

**Appendix 8.1: MON 810 Literature Review:
Food/Feed (June 2010)**

MON 810 literature review (June 2010)

Appendix 8.1 - Food/Feed

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Area of the environmental risk assessment: Food/Feed Safety - Molecular Characterisation

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Aguilera <i>et al.</i> , 2008)	<p>Objective: Assessment of the genetic and phenotypic stability of MON 810 trait in commercial maize varieties.</p> <p>Experimental design: Twenty-five MON 810 commercial varieties were assessed in the study. One MON 810 positive control (DK 513) and one non-GM wild type maize control (DK 512) were used. The genetic and phenotypic stability of the MON 810 construct was assessed by a combined qualitative approach using DNA and protein based analytical methods. Genomic DNA was isolated from fresh leaves. The presence of the MON 810 construct was confirmed by PCR for amplification of a 35S promoter fragment. The intact transgenic insert present in MON 810 varieties was amplified using primers annealing at 5'-flanking genomic sequence and primers annealing at 3'-flanking genomic sequence. Restriction fragment length polymorphism (RFLP) profiles were verified. Cry1Ab protein expression levels in MON 810 varieties were determined using a commercial ELISA kit. Total protein was isolated from leaf tissues.</p> <p>Results: High quality of genomic DNA was obtained from all 26 varieties. An amplicon specifically for 35S promoter sequence was observed for all varieties except for variety ARISTIS BT. The intact MON 810 construct was amplified from 24 out of 26 varieties and no amplification products were observed from varieties ARISTIS BT and CGS4540. ELISA analysis identified Cry1Ab protein present in 25 varieties. ARISTIS BT gave negative results in all assays, and the variety CGS4045 from Syngenta contained the necessary genetic elements for expressing Cry1Ab protein although giving negative results for the whole MON 810 PCR product. This study confirmed that 24 out of the 26 varieties including all Monsanto varieties met the expected stability features. The MON 810 construct in ARISTIS BT and CGS4045 varieties was altered, indicating that the MON 810 construct in these two varieties was not stable. Although CGS4045 was selected for testing, it was never submitted and approved for marketing in the EU.</p>	The authors concluded that the MON 810 trait was genetically stable in 24 out of 26 varieties including all 6 Monsanto varieties, but it was not stable in varieties ARISTIS BT and CGS4045 ¹ .	Environment	No adverse effects were detected in this study
			Observed parameter	Feedback on initial environmental risk assessment
			Genetic and phenotypic stability	There are no changes to the conclusions of safety of the initial risk assessment.

¹ Aguilera *et al.* (2008) has been commented by Brants *et al.* (2010) who concluded that there's no scientific evidence for MON 810 instability.

Area of the environmental risk assessment: Food/Feed Safety - Molecular Characterisation

