Antiserum test bleeds were taken from the ear veins 10–14 days after each booster injection and screened for the presence of antibodies to Cry1Ab toxin using a direct competitive chequerboard titration ELISA (immobilized antiserum and Cry1Ab toxin-biotin tracer).

2.2.2. Immuno-affinity cleanup of polyclonal antiserum

The polyclonal antibody was purified from the blood plasma obtained from the immunized rabbit, on an affinity column made by coupling Cry1Ab protein to CNBr-activated Sepharose-4B by a standard procedure (Pharmacia, Sweden). Briefly, one gram of CNBr-activated Sepharose-4B was rehydrated and washed using 1 mmol L^{-1} HCl at room tem-

perature. The coupling buffer (0.1 mol L^{-1} $NaHCO_3$, 0.5 mol L^{-1} NaCl pH 8.3) was added to the gel for prehydrolysis for 4 h in order to reduce the number of coupling groups on the matrix to preserve the structure of the binding site and to facilitate elution. Ten milligrams of Cry1Ab toxin dissolved in 5 mL of coupling buffer was mixed with the gel. The tube was immediately capped and rocked end-to-end for 1 h at room temperature on a rotor. The non-reacted Cry1Ab toxin in supernatant was washed away with 5 times gel volume of coupling buffer. In order to block the redundant active groups on the Sepharose-4B, the gel was further treated with 0.1 mol L^{-1} Tris–HCl buffer pH 8.0, for 2 h; and washed three cycles of alternating buffers: 0.1 mol L^{-1} acetate buffer (pH 4.0, containing 0.5 mol L^{-1} NaCl) followed by 0.1 mol L^{-1} Tris–HCl (pH 8.0, containing 0.5 mol L^{-1} NaCl). Then the coupled gel was extensively washed PBS and packed into a chromatography column.

The Sepharose-4B-Cry1Ab protein column was extensively washed with PBS before blood plasma sample of immunized rabbit was loaded onto the column. The sample was allowed to flow into the column slowly. Afterwards, the flow was stopped for 30 min to let antibody adsorb fully. The adsorbed impurities and low affinity antibodies were eluted with different elution buffers A, B, and C (elution buffer A, PBS pH 7.4; B, 0.5 mol L^{-1} NaSCN pH 8.0, and C, 0.1 mol L^{-1} glycine pH 3.5). The eluent was slowly collected under gravity at 2 mL per sample tube, and the optical density at 280 nm (A_{280}) of each collected fraction was measured with a biophotometer (Eppendorf). Elution buffer was changed when absorbance at 280 nm in the eluted fractions reached to the lowest constant value. Finally Cry1Ab protein specific antibody was eluted with 0.1 mol L^{-1} glycine pH 2.0 (elution buffer D) and immediately pH of eluted antibody solution is brought to 7 with 1 mol L^{-1} Tris–HCl (pH 8.0). Fractions containing antibody were pooled and dialyzed against PBS, diluted to 1 mg mL^{-1}, aliquoted, and stored frozen at −20 °C.

Protein concentration of purified antibody was determined by the Bicinchoninic acid (BCA) protein assay [28] adapted to microtitre plates using bovine serum albumin as standards.

2.3. Biotin labeling of purified polyclonal antibody

The purified antibody was dialyzed against PBS and labeled with biotin using *N*-hydroxysuccinimide biotin. One milliliter (210 μg) of the dialyzed antibody solution was reacted with 20 μL (20 μg) of freshly prepared NHS-x-biotin in *N*,*N*-dimethylformamide (DMF). After 4 h incubation at room temperature, the non-reacted biotin was blocked from further reaction by addition of 400 μL glycine (2 mg mL^{-1} in PBS) followed by 25 min incubation at room temperature. Non-reacted biotin containing reagent was separated, using Amicon Ultra 10 K centrifugal units from Millipore (Schwalbach, Germany). The concentration of biotinylated antibody was adjusted to 1 mg mL^{-1} in PBS.

2.4. Development and optimization of enzyme immunoassay

The basic sandwich enzyme immunoassay protocol is used with variation in a series of assays to select optimal antibody concentrations, choose an appropriate assay buffer and reduce plasma interference.

