Rapid degradation of the Cry3Bb1 protein from Diabrotica-resistant Bt-corn MON88017 during ensilation and fermentation in biogas production facilities

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Abstract

BACKGROUND: Insect-resistant transgenic corn is meeting a growing adoption in Europe. Corn is also the major substrate for biogas production, which has vastly increased in recent years. In areas with high insect pest pressure Bt-corn silage will consequently be fed into biogas production facilities. To assess the potential risks of transgenic plants as energy crops we investigated the fate of the coleopteran-specific protein Cry3Bb1 from MON88017 corn in the utilization chain of a farm-scale biogas production facility using ELISA.

RESULTS: The mean Cry3Bb1 protein content in chaffed MON88017 plant material was 15 µg g⁻¹. In silage the content decreased rapidly, with less than 20 ng g⁻¹ detectable after 8 months. From the start of fermentation in the biogas reactor, only trace amounts of Cry3Bb1 could be detected. Conventional corn harvested alongside MON88017 with the same equipment showed a presence of transgenic plant material of around 0.5%. Analyses performed at two laboratories showed consistent and systematic differences in the concentrations of Cry3Bb1 measured with ELISA due to methodical differences.

CONCLUSIONS: An extensive degradation of Cry3Bb1 was observed in silage despite differences in ELISA measurements. The potential risk associated with the use of MON88017 silage is discussed on this basis.

Keywords: Bt-corn; MON88017; Cry3Bb1; biogas production; silage; ELISA

INTRODUCTION

Genetically modified corn with insect resistance based on Bt-technology has gained growing acceptance worldwide. In Europe, the cultivation of Cry1Ab-expressing genetically modified maize lines almost doubled in 2007 compared with the previous year, reaching over 100 000 ha.1 In Germany, an area of 2751 ha was planted with Bt-corn MON810 in 2007, which meant an increase of almost 200% compared with 2006.2 MON810 and other Cry1Ab-expressing maize lines are resistant to the European corn borer Ostrinia nubilalis (Hübner) (Lepidoptera: Crambidae). In the future, Bt-corn lines expressing the protein Cry3Bb1, and thus resistant against the western corn rootworm (WCR) Diabrotica virgifera virgifera (LeConte) (Coleoptera: Chrysomelidae), will most likely become more important. WCR has been introduced to Europe and has spread rapidly, reaching economic thresholds in some Balkan states.3 It is generally regarded as a serious threat to European corn production. Genetically modified corn may be a key strategy to contain or eliminate WCR populations, since these are not readily controlled by current plant protection practices.4

With the introduction of the Renewable Energy Sources Act in Germany (EEG) energy producers were encouraged to generate electricity from organic materials and wastes.5–7 This has led to a rapid increase in the number of biogas production facilities and, consequently, in the acreage used for biomass-rich crops. The same trend can also be observed in other European countries. As a consequence, the demand for corn cultivation has increased immensely owing to its suitability for biogas production based on its high per-hectare methane yield.8,9

In areas of high infestation pressure by insect pests Bt-corn varieties will be cultivated and subsequently used for biogas production. Residual materials from the biomethanation process might then be used as fertilizer on agricultural areas. While organisms in...
and on the soil, e.g. earthworms, mites, Collembola and ground beetles, are known to be stimulated by the use of organic wastes, they may be exposed to considerable amounts of Bt-proteins when residual materials from the biomethanation process are used, depending on the concentration of Bt-protein in these materials. This may lead to a negative impact on the occurrence, abundance and function of a diverse range of non-target organisms. Work on the ecotoxicology of Cry3Bb1 with respect to the above-mentioned groups of soil organisms is limited. Ahmad et al. report no negative impact of the cultivation of Cry3Bb1 expressing corn on surface and below-ground non-target arthropods and earthworms. The actual exposure pathways and amounts of transgenic protein taken up by these organisms remain unclear, however, demonstrating a significant lack in knowledge.