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
<p>(Barros <i>et al.</i>, 2010)</p>	<p>Objective: The aim of this study was to evaluate the use of four non-targeted analytical methodologies in the detection of unintended effects that could be derived during genetic manipulation of crops.</p> <p>Experimental design: Two transgenic maize lines (MON 810 and NK603) and the near-isogenic non-GM variety CRN3505 were grown in two different sites in South Africa, over respectively three (2004, 2005 and 2006) and one growing seasons (2004). For the field trial performed in 2005, three replicate samples were available and the results were averaged prior to further analysis for all techniques. For all other field trials, one sample was analyzed. MON 810 and the control variety were also grown in an additional site under low-input system, which means that no fertilizer, no fungicide and no herbicide were applied throughout the growth of the plants (organic cultivation). Non-commercial 57K spots microarrays were used for RNA transcript profiling. Proteome profiling was made by two-dimensional gel electrophoresis and spots were matched and quantified. Maize powder extracts were analysed by H-NMR spectroscopy and metabolites were quantified. Metabolites were profiled using GC/MS. One-way ANOVA was performed in combination with Tukey’s HSD test to identify differences in the expression signals of transcript, protein content and metabolic compounds using H-NMR fingerprinting and GC/MS-based metabolite profiling. Differences at the level $P < 0.01$ were considered statistically significant.</p> <p>Results: The environment was shown to play an important effect in the protein, gene expression and metabolite levels of the maize samples tested. 5 proteins, 65 genes and 15 metabolites were differentially expressed. A distinct separation between the three growing seasons was found for all the samples grown in one location. Together, the environmental factors caused more variation in the different transcript/protein/metabolite profiles than the different genotypes². Whereas the influence of the cultivars could be clearly shown within the single years, combination of samples from all seasons revealed the environmental impact to be the most prominent impact factor.</p>	<p>The authors conclude that “potential unintended effects shown could very well fall within natural variability that exists among maize lines and that was beyond the scope of this study, such as different landraces, or more diverse locations and climates. Compared to the targeted methods, -omics methods can potentially give a much wider picture of food composition. Furthermore, these non-targeted methods enable the detection of unexpected or unintended changes caused by genetic modification, traditional breeding or various external factors from environmental conditions to agricultural regimen.”</p>	<p>Animal and human health</p>	<p>No adverse effects were detected in this study</p>
			<p>Observed parameter</p>	<p>Feedback on initial environmental risk assessment:</p>
			<p>Comparative assessment</p>	<p>There are no changes to the conclusions of safety of the initial risk assessment.</p>

² While differences across the 3 seasons, attributable to genotype, were found by Principal Component Analysis (PCA), those differences accounted for a relatively small amount of the variation in the data. In one-way ANOVA sub-analyses the most striking metabolomic effect (by H-NMR) attributable to genotype was a 13.8 fold increase in glucose, and a 6.9 fold increase in fructose for MON 810, as compared to conventional. However, those metabolites were not found using another metabolomic profiling method (GC/MS).

Area of the environmental risk assessment: Food/Feed Safety - Molecular Characterisation

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Coll <i>et al.</i> , 2010)	<p>Objective: The aim of the study was to assess the relative contribution of (1) the GMO character (using MON 810 as an example); (2) the variety and (3) the N treatment on the transcriptional patterns of maize plants grown in agricultural fields.</p> <p>Experimental design: A transcriptomic approach utilizing microarray was utilized to study the similiarity (or lack thereof) between MON 810 (Helen Bt) and near-isogenic non-GM varieties (Beles Sur) grown in a real agricultural environment (Spain). Seeds were grown under standard conditions for the region. 30 plants at vegetative tasseling stage were harvested for analysis. A maize genome array was used to search for transcriptome differences between MON 810 and near-isogenic varieties and nitrogen conditions. The array contains 17,000+ probe sets, representing 13,000+ maize genes (about 1/3 of genes of maize). 3 arrays were used for independent replicates per variety and nitrogen level. Sequences showing expression changes greater than 2-fold were defined as differentially expressed.</p> <p>Results: Natural variation explained most of the variability in gene expression among the samples. Up to 37.4 % (of the variability in gene expression) was dependent upon the variety (from conventional breeding) and 31.9% a result of the fertilization treatment. In contracts, the MON 810 GM character had a very minor effect (9.7%) on gene expression in the analyzed varieties and conditions, even though similar <i>CryIAb</i> expression levels were detected in the two MON 810 varieties and nitrogen treatments.</p>	This indicates that the extent of natural variation of gene expression is, in the varieties and conditions analyzed, larger than the variation due the insertion and expression of the MON 810 transgene. This emphasizes that such natural variation should be taken into account to assess the biological and/or toxicological relevance of observed differences between a GM and a comparable non-GM plant.	Animal and human health	No adverse effects were detected in this study
			Observed parameter	Feedback on initial environmental risk assessment:
			Comparative assessment	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Food/Feed Safety – Molecular characterisation

