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# Fate of Cry1Ab Protein in Agricultural Systems under Slurry Management of Cows Fed Genetically Modified Maize (*Zea mays* L.) MON810: A Quantitative Assessment

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**ABSTRACT:** The objective of the study was to track the fate of recombinant Cry1Ab protein in a liquid manure field trial when feeding GM maize MON810 to dairy cows. A validated ELISA was applied for quantification of Cry1Ab in the agricultural chain from GM maize plants, feed, liquid manure and soil to crops grown on manured fields. Starting with 23.7 $\mu$g of Cry1Ab $g^{-1}$ dry weight GM maize material, a rapid decline of Cry1Ab levels was observed as 2.6% and 0.9% of Cry1Ab from the GM plant were detected in feed and liquid manure, respectively. Half of this residual Cry1Ab persisted during slurry storage for 25 weeks. After application to experimental fields, final degradation of Cry1Ab to below detectable levels in soil was reported. Cry1Ab exhibited a higher rate of degradation compared to total protein in the agricultural processes. Immunoblotting revealed a degradation of the 65 kDa Cry1Ab into immunoreactive fragments of lower size in all analyzed materials.

**KEYWORDS:** GM maize, MON810, Cry1Ab protein, dairy cow, liquid manure, soil, *Zea mays*

## 1. INTRODUCTION

The global area planted with biotech crops comprised 148 million hectares in 2010 with genetically modified (GM) maize accounting for a major proportion as 47 million hectares were cultivated with GM maize.[1] The majority of GM maize crops used at present exhibit resistance against insect pests by expression of insecticidal proteins derived from delta-endotoxins of *Bacillus thuringiensis* (Bt). These Bt proteins are summarized as crystal (Cry) proteins as they are stored in crystalline inclusion bodies in the bacterial cell.[2,3] Each class of Cry proteins is characterized by a narrow range of susceptible target organism species as the toxin needs proteolytic activation in the gut and binding to selective receptors localized in the midgut epithelial cells of larvae to unfold its toxic effects.[4] This defined spectrum of insecticidal activity allows for target specific pest management and reduced application of insecticides when growing Bt crops.[5]

MON810 is a transgenic maize event based on the introduction of a modified *cry1Ab* gene into the plant genome enabling plants to synthesize a truncated form of the protein Cry1Ab. Cry1Ab is highly active against Lepidoptera and widely used to combat the European corn borer (*Ostrinia nubilalis* Hübner), a major pest in maize fields.[6] MON810 cultivars have been registered and commercialized in 1996 in the United States, followed by their approval in other countries all over the world including countries of the European Union in 1998.[7]

Since the development and application of the Bt-maize technology, safety concerns have been raised concerning potential adverse effects on human and animal health and the environment, though an extensive environmental risk assessment is prescribed and performed before the approval of any GM crop.[8,9] For Cry1Ab expressing maize these concerns were met by a broad range of studies dealing with the toxicity and allergenic potential of Cry proteins,[10−12] transfer of Cry1Ab from livestock feed into food for human consumption,[13] impact on nontarget organisms[14,15] and entry of Cry1Ab from Bt-maize plants into agricultural soils.[16−18] Although the current scientific knowledge about Bt crops gives no evidence for any significant risk to the environment or to human and animal health,[7,19] commercial Bt-maize (MON810) cropping was banned in some EU countries in the face of low consumer acceptance when GM crops are employed in food and feed production.[20]

Regarding the fate and metabolism of the insecticidal Cry1Ab protein in dairy cows fed Bt-maize, results from a 25 month long-term feeding study[21] revealed that immune detective Cry1b protein fragments are not transferred to body fluids as urine, blood plasma and milk.[22] However, as the protein is not completely degraded by digestive processes in the gastrointestinal tract (GIT), the Cry1Ab is still detectable in feces of Bt-maize fed cattle.[23] Considering these findings in view of manuring practice on dairy farms, the use of a mixture of feces and urine as liquid manure for fields implicates the introduction of the recombinant Cry1Ab protein into the agricultural environment, particularly the soil compartment, provided the fact that it is not totally degraded during liquid manure storage. Whereas the entry of Cry1Ab into soil via maize plant residues has been extensively studied,[18,24−27] the entry via liquid manure of Bt-maize fed cattle

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**Table 1. Time Schedule for Application of Liquid Manure to Experimental Field Lots and Collection of Samples for Assessing Cry1Ab Protein Contents in the Field Trial Carried Out in 2007**

| | date of liquid manure treatment or sampling | |
|---|---|---|
| | Grub[a] | Finsing[a] |
| 1st manure application grass | Apr 3 | Apr 3 |
| 1st manure application maize field | Apr 19 | Apr 18 |
| 1st grass cut | May 2 | May 4 |
| 2nd manure application grass | May 3 | May 4 |
| 2nd manure application maize field | May 15 | Jun 12 |
| 2nd grass cut | May 31 | Jun 4 |
| 3rd manure application grass | Jun 4 | Jun 4 |
| 3rd grass cut | Jul 16 | Jul 18 |
| 4th manure application grass | 20-Jul | 20-Jul |
| 4th grass cut | Aug 24 | Aug 27 |
| 5th manure application grassland | Aug 28 | Aug 28 |
| sampling of maize plants | Sep 19 | Sep 19 |
| soil sampling | Oct 8 | Oct 9 |

[a] Experimental field site.

has not been examined so far. To address this issue, an extensive field trial was performed in close association with a Bt-maize MON810 feeding study[13,21] to track the further fate of Cry1Ab in liquid manure.

In order to provide a set of quantitative Cry1Ab protein data reflecting the entire agricultural manuring process, this study investigated liquid manure storage, its soil entry and harvest of maize and grass crops from fields after application of manure from Bt-maize fed cows. Consequently, to meet the analytical requirements of the trial, an appropriate detection method to evaluate the presence of Cry1Ab in a range of samples with different matrix properties was necessary. We took advantage of a highly specific immunoaffinity purified polyclonal antibody based ELISA system for Cry1Ab toxin determination in animal liquids and animal byproducts,[23,28] that had been validated and successfully used for surveillance of transgenic protein in feces and urine of cows.[22] By adopting this ELISA system to other matrices and validating the detection system for the sample material investigated in the present liquid manure field trial, we aimed to keep track of the insecticidal Cry1Ab protein through different agricultural environments under comparable analytical conditions.

