Safety Assessment of MON 89034

1. Executive Summary

Corn *(Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. Insect pests in the corn fields, if not properly controlled, significantly reduce corn yields and grain quality. In 1997, Monsanto commercialized a biotechnology-derived corn product, YieldGard Corn Borer corn (i.e., MON 810) which contains the *Bacillus thuringiensis* (Bt) *crylilb* gene. The expression of Cryl Ab protein in corn plants provides effective protection against damage caused by lepidopteran insect pests, such as European corn borer (ECB). Since the launch of MON 810, several other Bt corn products have also been commercialized in the U.S., including YieldGard® Rootworm corn which expresses Cry3Bb1 protein that confers protection against coleopteran pests. In 2007, approximately 68 3 million acres of corn planting area in the U.S. (or 73% of the total U.S. corn acreage) was planted with biotechnology-derived corn seed, and 45.9 million acres (or 49% of the total corn acreage) were planted with corn seed possessing insect resistance (Bt) traits (USDA-NASS, 2008).

Monsanto has developed MON 89034 as a second generation insect protection corn product to provide enhanced benefits for the control of lepidopteran insect pests. MON 89034 produces the Cryl A.105 and Cry2Ab2 proteins derived from *Bacillus thuringiensis,* which are active against lepidopteran insect pests. Compared to MON 810, MON 89034 will better serve corn growers' need for controlling a wider spectrum of lepidopteran pests and help assure the durability of Bt corn. MON 89034 provides outstanding control of *Ostrinia* species such as European corn borer (ECB) and Asian corn borer (ACB), and *Diatraea* species such as southwestern corn borer (SWCB) and sugarcane borer (SCB). Control of these insects provided by MON 89034 is comparable to MON 810. MON 89034 also provides a high level control of fall armyworm (FAW) throughout the season, whereas MON 810 principally controls fall armyworm larvae during vegetative growth stage. Furthermore, MON 89034 provides significantly improved protection from damage caused by corn earworm (CEW) than MON 810.

In addition to the wider spectrum of insect control, the combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant, MON 89034, provides a much more effective insect resistance management (IRM) tool. Mathematical modeling indicates that biotechnology-derived plants expressing two Cry proteins will have significantly greater durability than plants producing either of the single proteins if the cross-resistance between the Cry proteins is low and the mortality of susceptible insects caused by each of the individual proteins is at least 90%. Comparative biophysical studies indicate that the Cry1A.105 and Cry2Ab2 proteins have important differences in their mode of action, specifically in the way in which they bind to the lepidopteran midgut. Therefore, the probability of cross-resistance between these two proteins is low. Furthermore, *in vitro* and *in planta* studies with Cry1A.105 and Cry2Ab2 demonstrate that both proteins are highly active against the primary lepidopteran pests of corn (ECB, SWCB, CEW, and FAW), particularly ECB, achieving close to or greater than the critical 95% level of control in all cases. With these properties, MON 89034 should be durable with a significantly smaller structured refuge than is necessary for Bt corn products producing a single insecticidal protein.

The data and information generated through multi-year tests and trials demonstrate that MON 89034 is safe as conventional corn as food and feed and safe to the environment. This conclusion is based on multiple lines of evidence. The first is the detailed molecular characterization of the inserted DNA. Results confirm the insertion of a single functional copy of *cryli4.105* and *cry24b2* expression cassettes at a single locus within the corn genome. The second is a detailed biochemical characterization of the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034. In addition, the data demonstrate that the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 are equivalent to the respective Cry1A.105 and Cry2Ab2 proteins produced by recombinant strains of *Escherichia coli,* which were used in the various safety assessment studies. The third line of evidence is an assessment of the toxicity and allergenicity potential of the Cryl A.105 and Cry2Ab2 proteins based on extensive information collected and studies performed on the two proteins. The results demonstrate with reasonable certainty that the Cry1A.105 and Cry2Ab2 proteins are unlikely to be allergens or toxins. The fourth line of evidence is the compositional and nutritional assessment which confirms that MON 89034 grain and forage are compositionally equivalent to and as safe as those of conventional corn. The fifth line of evidence is the extensive evaluation of the MON 89034 phenotypic and agronomic characteristics and ecological interactions, which demonstrates that MON 89034 is not likely to have an increased plant pest potential compared to the conventional corn. Finally, an assessment on the potential impact on non-target organisms (NTO) and endangered species, and on gene flow concludes that MON 89034 is unlikely to have adverse effects on these organisms and sexually compatible wild plant species under the conditions of use.

