SAFETY ASSESSMENT OF YIELDGARD ROOTWORM™ CORN

EXECUTIVE SUMMARY

Corn (*Zea mays* L.) is the world's third leading cereal crop, following wheat and rice. It is grown commercially in over 25 countries. In 2002, worldwide production of corn was approximately 594 million metric tons. In the United States (U.S.) its production covered 32 million hectares that yielded 229 million metric tons and had a net value of US$21.2 billion.

Corn yields are negatively impacted by a number of insect pests. One of the most pernicious in the U.S. Corn Belt is the corn rootworm (CRW). CRW larvae damage corn by feeding on the roots, reducing the ability of the plant to absorb water and nutrients from soil, and causing harvesting difficulties due to plant lodging. CRW is the most significant insect pest problem for corn production in the U.S. Corn Belt from the standpoint of chemical insecticide usage. Over 14 million acres of corn in the U.S. were treated with organophosphate, carbamate and pyrethroid insecticides to control CRW in 2000. CRW has been described as the billion dollar pest complex (Metcalf, 1986), based on costs associated with the application of soil insecticides and crop losses due to pest damage.

Monsanto Company has developed, through the use of recombinant DNA techniques, corn plants that are protected from damage due to CRW feeding. The tissues of these plants produce a *Bacillus thuringiensis* Cry3Bb1 protein that is selectively toxic to CRW species. A DNA vector containing the *cry3Bb1* gene was introduced into embryonic corn cells by microprojectile bombardment. Transformation event MON 863 was selected for development as *YieldGard Rootworm™ Corn*. Corn varieties containing transformation event MON 863 are afforded a level of protection from CRW feeding damage that is comparable or superior to that offered by currently available conventional insecticides.

Bacillus thuringiensis (B.t.) Cry proteins have a long history of safe and widespread use in agriculture. The Cry3Bb1 protein produced in *YieldGard Rootworm Corn* binds to specific receptors in the midgut of sensitive insects, but exerts no toxicity in species that lack these receptors. Cry3Bb1 protein has been shown to be selectively toxic to specific coleopteran insects such as corn rootworms.

™ YieldGard Rootworm is a trademark of Monsanto Technology LLC.

The food and feed safety of *YieldGard Rootworm Corn* has been established through the long history of safe use for *B.t.* Cry proteins, including members of the Cry3 class, compositional analyses of corn varieties containing event MON 863, Cry3Bb1 rodent toxicity bioassays, and biochemical fate analyses. The results of biochemical analyses of the grain and forage demonstrate the compositional equivalence of *YieldGard Rootworm Corn* to conventional corn varieties. Acute oral administration of Cry3Bb1 protein to laboratory mice was without effect at the highest dose attainable. Subchronic dietary administration of grain containing event MON 863 failed to produce any evidence of adverse health effects in rats. *In vitro* digestive fate studies with the Cry3Bb1 protein demonstrated that the protein is rapidly degraded to small pesticidally inactive fragments in a matter of seconds, the protein is not stable to heat, is not glycosylated, and it has no biologically relevant amino acid sequence similarity to known allergens and toxins. The Cry3Bb1 protein is present at low levels in grain and corn-based food and feed products. Using upper bound estimates of corn consumption for humans and livestock, the margin of safety for Cry3Bb1 in humans is $>5x10^4$ and in livestock is $>1x10^3$. Nutritional equivalence and comparative animal feeding performance of *YieldGard Rootworm* hybrids to conventional corn hybrids have been confirmed in feeding studies with broiler chickens, feedlot steers, dairy cattle and swine.

The environmental safety of *YieldGard Rootworm Corn* has been established through extensive laboratory and field testing of plant tissue or purified Cry3Bb1 protein with a wide range of nontarget species. No adverse effects have been observed in nontarget species exposed to maximum expected environmental concentrations of Cry3Bb1 protein. Furthermore, environmental fate studies demonstrate that Cry3Bb1 protein rapidly degrades in a variety of soil types. Agronomic, morphological and pest susceptibility observations have been recorded in multiple field trials conducted across major corn growing regions of the United States. Results from these trials confirm that *YieldGard Rootworm Corn* is phenotypically equivalent to conventional corn except for its tolerance to CRW and other coleopteran pests.

Collectively, the data summarized in this document support a conclusion that food and feed products derived from corn containing event MON 863 are as safe and nutritious for consumption as those derived from conventional corn varieties and that the use of this product poses no meaningful risk to the environment. In fact, use of this new technology in place of conventional insecticides will dramatically reduce overall environmental exposure. The introduction of *YieldGard Rootworm Corn* will offer U.S. farmers an environmentally sound and effective alternative to the use of chemical insecticides for control of the CRW pest.

Information and data contained within this document have been provided to regulatory authorities for review. Regulatory reviews continue as we update regulatory files and make submissions to additional countries globally.

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KEY TO ABBREVIATIONS

35S	Cauliflower mosaic virus (CaMV) promoter
~	Approximately
ADF	Acid detergent fiber
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists Society
AS1	Activating sequence-1
ATP	Adenosine triphosate
bp	Base pairs
B.t.	*Bacillus thuringiensis*
B.t.k.	*Bacillus thuringiensis* subspecies *kumamotoensis*
CaMV	Cauliflower mosaic virus
CFR	Code of Federal Regulations (U.S.)
C.I.	Confidence interval
CFU	Colony forming unit
CRW	Corn rootworm, *Diabrotica* sp.
Cry	Crystal protein, a diverse group of insecticidal proteins produced by *B.t.*
DDE	Daily dietary exposure
DNA	Deoxyribonucleic Acid
dw	Dry weight
E. coli	*Escherichia coli*
Eco RI	Restriction endonuclease that cuts DNA at specific locations
Eco RV	Restriction endonuclease that cuts DNA at specific locations
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency (U.S.)
FA	Fatty acid
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration (U.S.)
fw	Fresh weight
Hind III	Restriction endonuclease that cuts DNA at specific locations
kb	Kilobase pairs
kDa	Kilodaltons
LC_{50}	Median lethal concentration
Mlu I	Restriction endonuclease that cuts DNA at specific locations
MOS	Margin of safety
MON 863	Transgenic corn event that expresses the insecticidal protein Cry3Bb1
mRNA	Messenger RNA
MW	Molecular weight
NCGA	National Corn Growers Association
Nco I	Restriction endonuclease that cuts DNA at specific locations
NDF	Neutral detergent fiber
NOEC	No observable effect concentration

NOEL	No observable effect level
NOS 3’	Nopaline synthase 3’ transcription termination sequence
nptII	DNA sequence that encodes for the enzyme neomycin phosphotransferase type II
NPTII	Neomycin phosphotransferase type II enzyme
p	Probability
PCR	Polymerase chain reaction
ppm	Parts per million
ract1 intron	Intron from the rice actin gene
RNA	Ribonucleic acid
SD	Standard deviation
S.E.	Standard error of the mean
sp	Species
subsp.	Subspecies
tahsp17 3’	3’ nontranslated sequence of wheat heat shock protein 17.3
T-DNA	Transferred DNA
T.I.	Tolerance interval
TIU	Trypsin inhibitor units
U.S.	United States
U.S.C.	United States Code
USDA	United States Department of Agriculture
WHO	World Health Organization of the United Nations
wt CAB	5’ untranslated leader sequence of wheat chlorophyll a/b-binding protein
YGRW	*YieldGard Rootworm Corn*

Standard abbreviations (*e.g.*, units of measure) are used according to the format described in ‘Instructions to Authors’ in the Journal of Biological Chemistry.

I. Introduction

Corn (*Zea mays* L.), the world's third leading cereal crop, following wheat and rice, is grown commercially in over 25 countries. In 2002, worldwide production of corn was approximately 594 million metric tons (Corn Refiners Association, 2003). Corn is the largest U.S. crop in terms of acreage planted and net crop value. According to statistics compiled by the National Corn Growers Association (NCGA), the 2002 U.S. corn crop covered 79.1 million acres, had an overall yield of 9 billion bushels from an average yield of 130 bushels per harvested acre, and had a net value of US$21.2 billion (NCGA, 2003).

Corn yields are negatively impacted by a number of insect pests. One of the most pernicious insect pests in the U.S. Corn Belt is the CRW complex (Coleoptera, *Diabrotica* spp.), comprised primarily of the western corn rootworm and the northern corn rootworm. CRW larvae damage corn by feeding on the roots, which reduces the ability of the plant to absorb water and nutrients from the soil (Reidell, 1990), and causes harvesting difficulties due to plant lodging (Spike and Tollefson, 1991). CRW is the most significant insect pest problem for corn production in the U.S. Corn Belt from the standpoint of chemical insecticide use (Doane, 2001). Over 14 million acres of corn were treated with more than seven million pounds of organophosphate, carbamate and pyrethroid insecticides to control CRW in 2000. CRW has been described as the billion dollar pest complex, based on costs associated with the application of soil insecticides and crop losses due to CRW damage (Metcalf, 1986).

Monsanto Company has developed, through the use of recombinant DNA techniques, corn plants that are protected from damage due to CRW feeding. The tissues of these plants produce a *Bacillus thuringiensis* (subspecies *kumamotoensis*) Cry3Bb1 protein that is toxic to CRW species. A DNA vector containing the *cry3Bb1* gene was introduced into embryonic corn cells by microprojectile bombardment. Transformation event MON 863 was selected for development as *YieldGard Rootworm Corn*. Corn varieties containing transformation event MON 863 are afforded a level of protection from CRW feeding damage that is comparable or superior to that offered by currently available conventional insecticides.

Product characterization and protein toxicity studies have established the safety of *YieldGard Rootworm Corn* for human and animal consumption. Product characterization studies include: molecular characterization of the inserted DNA, estimation of Cry3Bb1 levels in key plant tissues, and compositional analysis of forage and grain. Safety studies for Cry3Bb1 include: acute oral toxicity in mice, digestive fate, protein characterization and amino acid sequence comparison to known toxins and allergens. The safety of the protein has been further confirmed by assessing the history of safe use and existing toxicology databases for structurally similar *B.t.* Cry3 proteins. Safety of *YieldGard Rootworm* grain has been demonstrated in subchronic feeding studies with rats and broiler chickens, and the nutritional equivalence of *YieldGard Rootworm* grain to conventional corn grain has been demonstrated in livestock feeding studies.

The concepts and approaches that Monsanto has employed in evaluating the safety and nutritional equivalence of *YieldGard Rootworm Corn* are derived from, and consistent with, the principles outlined by multiple international bodies such as the World Health Organization

(WHO, 1995) and the Food and Agriculture Organization (FAO, 1996).

II. Regulatory Evaluations

As part of the precommercial development process for *YieldGard Rootworm Corn*, Monsanto has taken the following steps to obtain appropriate government approvals:

- *YieldGard Rootworm Corn* falls within the scope of the U.S. Food and Drug Administration (FDA) policy statement concerning regulation of products derived from new plant varieties, including those produced through genetic engineering (FDA, 1992). Monsanto has voluntarily initiated and completed a consultation process with FDA (File BNF0075). This consultation process was concluded on December 31, 2001. The consultation process established that corn varieties containing event MON 863 are not materially different in composition, safety and other relevant parameters from corn currently on the market and that *YieldGard Rootworm Corn* does not raise issues that would require premarket review or approval by FDA.