2.4.1. *Optimizing the capture and biotinylated detection antibody concentrations*

In order to determine the optimum capture (coating) antibody and biotinylated detection antibody concentration, a sandwich ELISA was applied as described in immunoassay procedure. A dilution range of 0.008–0.1 μg per well of capture antibody and 0.063–2 ng per well of detection antibody was tested with the fixed concentration of 1 ng per well Cry1Ab toxin. The ELISA values (absorbance at 450 nm) obtained after substrate reaction were used for choosing the optimal concentration of antibody pairs. The optimal reagent concentration was defined as those, which gave the absorbance values around 1–1.5 at 450 nm with minimum reagent expense and lower than 0.2 value for negative antiserum (background).

2.4.2. *Antibody specificity*

The degree of antibody specificity within the Cry toxin family (i.e., the extent of cross-reactivity) was estimated analyzing parallel concentration (4–1000 pg per well) calibrators of different Cry protein along with the Cry1Ab protein (calibrators) calibration curve by means of a sandwich ELISA. Cross-reactivity was calculated as follows: ($C_{1–1000}$ of Cry1Ab protein/$C_{1–1000}$ of Cry toxins) × 100; where C_{1-1000} represents the concentrations of analyte binding to the capture and detection antibody (anti-Cry1Ab protein) and respective optical density values at 450 nm.

2.4.3. *Sandwich enzyme immunoassay*

Immuno-affinity purified capture antibody was immobilized in 96-well microtitre plates overnight at 4 °C by the addition of 100 μL antibody (0.05 μg diluted in coating buffer) to each well. Excess antibody was removed by decanting the plate contents. Remaining sites in well were blocked with incubating 300 μL per well blocking buffer (1% BSA in PBST) for 1 h at room temperature while shaking. After decantation of blocking buffer, the capture antibody coated and blocked plates were stored frozen at −20 °C until used in assay. At the time of assay frozen plates were thawed to room temperature and washed twice with assay buffer. Aliquots of 10 μL matrix-matched calibrators (0.4–100 ng mL^{-1}), controls (2.5, 20 and 60 ng mL^{-1}) and unknown plasma samples were added to respective wells of microtiter plate (in duplicates) followed by the addition of 90 μL assay buffer. Plates were incubated for 3 h at room temperature while shaking, and then washed six times with assay buffer. Then 100 μL biotinylated detection antibody (5 ng mL^{-1} diluted in assay buffer) was added to each well, and incubated at room temperature for 1 h on plate shaker, followed by four washing steps with assay buffer. Streptavidin-horseradish peroxidase enzyme conjugate (100 μL, diluted 1:15,000 times in assay buffer) was then added and the plates were incubated for 15 min at room temperature, followed by four washings with assay buffer. TMB enzyme substrate (150 μL) was added to each well and the plates were incubated for 40 min at room temperature in dark on plate shaker. Colour development was stopped by addition of 2 mol L^{-1} sulfuric acid (50 μL). The absorbance was read at 450 nm in microplate reader, a Cry1Ab toxin calibration curve was constructed using online Megal-

lan V6.1 software and the concentration of Cry1Ab toxin in unknown samples were determined by interpolation. All data are presented as ng of active Cry1Ab toxin mL^{-1} blood plasma.

2.5. *Plasma samples and assay validation*

The assay was validated according to the criteria specified in the recently adopted Commission Decision 2002/657/EC [27] for the performance and validation of screening and confirmatory analytical methods.

2.5.1. *Decision limit (CCα) and detection capability (CCβ)*

ELISA validation was carried out using 48 different blood plasma samples (blanks), known to be free of Cry1Ab toxin, collected from cows fed non-transgenic ration reared at three different farms (from three different breeds: 15 Brown Swiss, 18 Bavarian Fleckvieh and 15 Red Holstein cows). The samples were analyzed using sandwich ELISA as described above to demonstrate the range of blank matrix effects and the determination of CCα and CCβ. CCα is equal to three times the average signal to noise level recorded for the Cry1Ab protein in the blanks. CCβ was determined using the equation $CC\beta = CC\alpha + 1.64 \times S.D._S$; $S.D._S$ being the standard deviation obtained for above 48 blanks fortified at the spike concentration level of CCα. Calculation for α- and β-error were carried out from the Cry1Ab protein background noise level in 48 blanks and fortifying the same samples at concentration level of CCβ value. α-Error is the percentage of blank values exceeding the CCα value. β-Error is represented by the percentage of blank samples lying below the CCα value when fortified at the concentration level of CCβ.