The aim of this study was to assess the corresponding potential risks implicated by the use of Bt-transgenic plants as energy crops. To address this issue, we investigated the fate of the expression product Cry3Bb1 of the WCR-resistant corn variety MON88017 in the utilization chain of a farm-scale biogas production facility. Plants of this variety contain a transgenic cassette encoding for a variant of the coleopteran active Cry3Bb1 protein (trademark YieldGard® Rootworm) and for CP4 EPSPS, which confers herbicide tolerance against glyphosate. The on-farm utilization chain for corn consists of the plants on the field, silage stored over several months and fed into the biogas reactor, and residual materials from the biomethanation process. Based on a variety of factors, expression levels of Bt-proteins may vary considerably within different plant materials. Thus, samples were analysed all through the above process using double-antibody-sandwich enzyme-linked immunosorbent assays (DAS-ELISA). Silage samples from conventional corn harvested alongside MON88017 with the same equipment were tested to check for the degree of intermixing of the conventional with genetically modified corn. To assess the temporal stability of Cry3Bb1 during storage, plant samples were frozen at different temperatures over eight months, and then analysed. Selected samples were tested in parallel at a second laboratory to evaluate inter-lab differences with regard to sample preparation and test protocols.

**EXPERIMENTAL**

**Bt-corn cultivation and silage preparation**

Bt-corn MON88017 (Monsanto, St. Louis, MO, USA) and three conventional corn hybrids (DKC5143, DK315, Benicia) were cultivated in 2006 in a systematic plot design, near Wuerzburg in southern Germany. The total area of the field was 4 ha. All stages of corn cultivation, harvest and silage preparation were carried out according to common agricultural practice. For all corn hybrids the same harvester was used, with no intermittent cleaning of the machine. Two silage heaps were set up using different kippers: one containing only chaffed MON88017 plants and the other containing all conventional corn hybrids. The chaffed plant material was compacted and covered with plastic foil. No additives were used in the silage preparation.

The two laboratories concerned with sampling and subsequent ELISA measurements were the Laboratory for Biotechnological Crop Protection at the Agricultural Service Centre Palatinate (DLR Rheinpfalz) (hereafter called ‘Lab 1’) and the Institute of Environmental Research at the RWTH Aachen University (hereafter called ‘Lab 2’). Details on the storage conditions for the different sample materials and on the ELISA methodologies employed are summarized in Table 1 and given in the following.

**Plant sampling at BBCH83**

The sampling of whole plants and the subsequent tissue-specific analysis of Cry3Bb1 contents was done by ‘Lab 1’. Approximately three weeks before harvest, at the maturity level BBCH 83 (dry matter of kernels approximately 40%), entire corn plants were collected. Five Bt-corn plants were randomly sampled in each of five plots with MON88017. Two plants of the near-isogenic DKC5143 were collected from a single plot and used as control. The plants were cut into two parts, placed in a polystyrene cooling box and then brought to the laboratory. Leaves, stalk and kernels of each plant were separated and cut into small segments of about 0.5 cm size.

| Table 1. Storage conditions and details on the ELISA methodologies used at the two independent laboratories |
|--------------------|-----------------|-----------------|
| **Procedure**      | **‘Lab 1’**     | **‘Lab 2’**     |
| **Storage**        | **Temperature** | °C              | −80              | −50              |
|                    | °C              |                 | Room temperature | Household blender |
| **Extraction**     | **Temperature** | °C              | 4                | 10               |
|                    | **Device**      |                 | Ultra-Turrax homogenizer | Household blender |
|                    | **Time**        | min             | 30               | 15               |
|                    | **Sample amount** | g              | 5–10             | 10               |
|                    | **Volume**      | mL              | 5–10             | 100              |
| **Incubation**     | **Time**        | h               | 15               | 2                |
|                    | **Temperature** | °C              | 4                | Room temperature|
| **Measurement**    | **Calibration curve** | ng mL⁻¹      | 0.59, 0.89, 1.33, 2, 3 and 4.5 | 0.1, 0.2, 0.4, 0.8, 1.25, 2.5 and 5 |
|                    | **Wavelength**  | nm              | 450              | 450              |
samples were weighed to assess their proportion of the whole plant. All of the sample matter from the same plot was then pooled, mixed well, and stored at −80°C in portions of 5 g until further analysis.

Silage sampling
The sampling of plant material during harvest and ensilation of MON88017 and the main work in the ELISA analysis of these materials was performed by ‘Lab 2’. Aliquots of the freshly chaffed plant material sampled on the day of harvest were sent to ‘Lab 1’ and stored there at −20, −50 and −80°C to assess the temporal stability of Cry3Bb1 under different storage conditions. Subsequent samples were taken 4, 8, 16, 20, 26, and 31 weeks after harvest from the MON88017 silage, and after 4 and 8 weeks from the conventional silage. At each sampling date, three 0.5–1 kg samples were taken at different positions from the Bt silage heap and one sample from the conventional corn. To that end the plastic foil was cut, the silage sample was removed from a depth of approx. 20 cm, and the foil was then sealed again with duct tape. Each sampling point was only ever used once. At the last sampling date, material was taken from different strata of the MON88017 silage heap: the bottom, the approximate middle and the topmost layer. The aim of this sampling scheme was to compare the degree of degradation at different strata of the silage heap. All samples were thoroughly mixed on site. An aliquot from selected samples was sent for concurrent analyses to ‘Lab 1’. Samples were kept frozen at −80°C at ‘Lab 1’ and at −50°C at ‘Lab 2’, until analysis, which was performed within two weeks after sampling.