In the course of feed processing and passage through the GIT of the cow the Cry1Ab undergoes various proteolytic processes leading to the breakdown of the insecticidal protein into smaller fragments.[28−31] In agricultural manuring practice, further degradation may take place during the storage of liquid manure and by physicochemical breakdown and proteolytic microbial activity when liquid manure is applied to agricultural soil. Analysis of the fragmentation patterns of the Cry1Ab protein in the samples collected in a field trial could give important additional information considering the stability of the insecticidal protein and its fragments in the described processes.

Therefore, the objectives of this study were to develop, optimize and standardize extraction and immunological detection methods for Cry1Ab in liquid manure, soil, maize and grass crop samples. The methods were then applied to samples of a liquid manure field trial after feeding cows maize MON810 in order to assess the persistence and degradability of the insecticidal Cry1Ab protein in different stages of agricultural manure management.

## 2. MATERIALS AND METHODS

**2.1. Feeding Experiment and Liquid Manure Field Trial.** All handling of the dairy cows was performed under the approval of the Bavarian State Research Center of Agriculture (LfL, Grub, Germany) institutional animal care and use committee. A detailed description of the Bt-maize feeding experiment and of the feed composition in the daily diet of the cows is given in refs 21 and 13.

Five Simmental cows of the target group were fed a partial total mixed ration (PTMR) containing 63% (dry matter basis) GM maize MON810 whereas the control group received a substantially equivalent ration based on near-isogenic maize components. Cows were kept in a stable equipped with special slurry sampling facilities for a time period of six days. The entire stall manure of each group was collected and pooled. Storage was carried out according to agricultural practice at ambient temperature for further experimentation (stores 1 and 2, near-isogenic feed ration; stores 3 and 4, GM feed ration).

The liquid manure field trial took place in 2007 on two experimental field sites in Grub and Finsing, both located in Southern Bavaria, Germany. Whereas the grassland was under common permanent agricultural cultivation, maize fields were under experimental maize cultivation before the liquid manure field trial for seven years and one year, respectively. Experimental maize cultivation continued during the liquid manure trial in 2007 by growing the GM maize hybrid Kuratus (KWS-Saat, Einbeck, Germany) representing the transformation event MON810 and its near-isogenic maize hybrid Gavott (KWS-Saat, Einbeck, Germany) as control on four replicate plots of 750 $m^2$ size.

Liquid manure of the Bt-maize fed group and the control group was applied to four replicate small plots of 6 $m^2$ on grassland and within maize fields according to the time schedule shown in Table 1. On the maize fields, liquid manure of transgenic and non-transgenic origin was applied each to GM maize and near-isogenic maize plots, resulting in a total of 16 small plots of manure application. The manure was evenly applied with a watering can in practice-oriented maximum admissible amounts of 2.5 L/$m^2$ on grassland and 4 L/$m^2$ on maize fields.

**2.2. Sampling Procedures.** Representative PTMR feed samples based on GM maize and near-isogenic maize were collected daily within the period of liquid manure retrieval from the cows and were subsequently pooled and ground finely in liquid nitrogen using mortar and pestle.

Liquid manure samples were collected from slurry stores at the beginning of storage and at each date of manuring the experimental field lots (Table 1). Each sample was pooled from six subsamples taken with a 35 mm diameter sampling tube after exhaustive homogenization of the slurry from the tank.

At four time points, depicted in Table 1, the entire grass cut from each plot was harvested. Grass from each plot was mixed, one aliquot drawn from the core of the crop, chopped to small pieces and ground in liquid nitrogen.

For Western blot analysis, one set of maize plants of the varieties Kuratus (GM) and Gavott (near-isogenic) were grown in the glasshouse at 22 °C with a weekly nitrogen phosphorus potassium (NPK) treatment. The leaves of four plants of each variety were collected in the BBCH growth stages[32] of BBCH14 (5 leaves) and BBCH69 (end of tasseling) omitting the uppermost and undermost leaves. Leaves were immediately reduced to small pieces using a scalpel, ground in liquid nitrogen and frozen at −20 °C. Mature maize plants at growth stage of BBCH85 (ripening stage) were harvested by cutting with a sickle from each experimental field plot treated with liquid manure. Ten representative plants were selected and chaffed by a crop chopper to pieces of

approximately 5 cm size in diameter. Aliquots of the well-mixed chaffed material were ground in liquid nitrogen.

Finally, soil samples were taken at the end of the vegetation period in depths of 0−30 cm and 30−60 cm from maize field plots and 0−15 cm from grassland plots as mixed samples comprising ten drilling cores. Soil was homogenized and sieved at 2 mm mesh size. All sample material was stored at −20 °C until analyzed.

**2.3. Analysis of Substantial Equivalence: Influence of Different Types of Liquid Manure on Heterogeneous Parameters of Maize and Grass Crop.** Maize plants, grass crop and liquid manure were assessed for substantial equivalence by determining fourteen maize parameters (fresh matter, dry matter, starch, enzyme soluble organic substance, crude fiber, crude protein, crude fat, sugar, neutral soluble fiber, acid soluble fiber, acid soluble lignin, organic neutral soluble fiber, organic acid soluble fiber, in vitro digestible organic matter), six grass crop parameters (fresh matter, dry matter, crude fiber, crude protein, crude fat, crude ash) and nine parameters for liquid manure (dry matter, pH of liquid manure, pH of liquid manure extract, total nitrogen, $NH_4$−N, $P_2O_5$, $K_2O$, MgO, CaO; samples taken over the entire manure storage period). Statistical analysis investigating the influence of the two types of liquid manure (transgenic origin/non-transgenic origin) on various maize and grass parameters was performed by using ANOVA. As there were 14 or 6 individual tests conducted (one for each parameter), the threshold level of $\alpha$ was adjusted to multiple comparison to prevent the overall probability of type 1 error to be larger than 5%. The Bonferroni correction method was applied according to the formula $1 - (1 - \alpha)^{1/n}$. Only effects with obtained $p$-values <0.0037 for maize and <0.0083 for grass are considered significant. In addition, a multivariate analysis of variance (MANOVA) model was created using liquid manure as explanatory variable with the same set of response variables as in the ANOVA model. This test studied the effect of the factor liquid manure on all response variables simultaneously, using the overall probability of type one error set to 5% (no significant overall effect with the Wilks lambda statistic significance $p = 0.5181$).

**2.4. Reagents.** All reagents were of analytical grade and supplied by Merck (Darmstadt, Germany) unless otherwise stated. HPLC-purified trypsin-activated Cry1Ab protein was used as standard and for validation procedures. Development, purification and biotin-labeling of highly specific polyclonal anti-Cry1Ab antibodies were described in detail in ref 23. Assay buffer in the ELISA was phosphate-buffered saline (PBS; 8 mM $NaH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$ pH 7.4) containing 0,1% Tween 20.