MON 89034 was produced by *Agrobacterium-mediated* transformation of corn with PV-ZMIR245, which is a binary vector containing 2T-DNAs. The first T-DNA, designated as T-DNA I, contains the *cryli4.105* and the *cry24b2* expression cassettes. The second T-DNA, designated as T-DNA II, contains the *nptll* (neomycin phosphotransferase II) expression cassette. During transformation, both T-DNAs were inserted into the genome. The *nptll* gene was used as the selectable marker which was needed for selection of the transformed cells. Once the transgenic cells were identified, the selectable marker gene was no longer needed. Therefore, traditional breeding was used to isolate plants that only contain the *cryli4.105* and *cry24b2* expression cassettes (T-DNA I) and do not contain the *nptll* expression cassette (T-DNA II), thereby, producing marker-free corn MON 89034. Molecular characterization of MON 89034 by Southern blot analyses demonstrated that the DNA inserted into the corn genome is present at a single locus and contains one functional copy of the *cryli4.105* and the *cry24b2* expression cassettes. All genetic elements are present in the inserted DNA as expected with the exception that the *e35S* promoter, which regulates expression of the *cryli4.105* gene, has been modified and that the Right Border sequence present in PV-ZMIR245 was replaced by a Left Border sequence in MON 89034. No backbone plasmid DNA or *nptll* sequences were detected. PCR and DNA sequence analyses provided the complete DNA sequence of the insert and confirmed the organization of the elements within the insert.

The stability of the integrated DNA was demonstrated by the fact that the Southern blot fingerprint of MON 89034 was maintained for seven generations tested in the breeding history.

Additionally, T-DNA II analysis of multiple generations of MON 89034 indicated that there were no T-DNA II elements present other than those which are common to T-DNA I, including *35S* promoter, *nos* 3' end sequence, and Left Border sequence. Furthermore, these generations have been shown not to contain any backbone sequence from plasmid PV-ZMIR245. The stability was further confirmed by the fact that the inheritance of the lepidopteran protection trait in MON 89034 follows Mendelian segregation principles.

The expression levels of Cry1A.105 and Cry2Ab2 proteins were determined in MON 89034 tissues produced from multiple field sites in the major U.S. corn production regions. The results demonstrated that both Cry1A.105 and Cry2Ab2 proteins were expressed in all tissues collected, including leaf, root, forage, silk, pollen, grain and stover. The mean Cry1A.105 protein levels (µg/g dwt) across all test sites were 5.9 in grain, 42 in forage, 12 in pollen, 520 in leaves of plants at V2-V4 stage, 120 in leaves of plants at pre-VT stage, 12 in forage root, and 50 in stover. The mean Cry2Ab2 protein levels (µg/g dwt) across all test sites were 1.3 in grain, 38 in forage, 0.64 in pollen, 180 in leaves of plants at V2-V4 stage, 160 in leaves of plants at pre-VT stage, 21 in forage root, and 62 in stover.

Certain safety tests and studies require large amounts of the Cry1A.105 and Cry2Ab2 proteins. The expression levels of the two proteins in MON 89034 were too low to allow for purification of sufficient quantities of the two proteins directly from MON 89034 for use in the safety assessment studies. Therefore, it was necessary to produce the Cry1A.105 and Cry2Ab2 proteins in a high-expressing recombinant host organism, *E. coli*. The proteins produced by *E. coli* were engineered to match the amino acid sequences of their counterparts expressed in MON 89034. Thus, the physicochemical and functional equivalence of MON 89034-produced and *E.* coli-produced proteins was examined to ensure that the proteins from the two host sources were equivalent so that the *E.* coli-produced proteins could be used as surrogates in the studies. Small quantities of the Cry1A.105 and Cry2Ab2 proteins were purified from the grain of MON 89034. Large quantities of Cry1A.105 and Cry2Ab2 proteins were produced and purified from recombinant *E. coli*. The proteins from the two sources were characterized and the equivalence was evaluated based a panel of analytical tests and assays, including Western blot analysis; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS); N-terminal sequence analysis; glycosylation analysis; and insect activity bioassay. The results provide a detailed characterization of the Cry1A.105 and Cry2Ab2 proteins isolated from MON 89034 and confirmed their equivalence to the *E.* coli-produced Cry1A.105 and Cry2Ab2 proteins.