Reactor effluent sampling
All work done on reactor effluents was performed at ‘Lab 2’. Effluent samples were taken from the first day of MON88017 silage input into the reactor (negative control). Samples consisted of 250 mL effluent taken directly from the premixing chamber of the reactor. During silage feed-in, samples were taken every second day over a course of two weeks. After that, the interval was extended to every third to fourth day for another three weeks. Samples were stored at −50°C until analysis, which was performed within two weeks after sampling.

Cry3Bb1 protein measurements (DAS-ELISA)
The determination of the Cry3Bb1 content of samples was carried out at both laboratories with a commercially available ELISA kit (Agdia Inc., Elkhart, IN, USA; purchased via Linaris GmbH, Wertheim-Bettingen, Germany). Different procedures were used, however.

At ‘Lab 1’, all extraction steps were performed on ice or at 4°C. Five grams of each plant tissue sample (stalks and leaves) or 10 g of chopped plant material were soaked in 10 volumes of phosphate-buffered saline Tween 20 buffer (PBST, supplied with the kit) in 250 mL glass bottles (Fischer Scientific, Schwerte, Germany). Plant samples were homogenized using an Ultra-Turrax homogenizer (Kinematica, Lucerne, Switzerland) at 30 000 rpm for 1 min. The homogenates were kept on ice for 30 min and transferred to 1.5 mL Eppendorf tubes. To extract Cry3Bb1 from kernel samples, 5 g of kernel was soaked in 1 volume of PBST buffer and ground using mortar and pestle. The kernel extracts were transferred to 2 mL Eppendorf tubes. All extracts were then centrifuged at 5000 × g for 5 min. The supernatants were diluted with PBST buffer and analysed using ELISA. Extracts of non-transgenic plants were used as a negative control.

The test was conducted following the manufacturer’s instructions. In order to increase the binding capacity, the ELISA plate was incubated in a humid box for 15 h at 4°C. After washing the plate, 100 µL of the tetramethyl benzidine (TMB) substrate was added in each well and incubated at room temperature for 15 min. To stop colour development 3 mol L−1 H2SO4 was added. Absorbance was measured at 450 nm with a BioRad model 680 microplate reader (BioRad, Hercules, CA, USA) and data were analysed using the Microplate Manager 5.2.1 program (BioRad). A six-point standard curve of purified Cry3Bb1 (supplied with the kit) with concentrations of 0.59, 0.89, 1.33, 2, 3 and 4.5 ng mL−1 was used to quantify the Cry3Bb1 protein in the samples.

At ‘Lab 2’, silage samples were prepared as follows. The bulk samples were ground in a household blender and thoroughly mixed. A portion of 10 g was transferred to a beaker and extracted for 15 min with 100 mL PBST extraction buffer (ratio 1:10 w/v). Two millilitres of the sample extracts were centrifuged, and different dilutions were prepared from the supernatant. Reactor effluent samples were either used undiluted or mixed with extraction buffer at a ratio of 1:1, and extracted for 15 min. After centrifugation at 10 000 × g for 5 min, 100 µL of the supernatants were used for ELISA. All steps of the test procedure were carried out according to the manufacturer’s instructions. After addition of 50 µL of 3 mol L−1 sulfuric stop solution (Agdia) to each test well, samples were measured at 450 nm using an MRX plate reader (Dynatech, Boonville, IN, USA). All samples were measured in duplicate and in at least one assay. For quantitation of Cry3Bb1 content, the positive control of 40 ng Cry3Bb1 mL−1 provided with the kit was used to create a standard curve with concentrations of 0.1, 0.2, 0.4, 0.8, 1.25, 2.5 and 5 ng mL−1.

Negative control samples taken from conventional maize were used to assess interactions of the test antibodies with the different sample matrices. The
optical density (OD) values of these controls were subtracted from the OD values of test samples.