**2.5. Protein Extraction.** *2.5.1. Protein Extraction of Maize and Maize Feed (PTMR).* Protein was extracted by homogenization of ground maize plant and feed samples in ice cold extraction buffer (EB: 8 mM $NaH_2PO_4$, 137 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 0.1% Tween 20 pH 7.4) containing protease inhibitor (Roche, Germany) using a Fast Prep FP120 (MP Biomedicals, Germany) homogenization machine. Samples (100 mg) were weighed in FastPrep tubes filled with 200 mg of beads (Lysing Matrix D; MP Biomedicals, Germany) and homogenized five times at 5 m $s^{-1}$ for 20 s with a 5 min cooling step on ice in between each homogenization cycle. Protein from the samples was extracted by horizontal shaking at 225 rpm for 30 min at room temperature. The homogenate was then centrifuged at 15000*g* at 4 °C for 15 min, and 500 $\mu$L of the supernatant was subjected to a second centrifugation to receive a clear sample extract. Before Cry1Ab analysis, maize and PTMR extracts were diluted in EB in a ratio of 1:100 and 1:5 respectively.

*2.5.2. Protein Extraction of Liquid Manure.* Samples of 100 mg of thawed liquid manure were extracted by the FastPrep procedure as described above, with the exception of using 1 mL of ice cold EB containing protease inhibitors and 1.5% skim milk as extractant. In extracts prepared for total protein determination, skim milk was omitted in the extraction buffer.

*2.5.3. Protein Extraction of Grass.* Protein from finely ground grass samples was extracted applying the procedure stated above but using 400 mg of sample and 4 mL of ice cold EB containing protease inhibitors and 1.5% skim milk in a FastPrep-24 (MP Biomedicals, Germany) homogenization machine. The homogenate was centrifuged at 4,500 rpm for 15 min at 4 °C and 500 $\mu$L of the supernatant clarified by a second centrifugation step (15000*g*, 15 min, 4 °C) before ELISA analysis.

*2.5.4. Protein Extraction of Soil.* Soil samples (<2 mm) of 200 mg were extracted using 2 mL of EB containing 2.5 mM EDTA and 0.5% skim milk in plastic tubes by horizontal shaking at RT for 30 min at 225 rpm. A clear extract was gained after centrifugation at 4,500 rpm for 15 min at 4 °C followed by centrifugation at 15000*g* for 15 min at 4 °C.

**2.6. BCA Assay.** Total protein concentrations in PTMR, liquid manure and maize extracts was measured by bicinchoninic acid (BCA) assay[33] using BSA (SERVA, Heidelberg, Germany) in a concentration range of 0 to 1000 $\mu$g/mL PBST as protein standard.

**2.7. Cry1Ab Protein ELISA.** For the determination of the Cry1Ab concentration in the protein extracts, a previously developed and well-described sandwich ELISA system[23] was used whereas the incubation time of calibrators and unknown samples was changed from 3 to 15 h.

**2.8. Assay Validation.** A separated validation of the Cry1Ab protein ELISA was carried out for the matrices of plant material (maize and grass), soil and liquid manure according to the criteria specified in the recently adopted European Commission Decision 2002/657/EC34 for the performance and validation of screening and confirmatory analytical methods. A detailed description of the general standardization procedure is given in ref [23].

*2.8.1. Decision Limit (CC$\alpha$) and Detection Capability (CC$\beta$).* The validation procedure was carried out for the plant material by using 32 discrete grass and 16 near-isogenic maize samples (blanks) derived from sections of the experimental fields, where neither GM maize was planted nor liquid manure from GM maize feeding was applied. A number of 32 blank liquid manure samples, collected from cows fed the diet based on near-isogenic maize, were taken for standardization of the Cry1Ab ELISA in liquid manure. Accordingly 32 independent blank soil samples were drilled at least 300 m distant of GM maize cultivation on four experimental field sites including Grub and Finsing for validation of the Cry1Ab ELISA in soil.

These blank samples were analyzed using the Cry1Ab ELISA to demonstrate the range of blank matrix effects and to determine the decision limits (CC$\alpha$) and the detection capabilities (CC$\beta$) in the different sample materials. According to the EC guideline 2002/657/EC, CC$\alpha$ is defined as three times the average signal-to-noise level measured in the assay for Cry1Ab in the blanks. CC$\beta$ was determined by use of the equation $CC\beta = CC\alpha + 1.64 \times SD_s$, $SD_s$ being the standard deviation obtained for the blank samples fortified at the spike concentration level of CC$\alpha$. Whereas the $\alpha$-error is the percentage of blank samples exceeding the CC$\alpha$-value, $\beta$-error describes the percentage of blank samples spiked at Cry1Ab concentration of CC$\beta$ and falling below the CC$\alpha$-value.

*2.8.2. Precision and Recovery.* Precision of the Cry1Ab-ELISA was expressed by intra-assay and interassay coefficients of variation (CV) calculated from the analysis of Cry1Ab control samples dissolved in EB containing 1.5% skim milk at three concentrations of 0.3, 1.2, and 5.0 ng $mL^{-1}$ in nine independent assays.

The mean analytical recovery of Cry1Ab in each matrix was determined by spiking eight of the blank samples collected for assay validation at three different concentration levels of Cry1Ab (5, 20, and 60 ng $g^{-1}$ sample) and measuring Cry1Ab concentrations in the spiked samples by applying the Cry1Ab ELISA.

*2.8.3. Statistics.* Final data are presented as ng of Cry1Ab protein $g^{-1}$ wet weight sample and as $\mu$g of Cry1Ab protein per g total protein. Student's $t$ test was used to compare the means of Cry1Ab concentrations in maize plants, PTMR, and liquid manure of transgenic and non-transgenic origin, considering a $P$-value below 0.05 as significant.

**2.9. Immunoblotting Procedure.** The fragmentation of Cry1Ab protein from GM maize MON810 was recorded by immunoblot analyses of protein extracts from maize plants, PTMR samples and liquid

manure samples. Amounts of 5 (maize plants), 50 (PTMR) or 220 (liquid manure) μg total protein were applied to 12% reducing SDS−polyacrylamide gels, and proteins were resolved by gel electrophoresis at 120 V for 150 min, followed by blotting of the proteins onto nitrocellulose membranes (Protran AB 85, Whatman, Dassel, Germany). Immunodetection with polyclonal Cry1Ab specific antibodies (rabbit 0.1 μg/mL) and visualization of immunoactive Cry1Ab fragments was performed according to the procedures described earlier.[28] HPLC purified trypsin activated Cry1Ab protein (65 kDa) was used as a positive control for the presence of Cry1Ab in the respective samples.