The history of safe use and data from multiple studies support the safety of MON 89034 and the Cry1A.105 and Cry2Ab2 proteins. The two proteins belong to a family of Cry proteins from *Bacillus thuringiensis,* an organism which has been used commercially in the U.S. for over four decades to produce microbial pesticides. Bt corn expressing several Cryl proteins and Bt cotton expressing both Cryl and Cry2Ab2 proteins have been cultivated in large areas in the U.S. and other countries for over a decade. The extremely low mammalian toxicity of Bt-based microbial insecticides and Cry proteins has been demonstrated in numerous safety studies, and there are no confirmed cases of allergic reactions to Cry proteins. Cry1A.105 and Cry2Ab2 proteins do not share any amino acid sequence similarities with known allergens,

gliadins, glutenins, or protein toxins which have adverse effects to mammals. This has been shown by extensive assessments with bioinformatic tools, such as FASTA sequence alignment search and an eight-amino acid sliding window search. Cry1A.105 and Cry2Ab2 proteins are rapidly digestible in simulated gastric fluids (SGF). Greater than 95% to 99% of these proteins were digested in SGF in less than 30 seconds. Proteins that are rapidly digestible in mammalian gastrointestinal systems are unlikely to be allergens when consumed.

Mice acute oral toxicity studies demonstrate that the Cryl A.105 and Cry2Ab2 proteins are not acutely toxic and do not cause any adverse effects even at the highest does levels tested, which are 2072 and 2198 mg/kg body weight for Cry1A.105 and Cry2Ab2 proteins, respectively. The dietary safety assessment based on the acute toxicity data and corn product dietary pattern establishes that the margins of exposure (MOE) for the overall U.S. population are >199,000 and 981,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively. And the MOEs are >79,400 and 390,000 for the Cry1A.105 and Cry2Ab2 proteins, respectively, for children aged 3-5 years old, an age group with the highest corn consumption per unit body weight. For poultry and livestock, the MOEs range between 1,930-13,500 and 2,160-47,600 for the Cry1A.105 and Cry2Ab2 proteins, respectively. Taken together, these data indicate that food and feed derived from MON 89034 which contains the Cry1A.105 and Cry2Ab2 proteins are safe for consumption.

Compositional assessment of the grain and forage from multiple field sites in the major U.S. corn production regions demonstrate that MON 89034 is nutritionally and compositionally equivalent to, and as safe, nutritious and wholesome as its conventional counterpart. The compositional analyses were conducted on a total of 77 components, nine in forage and 68 in grain, including protein, fat, carbohydrate, fiber, ash, moisture, amino acids, fatty acids, vitamins, anti-nutrients, secondary metabolites and minerals. The results were within the range of data expected for these types of analyses. Of the 77 components analyzed, the results of 16 components were below the limit of quantitation (LOQ) for over half of the analyses conducted. As such, these 16 components were not included in the statistical analyses. Among the remaining 61 components, analyses of the data across all test sites (combined sites) indicate that there were no statistical differences for 58 components. The three analytes with statistical differences were phosphorus in forage, and stearic acid (C18:0) and arachidic acid (C20:0) in grain. The differences observed are generally small (3.4 — 19.2%) considering the range of natural variability, and the mean levels and ranges of these three analytes of MON 89034 are well within the ranges of values observed for the 15 reference commercial corn hybrids grown along the side of MON 89034, and within the ranges in the International Life Sciences Institute crop composition database, as well as within the published literature ranges. Therefore, it is concluded that the slight difference observed for these 3 components is not biologically meaningful, and MON 89034 and the control corn are compositionally equivalent. The nutritional wholesomeness of MON 89034 corn grain was also confirmed by a broiler chicken performance study.

The phenotypic, agronomic, and ecological interaction assessment indicates that MON 89034 is comparable to conventional corn and is unlikely to have any increased plant pest risk. An important element in assessing plant pest potential and environmental impact of MON 89034 is to compare MON 89034 to conventional corn. The assessment is based initially on the concept

of familiarity, which is based on the fact that the biotechnology-derived plant is developed from a conventional plant variety whose biological properties and plant pest potential are known to experts. Familiarity considers the biology of the crop, the introduced trait, the receiving environment and the interaction among these factors, and provides a basis for comparative risk assessment between a biotechnology-derived plant and its conventional counterpart.

Results from the phenotypic and agronomic characteristics assessments indicate that MON 89034 does not possess characteristics that would confer an increased plant pest risk compared to conventional corn. The assessments are based on a combination of laboratory experiments and field studies conducted by scientists who are familiar with the production and evaluation of corn. In each of these studies, MON 89034 was compared to an appropriate conventional corn hybrid which has a genetic background similar to MON 89034 but does not possess the lepidopteran-protection trait. In addition, multiple commercial corn hybrids were also employed to provide a range of values that are common to the commercial corn hybrids for each measured characteristic. These assessments included five seed germination parameters, two pollen characteristics, 14 plant growth and development characteristics, and more than 70 observations for each of the plant-insect, plant-disease and plant responses to abiotic stressor interactions.