RESULTS

Cry3Bb1 content in plants at growth stage BBCH83

The mean content of Cry3Bb1 (± standard deviation, SD) in leaves at the growth stage BBCH83 was 24.2 ± 3.7 µg g⁻¹ fresh weight (f.w., n = 5). The leaf content was 2.1- and 2.8-fold higher, respectively, than in the stalk (11.36 ± 3.29 µg g⁻¹ f.w., n = 5) and the kernel samples (8.61 ± 1.56 µg g⁻¹ f.w., n = 5, Fig. 1). No Cry3Bb1 protein was detected in any negative control sample.

Cry3Bb1 content in silage

The mean content of Cry3Bb1 (± SD) in the freshly chaffed Mon88017 plant material determined at ‘Lab 1’ was 15.0 ± 2.8 µg g⁻¹ f.w. (n = 2). At ‘Lab 2’, the same plant material yielded a mean Cry3Bb1 content of 3.5 ± 0.9 µg g⁻¹ f.w. (n = 6). This difference remained when the original, unprepared and unextracted samples were exchanged between the two labs and tested again.

Analyses of the subsequent silage samples at ‘Lab 2’ showed that the Bt-protein content declined rapidly (Fig. 2). After 4 weeks, the concentration of the transgenic protein was reduced to 55 ± 31 ng g⁻¹ f.w. (n = 3), which was less than 2% of the initial amount. Silage samples from the following timespan showed varying Cry3Bb1 contents, but no gradual decline of the protein concentration. After 8 months, however, the Cry3Bb1 content was down to 17 ± 5 ng g⁻¹ (n = 3). This was uniform for the lower, middle and upper strata of the silage heap. When the three strata samples were analysed at ‘Lab 1’, consistently higher contents of Cry3Bb1 were estimated, which indicated a gradient from higher contents in the topmost layer to lower concentrations at the bottom of the silage heap (Table 2). Overall, the relative extent of degradation of the transgenic protein was similar for both laboratories with 0.42% and 0.49% of the initial concentration still detectable (as measured at ‘Lab 1’ and ‘Lab 2’, respectively).

In the silage mixture of the three conventional corn lines, a Cry3Bb1 concentration of 17 ± 3 ng g⁻¹ f.w. would be present. This concentration is lower than the limit of detectability for the protein (Table 2). However, the Cry3Bb1 content in the silage samples was higher than the detectability limit for the protein (Table 2).

**Table 2.** Contents of Cry3Bb1 protein (ng g⁻¹ fresh weight) in selected sample materials analysed independently in the two laboratories

<table>
<thead>
<tr>
<th>Sample</th>
<th>‘Lab 1’ (ng g⁻¹ f.w.)</th>
<th>‘Lab 2’ (ng g⁻¹ f.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvested material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MON88017</td>
<td>15 000</td>
<td>3 500</td>
</tr>
<tr>
<td>Non-transgenic</td>
<td>40</td>
<td>17</td>
</tr>
<tr>
<td>Silage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>81</td>
<td>12</td>
</tr>
<tr>
<td>Middle</td>
<td>55</td>
<td>21</td>
</tr>
<tr>
<td>Lower</td>
<td>52</td>
<td>18</td>
</tr>
</tbody>
</table>

Figure 1. Contents of Cry3Bb1 protein in different plant tissues of corn variety MON88017 at growth stage BBCH83, three weeks before harvest (n = 5).

Figure 2. Contents of Cry3Bb1 protein (plus one standard deviation) in MON88017 silage at different times after harvest. The bar with diagonal lines represents the freshly harvested plant material (left ordinate); bars with dots represent silage samples taken weeks after harvest as indicated (right ordinate).
At ‘Lab 1’ 40 ng Cry3Bb1 g\(^{-1}\) f.w. which corresponds to a content of 0.5% MON88017. (\(n = 12\)) was found at ‘Lab 2’ directly after harvest, which corresponds to a content of 0.5% MON88017. At ‘Lab 1’ 40 ng Cry3Bb1 g\(^{-1}\) f.w. was detected (Table 2), corresponding to 0.27% MON88017, based on the initial concentration measured in this laboratory. Subsequent analyses at ‘Lab 2’ showed that after 4 weeks the concentration had declined to 9 ± 2 ng g\(^{-1}\) f.w. (\(n = 4\)), and after 8 weeks to 7 ± 5 ng g\(^{-1}\) f.w. (\(n = 9\)).