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(La Paz <i>et al.</i> , 2010)	<p>Objective: To characterize transcripts originating from the inserted Cry1Ab transgene in MON 810 commercial varieties.</p> <p>Experimental design: RNA was isolated from leaf tissue of 28 MON 810 commercial varieties and reverse transcribed. The resulting cDNA was subjected to quantitative PCR and Northern blot analysis using Cry1Ab specific probes. Isolated RNA was also subjected to RACE 3'-PCR using anchored-oligo(dT) and MON 810 specific sequences were amplified using primes to the dT and MON 810 forward probes. These were sized by capillary micro-electrophoresis, and cloned for sequencing.</p> <p>Results: Real-time PCR showed that the level of Cry1Ab mRNA was similar in all 28 MON 810 varieties. Northern blot analysis performed with <i>Cry1Ab</i> transcript-specific probes on mRNA from a subset of the MON 810 varieties and 2 non-GM lines revealed a 2.5 kb band with a smear above and 2 higher MW bands at ~3.0 and 3.3 kb in all. Transcripts of the HECT E3 ubiquitin-ligase gene (interrupted by the transgene and in the reverse direction) and the non-coding strand of the Cry1Ab gene were not detected. Transcript sizing indicated that transcription either terminated around the truncation site of the <i>Cry1Ab</i> coding region or extended ~200 to 1000 bases beyond with clusters at ~550-625 and 800-1000 3' of the <i>Cry1Ab</i> coding region. Sequencing of 8 of the RACE 3'-PCR products showed that the transcripts that extended beyond the <i>Cry1Ab</i> coding region all perfectly matched the genomic sequence with no insertions or deletions. Size profiles of RACE 3'-PCR products from 6 MON 810 varieties were essentially identical indicating that the mRNA species produced in the different varieties are the essentially the same.</p>	<p>The mRNA levels of <i>Cry1Ab</i> in 28 MON 810 varieties were shown to be similarly expressed. The transcript pattern of <i>Cry1Ab</i> mRNAs are similar in the 6 MON 810 varieties tested. It indicated that the transcription of the event is stable.</p>	Animal and human health	No adverse effects were detected in this study
			Observed parameter	Feedback on initial environmental risk assessment:
			Expression of the insert	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Food/Feed Safety – Molecular characterization

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Szekacs <i>et al.</i> , 2010)	<p>Objectives: To measure Cry1Ab content in the leaves of MON 810 and to assess Cry1Ab distribution among leaf levels and within individual leaves.</p> <p>Experimental design: Leaf samples were collected from MON 810 (DK-440 BTY) maize and its near isogenic variety (DK-440). Cry1Ab content was determined using a commercial ELISA kit. Cry1Ab concentration was determined using the calibration curve and by spiking known amounts of protein in the internal reference lyophilized corn leaf sample. (1) To measure Cry1Ab content, 5 mm wide samples were collected from middle section of each leaf. (2) To assess the distribution of Cry1Ab, 5 mm diameter leaf discs were collected from the middle part diagonally or leaf strips were cut at the middle between vein and perimeter. Leaf tips with or without necrotic tissue were also collected.</p> <p>Results: No substantial matrix effect was observed at the dilution tested. Similarly, the assay proved reliable in Cry1Ab determination across several years. (1) Cry1Ab was not detected in the near isogenic control line, where as the toxin content in the MON 810 maize ranged between 4,821 and 10,054 ng toxin/g fresh leaf. Variation in the toxin concentration between different samples from the same plant ranged between 19-21%, where as up to 22% variation was observed between samples from different plants. Levels of Cry1Ab were significantly lower at the lowest leaf level than the higher levels. (2) Within a single leaf, Cry1Ab levels were about 20% higher at or near the leaf vein over the leaf edge. Similarly toxin content was higher at the middle section of the leaf and decreased towards the leaf tip. When the leaf tips were tested for the toxin content with or without necrotic tissue, decreased Cry1Ab levels were observed with leaf tip necrotization. When the necrotic areas were removed, Cry1Ab levels at the leaf tip were statistically similar to the toxin levels at the lamella base and middle.</p>	<p>The Cry1Ab levels reported in this study are similar to the toxin concentrations reported in the official MON 810 documentation. Location of leaf samples taken from a single maize plant and within a single leaf affects the detectable Cry1Ab levels in the sample. Cry1Ab content showed higher variation across the length of the leaf and hence, diagonal middle sections are recommended for sampling.</p>	Environment	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Expression of the insert	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Food/Feed Safety – Animal Feeding Study