2.5.2. *Recovery and precision*

Recovery and precision were determined in accordance with Commission Decision 2002/657/EC by spiking blank plasma samples with Cry1Ab protein. Recovery was calculated for the six aliquots of blank plasma per spike concentration level for six different concentration levels (2.5, 3.75, 5, 10, 25 and 80 ng mL^{-1}).

Precision was expressed by inter- and intra-assay coefficients of variation, and calculated from the analysis of blank plasma aliquots fortified with Cry1Ab protein at three (controls) different concentration levels of 2.5, 20 and 60 ng mL^{-1} (three determinants per assay) in total 14 assays performed on different days.

2.6. *Application of developed ELISA for surveillance of Bt-toxin in blood plasma of cows fed transgenic maize (MON810)*

To investigate the possible transfer of Cry1Ab protein into blood plasma from the digestive tract of animals fed on a ration supplemented with transgenic maize (MON810), the blood samples from two groups of multiparous (second lactation) lactating cows fed transgenic (containing kernel, silage and cobs from MON810 as diet component; $n = 8$) and non-transgenic (containing kernel, silage and cobs from non-transgenic maize as diet component; $n = 7$) ration were analyzed for the presence of Cry1Ab protein. The daily diet contained a TMR (total mixed ration) consisting of forage (7.0 kg maize silage, 3.6 kg maize cobs and 3.0 kg grass silage as dry matter) and 3.5 kg concentrate (40.8% maize kernels, 52% rape-bruised grain extract, 5% mineral mixture and 2.2% urea) as dry matter. Blood samples were collected before, and after 1 and 2 months of continuous feeding transgenic and non-transgenic rations. Blood plasma was separated by centrifugation of blood samples at $2000 \times g$ for 15 min under refrigerated conditions and further stored at −80 °C until used in Cry1Ab protein ELISA.

3. Results and discussion

3.1. *Production and purification of antiserum*

All immunized rabbits produced antisera against Cry1Ab toxin with titers ranging from 1.5×10^{-4} to 6×10^{-5}. However in subsequent competition experiments, the antisera showed that quite different degrees of inhibition by the analyte for the calibration curve (data not shown). The one that exhibited the largest inhibition by the analyte was selected as the assay reagent for the Cry1Ab protein sandwich enzyme immunoassay. The selected antiserum of a rabbit with high titer of anti-Cry1Ab toxin and a sensitive calibration curve (with direct competitive assay) was further purified by immuno-affinity chromatography. Absorbance measurements (A_{280}, nm) for protein concentrations in eluted fractions for change of elution buffer during antibody purification are presented in Fig. 1. Cry1Ab toxin specific antibody was collected in the eluted fractions 28–32.

3.2. *Assay development and optimization*

The several parameters optimized for the development of a sandwich ELISA included, the concentrations of coating and

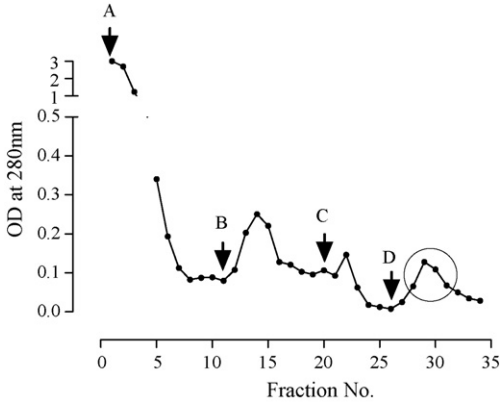

Fig. 1 – Immuno-affinity cleanup of antiserum collected from rabbit immunized against Cry1Ab protein. After applying antiserum onto affinity column, the column was washed with different wash buffers: A, PBS buffer pH 7.4; B, 0.5 mol L^{-1} NaSCN buffer pH 8.0 and C, 0.1 mol L^{-1} glycine buffer pH 3.5. Cry1Ab protein specific antibody (IgG) was eluted with buffer D (0.1 mol L^{-1} glycine buffer pH 2.0). Eluted fractions 28–31 (marked in circle) containing specific antibody were pooled and further concentrated after dialysis.

detection antibody, optimal pH of assay buffer, and the volume of plasma sample in assay. Standard curves were obtained by plotting absorbance value (450 nm) against the concentration of analyte.