## 3. RESULTS AND DISCUSSION

**3.1. Analysis of Substantial Equivalence: Influence of Different Types of Liquid Manure on Heterogeneous Parameters of Maize and Grass Crop.** The analysis of nine chemical parameters of the two types of liquid manure resulted in the following mean differences: dry matter, 5% ($n = 7$); pH of liquid manure, 1% ($n = 6$); pH of liquid manure extract, 3% ($n = 6$); total nitrogen, 2% ($n = 7$); $NH_4$−N, 8% ($n = 8$); $P_2O_5$, 4% ($n = 5$); $K_2O$, 4% ($n = 5$); MgO, 4% ($n = 5$); CaO, 0% ($n = 5$). These differences are minor regarding the substantial equivalence of the applied liquid manure. Statistical analysis of 14 nutrient and energy parameters in all maize samples and of six respective parameters in all grass samples revealed no significant effect of transgenic or non-transgenic liquid manure treatment onto these parameters in maize and grass crop (data not shown). The MANOVA revealed no significant overall effect of the factor liquid manure on all response variables simultaneously. These results are in close accordance with the Bt maize long-term feeding study where the compositional equivalence of GM and near-isogenic maize fed to the dairy cows was demonstrated with respect to nutrient concentration.[21]

**Table 2. Analytical Precision for ELISA in Cry1Ab Protein Control Samples: Coefficients of Variation at Three Different Concentrations (C1−C3) of Cry1Ab Protein (Three Determinants per Assay) in Nine Independent Assays**

| | Cry1Ab protein controls | | | |
|---|---|---|---|---|
| CV[a] | C1: 0.3 ng/mL | C2: 1.2 ng/mL | C3: 5.0 ng/mL | mean CV |
| intra-assay (%) | 11.0 | 2.2 | 4.6 | 5.9 |
| interassay (%) | 17.1 | 14.0 | 11.9 | 14.3 |

[a] Coefficient of variation.

**3.2. Analytical Assay Performance.** *3.2.1. Assay Precision.* Calibration curves of Cry1Ab standards in the respective extraction buffers allowed quantification of Cry1Ab in the extracts over the dynamic range from 0.04 to 20 ng $mL^{-1}$. The analytical limit of 0.04 ng $mL^{-1}$ of extract corresponds to 0.4 ng of Cry1Ab protein $g^{-1}$ wet sample for all the analyzed matrices. Cry1Ab control samples in concentrations of 0.3, 1.2, and 5.0 ng $mL^{-1}$ in nine independent assays indicated a good assay precision, with a mean intra-assay coefficient of variation of 5.9% and a mean interassay coefficient of variation of 14.6% (Table 2).

*3.2.2. Assay Standardization.* The assay standardization was performed according to the European Commission Decision 2002/657/EC[34] and resulted in distinct analytical limits for the different sample materials (Table 3). According to the European Commission Decision false compliant rates ($\beta$-error: samples below CCα, when spiked at a concentration level of CC$\beta$) of

**Table 3. Decision Limits (CCα), Detection Capabilites (CC$\beta$) and Recoveries Achieved for Cry1Ab Protein Determination in Spiked Samples of the Different Matrices Investigated**

| sample material | CCα [ng $g^{-1}$ wet wt] | CC$\beta$ [ng $g^{-1}$ wet wt] | α-error [%] | $\beta$-error [%] | mean recovery[a] [%] |
|---|---|---|---|---|---|
| feed (PTMR) | 4.02 | 5.96 | 0 | 0 | 78.9 ± 13.2[b] |
| liquid manure | 1.20 | 1.41 | 0 | 0 | 71.2 ± 5.1 |
| soil | 2.00 | 3.06 | 0[c] | 0[c] | 68.1 ± 9.9[c] |
| plant material | 1.38 | 2.00 | 0 | 3.1 | 98.8 ± 7.2 |

[a] Mean recovery ± SD after spiking with three concentrations of Cry1Ab protein (eight replicates per spike concentration). [b] As determined by Guertler et al.,[22] 2010. [c] Validation soil samples of the experimental field sites Grub and Finsing.

**Table 4. Cry1Ab Protein Concentration in Soil Samples Collected in the Field Trial[a]**

| field site | cultivation | origin of maize feed and liquid manure[b] | Cry1Ab protein [ng $g^{-1}$ wet wt] |
|---|---|---|---|
| Grub | GM maize MON810 | NT | 0.40 ± 0.00 |
| | GM maize MON810 | T | 0.40 ± 0.00 |
| | maize near-isogenic | NT | 0.40 ± 0.00 |
| | maize near-isogenic | T | 0.40 ± 0.00 |
| Finsing | GM maize MON810 | NT | 0.45 ± 0.09 |
| | GM maize MON810 | T | 0.50 ± 0.14 |
| | maize near-isogenic | NT | 0.40 ± 0.00 |
| | maize near-isogenic | T | 0.40 ± 0.01 |
| Grub | grassland | NT | 1.56 ± 0.32 |
| | grassland | T | 1.68 ± 0.21 |
| Finsing | grassland | NT | 2.45 ± 1.95 |
| | grassland | T | 3.42 ± 1.62 |

[a] Mean values and standard deviation of soil samples drilled from four replicate plots are shown. [b] T: feed and liquid manure of transgenic origin. NT: feed and liquid manure of non-transgenic origin.