- Prior to its deregulation by the U.S. Department of Agriculture (USDA), *YieldGard Rootworm Corn* was subject to regulations (7 CFR Part 340) administered by the Animal and Plant Health Inspection Service based on its authority under the Plant Protection Act. On May 15, 2001, Monsanto submitted to USDA a Petition for Determination of Nonregulated Status for the Regulated Article: Corn Rootworm Protected Corn Event MON 863. On October 8, 2002, the USDA granted Monanto's request for a determination of nonregulated status for corn event MON 863. A notice advising the public of USDA's determination that *YieldGard Rootworm Corn* and its progeny are no longer considered regulated articles under 7 CFR 340 was published in the *Federal Register* on October 23, 2002 (USDA, 2002).

- Substances that are pesticides as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. §136(u)) are subject to regulation by the U.S. Environmental Protection Agency (EPA). An application for registration of "*Bacillus thuringiensis* Cry3Bb1 Protein and the Genetic Material (Vector ZMIR13L) Necessary for its Production in Corn" was submitted to EPA on June 20, 2000. An application for an exemption from the requirement of a tolerance for *B.t.* Cry3Bb1 protein, pursuant to §408(d) of the Federal Food Drug and Cosmetic Act (21 U.S.C. §346a(d)), was also submitted to EPA (PP 7F4888). On May 11, 2001, EPA established an exemption from the requirement of a tolerance for Cry3Bb1 and the genetic material necessary for its production in all corn commodities (EPA, 2001). On February 24, 2003, EPA granted a FIFRA Section 3 registration for *YieldGard Rootworm Corn*. EPA previously established an exemption from the requirement of a tolerance for the NPTII protein and the genetic material necessary for its production in or on all agricultural commodities (40 CFR §180.1134).

- The Japanese Ministry of Health, Labor and Welfare and the Ministry of Agriculture, Forestries and Fisheries have cleared food and feed products containing event MON 863 for importation into Japan (February 2002), as well as environmental release (May 2001).

- The Canadian Food Inspection Agency and Health Canada have approved *YieldGard Rootworm Corn* for commercialization in Canada (March 2003).

- *YieldGard Rootworm* food and feed safety dossiers are currently under review by other regulatory agencies around the world (*e.g.*, EU, China, Australia, Russia, Taiwain, Mexico, South Korea, and the Philippines).

III. Description of the Transformation System

In 1991, Rupar *et al.* reported discovery of a novel *B.t.* subsp. *kumamotoensis* (*B.t.k.*) strain that produced a crystal protein with insecticidal activity against the southern corn rootworm (*Diabrotica undecimpunctata howardi*). Donovan *et al.* (1992) isolated and sequenced the gene encoding this crystal protein, which was designated as CryIIIB2. Following the adoption of standardized nomenclature for identifying *B.t.* crystal proteins, the protein isolated from this strain was renamed Cry3Bb1 (Crickmore *et al.*, 1998).

Recently developed molecular techniques have been directed to the design of genes that encode proteins with enhanced insecticidal activity. The wild type *cry3Bb1* gene (GenBank Accession No. M89794) from *B.t.k.* was modified to produce a protein with enhanced insecticidal activity against the coleopteran pest, CRW, and was codon optimized for expression in monocotyledonous plants (English *et al.,* 2000). The variant produced in *YieldGard Rootworm Corn* is virtually identical in structure to the Cry3Bb1 wild type protein with the exception of a small number of strategically placed amino acid substitutions that impact insecticidal activity. This Cry3Bb1 variant is approximately eight times more lethal to southern corn rootworm larvae than the wild type protein.

The Cry3Bb1 coding sequence is under the control of 5’ noncoding elements consisting of four repeats of activating sequence-1 (AS1) (Lam and Chua, 1990) and a single portion of the 35S promoter (Odell *et al*., 1985). The 35S promoter and AS1 are derived from cauliflower mosaic virus (CaMV). AS1 is a 21 base pair (bp) element identified from this promoter that has been associated with high levels of protein expression in roots (Lam *et al*., 1989). The promoter sequences are followed by a 5’ untranslated leader sequence from wheat chlorophyll a/b binding protein (wtCAB), which facilitates mRNA translation (Lamppa *et al*., 1985), and the first intron of the rice actin 1 sequence (ract1), which enhances DNA transcription (McElroy *et al*., 1990). All of these elements are located upstream of the *cry3Bb1* coding sequence. The *cry3Bb1* coding sequence is followed by a sequence from the 3’ nontranslated region of the gene encoding wheat heat shock protein 17.3 (tahsp 3’), which ends transcription and directs polyadenylation (McElwain and Spiker, 1989).

The *cry3Bb1* gene cassette was linked to a DNA cassette that encodes the selectable marker, neomycin phosphotransferase type II (NPTII). The selectable marker cassette contains *nptII* coding sequence under the control of a 35S CaMV promoter (Odell, *et al*., 1985). It is joined to the nopaline synthase 3’ nontranslated sequence (NOS 3’), from *Agrobacterium tumefaciens* T-DNA, which ends transcription and directs mRNA polyadenylation (Bevan *et al*., 1983).

NPTII functions as a dominant selectable marker in the initial laboratory stages of plant cell selection following transformation (Horsch *et al*., 1984; DeBlock *et al*., 1984). The NPTII enzyme encoded by the *nptII* cassette uses ATP to phosphorylate neomycin and related aminoglycoside antibiotics, thereby inactivating them. Cells that produce the NPTII enzyme selectively survive exposure to these aminoglycosides. The *nptII* coding sequence is derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982). The purpose of inserting *nptII* into corn cells along with the *cry3Bb1* cassette is to have an effective method for selecting cells that contain the inserted genes of interest and that can be used in bacterial selection during construction of the plasmid. In general, the frequency of cells that are transformed is low, ranging from $1x10^{-4}$ to $1x10^{-5}$ of cells treated (Fraley *et al.*, 1983). Therefore, the selectable marker, *nptII*, and the selection agent, paromomycin, are used to facilitate the screening process.

A purified DNA fragment containing the *cry3Bb1* and *nptII* gene cassettes was used in the transformation of embryonic corn cells to produce event MON 863. A diagrammatic representation of this DNA vector is displayed in Figure 1. This vector was introduced into corn tissue by a particle acceleration method described by Klein *et al.* (1987) and Gordon-Kamm *et al.* (1990). The introduced DNA contained nucleotide sequence encoding resistance to the antibiotic, paromomycin (*i.e.*, *nptII*). When grown in the presence of paromomycin, only genetically transformed cells continued to grow. Plants were regenerated from the tolerant callus tissue and assayed for the presence of Cry3Bb1 protein by enzyme-linked immunosorbent assay (ELISA).

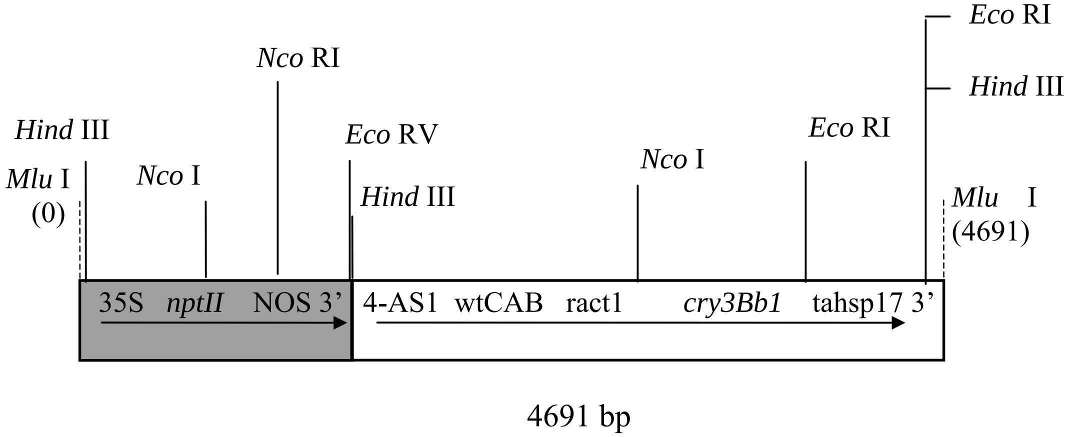

Figure 1. Linear map of DNA vector and restriction sites used in transformation of corn. This purified linear fragment was introduced into corn tissue by particle acceleration technology to produce transgenic event MON 863.

IV. Molecular Characterization of *YieldGard Rootworm Corn*

Molecular analysis was performed to characterize the DNA inserted into corn to produce event MON 863. Genomic DNA was analyzed for the number of insertion sites in the plant genome; the copy number of the inserted DNA; the nucleotide sequence of the inserted DNA; the integrity of the inserted promoters, coding regions, and terminators; and for the presence of any plasmid backbone sequence. DNA extracted from *YieldGard Rootworm* tissue was digested with a variety of restriction endonucleases and subjected to Southern blot hybridization analysis. Control genomic DNA was digested with the same restriction enzymes as used for *YieldGard Rootworm Corn*. Digested DNA was separated by means of agarose gel electrophoresis. Polymerase chain reactions (PCR) were performed to verify the 5' and 3' insert-to-plant junctions, as well as to determine whether the 5' and 3' ends of the insert were intact. Nontransgenic corn of comparable germplasm served as a control.

Southern blot analyses confirmed that *YieldGard Rootworm Corn* contains one DNA insert located on a 5.0 kb restriction fragment. This insert contains one copy of the DNA vector used in transformation. No additional elements from the DNA vector used in transformation, linked or unlinked to intact cassettes, were detected in the genome. All components of the inserted DNA cassette are intact. These results were confirmed by sequencing the inserted DNA. A summary of the molecular findings is presented in Table 1. PCR and DNA sequencing were used to verify the 5' and 3' junction sequences of the insert with the plant genome, as well as the intactness of the 5' and 3' ends of the insert. These data support the conclusion that only the two full-length proteins, Cry3Bb1 and NPTII, are encoded by the insert in *YieldGard Rootworm Corn*.

Table 1. Summary of molecular characterization findings for *YieldGard Rootworm Corn*.

Genetic Element	**Findings for MON 863**
# of transgene insertions	1
# of copies of *cry3Bb1* cassette	1
# of copies of *nptII* cassette	1
4-AS1 + wtCAB + ract 1	Intact
cry3Bb1 coding sequence	Intact
tahsp17 3' transcriptional terminator	Intact
35S promoter	Intact
nptII coding sequence	Intact
NOS 3' transcriptional terminator	Intact

Chi square analysis of Mendelian inheritance data over five generations was performed to evaluate the heritability and stability of the *cry3Bb1* gene in *YieldGard Rootworm Corn*. Plants were identified as being positive for the CRW-protected phenotype based on the presence of Cry3Bb1 protein as determined by ELISA. The results of this analysis are consistent with the finding of a single active site of insertion of the *cry3Bb1* gene that segregates according to Mendel's laws of genetics. The stability of the insert has been demonstrated through three generations of cross-fertilization and two generations of self-pollination. Southern blot fingerprint analyses of DNA extracted from plants spanning multiple generations were also conducted to evaluate the stability of the inserted DNA in *YieldGard Rootworm Corn*. These results demonstrate the stability of the inserted DNA in *YieldGard Rootworm Corn* across multiple generations.