3.2.1. *Optimizing the capture and biotinylated detection antibody concentrations*

The absorbance values were influenced significantly by the capture and detection antibody concentrations. It revealed that higher concentrations exhibits higher absorbance at 450 nm. According to the principle, we choose the concentrations when the absorbance values (A_{max}) were around 1.0–1.5 and the ELISA (absorbance) value of negative antiserum was lower than 0.1. As a result, the optimal concentrations of antigen capture and biotin-labeled detection antibodies were 0.5 $\mu g\,mL^{-1}$ and 5 $ng\,mL^{-1}$, respectively. The optimized antibody concentrations for pairing Cry1Ab protein were subsequently used in the sandwich ELISA to get a sensitive calibration curve.

3.2.2. *Antibody specificity*

Immuno-affinity purified Cry1Ab protein antibody has not shown any cross-reaction with other Cry toxins: Cry3Bb1, Cry1C, Cry9C, Cry2A, and Cry3A when used in a sandwich ELISA (Fig. 2). These results indicate that the developed immunoassay is highly specific for the Cry1Ab toxin and confirms the suitability of immuno-affinity purified antibody in assay at $ng\,mL^{-1}$ detection level.

3.2.3. *Matrix interference and assay sensitivity*

One of the common challenges of immunoassays is the matrix interference. These interferences can be reduced by either dilution with some buffers or by using a matrix-matched calibration curve. Hence, the influence of the blood plasma volume on the assay was evaluated by using Cry1Ab toxin calibration curves in assay buffer, compared with the addition of different volumes of blood plasma (10, 20 and 40 μL). The results indicated that the increasing blood plasma volumes decreased the A_{max} and the sensitivity of the calibration curve. As a result, 10 μL sample size (plasma volume) was used in assay to nullify the matrix effect by using a matrix-matched calibration curve and achieved good assay sensitivity.

A typical matrix-matched Cry1Ab protein calibration curve using optimized ELISA conditions is shown in Fig. 3. The developed sandwich assay allowed the determination of Cry1Ab toxin over the dynamic range (<20% CV between the replicates of calibrators) from 0.4 to 100 $ng\,mL^{-1}$ with an analytical limit 0.4 $ng\,mL^{-1}$.

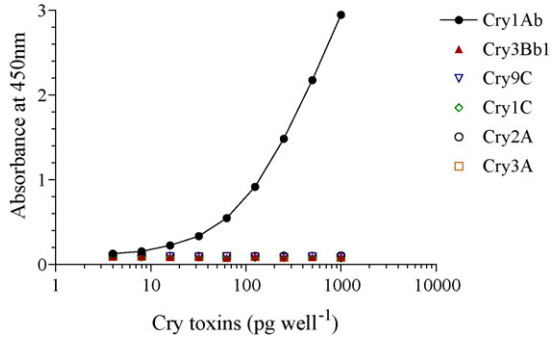

Fig. 2 – Cross-reactions of Cry toxins with Cry1Ab antibody using parallel standard concentrations in assay buffer in ELISA.

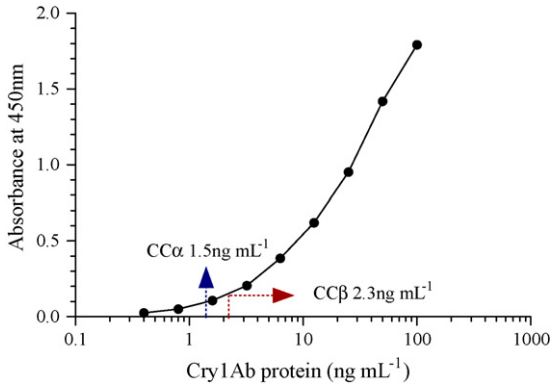

Fig. 3 – A typical matrix-matched calibration curve for Cry1Ab protein in bovine blood plasma.