Table 5. Quantitative Degradation of Cry1Ab Protein in Agricultural Processes Relevant for Liquid Manure Management[a]

| | | Cry1Ab protein content | | Cry1Ab protein concn/total protein | |
|---|---|---|---|---|---|
| | $n$ | [ng of Cry1Ab $g^{-1}$ dry wt] | [%] of Bt maize plant | [$\mu$g of Cry1Ab $g^{-1}$ of total protein] | [%] of Bt maize plant |
| maize near-isogenic | 32 | <CCα[b] | –[c] | – | – |
| GM maize MON810 | 32 | 23 677 ± 1934.6 | 100 | 457.9 ± 46.5 | 100 |
| feed (NT[d]) | 8 | <CCα | – | – | – |
| feed (T) | 8 | 611.3 ± 64.4 | 2.6 | 10.6 ± 1.2 | 2.3 |
| liquid manure[e] (NT) | 8 | <CCα | – | – | – |
| liquid manure[e] (T) | 8 | 204.6 ± 20.4 | 0.9 | 1.9 ± 0.4 | 0.4 |
| liquid manure[f] (NT) | 8 | <CCα | – | – | |
| liquid manure[f] (T) | 8 | 104.5 ± 21.5 | 0.4 | 0.9 ± 0.2 | 0.2 |
| soil[g] (NT) | 16 | <CCα | – | – | |
| soil[g] (T) | 16 | <CCα | – | – | – |
| near-isogenic maize crop (NT) | 16 | <CCα | – | – | – |
| near-isogenic maize crop (T) | 16 | <CCα | – | – | – |
| grass crop (NT) | 32 | <CCα | – | – | |
| grass crop (T) | 32 | <CCα | – | – | – |

[a] Cry1Ab concentrations are given in mean values ± standard deviation. Column 3 shows Cry1Ab contents on dry weight basis. In column 5 Cry1Ab in relation to total protein contents is depicted. The quantitative decrease of the recombinant protein by each degradation step is shown by the percentages of Cry1Ab protein recovered in a sample in relation to Cry1Ab quantities in the primary GM maize plant material. [b] <CCα: any sample analyzed revealed Cry1Ab measurements below the decision limit CCα determined for the respective sample matrix. [c] –: not calculable as Cry1Ab was not detected in the samples. [d] T: feed and liquid manure of transgenic origin. NT: feed and liquid manure of non-transgenic origin. [e] Week 1 of slurry storage. [f] Weeks 24 and 25 of slurry storage. [g] Maize field plots.

<5% are prescribed for screening assays. Similarly, <5% false non-compliant results (α-error: blanks exceeding the CCα value) are accepted. As depicted in Table 3, the 5% limit for α-error and $\beta$-error was under-run in all investigated matrices. Thus, the CCα and CC$\beta$ values satisfy the criteria for the performance and validation of screening and quantitative analytical methods of this guideline in the sample materials of PTMR, liquid manure, soil and plant material collected in the liquid manure field trial.

*3.2.3. Analytical Recovery.* For each of the different sample materials analyzed, the analytical performance of the assay was assessed by spiking matrix samples of non-transgenic origin with Cry1Ab protein. The immunoassay operated well for the investigated matrices with mean analytical recovery rates of 68% for soil; 71% for liquid manure, 79% for PTMR and 99% for plant material (Table 3). Recovery of 68% Cry1Ab protein from the soil matrix is in the range of 49−89% shown for different soils[16] and compares favorably with extraction efficiencies between 27 and 60% reported in earlier studies for Cry proteins.[18,27,35] For PTMR 79% Cry1Ab recovery was documented earlier by ref 22.

**3.3. Cry1Ab Protein Contents.** *3.3.1. Cry1Ab Protein Contents in Feed (PTMR).* As depicted in Table 5, no Cry1Ab was detected in the non-transgenic feed samples of the control group. In the transgenic PTMR, Cry1Ab was reported in amounts of 278 ± 29 ng of Cry1Ab $g^{-1}$ wet feed sample. This corresponds well with amounts of 246 ng of Cry1Ab $g^{-1}$ feed sample reported for samples analyzed in course of the associated Bt-maize feeding study.[28]

*3.3.2. Cry1Ab Contents in Liquid Manure.* During the 29 weeks of the liquid manure field trial, slurry samples of control manure (cows fed on non-transgenic ration) and the manure of cows fed a transgenic ration were collected with proceeding time of storage from the slurry stores. In total, 22 samples were analyzed for the presence of Cry1Ab using the validated ELISA system. No liquid manure sample of the cows fed a non-transgenic ration was positive for the presence of Cry1Ab at the decision limit CCα of 1.2 ng of Cry1Ab $g^{-1}$ wet slurry (Figure 1). In the stores 3 and 4 filled with slurry from the transgenic ration-fed cows, Cry1Ab was detected in all samples taken at different time points with initial concentrations of 23.5 ± 0.9 and 18.2 ± 1.0 ng of Cry1Ab $g^{-1}$ slurry in stores 3 and 4, respectively. After feed ingestion, microbial activity in the rumen leads to degradation of feed protein, but a fraction of protein passes through the rumen and is partly degraded in the small intestine. Thus, immunoactive Cry1Ab protein fragments were found in contents of the GIT and in the feces of cows and pigs fed GM feed.[28,31,36,37] Previous work reported the presence of Cry1Ab in feces, but the absence of Cry1Ab in urine of maize MON810 fed dairy cows.[22] Thus in liquid manure, as a mixture of both, the Cry1Ab detection can be attributed to poorly digested GM maize plant feed material in the feces. Apparently, the Cry1Ab protein was protected from degradation by proteolytic enzymes in the GIT when enclosed in the heavy digestible parts of the maize plant. In contrast easily accessible purified Cry1Ab, isolated from GM maize plants, was shown to be very rapidly degraded by pepsin in in vitro studies.[38]

During further storage, Cry1Ab concentrations in samples taken from the tanks continuously decreased to Cry1Ab contents of 9.2 ± 1.1 and 8.6 ± 2.1 ng $g^{-1}$ slurry in stores 3 and 4. This implies a significant decrease ($P < 0.01$) in Cry1Ab concentration to a mean of 51% of the initial values within 25 weeks of storing liquid manure. Thus, a proportion of Cry1Ab will persist in liquid manure within the time period of slurry storage in common agricultural practice. It has to be assumed that minor amounts of Cry1Ab will be applied to agricultural fields by slurry management of dairy farms feeding GM maize MON810. In the described field experiment, transgenic protein in one liquid manure application added up to diminutive amounts of 431 mg of Cry1Ab $ha^{-1}$ for grassland and 690 mg of Cry1Ab $ha^{-1}$ for maize fields. Evidently, these amounts are very small in comparison to the amounts of Cry proteins added to soil by GM maize plant residues remaining on the fields after harvest.[16] From the

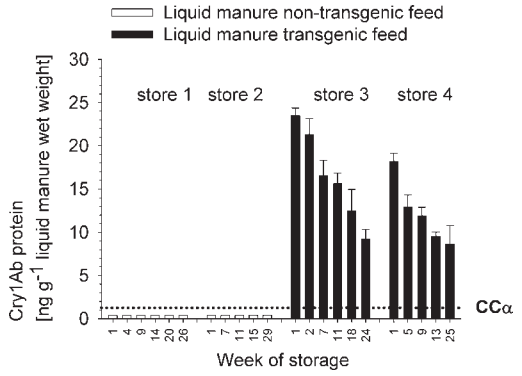


**Figure 1.** Detection and quantification of Cry1Ab protein in liquid manure samples derived from cows fed partial total mixed ration containing near-isogenic maize (store 1, store 2) or GM maize MON810 (store 3, store 4). Samples were analyzed with proceeding time of storage in the slurry stores. The data are presented as mean ± SD values ($n$ = 4).