V. Safety of the New Corn Variety

Corn, *Zea mays* L., originated in Mexico and was grown as a food crop as early as 2700 B.C. (Salvador, 1997). It is now grown on more than 296 million acres globally. The history of corn has been studied extensively and multiple hypotheses for its origin and parentage have been advanced (Mangelsdorf, 1974). The preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988).

A. CORN PRODUCTION AND USEAGE

Corn, also referred to as maize, has been a staple of the human diet for centuries. Corn grain and its processed fractions are consumed in a multitude of food and animal feed products. Corn forage is extensively consumed as feed by ruminants. Corn does not produce significant quantities of toxins, allergens or anti-nutritional factors warranting analytical or toxicological tests (Watson, 1982; White and Pollak, 1995). Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2002, its production covered 79.1 million acres that yielded 9 billion bushels and had a net value of US$21.2 billion (NCGA, 2003).

Hybrid corn is an extremely productive crop, yielding an average of 130 bushels per acre in the U.S. during 2002 (NCGA, 2003). Its high yield makes it one of the most economical sources of metabolizable energy for feeds, and of starch and sugar for food and industrial products. Approximately two-thirds of the corn produced in the U.S. is fed to livestock. Therefore, indirect consumption is much greater than direct consumption for humans.

In spite of its great value as a source of energy, little whole kernel corn is consumed by humans when compared to corn-based food ingredients (Hodge, 1982; Watson, 1988). The low price and ready availability of processed corn products has resulted in the development of large volume food and industrial uses. Corn is an excellent raw material for the manufacture of starch (Anderson and Watson, 1982). Nearly one quarter of corn starch produced is sold as starch products, whereas three quarters of the starch is converted to a variety of sweetner and fermentation products including high fructose corn syrup and ethanol (Watson, 1988; NCGA, 2003; Anderson and Watson, 1982; White and Pollack, 1995). Additionally, corn oil is commercially processed from the germ and accounts for approximately 9% of domestic vegetable oil production (Orthoefer and Sinram, 1987). Each of these materials is a component of many foods including bakery and dairy goods, beverages, confections and meat products.

Animal feeding represents the largest use of corn in the U.S. with approximately two-thirds of annual production being fed to cattle, chicken and swine (Hodge, 1982; Perry, 1988; Watson, 1988). Approximately 100 million metric tons of grain are fed annually to livestock directly as grain. Another 1.5 to 2 million metric tons of wet and dry milling by-products (primarily corn gluten meal and feed) are fed directly or in formulated feeds (Perry, 1988).

B. COMPOSITIONAL ANALYSIS OF *YIELDGARD ROOTWORM CORN*

Compositional analyses were performed on grain and forage collected from *YieldGard Rootworm Corn*, its nontransgenic parental control line, and 18 commercial corn hybrids grown under field conditions (George *et al.*, 2003). Field trials were conducted in the U.S. in 1999 at four replicated sites located in Monmouth, Illinois; Richland, Iowa; Van Horne, Iowa; and York, Nebraska. *YieldGard Rootworm Corn* and its parental control line were planted at all sites. Four commercial reference hybrids (nontransgenic) were planted at two sites and five different commercial reference hybrids were planted at the remaining two sites to give a total of 18 different reference hybrids.

Forage and grain were collected from all sites. Compositional analyses were conducted to measure proximates (protein, fat, ash, moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), amino acids, fatty acids, vitamin E, minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc), phytic acid and trypsin inhibitor content of grain; and to measure proximates, ADF and NDF content of forage. In addition, the content of carbohydrates in forage and grain was determined by calculation. In all, 51 different components (seven in forage and 44 in grain) were evaluated as part of the safety and nutritional assessment of *YieldGard Rootworm Corn*.

Statistical analyses of the compositional data were conducted using a randomized complete block model analysis of variance for five sets of comparisons: analyses of data from each of the four replicated trials and data from a combination of all four trials. As there were a total of 51 components evaluated, a total of 255 comparisons were made: 51 comparisons for each of the five statistical analyses. *YieldGard Rootworm Corn* results were compared to the parental control line to identify any statistically significant differences at $p<0.05$. In addition, the comparison of *YieldGard Rootworm Corn* to the 95% tolerance interval for the commercial reference varieties was conducted to determine whether the range of values for *YieldGard Rootworm Corn* fell within the population of commercial corn.

The results of compositional analyses demonstrated that all of the 51 components measured in *YieldGard Rootworm Corn* were within the range observed for commercial corn lines planted at the same U.S. sites in 1999. Futhermore, all 51 components were within published literature ranges (Jugenheimer, 1976; Watson, 1982; Watson, 1987) or historical ranges for nontransgenic corn varieties (Sidhu *et al.*, 2000). Tables A-1 through A-5 in Appendix A provide a summary of grain compositional results for all four sites combined. Table A-6 in Appendix A provides a summary of forage compositional results for all four sites combined.

There were no statistically significant differences in 224 of the 255 comparisons made between *YieldGard Rootworm Corn* and the parental control line which included forage (fat, protein, ash, carbohydrate, ADF and NDF) and grain (ash, ADF, NDF, 12 of 18 amino acids, six of eight fatty acids, potassium, and trypsin inhibitor) components. Differences computed for the remaining 31 comparisons were found to be statistically significant. These differences between *YieldGard Rootworm Corn* and its parental control, expressed as a percent of the control values, ranged from 1.38%-15.52%. Five percent or approximately thirteen (0.05×255) of these significant differences were expected to be false positives based on chance alone. Differences that were observed for only one to three of these comparisons, and not consistently across all five comparisons, are unlikely to be of biological significance.

The range of values for those parameters associated with small statistically significant differences was found to all fall within the 95% tolerance interval for commercial varieties planted at the same U.S. sites in 1999. This demonstrates, with a confidence level of 95%, that the levels of key nutrients and other compositional components for MON 863 were within the same population as expected for nontransgenic commercial reference corn used in this study. Therefore, these minor differences are unlikely to be biologically meaningful, and the grain and forage from *YieldGard Rootworm Corn* are considered compositionally equivalent to that of conventional corn grain and forage.

In an analysis of variance for combined location data, the differences between *YieldGard Rootworm Corn* and concurrent control means were found to be statistically significant ($p<0.05$) for five of the 51 parameters measured. Table 2 displays the *YieldGard Rootworm Corn* and concurrent control mean values for these parameters, as well as a range of values for these parameters obtained from the scientific literature, Monsanto's historical control database, and 18 commercial corn hybrids that served as reference materials in the *YieldGard Rootworm Corn* study.

Table 2. A comparison of selected *YieldGard Rootworm* grain composition values to parental, commercial, literature and historical control values. This table includes only the five parameters for which the difference between *YieldGard Rootworm* and parental control means was found to be statistically significant at $p<0.05$ in a combined location analysis of variance.

Parameter	***YGRW***	**Parental Control**[a]	**Mean Δ**[b]	**Control Range**	**Comm. Hybrid Range**[c]	**Literature Range**[d]	**Historical Control Range**[e]
Arginine (% total AA)	4.43	4.33	0.10	4.09 – 4.63	3.86 – 4.83	2.9 – 5.9	3.5 – 5.0
Cystine (% total AA)	2.20	2.09	0.11	1.99 - 2.29	1.84 – 2.35	1.2 – 1.6	1.8 – 2.7
Leucine (% total AA)	13.36	13.65	-0.29	13.27 – 14.17	11.94 – 14.47	7.8 – 15.2	12.0 – 15.8
Phytic Acid (% dw)	1.11	1.23	-0.12	1.01 – 1.37	0.73 – 1.17	na	0.55 – 1.37
Vitamin E (mg/g dw)	0.011	0.013	-0.0015	0.0088 – 0.016	0.0041 – 0.014	0.017 – 0.047	0.008 – 0.015

a - nontransgenic control line which is of comparable germplasm to that of *YieldGard Rootworm Corn*

b - *YGRW* mean minus parental control mean

c - Range of values for grain harvested from 18 commercial nontransgenic hybrid corn lines that were planted as reference materials.

d - Range of values reported by Watson, 1982 (amino acids and Vitamin E); Watson, 1987 (minerals, moisture and protein); and Jugenheimer, 1976 (protein).

e - Range of values reported for concurrent control lines used in corn composition studies conducted by Monsanto.

For each of these five parameters, the difference between *YieldGard Rootworm Corn* and its concurrent control mean is very small. Each of the *YieldGard Rootworm* means fall within the range of concurrent control values. These means also fall within the range of recorded values for the 18 commercial reference hybrids and the range of historical control values measured in multiple compositional analysis studies conducted by Monsanto. Two of the values fall slightly outside of reported literature ranges, but many factors could account for this (*e.g.,* different analytical methodology, etc.). Collectively, these observations support a conclusion that the five combined site compositional differences represent normal biological and analytical variability. Furthermore, they are unlikely to be of any nutritional significance for humans or animals.

Extensive analyses have been performed to compare the composition of grain and forage from *YieldGard Rootworm Corn* to that of nontransgenic varieties. The results of these analyses demonstrate that grain and forage from *YieldGard Rootworm Corn* are substantially equivalent to nontransgenic corn and therefore, should be considered as safe as conventional corn varieties.

C. SAFETY OF THE CRY3BB1 AND NPTII PROTEINS

The safety assessment for Cry3Bb1 and NPTII proteins includes biochemical characterization of the proteins, their digestive fate, heat stability, acute oral toxicity, amino acid sequence comparison to known toxins and allergens, and bioassays with nontarget organisms, specifically for the Cry3Bb1 protein.

The *cry3Bb1* gene was isolated from *Bacillus thuringiensis* subspecies *kumamotoensis,* a spore-forming, gram-positive bacterium that is found naturally in soil. Many strains of *B.t.* have been shown to produce protein crystals, or parasporal inclusions, that are selectively toxic to certain orders and species of insects. *B.t.* toxin proteins are classified based on their specific insecticidal activity; for example, Cry1, Cry2, Cry3, and Cry4 proteins are toxic to lepidopteran, lepidopteran/dipteran, coleopteran, and dipteran pests, respectively (Bravo, 1997; Höfte and Whitely, 1989). *B.t.* strains have been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988).

An exemption from the requirement of a tolerance for the first microbial *B.t.* product was granted in 1960 by the FDA after an extensive toxicity and infectivity evaluation program. The testing program consisted of acute, subchronic, and chronic toxicity studies, which resembled the testing required for conventional chemical pesticides. Registration was granted by the USDA later that same year. In 1971, EPA assumed responsibility for pesticide tolerance exemptions for microbial *B.t.* products. Since then, a variety of naturally-occurring and genetically-modified microbial *B.t.* products have been registered and covered under these tolerance exemptions. EPA has established separate tolerance exemptions by amendment for individual Cry proteins (*e.g.*, Cry1Ab, Cry1Ac, Cry2Ab2, Cry3Bb1 and Cry3Aa) expressed in genetically modified food crops (EPA 1995a, 1995b, 1995c, 1996, 1997 and 2001). The conclusion of reasonable certainty of no harm and the resultant tolerance exemptions for this wide array of *B.t.* mixtures and Cry proteins in food or feed is based on the lack of adverse effects to mammals in numerous toxicological studies. This conclusion is supported by a history of safe use in agriculture for over 40 years (McClintock *et al.*, 1995). There are no adverse effects known to have occurred in humans during this prolonged period of use (EPA, 1998).