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(de Vendomois <i>et al.</i> , 2009)	<p>Objective: To conduct a statistical re-evaluation of data from three 90 day rat feeding studies with GM maize varieties MON 810, MON 863 and NK603.</p> <p>Experimental design: No new studies were performed; the authors repeated the statistical analysis performed by Monsanto on the aforementioned products. A variety of different statistical tests than performed by Monsanto including pair wise comparisons between the near isogenic control and the references groups were carried out. A False Discovery Rate approach was used to perform multiple pair-wise comparisons to calculate adjusted p-values and Principal Component Analysis to study the scattering of the different factors (sex, period, diet, dose, group). Paired tests corresponding to temporal changes between the two feeding periods (kinetic plots) were established for each group and parameter.</p> <p>Results: Similar results as Monsanto were obtained when the Monsanto protocol for statistical analysis of the data was followed. According to the authors’s statistical analysis, more statistically significant differences were found than previously reported by Monsanto. For NK603, sex differences were reported for some of the parameters based on Principal Component Analysis. Evidence of bone marrow, heart, liver and kidney toxicity were reported. Similar findings were reported for MON 810 with the addition of the spleen. For MON 863, an earlier analysis by the authors indicating sex dependency for toxicity in the kidney and liver was mentioned. Through the use of kinetic plots, significant time variations were reported for certain measured parameters which were interpreted as evidence of physiological disturbances in rats fed GM crops.</p>	By carrying out a comparative analysis of blood and organ system data for the three different studies, authors claimed to find evidence of kidney and liver toxicity across the three studies which were sex and often dose-dependent. Authors also claimed to find evidence of adverse effects in the heart, adrenal glands, spleen and hematopoietic systems. ³	Animal health	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Toxicology	There are no changes to the conclusions of safety of the initial risk assessment.

³ While the authors claimed to find evidence of adverse effects in various organ systems, and common findings of liver and kidney toxicity across the three studies, these claims are not supportable by the analysis presented. EFSA (EFSA 2010 - <http://www.efsa.europa.eu/en/events/event/gmo100127.htm>), following review of the publication concluded that there was no new evidence of harmful effects in these studies. Specifically, EFSA had fundamental criticisms of the statistical analysis methods used and how they were applied to interpret the data. EFSA had criticized similar statistical methods used in a previous publication by some of the same authors. They failed to put into proper context the biological relevance of the statistical differences they reported as they did not use all of the control data generated in the studies. The French High Commission on Biotechnology (HCB - http://www.ogm.gouv.fr/article.php3?id_article=115) pointed out after review of the paper that a statistical difference does not necessarily imply the existence of a biological disorder. This principle is universally accepted by institutions that are responsible for the evaluation of toxicological risks. Thus the exercise of the authors to simply count the number of statistical differences between test and control animals is not acceptable. As a consequence of the insufficiencies and errors by the author’s interpretation of the data, they did not establish any evidence of treatment adverse effects in any of the studies they analyzed.

Area of the environmental risk assessment: Food/Feed Safety – Animal Feeding Study