Cry1Ab amounts determined in GM maize chaff analyzed in this study, a calculated maximum of 462 g of Cry1Ab ha$^{-1}$ could be introduced into soil by maize crop residues. These estimates are confirmed by a maximum theoretical load of 480 g of Cry1Ab ha$^{-1}$ calculated for recombinant protein from GM maize MON810 by ref 39. Thus, the theoretical Cry1Ab protein load applied to agricultural fields by way of liquid manure from GM maize fed cows adds up to less than 0.15% of Cry1Ab protein introduced directly by plant residues.

Cry1Ab biotoxicity studies provide a basis to estimate the toxicological relevance of the Cry1Ab concentrations arising in liquid manure. The Cry1Ab protein susceptibility of the target organism *Ostrinia nubilalis* was described by a $LC_{50}$ ranging from 0.10 to 0.34 μg of Cry1Ab per gram of diet.[40] Both free and humic acid bound Cry1Ab protein was insecticidal to larvae of the lepidopteran tobacco hornworm *Manduca sexta* with $LC_{50}$ values between 215 and 304 ng of 100 μL$^{-1}$.[41] Thus the Cry1Ab concentrations detected in this study were clearly below any concentration that would cause a direct toxic effect on nontarget organisms such as *Manduca sexta* or *Caenorhabditis elegans*.[42]

*3.3.3. Cry1Ab Contents in Soil.* At the experimental field sites Grub and Finsing, 32 soil samples in total were collected from maize field and grassland lots treated with liquid manure obtained from cows receiving non-transgenic or transgenic feed rations. In the soil samples collected from maize lots, no Cry1Ab was detectable as ELISA analysis revealed concentrations below the CCα of 2.0 ng of Cry1Ab g$^{-1}$ of soil (Table 4). For the maize field, this gives evidence for degradation of the insecticidal Cry1Ab protein when introduced into soil by way of liquid manuring. Cry1Ab values of soil under grassland management at the field site Grub were below CCα of 2.0 ng of Cry1Ab g$^{-1}$ of soil in both manuring variants showing that the transgenic protein applied with the slurry is degraded to nondetectable levels in this soil. For soil samples collected at the Finsing field site, values exceeding CCα of 2.0 ng of Cry1Ab g$^{-1}$ of soil were determined on the lots treated with slurry from non-transgenic and transgenic origin. This can be due to a cross reaction of the polyclonal antibody used in the ELISA with organic substances in this soil horizon, which is rich in fine grass roots and organic material. Natural organic matter and in particular humic substances were identified as commonly interfering substances when environmental samples were analyzed by ELISA before.[43−45] Beyond the finding that there is no significant difference in soil samples of Finsing undergoing each slurry treatment, further methodological development is necessary to give final evidence for the presence or absence of Cry1Ab protein in this special soil matrix rich in organic substances. In summary the absence of detectable Cry1Ab in the soil samples indicates that, after their release from the conserving environment in liquid manure,[46] Cry1Ab in undigested GM plant residues will undergo a fast degradation in the well-aerated and microbially active soil matrix. These results are consistent with the rapid biodegradation reported for Cry1Ab introduced directly into the soil compartment by Bt maize plant residues after harvest.[16,18]

*3.3.4. Cry1Ab Contents in Grass.* Grass crop was sampled at four cutting dates keeping a minimum time interval of 28 days to the preceding manure application as commonly practiced in grassland management. All grass samples analyzed were below CCα of 1.4 ng of Cry1Ab g$^{-1}$ of plant material investigated in this study (Table 5). Thus, no Cry1Ab was detected in any of the grass samples collected from grassland lots treated with slurry from cows fed GM maize or from the control group receiving non-transgenic feed.

*3.3.5. Cry1Ab Contents in Maize.* In the field trial, GM maize Kuratus and the corresponding near-isogenic variety Gavott were grown and manured twice during the vegetation period using slurry from feeding with non-transgenic or transgenic feed ration. Analysis of chopped plant material revealed that all near-isogenic maize samples were below the CCα of 1.4 ng of Cry1Ab g$^{-1}$ of plant material irrespective of the origin of liquid manure applied to the plants (Figure 2). This implies that no residual traces of the transgenic protein from undigested GM maize plant material in liquid manure were dispersed to and persisted during the growth of the maize plants. According to these findings conventional maize plants harvested from fields manured with slurry containing traceable amounts of Cry1Ab originating from GM maize feed can be regarded as free of Cry1Ab toxin. These results are important regarding concerns about Cry1Ab protein remains on crops grown in soils, in which Bt-maize has been cultivated before. It was demonstrated earlier that Cry1Ab released to soil in root exudates of Bt-maize, from the degradation of the biomass of Bt-maize, or as purified protein, was not taken up from soil or from hydroponic culture by conventional maize plants.[47]

Analysis of the GM maize plants revealed mean Cry1Ab concentrations ranging from 6.7 to 8.4 μg of Cry1Ab g$^{-1}$ of plant material. This is consistent with data on Cry1Ab protein contents of GM maize MON810 plants reported before.[48]

**3.4. Degradation of Cry1Ab Protein in Relation to Total Protein Contents.** Cry1Ab concentrations of PTMR, liquid manure and maize plants of transgenic origin were also calculated on the base of the respective total protein contents to investigate the degradation of Cry1Ab in comparison to the entirety of other proteins.