The *nptII* gene was isolated from prokaryotic transposon Tn5 present in *Escherichia coli.* The enzyme encoded by this gene (*i.e.,* NPTII) is a commonly used selectable marker in the development of genetically improved plants for agriculture. The bacterium *E. coli* is ubiquitous in the environment and found in the digestive tracts of vertebrate species, including humans

(Jefferson *et al.*, 1986). *E. coli* strains are used as protein production systems in many commercial applications (Bogosian and Kane, 1991). The safety of the donor organism, *E. coli*, has previously been assessed by FDA as part of the consultation process for other transformed crops that contain the same *nptII* gene (FDA, 1998).

1. Expression of the Inserted *cry3Bb1* and *nptII* Genes

Levels of Cry3Bb1 and NPTII proteins were evaluated in tissues collected from *YieldGard Rootworm Corn* plants grown under field conditions at multiple sites. Tissue samples from nontransgenic plants of comparable germplasm served as controls and were analyzed for the presence of both proteins.

Tissue samples were collected from plants grown in four U.S. field trials conducted in Iowa (two sites), Nebraska, and Illinois during the 1999 growing season. Three additional sites in Argentina were planted for harvesting of pollen during the winter of 2000. Collectively these sites provided a variety of environmental conditions representative of regions where corn rootworm protected corn lines would be grown as commercial products. MON 863 and parental controls were planted in four plots at each location.

Composite samples of young leaf (V4 stage), forage, mature root and grain were collected from each replicate at the four U.S. sites. At three of the U.S. sites, single plot composite samples of leaf, whole plant and root were collected throughout the growing season and analyzed. A composite sample of silk was analyzed from one U.S. site. Composite samples of pollen were analyzed from one U.S. site and from all three sites (12 plots) planted in Argentina. Cry3Bb1 protein levels were measured in all tissues. NPTII protein levels were measured only in samples of young leaf, forage and grain taken from all four U.S. sites.

Direct double antibody sandwich ELISA methods were developed and validated to quantify the levels of Cry3Bb1 and NPTII proteins in tissue extracts of *YieldGard Rootworm* and control corn. Values are expressed as micrograms (µg) of protein per gram (g) of tissue on a fresh weight (fw) basis. All values have been corrected for assay bias as determined during the method validation.

Table 3 presents a summary of Cry3Bb1 and NPTII protein levels found in *YieldGard Rootworm Corn* tissues collected from multiple field sites. Table 4 presents a summary of Cry3Bb1 protein levels in selected *YieldGard Rootworm* tissues sampled over the course of a growing season. As expected, Cry3Bb1 and NPTII levels were below the limit of detection in control plant tissues.

Mean levels of Cry3Bb1 protein in *YieldGard Rootworm Corn* plants were 81 µg/g in young leaf, 70 µg/g in grain, 41 µg/g in root, and 39 µg/g in forage tissues. Mean levels of Cry3Bb1 protein declined during the growing season in leaf tissue, whole plant and root tissue of *YieldGArd Rootworm Corn*. Mean levels in root tissue ranged from a high of 58 µg/g in young plants to a low of 24 µg/g in senescent plants. These levels were sufficient to confer protection against CRW root feeding damage during the critical early periods of root development. Mean Cry3Bb1 levels in pollen and silk were 62 and 10 µg/g, respectively. NPTII protein levels in all tissues tested ranged from nondetectable (<0.076 µg/g) to 1.4 µg/g.

Molecular analysis of DNA (*i.e.,* Southern blots) from *YieldGard Rootworm* leaf and grain tissues confirmed the preservation of event identity across all test sites. Molecular analysis also confirmed the expected absence of *cry3Bb1* and *nptII* coding sequences in the control plants.

Table 3. Summary of Cry3Bb1 and NPTII protein levels measured in *YieldGard Rootworm* tissue samples collected from multiple field sites.

Tissue (Days post-planting)	**Parameter***	**Cry3Bb1 (µg/g fw)**	**NPTII (µg/g fw)**
Young Leaf (21 days)	Mean ± SD Range (n)	81 ± 11 65 – 93 (4)	0.98 ± 0.27 0.74 – 1.4 (4)
Forage (90 days)	Mean ± SD Range (n)	39 ± 10 24 – 45 (4)	0.19 ± 0.03 0.17 – 0.23 (4)
Mature Root (90 days)	Mean ± SD Range (n)	41 ± 13 25 – 56 (4)	Not Analyzed
Grain (125 days)	Mean ± SD Range (n)	70 ± 17 49 – 86 (4)	<0.076[†] n/a (4)
Silk (58 days)	Mean ± SD (n)	10 n/a (1)	Not Analyzed
Pollen (60 days)	Mean ± SD Range (n)	62 ± 18 30 – 93 (13)	Not Analyzed

* SD = standard deviation of the mean; n = number of replicates analyzed; fw = fresh weight; n/a = not applicable

[†] Limit of detection for corn grain = 0.076 µg/g fw

Table 4. Summary of Cry3Bb1 protein levels measured in *YieldGard Rootworm* tissue samples collected over the 1999 growing season.

Days Post-planting	Parameter*	Cry3Bb1 in Leaf (µg/g fw)	Cry3Bb1 in Whole Plant (µg/g fw) †	Cry3Bb1 in Root (µg/g fw) †
21 days	Mean ± SD Range (n)	81 ± 14 65 – 93 (3)	NC	NC
35 days	Mean ± SD Range (n)	79 ± 6.4 72 – 84 (3)	46 ± 7.8 38 – 54 (3)	58 ± 10 46 – 66 (3)
49 days	Mean ± SD Range (n)	43 ± 18 30 – 56 (2)	31 ± 3.3 28 – 33 (2)	57 ± 3.8 54 – 59 (2)
90 days	Mean ± SD Range (n)	NC	37 ± 12 24 – 45 (3)	37 ± 11 25 – 47 (3)
126 days	Mean ± SD Range (n)	NC	25 ± 11 13 – 35 (3)	24 ± 18 3.2 – 36 (3)

* SD = standard deviation of the mean; n = number of replicates analyzed
† NC = not collected; fw = fresh weight

2. Human and Animal Safety of Cry3Bb1 Protein

Numerous factors have been considered in the safety assessment of the Cry3Bb1 protein that is expressed in *YieldGard Rootworm Corn*: 1) the biological mode of action for *B.t.* Cry proteins; 2) the results of extensive animal toxicology tests conducted with *B.t.* Cry proteins; 3) the results of toxicology studies conducted with Cry3Bb1 protein; 4) amino acid sequence comparisons of Cry3Bb1 to known toxins and allergens; and 5) the margin of exposure for consumption of Cry3Bb1 protein in food and feed derived from *YieldGard Rootworm Corn*.

A generalized mode of action for Cry proteins has been described by English and Slatin (1992). It includes ingestion of the crystals by insects, solubilization of the crystals in the insect midgut and proteolytic processing of the released Cry protein by digestive enzymes, sometimes with partial digestion ‘activating’ the toxin. The activated protein diffuses through the peritrophic membrane of the insect to the midgut epithelium. There it binds to specific high-affinity receptors on the surface of the midgut epithelium of target insects (Hoffman *et al.*, 1988a, Hoffman *et al.*, 1988b; Van Rie *et al.*, 1989; Van Rie *et al.*, 1990; Wolfersberger *et al.*, 1986).

Pores are formed in the membrane, leading to leakage of intracellular contents (*e.g.,* K^+) into the gut lumen and water into the epithelial gut cells (Sacchi *et al.*, 1986). The larval gut epithelial cells swell due to osmotic pressure and lyse. The gut becomes paralyzed as a consequence of changes in electrolytes and pH, causing the larval insect to quit eating and die.

Receptor binding is a critical step in the mechanism of action for Cry proteins. Irreversible binding of these proteins to midgut receptors appears to be correlated with insect susceptibility to the toxin (Schnepf *et al.*, 1998). This observation is relevant to assessing the safety of Cry proteins for humans since no receptors for these proteins have been identified on intestinal cells of mammals (Noteborn, 1994; Sacchi *et al.*, 1986; Van Mellaert *et al.*, 1988). This would explain, in part, the absence of any reported adverse effects for *B.t.* products in humans.

B.t. has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). The extremely low mammalian toxicity of *B.t.*-based insecticide products has been demonstrated in numerous safety studies (McClintock *et al.*, 1995).

Data requirements for *B.t.* proteins produced in genetically modified crops include acute oral toxicity and *in vitro* digestibility studies. These requirements are based on the fact that oral ingestion is the predominant route of exposure for humans to Cry proteins in genetically improved crops. Furthermore, when proteins are toxic they are known to act *via* acute mechanisms and generally at very low dose levels (Sjoblad *et al.*, 1992). The results of rodent acute oral toxicity tests conducted with Cry3 proteins, including the Cry3Bb1 variant produced in *YieldGard Rootworm Corn*, are summarized in Table 5. In each rodent bioassay there was no evidence of treatment related adverse effects even at the highest achievable dose level which was, therefore, considered to be a no observable effect level (NOEL).

Table 5. Acute oral NOELs for various *B.t.* and plant-produced Cry3 proteins.

Cry3 Protein	**NOEL (mg/kg)** [a]	**Reference**
Cry3Bb1 – *YGRW*	≥ 3200	Monsanto data
Cry3Bb1 – Wild type present in *Raven® Oil Flowable Bioinsecticide* [b]	≥ 30	Baum *et al.*, 1996
Cry3Aa4 - *NewLeaf® Potato*	≥ 5000	Lavrik *et al.*, 1995

a - NOEL in rodent acute gavage study. In all instances, the highest dose tested was the NOEL.

b - *Raven* contains a mixture of Cry3Bb1, Cry3Aa4 and CrylAc proteins. In the batch tested, Cry3 proteins constituted 40% (w/w) active ingredient; Cry3Bbl protein represents 66-75% of the Cry3 proteins present in *Raven*. The highest dose tested was 10^8 CFU/rat, which is approximately a 100 mg/kg body weight dose of total active ingredients.

Cry3Bb1 protein extracted and purified from a heterologous *E. coli* fermentation system was used as the test material for an acute oral toxicity study with mice. The identical *cry3Bb1* coding sequence used in the transformation of event MON 863 was used in the transformation of *E. coli*.

® NewLeaf is a registered trademark of Monsanto Technology LLC; Raven is a registered trademark of Ecogen Inc.

The equivalence of the plant and *E. coli*-produced proteins was assessed by a comparison of results obtained from multiple analytical methods, including matrix assisted laser desorption ionization-time of flight mass spectrometry, N-terminal sequencing, immunoblotting, insect bioassay, gel electrophoresis, glycosylation analysis, and amino acid compositional analyses. The Cry3Bb1 protein purified from this fermentation system was found to be physicochemically and functionally equivalent to the protein produced in *YieldGard Rootworm Corn*. In the acute oral toxicity study performed with laboratory mice, no mortality or grossly observable adverse effects were noted at any dose level tested. The NOEL was determined to be ≥3,200 mg/kg, which was the highest achievable dose.