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Sissener <i>et al.</i> , 2010)	<p>Objective: To compare growth, performance and liver transcription of mRNA coding for different proteins involved in cellular and oxidative stress between fish fed the non-GM and GM varieties of maize and soy using zebrafish. A second objective was to detect transgenic DNA fragments and rubisco, a chloroplast gene present in plants in high copy numbers, in different tissues of the fish.</p> <p>Experimental design: A randomized design with a 2x2 factorial arrangement of treatments (maize and soy <i>vs.</i> GM and non-GM) was used. GM feeds were 40-3-2 soy and MON 810 maize. Diets had 20% maize and 25% full-fat soybean. An additional experimental diet was included as a fifth treatment and did not include similar ingredients or dietary formulation⁴. At the beginning of the study fish weighed 149 mg. There were 17 fish/tank with 3 tanks/diet. Fish were fed experimental diets for 20 days. Growth and feed intake were measured and, samples were collected for determining DNA fragments. Hepatic mRNA was sampled for determining mRNA that codes for proteins related to cellular and oxidative stress. Tank was the experimental unit and data were analyzed using a nested ANOVA.</p> <p>Results: The commercial (reference) diet had significantly better growth, observed by a higher growth rate and final weight, than the diets in the 2x2 arrangement⁵. Weight was greater for fish fed GM maize than non-GM maize and feed intake was reduced for fish fed GM soy⁶. Transcription levels of superoxide dismutase were elevated in fish fed non-GM maize. DNA from 40-3-2 soy was undetectable and DNA from MON 810 maize was only detected in one liver sample.</p>	<p>Interaction between soy variety and sex of fish for RNA yield suggests some component affecting differently males and females was present at different level between GM and non-GM soy. The authors also conclude that stress response was higher in fish fed non-GM maize. Finally, the authors conclude on the use of zebrafish as a model organism: “Dietary DNA seemed to behave similarly to what has been reported from other species, while the different responses to GM maize compared with salmon are hard to explain.”</p>	Animal health	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Toxicology	There are no changes to the conclusions of safety of the initial risk assessment.

⁴ The stat analysis does not appear to be done correctly due to the 2x2 + added 5th diet. This likely results in some Type I errors.

⁵ The greater growth with the commercial diet suggests that the diets in the experimental 2x2 arrangement were not balanced for optimal intake and/or growth.

⁶ If real, these differences would both suggest that the GM feed did not negatively affect the fish

Area of the environmental risk assessment: Food/Feed Safety – Toxicology / Allergenicity

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(de Luis <i>et al.</i> , 2010)	<p>Objective: To evaluate the <i>in vitro</i> digestion of Cry1Ab protein from transgenic maize by pepsin using electrophoresis, mass spectrometry and immunochemical techniques.</p> <p>Experimental design: Cry1Ab was purified from maize (MON 810) using an immunoaffinity column of Cry1Ab polyclonal antibodies. Cry1Ab was digested with porcine pepsin for 30 min, 2 hr and 4 hr. The rate of degradation was assessed using sandwich ELISA and compared to protein isolated from <i>B. thuringiensis</i>. The resulting transiently stable fragments were examined by SDS-PAGE, Western Blot using Cry1Ab polyclonal antibodies and Maldi-TOF/TOF mass spectrometry.</p> <p>Results: Approximately 25% of maize Cry1Ab was immunoreactive by ELISA after 30 min remaining constant through 4 hours, while 70% of corresponding <i>B. thuringiensis</i> Cry1Ab was detected at 30 min, with the <i>B. thuringiensis</i> Cry1Ab protein decreasing to 23% reactive by 2h and then remaining constant. By western blot, no immunoreaction to full length protein or transiently stable fragments was observed for either protein after 30 sec exposure to pepsin. Transiently stable fragments were seen in both maize and <i>B. thuringiensis</i> samples, however, no investigation of identity of transiently stable fragments was performed.</p>	<p>The authors conclude that the Cry1Ab fraction purified from transgenic maize is rapidly and extensively degraded by pepsin, giving peptides of low molecular mass as reported by other publications. Most allergenic proteins do not have such properties.</p>	Human health	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Allergenicity	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Food/Feed Safety – Toxicology / Allergenicity