Total protein contents of the chaffed maize plants were found to be between 16.2 and 18.2 mg of total protein g$^{-1}$ of crop wet weight without significant differences between field sites, GM and near-isogenic variety and origin of liquid manure applied to maize lots ($P$ = 0.05). The mean Cry1Ab concentrations in maize plants were in the range of 391 to 499 μg of Cry1Ab g$^{-1}$ of total protein with a mean level of 458 μg of Cry1Ab g$^{-1}$ of total protein (Table 5). The corresponding concentration in the transgenic feed ration was 10.6 μg of Cry1Ab g$^{-1}$ of total protein in the PTMR. Thus, in relation to the initial concentrations of the

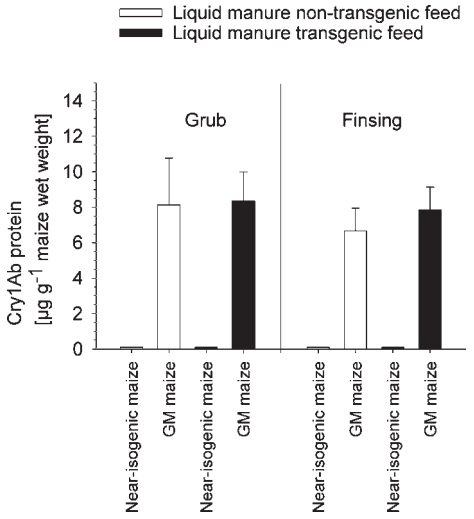


**Figure 2.** Cry1Ab contents in GM maize and near-isogenic maize plants harvested from experimental field lots under slurry management when feeding near-isogenic maize (blank bars) and GM maize MON810 (black bars).

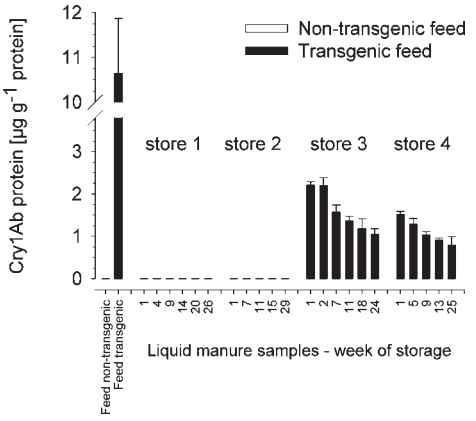


**Figure 3.** Concentration of Cry1Ab protein in feed (PTMR) and liquid manure of dairy cows fed either GM maize MON810 (black bars) or near-isogenic maize (blank bars). Data represent mean ± SD values ($n = 4$) in $\mu$g of Cry1Ab protein $g^{-1}$ of total protein in PTMR and liquid manure after successive time of storage.

transgenic protein in GM maize MON810 plants, only 2.3% of Cry1Ab $g^{-1}$ of protein was present in transgenic PTMR. This decline can be explained both by the addition of up to 40% of conventional feed components to the mixed feed ration[22] and by the ensiling and processing of maize plants to feed leading to heat denaturation and disintegration of the Cry1Ab protein.[30,49,50]

In liquid manure, mean levels of 2.0 $\mu$g of Cry1Ab $g^{-1}$ of total protein were observed at the time point of filling the slurry stores (Figure 3). Accordingly, the Cry1Ab concentration in liquid manure is decreased to 18% of the initial value of 10.6 $\mu$g of Cry1Ab $g^{-1}$ of total protein reported in the transgenic PTMR. A previous study[28] reported consistent findings with a Cry1Ab concentration decline of 44% when feed containing GM maize MON810 and freshly collected feces were analyzed. The decline observed in both studies can be explained by two reasons: First, additional protein of animal and microbial origin is added to the feed protein during the passage through the bovine gastrointestinal tract (GIT). Second, the marked reduction of Cry1Ab protein in relation to total protein points to a faster degradation of Cry1Ab in the GIT compared to the rest of the total protein, suggesting that Cry1Ab exhibits no greater stability compared to the entirety of other proteins in feed. Analysis of the Cry1Ab concentrations in liquid manure samples with increasing time of storage showed a further decline of Cry1Ab $g^{-1}$ of total protein (Figure 3). Within 23 weeks of storage decreasing concentrations of 2.20, 2.19, 1.57, 1.36, 1.18, and 1.05 were reported in slurry store 3 and a decline over 1.51, 1.28, 1.02, 0.91, and 0.79 $\mu$g of Cry1Ab $g^{-1}$ of total protein within 24 weeks in slurry store 4. On average, the Cry1Ab concentrations were 50% of the initial levels of Cry1Ab $g^{-1}$ of total protein after 23 or 24 weeks of storage. Compared to this, total protein levels, averaged over the four slurry tanks, declined from 12.4 ± 1.3 to 9.3 ± 1.1 mg of protein $g^{-1}$ wet slurry corresponding to a mean loss of 25% of the total protein during storage. These findings point out that, in comparison to total protein in liquid manure, Cry1Ab protein undergoes an even faster degradation process and can be assigned to the less stable group of proteins in liquid manure.

**3.5. Quantitative Balance of Cry1Ab Protein Turnover in Feeding and Liquid Manure Management.** The data collected in the study allow for balancing of Cry1Ab contents over the whole pathway from GM maize MON810 crop over feed and liquid manure into soil and the following crop. The quantitative turnover of the recombinant Cry1Ab protein and its immunoreactive fragments is summarized in Table 5. The major fraction of Cry1Ab is degraded by the processing of the GM maize plants to feed, as only 2.6% of the novel protein determined in plant material was recovered in the transgenic feed ration. Digestive processes during the GIT passage led to a further reduction of Cry1Ab levels in a way that 0.9% of the Cry1Ab content in the GM maize plant was detected in liquid manure. Half of this residual immunoactive Cry1Ab in slurry persisted during slurry storage for 25 weeks. When applied to agricultural soil, final degradation of Cry1Ab protein to below detectable levels in soil was reported. Cry1Ab concentrations in relation to total protein give further information about the degradation process and elucidate the proteolytic stability of the novel protein and its immunoactive fragments compared to other proteins. The overall decline of the Cry1Ab concentrations related to total protein (Table 5, column 6) suggests that Cry1Ab protein exhibits a lower stability compared to the entirety of other proteins in the GM maize plant and the feed components.

**3.6. Western Blot Analysis.** By Western blot analysis, no Cry1Ab protein specific protein bands could be detected in maize plants of the near-isogenic variety. The investigation of GM maize plant material, transgenic feed and liquid manure by immunoblot analyses revealed a fragmentation of the full-sized (65 kDa) Cry1Ab protein into smaller immunoreactive fragments in each of the analyzed materials.