On May 11, 2001, an exemption from the requirement for a tolerance was established by EPA for Cry3Bb1 protein and the genetic material necessary for its production in corn (EPA, 2001). As part of the decision to grant this tolerance exemption EPA stated: “The lack of mammalian toxicity at high levels of exposure to the Cry3Bb1….proteins demonstrate the safety of the product at levels well above maximum exposure levels anticipated in the crop”. The EPA further stated: “There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry3Bb1….proteins and the genetic material necessary for their production. This includes all anticipated dietary exposures and other exposures for which there is reliable information.”

Proteins of many sizes and function comprise a significant portion of the human diet. Only rarely do any of these tens of thousands of proteins elicit an allergic response when ingested (Taylor, 1992). Although there are currently no predictive bioassays available with which to assess the allergenic potential of proteins consumed by humans, a comparison of Cry3Bb1 structure to that of known allergens, toxins and pharmacologically active proteins can provide a basis for predicting whether ingestion of *YieldGard Rootworm Corn* would elicit an adverse response in humans. Protein sequence databases were assembled for this purpose and included allergen and gliadin, toxin, and the public domain sequence databases. A sequence alignment tool was used to assess structural similarity between Cry3Bb1 and proteins in these databases. Proteins that share a high degree of similarity throughout the entire length are often homologous. Proteins homologous to allergens are more likely to share cross-reactive allergenic epitopes than are unrelated proteins.

Structural similarities between the Cry3Bb1 protein sequence and the aligned database sequences were examined. In these analyses, sequences of eight or more linearly contiguous and identical amino acids were defined as immunologically relevant (Metcalfe *et al.*, 1996; Hileman *et al.*, 2002). The presence of such identities may point to the presence of potentially cross-reactive allergenic epitopes. No biologically relevant structural similarities were observed between any known allergen or toxin and the Cry3Bb1 protein produced in corn event MON 863. Further, no immunologically relevant sequence similarities were observed between the Cry3Bb1 protein and proteins in the allergen and gliadin database. These data demonstrate the absence of both structurally and immunologically relevant similarities between allergens and the Cry3Bb1 protein produced in *YieldGard Rootworm Corn*.

Apart from expected similarities to other known crystal proteins found in *B.t.* and related species, no additional significant structural similarities were observed. The results of these bioinformatics analyses indicate that the Cry3Bb1 protein produced by *YieldGard Rootworm Corn* is not similar to known allergens, toxins or other pharmacologically active proteins relevant to animal or human health.

Current scientific knowledge suggests that food allergens are abundant, resistant to pepsin digestion, may be resistant to acid or heat, and can be glycosylated (Metcalfe *et al.*, 1996; Astwood *et al.*, 1996). The results of an *in vitro* digestive fate study demonstrate that Cry3Bb1 protein degrades to nondetectable levels in simulated gastric fluid within 15 seconds. In simulated intestinal fluid, Cry3Bb1 is observed to degrade within one minute from a size of approximately 74 kDa to smaller fragments with approximate molecular weights of 68 and 57 kDa. Continued exposure to simulated intestinal fluid resulted in the formation of a single stable fragment with an approximate molecular weight of 57 kDa, which is the expected size of the trypitic core for Cry3 proteins. Neither the Cry3Bb1 protein produced by *E. coli* nor *YieldGard Rootworm Corn* is glycosylated. Cry3Bb1 is not detectable in MON 863 grain following baking at 204°C for 30 minutes.

Collectively, these data demonstrate that ingestion of Cry3Bb1-containing corn is unlikely to produce an allergic or toxic response in humans. There are also no confirmed cases of allergic reactions to Cry proteins in applicators of microbial-derived *B.t.* products during 40 years of use (McClintock *et al.*, 1995).

High-dose acute exposure studies are considered appropriate for assessing the potential toxicity of Cry proteins to mammals. EPA scientists have stated that: "if toxic - proteins are known to act through acute mechanisms. Also, laboratory animals show acute toxic effects from exposure to proteins known to be toxic to humans" (Sjoblad *et al.*, 1992). The potential for human exposure to Cry3Bb1 will occur through consumption of corn or corn products containing the protein. Exposure *via* the dermal or inhalation route is unlikely since the protein is contained within the plant tissue. Thus, there appears to be little opportunity for occupational exposure to the protein. The protein has been found to be short-lived in soil, thus runoff to drinking water sources will be negligible. A margin of safety (MOS) for Cry3Bb1 protein has been computed based on estimates of corn dietary intake. The MOS is defined as the ratio of an appropriate NOEL to an estimate of human daily dietary exposure (DDE). An upper bound estimate (90th percentile) of human daily corn consumption has been obtained from the Dietary Exposure Estimation Model[1]. The estimate of daily human corn consumption is 0.78 g corn per kg body weight and the mean level of Cry3Bb1 found in samples of corn grain is 0.070 mg protein/g of grain.

The MOS is computed as follows:

Daily corn consumption (g/kg) x Cry3Bb1 grain concentration (mg/g) = DDE (mg/kg)
NOEL (mg/kg) ÷ DDE (mg/kg) = **MOS**

Computation of the MOS for Cry3Bb1 in *YieldGard Rootworm* grain is as follows:

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[1] DEEM Version 7.76, Novigen, Inc.

$$0.78 \text{ g/kg} \times 0.070 \text{ mg/g} = 0.055 \text{ mg/kg}$$
$$3{,}200 \text{ mg/kg} \div 0.055 \text{ mg/kg} = \mathbf{5.8 \times 10^4}$$

A MOS of ≥100 is generally regarded as being protective of human health (Johannsen, 1990). There is a four order of magnitude difference between the acute oral NOEL and the upper bound estimate of human dietary exposure to Cry3Bb1 in *YieldGard Rootworm Corn*. The MOS for livestock consuming *YieldGard* Rootworm grain and forage is calculated to be $>1\text{x}10^{\mathbf{3}}$. These large margins of safety ensure a reasonable certainty of no harm for humans and other mammals exposed to the product.

3. Human and Animal Safety of NPTII Protein

The *nptII* coding sequence present in *YieldGard Rootworm Corn* was isolated from prokaryotic transposon Tn5 present in the enterobacteria, *E. coli* (Beck *et al.*, 1982). This bacterium is ubiquitous in nature and present in the digestive tracts of vertebrate species, including humans (Jefferson *et al.*, 1986). Safety questions associated with use of *nptII* and the NPTII protein have previously been examined by the U.S. FDA in their ruling that authorized use of this gene product as a processing aid food additive for the development of transgenic tomatos, cotton and oilseed rape (FDA, 1994). This ruling was reviewed by a panel of scientific experts who concluded that the approach taken by FDA in evaluating the safety of *nptII* and the protein it expresses was scientifically sound and included all relevant parameters (FDA, 1998). The safety of NPTII has been addressed in multiple publications (Fuchs *et al.,* 1993a & 1993b; Flavel *et al.,* 1992; and Nap *et al.,* 1992). An acute oral NOEL ≥5,000 mg/kg has been established for NPTII protein in mice. The computed MOS for potential NPTII human exposure through consumption of *YieldGard Rootworm* grain exceeds $8\text{x}10^7$.

In addition, EPA has established an exemption from the requirement of a tolerance for NPTII and the genetic material necessary for its expression in or on raw agricultural commodities (40 CFR §180.1134). Collectively, these regulatory actions confirm the safety of the NPTII protein.

A variety of global scientific committees and commissions have issued statements confirming the extremely low risk of using NPTII as a selectable marker in genetically improved crops (*e.g.*, OECD, FAO/WHO, EU Scientific Committee on Plants, *etc*.). A recently issued preliminary report by the European Network on Safety Assessment of Genetically Modified Food Crops stated that: “Marker genes coding for neomycin phoshotransferase (*nptII*) or hygromycin phosphotransferase (*hpt*) can be used without the risk of compromising human or animal health” (ENTRANSFOOD, 2003).

4. Horizontal Gene Transfer

Horizontal gene transfer is defined as the transfer of DNA from one species to another. With respect to crop plants that are developed through biotechnology, a number of assessments have been performed to evaluate the possibility that antibiotic resistance marker genes used to facilitate the selection of the transformed plants, such as *nptII*, might be transferred to bacteria either in the field or in animals that have consumed the crop.

Factors affecting possible horizontal gene transfer between genetically modified plants expressing antibiotic resistance marker genes and microorganisms in the environment have been extensively studied (Prins and Zadoks, 1994; Schlüter *et al.*, 1995; Nielsen *et al.*, 1998; Smalla *et. al.*, 2000). To date, there is no experimental evidence that any antibiotic resistance marker gene from a plant has transformed bacteria either under laboratory conditions or in the field (Broer *et al.*, 1996; Schlüter *et al.*, 1995; Nielsen *et al.*, 1997). Most bacteria present in the environment are not competent to accept plant DNA. Even under laboratory conditions, studies specifically designed to detect the transfer of functional marker genes from plants into bacteria have failed to demonstrate such an occurrence. USDA has previously stated in an interpretive ruling for a crop containing *nptII* that: "There is no published evidence for the existence of any mechanism, other than sexual crossing by which genes can be transferred from plants to other organisms" (USDA, 1992).

Several studies have been conducted to assess the potential for horizontal transfer of antibiotic selectable marker genes from transgenic plants to microflora in the gut of humans, ruminants or other animals. The results of these studies indicate that there is virtually no possibility for such a transfer to take place (Prins and Zadoks, 1994; Schlüter *et al.*, 1995; Nielsen *et al.*, 1998; Beever and Kempe, 2000).

The aminoglycoside antibiotics that are inactivated by NPTII, kanamycin and neomycin, are only used to a limited extent in medicine, having been replaced by newer and more effective drugs. Resistance to kanamycin and neomycin is already widespread amongst pathogenic and environmental bacteria. The conferring of aminoglycoside resistance from corn to bacteria *via nptII* transfer, no matter how unlikely, has no practical implications for human and animal health. As such, any risk associated with having an antibiotic resistant marker in *YieldGard Rootworm Corn* is considered insignificant.

5. Nutritional Assessment of *YieldGard Rootworm* Grain

Establishing the nutritional and toxicological equivalence of *YieldGard Rootworm Corn* to conventional corn is a key part of this safety assessment. Being able to establish the nutritional equivalence of *YieldGard Rootworm* corn varieties to conventional varieties is important for corn growers since the majority of harvested corn in the U.S. is consumed as animal feed. Feeding studies incorporating *YieldGard Rootworm* grain have been conducted in multiple species, including rats, broiler chickens, dairy cattle, finishing steers and swine.

Young male and female rats were fed *YieldGard Rootworm* grain at up to 33% of their diet for 13 weeks. When compared to rats fed a nontransgenic parental line of corn, or six reference commercial lines, there were no MON 863 related impacts on animal health, growth, survival, blood chemistry or postmortem findings. The highest dose tested was established as a subchronic NOEL.

Broiler chickens are highly sensitive to small nutrient changes in their diets because of their rapid growth (Cockburn, 2002). Feeding on diets composed of 55-60% *YieldGard Rootworm Corn* grain, nontransgenic parental line corn, or six nontransgenic reference varieties for six weeks revealed no biologically significant differences between treatments in bird growth or carcass quality parameters (Taylor *et al.*, 2003). Feeding of *YieldGard Rootworm* grain at 27%

of the total diet was without adverse effect on milk production and composition, or feed intake in lactating dairy cattle when compared to feeding on nontransgenic parental line corn or two nontransgenic commercial reference lines (Grant *et al.*, 2003). There were no differences in growth performance and carcass measurements in feedlot cattle fed diets containing either *YieldGard Rootworm*, nontransgenic parental line corn or two nontransgenic commercial reference lines (Vander Pol *et al*., 2003). There were no differences in growth performance and carcass measurements in growing pigs fed diets containing either *YieldGard Rootworm*, nontransgenic parental line corn or two nontransgenic commercial reference lines.