3.3. *Plasma samples and assay validation*

3.3.1. *Decision limits (CCα) and detection capability (CCβ)*

Analysis of 48 blank blood plasma samples by ELISA yielded the background values for the Cry1Ab protein ranging from 0.4 to 1.97 $ng\,mL^{-1}$ (mean 0.51 $ng\,mL^{-1}$). The decision limit (CCα) calculated from the mean signal to noise level was 1.53 $ng\,mL^{-1}$. When same 48 blanks were fortified with Cry1Ab protein at the concentration level of CCα (1.5 $ng\,mL^{-1}$), the values ranged from 1.21 to 3.51 $ng\,mL^{-1}$ (mean 1.57 $ng\,mL^{-1}$; $S.D._S$ 0.49 $ng\,mL^{-1}$). The detection capability (CCβ) calculated from the equation $CC\beta = CC\alpha + 1.64 \times S.D._S$ was 2.30 $ng\,mL^{-1}$ (Fig. 4(a)). The observed Cry1Ab protein values for the blanks fortified at concentration level of CCβ (2.30 $ng\,mL^{-1}$) ranged from 1.81 to 5.53 $ng\,mL^{-1}$ (mean 2.46 $ng\,mL^{-1}$). There was no overlap between the blanks and fortified samples (Fig. 4(b)). Detection capability (2.30 $ng\,mL^{-1}$) and threshold value of 1.81 $ng\,mL^{-1}$ (the lowest observed 2.30 $ng\,mL^{-1}$ fortified sample) laid the basis for selection of the samples for confirmatory analysis. Hence, samples with a concentration level at and above 1.81 $ng\,mL^{-1}$ must be analyzed by any other confirmatory method to draw a final conclusion. The assay β-error is zero since no false negative (false compliant) results were obtained for 2.30 $ng\,mL^{-1}$ fortified blood plasma samples. This satisfies Decision 2002/657/EC [27] which states that screening assays must "*have a false compliant rate of <5% (β-error) at the level of interest*". Similarly, the α-error (false non-compliant) is <5% as 2 blank values exceeded the CCα (1.53 $ng\,mL^{-1}$) value. Both CCα and CCβ values satisfy 2002/657/EC Commission Decision, the criteria for the performance and validation of screening and quantitative analytical methods.

3.3.2. *Recovery and precision*

The analytical performance of the developed enzyme immunoassay was assessed by spiking matrix samples (blank plasma) with the Cry1Ab protein. The immunoassay performed well when it was applied to spiked plasma samples