In all of the GM maize MON810 plant material, Cry1Ab protein fragments of 42 kDa, 34 kDa and 17 kDa are present in addition to the full-sized 65 kDa protein (Figure 4). However, the smallest 17 kDa fragment was not detected in leaves of BBCH14 plants, and signals for this fragment detected in leaves of BBCH69 and mature BBCH85 plants were weak compared to the more prominent bands of higher molecular weight Cry1Ab fragments. The fragmentation pattern looks similar in the developmental stages of BBCH14, BBCH69 and BBCH86 suggesting a continuous synthesis and degradation of Cry1Ab in the GM

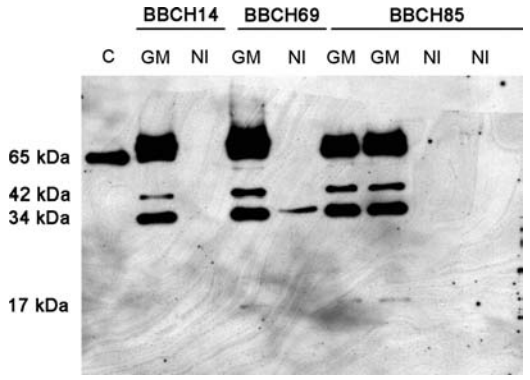


**Figure 4.** Western blot analysis of Cry1Ab protein in maize plants. Immunoreactive Cry1Ab protein fragments of genetically modified (GM) and near-isogenic (NI) maize plants in successive developmental stages of BBCH14, BBCH69 (plant leaves) and BBCH85 (chaffed maize crop) are presented. 500 pg of trypsin activated HPLC purified Cry1Ab protein served as positive control (C).

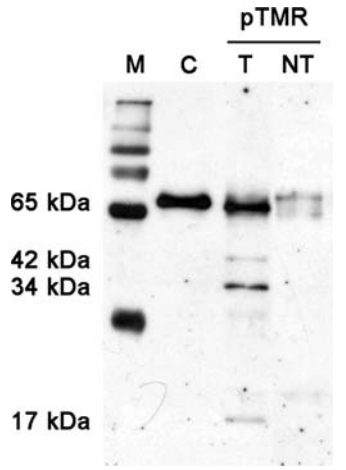


**Figure 5.** Western blot showing immunoactive Cry1Ab protein fragments in feed. Protein extracts of partial total mixed ration of transgenic (T) and non-transgenic (NT) origin were analyzed. Trypsin treated and HPLC purified Cry1Ab protein (100 pg) was included as a positive control (C).

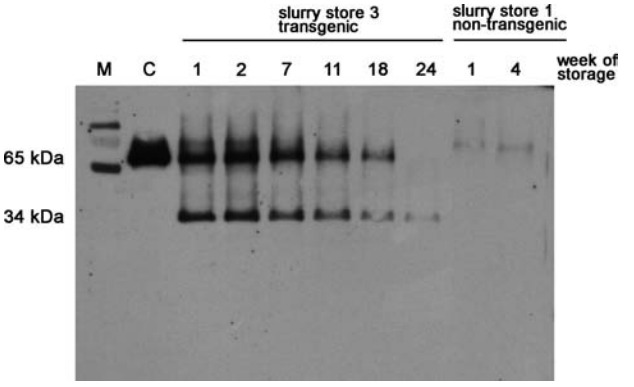


**Figure 6.** Western blot showing immunoreactive Cry1Ab protein fragments in liquid manure from GM maize MON810 fed cows with proceeding time of storage. Samples from slurry store 3 show a degradation of Cry1Ab protein in liquid manure of the GM maize fed animals from week 1 to 24. Liquid manure samples from the control group fed non-transgenic ration (store 1) was analyzed at weeks 1 and 4. As a positive control (C), 500 pg of trypsin treated and HPLC purified Cry1Ab protein was used.

plant. These findings are in accordance with studies using Cry1Ab purified from GM maize plants for in vitro degradation experiments. There, a fragmentation of the recombinant Cry1Ab plant protein into fragments of molecular size between 20 and 70 kDa was reported before addition of pepsin as the proteolytic agent.[38] The observation that Cry1Ab produced by the GM plant is partially degraded in any stage of development indicates a continuous breakdown of the recombinant Cry1Ab protein by endogenous plant proteases.

In transgenic feed, the full sized Cry1Ab protein and immunoactive fragments of 42 kDa, 34 kDa and 17 kDa were detected by immunoblot analyses (Figure 5), whereas none of these fragments were recorded in non-transgenic feed. The closely related pattern of Cry1Ab protein fragmentation in mature transgenic maize plants and processed feed indicates that the degradation of the recombinant Cry1Ab during feed processing affects immunoactive fragments of any size. Accordingly, the 65 kDa protein and the 42 kDa, 34 kDa and 17 kDa immunoactive fragments were detected by blot analyses in transgenic maize kernels and transgenic maize cobs,[28] which were components of the total mixed ration fed to the cows in the long-term feeding study using GM maize MON810. However, in this study, no 17 kDa could be detected in PTMR as the concentrations of this fragment in the PTMR were too low to be detected by Western blot analyses.

Immunoblot analysis of liquid manure obtained from cows fed GM maize MON810 revealed Cry1Ab protein fragments in the size of 65 kDa and 34 kDa (Figure 6), whereas the 42 kDa fragment detected in GM plant and feed samples was not recorded in liquid manure. These findings indicate a progressive microbial and enzymatic proteolysis of the Cry1Ab protein fragments in the bovine digestive tract. Previous reports of Cry1Ab degradation in the bovine digestive tract are in accordance with the Cry1Ab fragmentation patterns shown in the present study.[28,29,31] During slurry storage further degradation of the 65 kDa and 34 kDa immunoactive Cry1Ab protein fragments takes place as can be deduced from decreasing band intensities with proceeding time of storage. After 24 weeks of storage the 65 kDa Cry1Ab protein was not detected any more, suggesting a gradual degradation into the smaller 34 kDa fragment. This 34 kDa fragment was reported to be the most prominent immunoactive fragment in transgenic feed, digesta and feces before,[28] which could be a sign of a higher proteolytic stability of this degradation product. The fragmentation pattern of the Cry1Ab protein in addition to Cry1Ab quantity is an important criterion regarding effects of Cry1Ab introduced into the environment by agricultural processes. It should be considered that the insecticidal pathway of the recombinant Cry1Ab is closely related to the presence of all functional domains of the whole trypsin resistant 65 kDa protein.[5,51−53] To our knowledge toxic activity of Cry1Ab fragments smaller than 65 kDa has not been reported so far. Finally, the presence of Cry1Ab or Cry1Ab fragments exhibiting insecticidal activity can only be determined using sensitive organism species in biotoxicity assays at present.

In conclusion, the field trial keeps record about the extensive and, compared to other proteins, rapid degradation of recombinant Cry1Ab protein during processes in the liquid manure

management of a dairy farm feeding GM maize (event MON810), leading to nondetectable levels in soil and the following crop.

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## NOTE ADDED AFTER ASAP PUBLICATION

There was an error in section 3.2.2. in the version of this paper published June 8, 2011. The correct version published June 15, 2011.