6. Environmental Safety

A substantial database of information has been developed which establishes that the environmental risk posed by *YieldGard Rootworm Corn* is no greater than the environmental risk posed by conventional corn. Extensive phenotypic measurements and pest susceptibility observations confirm that the genetically improved corn is physiologically and agronomically equivalent to nontransgenic corn except for its tolerance to CRW larval feeding damage. Minimal risk to nontarget organisms has been established through a combination of laboratory and field studies with purified Cry3Bb1 protein or tissue samples from corn hybrids containing event MON 863. No adverse effects have been observed in a wide range of nontarget species exposed at levels exceeding the maximum expected environmental concentrations of Cry3Bb1 protein. Furthermore, environmental fate studies demonstrate that the protein rapidly degrades in a variety of soil types.

Replicated field trials at multiple locations have been conducted to compare the phenotypic characteristics of *YieldGard Rootworm* hybrids to conventional corn hyrbrids. The parameters evaluated and the timing of these evaluations were: seedling vigor (growth stage V2-V3); early stand count (V4-V6); growing degree units to 50% pollen shed; growing degree units to 50% silk emergence; ear height at maturity; plant height at maturity; final stand count at harvest; test weight at harvest; grain moisture at harvest; and yield at harvest. Data collected for each of these parameters from all locations were statistically analyzed. With only a small number of exceptions, there were no statistically significant differences between transgenic and nontransgenic hybrids observed for these parameters. The few of statistically significant differences that were observed were uniformly small, not consistently observed across the six hybrids tested, and none of the differences are considered to be of adverse agronomic consequence.

In addition to the agronomic equivalency assessment, qualitative visual observations for pest and disease susceptibility were made during *YieldGard* Rootworm field trials. Insects observed in one or more of these field trials included: European corn borer adults (*Ostrinia nubilalis*), thrips (*Anaphothrips* and *Frankliniella* sp.), corn earworm (*Heliothis zea*), corn rootworm beetles (*Diabrotica* sp.), beet armyworm (*Spodoptera* sp.), fall armyworm (*Spodoptera frugiperda*), Chinese Rose beetle (*Adoretus sinicus)*, leaf hoppers, ladybird beetle (*Hippodamia convergens*), picnic beetle, also referred to as 4-spotted sap beetle (*Glischrochilus quadrisignatus*) and aphids (*Rhopalosiphum maidis*). Diseases observed in one or more of these trials included: maize mosaic virus, *Psuedomonas* sp., southern corn leaf blight (*Cochliobolus heterostrophus)*, northern corn leaf blight (*Setophaeria turcica*), maize dwarf mosaic virus, rust (caused by

Puccinia sorghi), gray leaf spot disease (*Cercospora zeae-maydis*), and southern rust (caused by *Puccinia polysora*). No discernible differences in the level of insect infestation or disease severity were observed between *YieldGard Rootworm* and nontransgenic control plants.

Potential adverse effects on nontarget organisms resulting from exposure to Cry3Bb1 have been evaluated in a series of studies with representative avian, aquatic and terrestrial beneficial invertebrate species. These nontarget organisms were exposed to high doses of leaf tissue, grain or pollen containing event MON 863 or to an artificial diet containing laboratory-produced Cry3Bb1 protein. The results of these laboratory bioassays are summarized in Table 6. In each case a no observable effect concentration (NOEC) for Cry3Bb1 was established.

No adverse effects were observed at levels that exceeded the maximum expected environmental concentration (MEEC) of Cyr3Bb1 to which these nontarget beneficial organisms would be exposed. The MEEC for organisms feeding on corn plants is predicted to be 93 µg/g based on the highest level of Cry3Bb1 found in leaf and pollen tissue of *YieldGard Rootworm Corn* (see Table 3). Ladybird beetles can consume up to 50% of their diet as corn pollen (Hoffman and Frodsham, 1993), therefore, the MEEC for ladybird beetles exposed to pollen was set at ≤50% of their diet. For monarch butterflies, the MEEC for pollen deposition was set at 300 grains/cm^2, which is the upper 95th percentile rate for pollen deposition on milkweed leaves within a corn field (Pleasants *et al.*, 2001). The MEEC for earthworms is predicted to be 8.33 mg/kg based on an assumption that corn plants are tilled into the top six inches of soil at the time of maximum Cry3Bb1 concentration in plants (*i.e.*, 93 µg/g). The soil MEEC derived for earthworms could be used for assessing risk to Collembola, however, Collembola are less likely to consume large amounts of soil than are earthworms. Therefore, the MEEC for Collembola was set at the maximum plant expression level of 93 µg/g tissue fresh weight. The MEEC for *Daphnia magna* organisms is predicted to be 0.016 µg/l based on the following assumptions: the pollen concentration of Cry3Bb1 is 93 µg/g, the edge of field deposition rate for pollen is 0.02-0.03 mg/cm^2, and that the pollen drifts into a body of water 2 m deep.

These studies demonstrate that Cry3Bb1 proteins pose no significant risk for harm to nontarget organism populations. In all studies conducted, a NOEC was established and found to exceed predicted maximum environmental concentrations. Where possible, the NOEC for each test organism was compared directly to the MEEC and found to exceed it by nine to 141-fold, clearly demonstrating an adequate margin of safety for these organisms. Where it was not possible to make this direct comparison, for example in the pollen and grain feeding bioassays, it is reasonable to assume that the absence of adverse effects following exposure to a diet comprised largely of pollen or grain is indicative of no significant risk. The results of a two-year field monitoring study corroborate these laboratory findings. The abundance of prominent beneficial nontarget invertebrate species was found to be comparable in conventional and *YieldGard Rootworm Corn* fields, and in some cases their abundance was higher than in fields managed with conventional synthetic insecticides.

Table 6. Summary of results from ecological effects tests with Cry3Bb1 protein. Risk conclusions are based on protein concentrations measured in plant tissues of *YieldGard Rootworm Corn*.

Test Organism	Test Substance	Results [a]	Conclusions [b]
Adult Honey Bee (*Apis mellifera*)	Purified protein	NOEC ≥ 2,590 µg/ml	NOEC ≥ 28x maximum pollen level
Larval Honey Bee (*Apis mellifera*)	Purified protein	NOEC ≥ 2,550 µg/ml as a single dose	NOEC ≥ 27x maximum pollen level
Adult Ladybird Beetle (*Hippodamia convergens*)	Purified protein	NOEC ≥ 8,000 µg/g	NOEC ≥ 86x maximum pollen level
Adult Ladybird Beetle (*Hippodamia convergens*)	Pollen	50% of diet	No significant risk (MEEC ≤ 50% pollen in lady bird beetle diet)
Larval Ladybird Beetle (*Coleomegilla maculata*)	Pollen	50% of diet	No significant risk (MEEC ≤ 50% pollen in lady bird beetle diet)
Adult Ladybird Beetle (*Coleomegilla maculata*)	Pollen	50% of diet	No significant risk (MEEC ≤ 50% pollen in lady bird beetle diet)
Green Lacewing Larvae (*Chrysoperla carnea*)	Purified protein	NOEC ≥ 8,000 µg/g	NOEC ≥ 86x maximum pollen level
Parasitic Hymenoptera (*Nasonia vitripennis*)	Purified protein	NOEC ≥ 1860 µg/ml	NOEC ≥ 20x maximum pollen level
Monarch Butterfly Larvae (*Danus plexippus*)	Pollen	NOEC > 3,200 grain/cm^2	NOEC > 11x MEEC
Collembola (*Folsomia candida*)	Leaf	NOEC ≥ 872.5 µg/g	NOEC ≥ 9.4x MEEC
Earthworm (*Eisenia fetifa*)	Purified protein	NOEC = 167 mg/kg	NOEC ≥ 20x MEEC
Bobwhite Quail (*Colinus virginianus*)	Grain	10% of diet	No significant risk at levels exposed
Cladoceran (*Daphnia magna*)	Pollen	NOEC ≥ 2.26 µg/l [c]	NOEC ≥ 141x surface water MEEC [c]
Channel Catfish (*Ictalurus punctatus*)	Grain	No effect on growth or survival at 35% of diet	No significant risk at level exposed

a: NOEC – no observable effect concentration; b: MEEC - maximum expected environmental concentration; c: assumes Cry3Bb1 in pollen is bioavailable in an aqueous environment

Since Cry3Bb1 protein is essentially nontoxic to noninsect species, it poses no risk to endangered mammals, birds, noninsect aquatic organisms and noninsect soil dwelling organisms. Cry3Bb1 has shown a high degree of specificity among insects it affects. Only insects in the order Coleoptera (beetles) have been found to be sensitive to Cry3Bb1 protein and this sensitivity has thus far been limited to beetles of the family, Chrysomelidae. There are no endangered beetles in the Chrysomelidae family; therefore, no adverse effects on endangered beetles are expected.

The results of an aerobic soil degradation study demonstrate that Cry3Bb1 dissipates very quickly in the environment. Analysis of soil Cry3Bb1 concentration by insect bioassay and ELISA methods established rates for 50% degradation of 2.37 and 2.76 days, respectively, and rates for 90% degradation of 7.87 and 9.16 days, respectively. The rapid dissipation of Cry3Bb1 ensures exposure risk for soil dwelling organisms will be minimal.

The results of agronomic equivalency, environmental fate, field monitoring and nontarget organism toxicity studies support a conclusion that Cry3Bb1 protein present in *YieldGard Rootworm Corn* poses no significant risk to the environment.

D. SAFETY ASSESSMENT CONCLUSIONS FOR *YIELDGARD ROOTWORM CORN*

Corn hybrids containing the *YieldGard Rootworm* trait are comparable in composition, safety and agronomic characteristics to conventional lines of corn. *YieldGard Rootworm Corn* differs from conventional corn varieties only in its resistance to corn rootworm larval feeding damage.

VI. Benefits of *YieldGard Rootworm Corn*

There are many benefits associated with the commercialization of corn varieties containing event MON 863. Recipients of these benefits will be growers, consumers and the environment. Monsanto will be offering this trait in combination with a low rate of a seed-applied insecticide. Cry3Bb1 protects the root structure from corn rootworm larval feeding and the seed treatment provides control of other soil pests that are of secondary economic importance in corn crops (*e.g.,* wireworms, white grubs, and seed corn maggot). This product combination provides growers with a complete 'in the bag' solution for management of soil insect pests; the product has a technical fit for all corn rootworm infested acres. Varieties containing the *YieldGard Rootworm* trait may also be stacked through conventional breeding with other genetically enhanced corn varieties, such as those with herbicide tolerance or other insect protection.