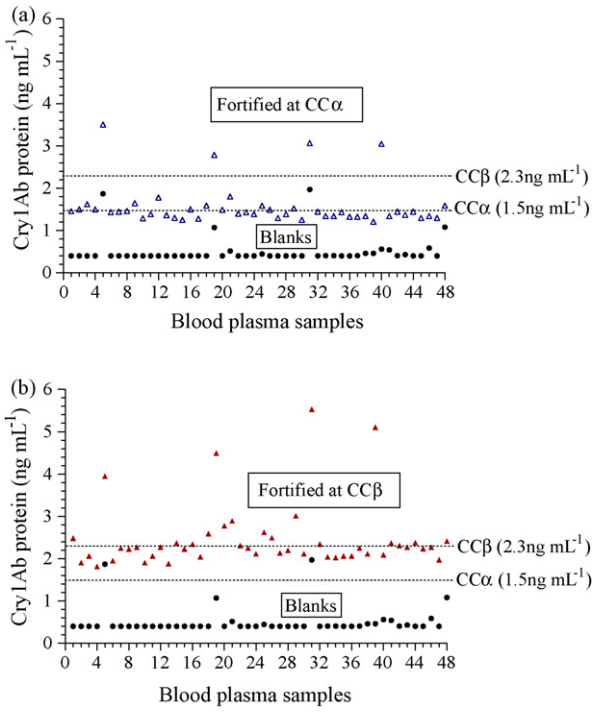

Fig. 4 – (a) Determination of detection capability (CCβ) of the Cry1Ab protein ELISA in bovine blood plasma. Detection capability was calculated from the Cry1Ab protein fortified blanks (△: $n = 48$) at the concentration level of decision limit (CCα 1.5 ng mL^{-1}). CCα calculated as three times S/N ratio from the blanks (●: blood plasma collected from 48 cows fed non-transgenic ration). (b) Determination of α- and β-errors for the Cry1Ab protein ELISA in bovine blood plasma. α-Error and β-error was calculated from the Cry1Ab toxin fortified blanks. Percentage of blanks (●: blood plasma from 48 cows fed non-transgenic ration) lying above the decision limit (CCα 1.5 ng mL^{-1}) indicated the α-error (>5%). β-error (zero) was indicated by the spiked blanks (▲: $n = 48$) at concentration level of 2.3 ng mL^{-1} (CCβ).

and recoveries ranged from 89 to 106% (mean value of 98%) in plasma (Table 1). The Cry1Ab protein fortified blanks at spike concentrations of 2.5, 20 and 60 ng mL^{-1} (three determinants per assay) in 14 independent assays showed a good assay precision, with intra- and inter-assay coefficients of variation below 10% (8.2 and 9.1%, respectively; Table 2).

Table 1 – Recoveries achieved for Cry1Ab protein determination in spiked bovine blood plasma[a]

Cry1Ab protein		Recovery (%)
Amount added (ng mL^{-1})	Amount measured (ng mL^{-1})[b]	
2.50	2.66 ± 0.17	106.4
3.75	3.87 ± 0.20	103.4
5	4.93 ± 0.19	98.5
10	9.99 ± 0.27	99.9
25	22.22 ± 1.15	88.9
80	73.92 ± 4.47	92.4
Mean recovery (%)		98.3[c]

[a] Blood plasma samples collected from non-transgenic ration fed cows.
[b] Mean value ± standard deviation (six replicates).
[c] Mean recovery (%).

The analytical performance of the assay indicates that it can be used for monitoring concentration levels of Cry1Ab protein in bovine blood plasma.

3.4. *Application of developed ELISA for the surveillance of Bt-toxin in blood plasma of cows fed transgenic maize (MON810)*

In total 45 blood plasma samples (collected before and, after 1 and 2 months of feeding) were analyzed for the presence of the Cry1Ab protein from both, transgenic ($n = 8$) and non-transgenic ($n = 7$) ration fed cows. There was no difference amongst both groups (all plasma samples were below 1.5 ng mL^{-1}; CCα value). No sample was positive for the presence of the Cry1Ab protein at the decision limit (CCα) and detection capability (CCβ) of the assay (Fig. 5). The absence of transgenic (Cry1Ab) protein from Bt-maize in blood plasma of cows is supported by the other findings reporting the lack of transgenic protein in the tissues (liver, spleen, kidney, lymph nodes and muscles) from livestock that had consumed GM corn [18,19,29]. The reasons for the absence of the Cry1Ab protein from Bt-maize in the blood plasma of cows fed transgenic ration could be the lack of the absorption mechanism involved in the transfer of this toxin from the gut into the blood stream. This could be further supported by the findings reporting the lack of Cry1Ab toxin specific receptors on bovine intestinal epithelium [13,14] and in vivo degradation of trans-

Table 2 – Precision for Cry1Ab protein ELISA in spiked bovine blood plasma[a]

Coefficient of variation	Spiked Cry1Ab (controls)			Mean CV (%)
	C1 (2.5 ng mL^{-1})[b]	C2 (20 ng mL^{-1})[c]	C3 (60 ng mL^{-1})[d]	
Intra-assay (%)	8.9	7.0	8.5	8.2
Inter-assay (%)	10.8	7.9	8.7	9.1

[a] Blood plasma samples collected from non-transgenic ration fed cows.
[b] Coefficients of variation at spike concentration of Cry1Ab protein 2.5 ng mL^{-1} (three determinants per assay) in 14 independent assays.
[c] Coefficients of variation at spike concentration of Cry1Ab protein 20 ng mL^{-1} (three determinants per assay) in 14 independent assays.
[d] Coefficients of variation at spike concentration of Cry1Ab protein 60 ng mL^{-1} (three determinants per assay) in 14 independent assays.