The limit of detection (LOD) for corn silage, calculated as the OD of the negative control plus three times its standard deviation, was 2.5 ng Cry3Bb1 g\(^{-1}\) f.w. The limit of quantitation (LOQ, equals OD plus six times the standard deviation) was 4.4 ng Cry3Bb1 g\(^{-1}\) f.w.

The sample taken before the feed-in of MON88017 for the reactor effluents was generally low: the Cry3Bb1 content in reactor effluents was measured with 10\(^{16}\) ng Cry3Bb1 mL\(^{-1}\) (Table 2), corresponding to 0.27% MON88017, based on the initial concentration measured in this laboratory. Subsequent analyses at ‘Lab 2’ showed that after 4 weeks the concentration had declined to 9 ± 2 ng g\(^{-1}\) f.w. (\(n = 4\)), and after 8 weeks to 7 ± 5 ng g\(^{-1}\) f.w. (\(n = 9\)).

The sample taken before the feed-in of MON88017 was observed. After 8 months of storage at −20 and −80 °C, protein concentrations were measured with 10.24 ± 0.33 μg Cry3Bb1 g\(^{-1}\) and 9.7 ± 0.64 μg Cry3Bb1 g\(^{-1}\) (\(n = 3\) in both cases). This is in the range of 68% and 65%, respectively, of the content originally measured directly after harvest.

### Cry3Bb1 content in reactor effluent

The sample taken before the feed-in of MON88017 into the reactor served as the background negative control for the reactor effluents. The concentration of Cry3Bb1 in the reactor effluents was generally low: only four out of 12 samples tested positive. Values ranged from 0.15 to 0.48 ng Cry3Bb1 mL\(^{-1}\), with a mean of 0.27 ± 0.16 ng Cry3Bb1 mL\(^{-1}\). There was no increase in the concentration of Cry3Bb1 in the effluents during silage feed-in. The LOD for this matrix was 0.09 ng Cry3Bb1 mL\(^{-1}\) effluent. The LOQ was 0.10 ng Cry3Bb1 mL\(^{-1}\) effluent.

### DISCUSSION AND CONCLUSIONS

#### Protein content in the utilization chain

The estimated mean content of Cry3Bb1 protein in the whole plant at the ripening stage was 14.7 ± 1.6 μg g\(^{-1}\) f.w. based on the individual expression levels in leaves, stalk and kernels and their respective weight proportions. The Cry3Bb1 protein content in leaves was consistently higher than in stalks and grains. Expression levels of the separately assayed plant tissues were highly variable. An analysis of the tissue-specific expression of Cry1Ab in transgenic MON810 over a course of three years has yielded similar expression patterns.\(^{17}\) The highest levels of Cry1Ab protein expression were also found in the leaves, but significant differences in Cry1Ab protein contents were observed. Major influencing factors are the plant tissue and ripening stage, the local environment and climatic conditions. It is important to study individual transgenic events for their production of transgenic proteins in multiple years and different environments to fully assess the full variability underlying protein expression.

The Cry3Bb1 protein content of freshly chaffed Mon88017 plant material was not significantly different from that of whole plants (15.0 ± 2.8 μg g\(^{-1}\) f.w.). In silage, the transgenic protein degraded rapidly. After a period of 4 weeks only a fraction of less than 2% of the initial protein concentration remained. In the following weeks, the Cry3Bb1 concentration varied strongly among samples from different dates. On one hand, this is probably due to the inherent differences in the protein content of different plant parts, and on the other hand to the heterogeneity of the silage and the small-scale conditions in the silage heap. Approximately 8 months after harvest, the Cry3Bb1 content had declined to about 0.5% of the initial value.

In the frozen samples (−80 and −20 °C), no such strong degradation of Cry3Bb1 over a similar timespan was observed. The difference in the Cry3Bb1 content measured at ‘Lab 1’ for the different strata is very small relative to the absolute decline from the initial concentration in the freshly chaffed corn. Therefore, it does not conflict with the assumption of a uniform and strong degradation. This led to the conclusion that Cry3Bb1 degraded in the whole silage heap rather uniformly and that local differences (such as pressure, moisture content, trapped air) played only a minor role in the degradation of this protein. A similar course of degradation was observed in silage of the corn hybrid Bt176, where Cry1Ab could not be traced after only four months of ensiling.\(^{19}\) For MON810, another Cry1Ab expressing corn, there was no clear-cut pattern of degradation.\(^{20}\) The reasons for these conflicting results on the same Bt-protein are unknown.