Corn hybrids containing the *YieldGard Rootworm* trait are more efficacious than soil-applied insecticides in protecting roots from larval feeding damage. The Cry3Bb1 toxin is root-incorporated, it does not require activation (as many conventional insecticides do), and its performance is unlikely to be impacted by severe environmental conditions. Superior performance and consistency of control are expected to result in a significant yield advantage for growers planting *YieldGard Rootworm* hybrids. Preliminary estimates place this yield advantage at up to 4.5%/ac. For a reasonable range of prices and yields, the value of this yield benefit to growers is \$4-\$12/ac relative to the use of a soil-applied insecticide, depending on corn rootworm pressure (Mitchell, 2002). Another estimate places the net economic benefit to adopters of this technology at $6.56/ac (EPA, 2003a).

YieldGard Rootworm hybrids also provide growers with tremendous operational benefits. For corn, early planting usually results in a longer growing season and higher yield. However, early planting can result in insecticide performance failures because of chemical dissipation prior to larval hatch. Root protection for *YieldGard Rootworm* hybrids will not diminish with early planting. In addition, growers are able to plant their crop in a shorter period of time because there isn't a need to continually stop and refill insecticide applicators. Reducing the time required for growers to complete the planting operation increases the likelihood that the crop can be planted during optimal weather conditions.

The quantity of conventional insecticides used to control corn rootworms annually exceeds the quantity applied to control any other targeted pest in any other crop (Doane, 2003). Adoption of *YieldGard Rootworm Corn* hybrids will provide an opportunity to significantly reduce the costs, use of natural resources, and occupational and environmental exposure associated with the manufacture, transportation, storage, handling, application and disposal of conventional chemical insecticides.

In year 2000, 7.8 million pounds of insecticide active ingredient were applied to 14.2 million acres of corn for control of corn rootworm (Doane, 2001). The U.S. EPA estimates that the corn rootworm pest accounts for one out of every seven conventional insecticide applications made annually to agricultural crops (EPA, 2003b). Numerous factors, including competitive product offerings, market acceptance, export restrictions, and hybrid availability, will influence grower decisions to replace conventional insecticide applications with plantings of hybrids containing event MON 863. At product maturity, utilization of *YieldGard Rootworm Corn* by growers has the potential to annually eliminate the use of millions of pounds of conventional insecticides.

VII. Insect Resistance Management

Effective insect resistance management (IRM) programs for *B.t.* crops are a vital part of responsible product stewardship and should be instituted based on the best available knowledge, employing what is known about the trait, the mode of action, the targeted insects and the environment in which the product is introduced, while being properly respectful of uncertainties so as to make *B.t.* technologies available to growers as an additional pest management tool. Such programs must strike a balance between available knowledge and practicality, with grower acceptance and implementation of the plan as critical components. Monsanto supports the development and implementation of an effective and practical IRM plan for all *B.t.* crops in all markets where these products are introduced. Each plan includes the following elements:

- Baseline susceptibility determination for the target pests and surveillance for changes in susceptibility;
- An adequate supply of susceptible insects to mate with any resistant insects (achieved through appropriate practical programs such as structured refuge, natural or cultivated alternate hosts, grower practices, etc.);
- Mitigation plans; and

- Grower awareness, education of IRM concepts, and some means of assessing grower behavior when particular IRM practices are required of them.

These plans vary according to geography, pest and overlapping crops, and are reviewed on a regular basis with updated information available from interested stakeholders.

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APPENDIX A

Summary of Grain and Forage Composition Results

Key to interpreting data presented in Tables A-1 to A-6:

- *YGRW = YieldGard Rootworm Corn*
- *YGRW* and parental control mean values are for 16 replicate samples collected from four sites
- S.E. = standard error of the mean
- C.I. = confidence interval
- Comm. = commercial value; the range of sample values for commercial reference lines grown at the same field sites in these 1999 trials
- T.I. = tolerance interval, specified to contain 95% of the commercial line population, negative limits set to zero
- Lit. = literature ranges (Jugenheimer, 1976; Watson, 1982; Watson, 1987)
- Historical range for control lines analyzed in Monsanto composition studies
- na = not available

Table A-1. Combined site statistical comparison of amino acid levels in *YGRW* and control grain.

	YGRW	**Control**	**Difference (*YGRW* minus Control)**			**Comm. Range**		
Amino Acid (% of total)	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	***p-value***	**95% C.I. (Lower, Upper)**	**(95% T.I. Lower, Upper)**	**Literature Range**	**Historical Range**
Alanine	7.74 ± 0.032 (7.65 - 7.85)	7.79 ± 0.032 (7.46 - 7.98)	-0.045 ± 0.031 (-0.23 – 0.24)	0.247	-0.14, 0.055	7.30 - 8.06 (6.94, 8.46)	6.4-9.9	7.2-8.8
Arginine	4.43 ± 0.062 (4.21 - 4.68)	4.33 ± 0.062 (4.09 - 4.63)	0.10 ± 0.044 (-0.16 – 0.51)	0.030	-0.0099, 0.19	3.86 - 4.83 (3.38, 5.22)	2.9-5.9	3.5-5.0
Aspartic acid	6.51 ± 0.053 (6.38 - 6.72)	6.45 ± 0.053 (6.30 - 6.67)	0.061 ± 0.021 (-0.11 – 0.23)	0.064	-0.0070, 0.13	6.05 - 7.14 (5.54, 7.65)	5.8-7.2	6.3-7.5
Cystine	2.20 ± 0.027 (1.98 - 2.40)	2.09 ± 0.027 (1.99 - 2.29)	0.11 ± 0.029 (-0.15 – 0.39)	<0.001	0.054, 0.17	1.84 - 2.35 (1.59, 2.65)	1.2-1.6	1.8-2.7
Glutamic acid	19.39 ± 0.16 (18.99 – 19.91)	19.56 ± 0.16 (18.97 - 20.26)	-0.17 ± 0.090 (-0.76 – 0.24)	0.157	-0.46, 0.12	18.31 - 20.25 (17.55, 21.25)	12.4-19.6	18.6-22.8
Glycine	3.60 ± 0.048 (3.45 - 3.74)	3.53 ± 0.048 (3.32 - 3.72)	0.072 ± 0.030 (-0.075 - 0.31)	0.100	-0.025, 0.17	3.20 - 4.13 (2.81, 4.46)	2.6-4.7	3.2-4.2
Histidine	2.84 ± 0.032 (2.70 – 2.95)	2.83 ± 0.032 (2.72 - 2.94)	0.011 ± 0.023 (-0.082 - 0.24)	0.665	-0.063, 0.085	2.60 - 3.20 (2.37, 3.35)	2.0-2.8	2.8-3.4
Isoleucine	3.67 ± 0.033 (3.45 – 3.89)	3.74 ± 0.033 (3.61 - 3.87)	-0.064 ± 0.033 (-0.33 – 0.15)	0.072	-0.13, 0.0065	3.47 - 3.94 (3.20, 4.17)	2.6-4.0	3.2-4.3
Leucine	13.36 ± 0.081 (12.88 – 13.65)	13.65 ± 0.081 (13.27 - 14.17)	-0.29 ± 0.084 (-0.75 – 0.13)	0.039	-0.56, -0.026	11.94 - 14.47 (11.30, 15.63)	7.8-15.2	12.0-15.8

(continued next page)

Table A-1 (cont.). Combined site statistical comparison of amino acid levels in *YGRW* and control grain.

	YGRW	**Control**	**Difference (*YGRW* minus Control)**			**Comm. Range**		
Amino Acid (% of total)	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	***p-value***	**95% C.I. (Lower, Upper)**	**(95% T.I. Lower, Upper)**	**Lit. Range**	**Historical Range**
Lysine	2.92 ± 0.061 (2.65 - 3.26)	2.88 ± 0.061 (2.67 - 3.08)	0.042 ± 0.036 (-0.19 – 0.32)	0.328	-0.073, 0.16	2.40 - 3.52 (1.87, 3.89)	2.0-3.8	2.6-3.5
Methionine	2.28 ± 0.060 (1.89 - 2.49)	2.24 ± 0.060 (1.96 - 2.58)	0.034 ± 0.035 (-0.20 – 0.25)	0.348	-0.040, 0.11	1.61 - 2.29 (1.34, 2.74)	1.0-2.1	1.3-2.6
Phenylalanine	4.99 ± 0.015 (4.93 - 5.06)	5.04 ± 0.015 (4.95 - 5.23)	-0.048 ± 0.017 (-0.17 – 0.041)	0.052	-0.096, 0.0010	4.80 - 5.35 (4.53, 5.66)	2.9-5.7	4.9-6.1
Proline	8.73 ± 0.054 (8.30 - 9.21)	8.78 ± 0.054 (8.60 - 9.05)	-0.052 ± 0.046 (-0.32 – 0.38)	0.267	-0.15, 0.045	8.57 - 9.61 (8.04, 10.35)	6.6-10.3	8.7-10.1
Serine	4.70 ± 0.11 (3.93 - 5.09)	4.67 ± 0.11 (4.20 - 4.94)	0.031 ± 0.094 (-0.77 - 0.89)	0.743	-0.17, 0.23	4.24 - 4.99 (3.76, 5.69)	4.2-5.5	4.9-6.0
Threonine	3.41 ± 0.035 (3.16 - 3.60)	3.36 ± 0.035 (3.16 - 3.49)	0.049 ± 0.024 (-0.15 - 0.23)	0.056	-0.0016, 0.099	3.19 - 3.59 (2.93, 3.83)	2.9-3.9	3.3-4.2
Tryptophan	0.66 ± 0.015 (0.60 - 0.83)	0.65 ± 0.015 (0.60 - 0.68)	0.013 ± 0.012 (-0.043 - 0.17)	0.295	-0.013, 0.039	0.54 - 0.82 (0.37, 0.90)	0.5-1.2	0.4-1.0
Tyrosine	3.63 ± 0.057 (3.33 - 3.77)	3.48 ± 0.057 (2.71 - 3.82)	0.15 ± 0.078 (-0.14 - 0.92)	0.073	-0.016, 0.32	2.60 - 3.73 (2.15, 4.65)	2.9-4.7	3.7-4.3
Valine	4.94 ± 0.043 (4.71 - 5.13)	4.94 ± 0.043 (4.64 - 5.12)	-0.0091 ± 0.043 (-0.36 - 0.50)	0.833	-0.097, 0.079	4.49 - 5.30 (4.15, 5.63)	2.1-5.2	4.2-5.3

Table A-2. Combined site statistical comparison of fatty acid levels in *YGRW* and control grain.