Seed dormancy and germination characterization observed no viable hard seed in any of the temperature regimes tested. No statistically significant differences were detected between MON 89034 and the control corn for pollen diameter or viability. The phenotypic and agronomic characteristics data collected from 18 field test sites in 2004 and 2005 demonstrate that no significant differences were detected between MON 89034 and the control corn for seedling vigor, early stand count, final stand count, days to 50% pollen shed, days to 50% silking, stay green, ear height, dropped ears, root lodged plants, grain moisture, test weight and yield. Differences in plant height and number of stalk lodged plants were detected in 2004 trials but not in 2005 trials. In 2004 trials, plant height was slightly lower for MON 89034 compared to control (84.1 vs. 85.4 inches), and stalk lodged plants were less for MON 89034 than for the control (0.8 vs. 2.4 per plot). The magnitude of these differences are generally small, similar differences are not detected in 2005 trials, and the mean values of MON 89034 fall well within the ranges of values observed for the 23 reference commercial corn hybrids grown along the side of MON 89034. Therefore, these two differences observed in 2004 field trials are not considered biologically meaningful and are unlikely to contribute to increased plant pest potential.

In addition to the phenotypic and agronomic characteristics, observational data on the presence of and differential response to biotic (insects, diseases) and abiotic (drought, wind, nutrient deficiency) stressors were collected in the two years of field trials to examine the ecological interactions of MON 89034 compared with those of the conventional control corn. Based on 255 comparative observations recorded over two years, no repeatable differences were observed across sites between MON 89034 and the control in their susceptibility or tolerance to the ecological stressors assessed. The result supports the conclusion that compared to conventional corn, the ecological interactions between MON 89034 and insects, diseases, and abiotic stressors were not altered except for the introduced lepidopteran-protection trait.

The environmental assessment of MON 89034 and Cry1A.105 and Cry2Ab2 proteins indicates that these two proteins pose no adverse effect on non-target organisms (NTOs) and endangered species under the conditions of use. The assessment took into consideration several components, including the familiarity with the mode of action of Cry proteins, the activity spectra of the Cry1A.105 and Cry2Ab2 proteins, the expression levels of the two proteins in MON 89034, the environmental fate of the proteins, the lack of interaction between the two proteins, and feeding tests of the two proteins or MON 89034 corn materials to representative NTOs. The tested NTOs include one mammalian species (mice), two avian species (broiler chicken and bobwhite quail), one aquatic species (Daphnia), two species of soil decomposers (Collembola and earthworm), and four beneficial insect species (honeybee, minute pirate bugs, ladybird beetle, and parasitic wasp). The estimates of MOEs for the non-target insects exposed to Cry1A.105 and Cry2Ab2 proteins are >14.

Risk assessments of potential effects on the federally listed threatened or endangered species indicated that only Karner blue butterfly *(Lycaeides melissa samuelis)* has the potential to occur in proximity to corn fields. There are only two Wisconsin counties in the Midwestern Corn Belt where potential temporal overlap between Karner blue butterfly larvae and corn pollen shed is likely. Based on a conservative risk analysis model, it is shown that the margin of safety for Karner blue is >12 fold using the highest possible pollen concentration from MON 89034. Taken together, these data support the conclusion that MON 89034 is unlikely to have adverse effects on NTOs and endangered species under the conditions of use.

The potential for MON 89034 outcrossing to sexually compatible species is unlikely in the U.S. Corn and annual teosinte *(Zea mays* subsp. *mexicana)* are genetically compatible, wind-pollinated and, in areas of Mexico and Guatemala, freely hybridize when in close proximity to each other. However, teosinte is not present in the U.S. other than as an occasional botanical garden specimen. Differences in factors such as flowering time, geographical separation and development factors make natural crosses in the U.S. highly unlikely. In contrast with corn and teosinte, special techniques are required to hybridize corn and *Tripsacum.* With the exception of *Tripsacum floridanum*, it is difficult to cross *Tripsacum* with corn, and the open literature indicates that the offspring of the cross show varying levels of sterility. *Tripsacum-corn* hybrids have not been observed in the field. Therefore, the environmental consequence of pollen transfer from MON 89034 to other wild plant species is considered negligible.

The data and information presented in this summary demonstrate the food, feed and environmental safety of MON 89034. The safety of MON 89034 has been confirmed by a number of independent product safety reviews conducted by governmental regulatory agencies. As of January 2009, MON 89034 safety has been confirmed by the United States Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), the Department of Agriculture (USDA), Health Canada, Canadian Food Inspection Agency (CFIA), Japan Ministry of Health Labor and Welfare (MHLW), Japan Ministry of Agriculture, Forestry and Fisheries (MAFF), Food Standards of Australia New Zealand (FSANZ), Mexico Ministry of Health, Taiwan Department of Health, Colombia Ministry of Agriculture and Rural Development, and the European Food Safety Authority (EFSA).

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