Publication	Summary of research and results	Conclusion	Protection Goal	Adverse effects
(Guimaraes <i>et al.</i> , 2010)	<p>Objective: Investigate the stability of Cry1Ab protoxin and toxin in different conditions of pepsin digestion.</p> <p>Experimental design: Cry1Ab protoxin, toxin, and β-casein (as a digestibility control) were digested using a pepsin:test protein ratio of 3:1 at pH 1.2 or pH 2.0. The same test proteins were also tested under what were referred to as “more physiologically relevant” conditions: pH 2.5, and pepsin:test protein ratio of 1:20, 1:1, or 20:1, in the presence or absence of phosphatidylcholine (PC). Digestibility of test protein was assessed by SDS-PAGE stained gel and by western blot. In addition, ability of digested protein to compete immunologically with undigested protein was assessed, as was IgE and IgG1 binding to digested protein.</p> <p>Results: By SDS-PAGE, Cry1Ab protoxin was rapidly digested under the standard condition. A slight amount of intact protein was detected by western blot. At pH 2.0, little protoxin digestion was observed. β-casein was digested in both pH conditions. Under the pH 2.5 conditions, at a 1:20 ratio of pepsin:test protein, no change in protein profile was observed. Increasing the pepsin:test protein ratio to 1:1 or 20:1 resulted in some digestion by the 60 min time point. In these cases, the digestion was apparently slowed by the presence of PC. Full-length β-casein was digested in 10 min at the 1:20 ratio, although some fragments remained. Cry 1Ab toxin (~65 kDa) appeared to be partially digested at pepsin:test protein ratios of 1:1 and 20:1 at pH 2.5, although intact protein was still visible by western blot. Under the standard condition, IgE and IgG1 binding were rapidly lost, (consistent with loss of the protoxin protein). At pH 2.0, IgE and IgG1 binding were affected somewhat by exposure of the protein to acidic conditions, and somewhat by digestion. Similar results were observed at pH 2.5.</p>	<p>Cry1Ab protoxin shows a different response to pepsin digestion as pH and pepsin:test protein ratios are altered, with some conditions showing significant lack of pepsin digestion. Digestion of control protein indicates that pepsin has at least some activity in these test conditions. The authors consider some of their test conditions more physiologically relevant than the standard conditions used to assess digestibility, and suggest that digestibility of newly expressed proteins should be assessed using more test conditions than the one presently in use.</p>	Human health	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment
			Allergenicity	There are no changes to the conclusions of safety of the initial risk assessment.

Area of the environmental risk assessment: Food/Feed Safety – Composition/Nutrition studies

Publication	Summary of research and results	Conclusion	Protection goal	Adverse effects
(Swiatkiewicz <i>et al.</i> , 2010)	<p>Objective: To evaluate the effect of genetically-modified (GM) corn (MON 810) and GM soybean meal (40-30-2), used as the main dietary components for broilers, on bird performance, carcass characteristics, and the chemical composition of the breast muscle.</p> <p>Experimental Design: A total of 640 Ross 308 broilers were fed corn-soybean meal based diets in a 42-day floor pen experiment. A randomised complete block design utilized four replicates (pens) of 40 birds (half male and half female) per treatment to evaluate four dietary treatments. All the experimental diets met the nutrient requirements of broilers; they were all isonitrogenous and isoenergetic, and contained non-modified corn and soybean meal (group I), non-modified corn and GM soybean (group II), GM corn and non-modified soybean meal (group III), or GM corn and GM soybean meal (group IV), respectively. The data were subjected to a one-way factorial analysis of variance. Differences between treatment means were determined using Duncan’s multiple range test ($P \leq 0.05$ significance level).</p> <p>Results: Final live weight, average daily weight gain, feed intake, feed conversion (feed:gain ratio), and mortality rate did not differ statistically ($P > 0.05$) across the dietary treatments. No statistical differences ($P > 0.05$) were found in the results of slaughter analysis (carcass yield, meat yield, abdominal fat pad, and relative weight of the liver, gizzard, and spleen) and chemical composition of the breast muscles.</p>	In agreement with several cited previously published reports, MON 810 and soybean meal produced from 40-30-2 are nutritionally equivalent to conventional feeds and can be used as components of broiler diets with no adverse effect on bird performance or carcass quality.	Animal health	No adverse effects were detected in this study.
			Observed parameter	Feedback on initial environmental risk assessment:
			Nutritional assessment of the GM food/feed	There are no changes to the conclusions of safety of the initial risk assessment.

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