	YGRW	**Control**	**Difference (*YGRW* minus Control)**			**Comm. Range**		
Fatty Acid (% of total)	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	***p-value***	**95% C.I. (Lower, Upper)**	**(95% T.I. Lower, Upper)**	**Literature Range**	**Historical Range**
16:0 palmitic acid	12.01 ± 0.11 (11.61 – 12.56)	11.88 ± 0.11 (11.66 - 12.20)	0.12 ± 0.11 (-0.21 - 0.79)	0.337	-0.22, 0.47	9.07 - 12.14 (7.74, 13.87)	7-19	9.9-12.0
18:0 stearic acid	1.66 ± 0.083 (1.40 - 1.86)	1.66 ± 0.083 (1.33 - 1.81)	0.0044 ± 0.013 (-0.087 - 0.078)	0.738	-0.023, 0.032	1.44 - 2.40 (1.04, 2.68)	1-3	1.4-2.2
18:1 oleic acid	22.00 ± 0.36 (20.97 – 23.55)	21.87 ± 0.36 (21.00 - 22.53)	0.13 ± 0.12 (-0.16 - 1.05)	0.365	-0.26, 0.52	21.26 - 32.06 (13.28, 36.31)	20-46	20.6-27.5
18:2 linoleic acid	62.23 ± 0.38 (60.02 – 63.21)	62.47 ± 0.38 (61.55 - 63.60)	-0.23 ± 0.18 (-1.83 - 0.32)	0.293	-0.81, 0.35	54.15 - 63.64 (50.21, 70.86)	35-70	55.9-66.1
18:3 linolenic acid	1.20 ± 0.020 (1.13 - 1.29)	1.24 ± 0.020 (1.09 - 1.45)	-0.037 ± 0.021 (-0.30 - 0.071)	0.079	-0.080, 0.0047	0.97 - 1.36 (0.75, 1.51)	0.8-2	0.8-1.1
20:0 arachidic acid	0.41 ± 0.0068 (0.39 - 0.44)	0.40 ± 0.0068 (0.39 - 0.42)	0.0052 ± 0.0062 (-0.017 - 0.027)	0.460	-0.014, 0.025	0.35 - 0.45 (0.30, 0.51)	0.1-2	0.3-0.5
20:1 eicosenoic acid	0.30 ± 0.011 (0.28 - 0.35)	0.30 ± 0.011 (0.28 - 0.35)	0.0011 ± 0.0037 (-0.039 - 0.040)	0.783	-0.011, 0.013	0.25 - 0.39 (0.18. 0.42)	na	0.2-0.3
22:0 behenic acid	0.18 ± 0.0068 (0.17 - 0.21)	0.18 ± 0.0068 (0.15 - 0.21)	0.0043 ± 0.0056 (-0.023 - 0.029)	0.498	-0.013, 0.222	0.089 - 0.21 (0.055, 0.30)	na	0.1-0.3

Table A-3. Combined site statistical comparison of mineral levels in *YGRW* and control grain.

Mineral	*YGRW* Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (*YGRW* minus Control)			Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
			Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)			
Calcium (% dw)	0.0052 ± 0.00041 (0.0041 – 0.0064)	0.0053 ± 0.00041 (0.0043 - 0.0089)	-0.00013 ± 0.00020 (-0.0027 - 0.00081)	0.538	-0.00056, 0.00031	0.0039 - 0.0060 (0.0022, 0.0073)	0.01-0.1	0.003-0.006
Copper (mg/kg dw)	2.26 ± 0.17 (1.72 - 3.18)	2.19 ± 0.17 (1.60 - 2.88)	0.078 ± 0.076 (-0.58 - 1.10)	0.315	-0.078, 0.23	1.03 - 2.15 (0.25, 2.70)	0.9-10	na
Iron (mg/kg dw)	23.55 ± 1.16 (21.13 – 26.36)	24.18 ± 1.16 (20.57 - 28.16)	-0.63 ± 0.80 (-3.92 - 1.83)	0.490	-3.18, 1.92	16.74 - 28.69 (12.52, 35.06)	1-100	na
Magnesium (% dw)	0.13 ± 0.0034 (0.12 - 0.14)	0.14 ± 0.0034 (0.12 - 0.16)	-0.0049 ± 0.0024 (-0.018 - 0.0049)	0.135	-0.013, 0.0028	0.091 - 0.14 (0.082, 0.17)	0.09-1.0	na
Manganese (mg/kg dw)	5.81 ± 0.78 (3.75 - 7.40)	6.15 ± 0.78 (4.01 - 8.28)	-0.34 ± 0.16 (-0.94 - 0.58)	0.122	-0.84, 0.17	3.51 - 9.80 (0, 12.84)	0.7-54	na
Phosphorus (% dw)	0.40 ± 0.0068 (0.37 - 0.45)	0.42 ± 0.0068 (0.39 - 0.46)	-0.022 ± 0.0094 (-0.070 - 0.019)	0.065	-0.045, 0.0020	0.27 - 0.41 (0.21, 0.47)	0.26-0.75	0.288-0.363
Potassium (% dw)	0.43 ± 0.0088 (0.40 - 0.48)	0.44 ± 0.0088 (0.39 - 0.48)	-0.0074 ± 0.0087 (-0.056 - 0.037)	0.457	-0.035, 0.020	0.33 - 0.43 (0.28, 0.48)	0.32-0.72	na
Zinc (mg/kg dw)	22.15 ± 1.44 (17.95 – 25.25)	23.68 ± 1.44 (18.77 – 28.14)	-1.53 ± 0.69 (-4.60 - 0.90)	0.112	-3.73, 0.66	12.84 - 31.22 (6.31, 37.95)	12-30	na

Table A-4. Combined site statistical comparison of fiber and proximate content in *YGRW* and control grain.

	YGRW	**Control**	**Difference (*YGRW* minus Control)**			**Comm. Range**		
Fiber & Proximates	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**p-value**	**95% C.I. (Lower, Upper)**	**(95% T.I. Lower, Upper)**	**Literature Range**	**Historical Range**
Ash (% dw)	1.35 ± 0.12 (0.84 - 1.71)	1.41 ± 0.12 (0.89 - 1.89)	-0.064 ± 0.047 (-0.45 – 0.31)	0.196	-0.17, 0.037	0.62 - 1.53 (0.26, 2.06)	1.1-3.9	1.2-1.8
Carbohydrates (% dw)	83.30 ± 0.56 (81.83 – 85.00)	82.76 ± 0.56 (80.70 - 84.80)	0.54 ± 0.27 (-0.78 - 2.43)	0.138	-0.32, 1.40	82.51 - 87.84 (78.97, 90.36)	na	81.7-86.3
Acid detergent fiber (% dw)	4.45 ± 0.15 (3.49 - 5.23)	4.50 ± 0.15 (3.62 - 5.89)	-0.050 ± 0.18 (-1.77 - 1.16)	0.778	-0.43, 0.33	3.65 - 6.09 (1.98, 6.62)	3.3 - 4.3	3.1 - 5.3
Neutral detergent fiber (% dw)	11.64 ± 0.54 (9.21 – 13.47)	12.02 ± 0.54 (10.31 - 15.82)	-0.37 ± 0.61 (-4.32 - 2.30)	0.585	-2.33, 1.58	9.50 - 14.95 (6.51, 16.28)	8.3-11.9	9.6 - 15.3
Moisture (% fw)	10.03 ± 0.50 (8.54 – 11.20)	10.23 ± 0.50 (8.60 - 11.40)	-0.20 ± 0.13 (-0.90 - 0.26)	0.216	-0.61, 0.21	8.75 - 15.70 (5.09, 18.62)	7-23	9.4 - 15.8
Total fat (% dw)	3.77 ± 0.20 (3.00 - 4.56)	3.64 ± 0.20 (3.05 - 4.29)	0.13 ± 0.18 (-0.77 - 1.02)	0.520	-0.44, 0.70	2.18 - 3.86 (1.68, 4.64)	3.1-5.7, 2.9-6.1	2.4-4.2
Protein (% dw)	11.60 ± 0.48 (10.43 – 12.82)	12.19 ± 0.48 (10.45 - 13.80)	-0.59 ± 0.22 (-1.52 - 0.12)	0.071	-1.28, 0.097`	- 13.83 (5.47, 16.57)	6.0 - 12.0, 9.7 – 16.1	9.0 - 13.6

Table A-5. Combined site statistical comparison of nutrient and anti-nutrient factor levels in *YGRW* and control grain.

Component	*YGRW* Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (*YGRW* minus Control) Mean ± S.E. (Range)	*p-value*	95% C.I. (Lower, Upper)	Comm. Range (95% T.I. Lower, Upper)	Literature Range	Historical Range
Phytic Acid (% dw)	1.11 ± 0.033 (0.92 - 1.28)	1.23 ± 0.033 (1.01 - 1.37)	-0.12 ± 0.034 (-0.31 - 0.19)	0.001	-0.19, -0.050	0.73 - 1.17 (0.39, 1.33)	to 0.9%	na
Trypsin Inhibitor (TIU/mg dw)	2.30 ± 0.16 (0.56 - 3.10)	2.48 ± 0.16 (1.91 - 3.45)	-0.18 ± 0.16 (-1.70 - 0.63)	0.288	-0.53, 0.17	0.58 - 3.05 (0, 4.25)	na	na
Vitamin E (mg/gdw)	0.011 ± 0.0012 (0.0062 – 0.014)	0.013 ± 0.0012 (0.0088 - 0.016)	-0.0015 ± 0.00047 (-0.0077 - 0.00090)	0.002	-0.0025, -0.00058	0.0041 - 0.014 (0, 0.019)	0.017-0.047	0.008-0.015

Table A-6. Combined site statistical comparison of fiber and proximate content in *YGRW* and control forage.

	YGRW	**Control**	**Difference (*YGRW* minus Control)**			**Comm. Range**	
Component	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	**Mean ± S.E. (Range)**	***p-value***	**95% C.I. (Lower, Upper)**	**(95% T.I. Lower, Upper)**	**Historical Range**
Ash (% dw)	4.73 ± 0.22 (3.62 - 5.65)	5.00 ± 0.22 (3.81 - 6.27)	-0.27 ± 0.16 (-1.29 - 1.09)	0.106	-0.61, 0.066	3.74 - 5.02 (3.04, 5.58)	2.9 - 5.1
Carbohydrates (% dw)	84.24 ± 0.53 (82.29 - 86.32)	84.32 ± 0.53 (80.78 - 87.21)	-0.084 ± 0.43 (-2.70 - 2.52)	0.859	-1.47, 1.30	82.59 - 87.10 (81.22, 88.97)	84.6 - 89.1
Acid detergent Fiber (% dw)	28.67 ± 1.66 (21.74 - 43.30)	28.41 ± 1.66 (23.39 - 32.08)	0.26 ± 2.06 (-7.90 - 14.03)	0.907	-6.29, 6.81	19.78 - 39.00 (9.33, 45.44)	21.4 - 29.2
Neutral detergent Fiber (% dw)	43.25 ± 1.26 (37.97 - 49.67)	42.94 ± 1.26 (37.32 - 51.85)	0.31 ± 1.25 (-10.81 - 12.34)	0.807	-2.25, 2.87	30.30 - 47.75 (22.71, 56.02)	39.9 - 46.6
Moisture (% fw)	71.09 ± 0.46 (69.30 - 73.10)	71.68 ± 0.46 (69.80 - 74.50)	-0.58 ± 0.43 (-3.70 - 2.90)	0.269	-1.95, 0.79	67.00 - 74.10 (62.70, 77.69)	68.7 - 73.5
Protein (% dw)	8.62 ± 0.53 (6.91 - 10.40)	8.33 ± 0.53 (5.99 - 10.55)	0.30 ± 0.37 (-2.54 - 2.42)	0.478	-0.87, 1.47	6.45 - 10.14 (4.94, 11.97)	4.8 - 8.4
Total fat (% dw)	2.40 ± 0.23 (0.92 - 3.16)	2.35 ± 0.23 (1.30 - 3.33)	0.053 ± 0.15 (-0.91 - 1.14)	0.721	-0.26, 0.36	1.39 - 2.62 (1.03, 3.24)	1.4 - 2.1