The presence of Cry3Bb1 in conventional silage may be hypothesized to be based on two factors. The first factor is the expression of the Cry3Bb1 gene in embryos derived from an outcrossing event. Developing embryos express genes already after some days following pollination.\(^{21}\) Cross-fertilized embryos might therefore also express the Cry3Bb1 protein, albeit in small quantities. The second factor is the use of shared equipment during harvest. Plant material from a plot with Bt-corn retained in the chopper was mixed with material from the other corn varieties while these were harvested. In consequence, there was a continuous source of technically unavoidable admixture with genetically modified plant material. The measured concentrations correspond to a content of 0.5% MON88017 in the conventional silage. While this proportion is still below the 0.9% threshold set for compulsory declaration in the EU, the threshold may be breached when further sources of admixture arise. Measures need to be taken to minimize such admixture and to reduce the presence of transgenic material in conventional products.

On the other hand, the rapid degradation of Cry3Bb1 in silage may mean that its presence will go unnoticed after some time of silage storage. DNA also degrades very rapidly in silage, so that a PCR
aimed at detecting GMOs in a silage sample will not necessarily yield a positive result, even if the materials were genetically modified.22–25 Consequently, there will be no way of telling that (parts of) a product had a genetically modified source. This loss of detectability has implications for labelling and safety assessment and needs to be considered when Bt-corn-derived materials are used as feed or food.

In reactor effluents, only trace amounts of Cry3Bb1 were detected. This means that either the concentration of Cry3Bb1 in the silage was already too low as to lead to a significant concentration in the reactor effluents and/or the remaining Cry3Bb1 was further degraded during the fermentation process. The latter would be in line with the results of Rauschen and Schuphan,20 who showed that Cry1Ab from MON810 degraded rapidly during the fermentation process. When the silage of MON88017 was fermented there were already only small amounts of Cry3Bb1 left. Notwithstanding, Cry3Bb1 could still be traced in some reactor effluent samples. Hence the extensive degradation of Cry3Bb1 in silage continued in the biogas reactor, leading to an almost complete depletion of this protein. The extremely low concentrations in the reactor effluents suggest that there is negligible to no risk to the environment, especially the soil ecosystem, when effluents are used as fertilizer for agricultural areas.

Differences between the results from the two laboratories

A comprehensive monitoring of the temporal expression of transgenic proteins in genetically modified plants is the basis for investigations into the fate of these proteins in the environment and plant products, the possible exposure of non-target arthropods in the field and the uptake of Bt-proteins by livestock fed with these plants. A reliable quantitation of transgenic proteins is paramount in this context. Hence, selected samples in our study were exchanged between two laboratories to assess reproducibility and comparability of test results. The values from the two laboratories differed on average by a factor of 4. This indicated consistent differences of ELISA measurements due to the particular sample preparation and testing procedures of each laboratory. Different equipment and ELISA protocols lead to different absolute amounts of Cry3Bb1 being measured. A round robin test recently performed by six laboratories, including those at the DLR Rheinfallz and the RWTH Aachen, yielded considerable variability in the measured Cry3Bb1 concentrations, although purified and standardized protein solutions were used as samples.26 Similar variation was found by Crespo et al.,27 who reported on a comparison and validation of methods to quantitate Cry1Ab. They found that the coefficient of variation for estimates obtained by ELISA ranged from 12.8% to 26.5%. Icoz and Stotzky28 investigated the persistence of Cry3Bb1 in soil using the same ELISA kit as the work on hand. They report marked differences between their measurements regarding Cry3Bb1 content in plant materials and earlier published works. In general, the extraction efficiency during sample preparation seems to be a major factor in the determination of protein from a sample, as recently shown with Cry3Bb1 and the ELISA kit used in this work.14,15

As the observed differences in the protein content in our study were consistent, this resulted in similar estimates for the relative degradation of Cry3Bb1 in corn silage. The concurrent analyses clearly and unambiguously identified a strong degradation of Cry3Bb1, with a final quantity of just below 0.5% of the original Cry3Bb1 remaining. Different absolute values measured in the ELISA therefore did not lead to a different interpretation of the test results. The very low concentration of Cry3Bb1 in reactor effluents suggests an inconsequential ecological risk of the use of these materials as fertilizer in agriculture.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of the German Federal Ministry of Education and Research under grant 0313279.

REFERENCES