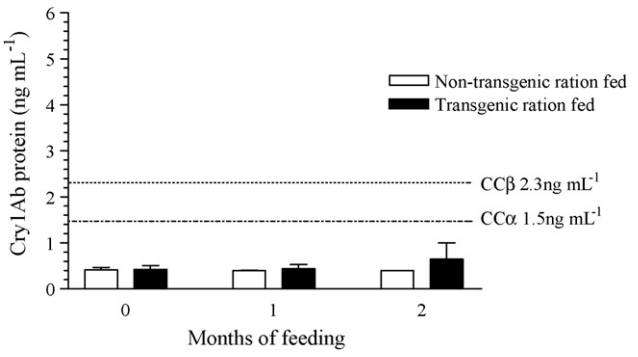

Fig. 5 – Cry1Ab toxin ELISA background signals in blood plasma of cows fed transgenic ($n = 8$) and non-transgenic ration ($n = 7$). Blood samples collected before and, after 1 and 2 months of feeding were investigated for the presence of the Cry1Ab protein. No sample found positive for the presence of Cry1Ab toxin in plasma and all the values were below the decision limit (1.5 ng mL^{-1}) and detection capability (2.3 ng mL^{-1}). The data is presented as mean (±S.D.) values.

genic protein during ruminal digestion [17]. The absence of novel protein in blood plasma of cows fed Bt-maize may consider it as a safe animal feed. The previous findings [16] of a short-term Bt11 maize feeding study on calves demonstrating no negative effects on development of any discernible clinical symptoms, growth rate, hematology, blood biochemistry, and rumen functions further supports Bt-maize as a safe animal feed. In addition the animal performance studies on lactating cows fed GM corn has depicted no changes in animal health, behavior, milk yield and milk composition in comparison to non-GM corn fed cows [30–32]. Though the short-term feeding studies consider GM corn as a safe animal feed, however, before drawing any definitive conclusions a long-term feeding study is warranted.

The enzyme immunoassay for the surveillance of transgenic protein in animal blood plasma is an attractive technique to answer questions like the possibility of transfer of this recombinant protein into animal blood during digestion. However, the developed immunoassay should consider the matrix interferences and all validation criteria (sensitivity, specificity, precision, recovery, and detection capability) of the performance of an analytical method. Such performance criteria are crucial for the detection of low levels of Cry1Ab toxin in blood plasma, if survived through the rumen digestion and crossing the intestinal mucosa barrier.

This is, to our knowledge, the first immunoassay for a specific detection of Cry1Ab toxin in the blood plasma of cows fed transgenic maize. The assay also fulfills all the validation criteria as prescribed in the guidelines of EU-Decision 2002/657/EC. Though the commercially available Cry1Ab protein ELISA kits (QuantiPlate kit for Cry1Ab/Cry1Ac, Envirologix and Agdia) were reported to detect Cry1Ab protein down to 1 ng mL^{-1} of spiked blood [26], however, the study missed the most important assay validation part. Further, in another study [19] the same ELISA kit (Envirologix) did not work for the analysis of blood plasma for the surveillance of transgenic protein.

4. Conclusions

A sandwich ELISA based on immuno-affinity purified polyclonal native capture and biotin-labeled detection antibody has been developed for the Cry1Ab toxin determination at low levels (CCβ, 2.3 ng mL^{-1}) in bovine blood plasma. The developed ELISA satisfied the performance and validation criteria laid down by Commission Decision 2002/657/EC. The immunoassay performed well with the spiked plasma samples and recoveries ranged from 89 to 106% (mean value of 98%). When applied for the surveillance of transgenic Cry1Ab toxin from Bt-maize in blood plasma of cows fed transgenic ration for a short-term, no sample was positive for the presence of Cry1Ab protein.

Further work can be carried out to apply and validate this ELISA for surveillance of Cry1Ab toxin in blood plasma and other matrices like milk, urine and faeces collected from the animals fed for long-term on transgenic ration. Most probably it could answer questions like digestive fate of transgenic protein and possible entry into the blood stream, if successful in breaking the digestive barrier of animals fed for long-term on GM plant and plant by-products.

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